A CRISPR Screen of HIV Dependency Factors to Identify Host Proteins Necessary for Activation of Latent HIV Proviruses

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Abstract

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The human immunodeficiency virus (HIV) integrates into host DNA and therefore persists as a long-lived pathogen in people living with HIV (PLHIV). One of the greatest challenges to achieving a cure for human immunodeficiency virus (HIV) is the presence of latently infected long-lived immune cells that endure throughout the course of antiretroviral treatment. If antiretroviral therapy is stopped, these immune cells will sporadically produce virions which contribute to viral rebound and progressive infection. There are several approaches to targeting the latently infected cell population. One approach, called “shock and kill”, relies on small molecule drugs called latency reversal agents (LRAs) that stimulate transcription of HIV proviral genes which could lead to the infected cells being targeted by the immune system for elimination. The second approach, which is predominantly the focus of this thesis, is called “block and lock” which seeks to permanently silence the viral reservoir to prevent viral reactivation from the latent cell population. In this thesis, I performed a CRISPR screen in

latently infected Jurkat T cell lymphocytes to identify host factors required for latency reactivation using a gene set of putative HIV dependency factors previously described. The goal was to broadly screen for these factors in Jurkat T cells but ultimately validate these hits in primary CD4+ T cells. I identified several factors that are novel for reactivation from latency including ALYREF, UBE2M, TBL1XR1 and AMBRA1. The top hit, Cyclin T1 (CCNT1) is a member of the P-TEFb complex with cyclin-dependent kinase 9 (CDK9) known to required for HIV transcription elongation but also for host gene transcriptional elongation. I found that CCNT1 knockout prevents reactivation using LRAs that target a broad spectrum of pathways, and that CCNT1 knockout in primary CD4+ T cells dramatically prevents latency reactivation without affecting T cell receptor activation. RNA sequencing of CCNT1 knockout cells revealed minimal effects on host gene transcription but dramatic effects on HIV transcription. I hypothesize that CCNT1 knockout is compensated by its paralogs CCNT2 or CCNK for host cell function, and thus CCNT1 may be a promising target in a block and lock model of HIV cure. Moreover, I hypothesize that HIV dependency factors also play a key role in latency reactivation.

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Acknowledgements

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Chapter 1. Introduction

The role of HIV as a global issue cannot be understated; at the end of 2022, the UNAIDS/WHO estimated that there were 39 million people living with HIV globally, and that 630,000 people died from HIV-related causes [7]. The advent of antiretroviral therapy (ART) has dramatically increased the outcome of people living with HIV (PLHIV), but is limited by access and a need to promptly and consistently administer treatment throughout an individual's lifetime. Thus, treatment is difficult for those living in more remote locations and for whom it is a challenge to regularly obtain and/or afford treatment, including those with substance use disorders.

A major challenge for finding a cure for HIV is the existence of a latent reservoir. HIV integrates its genome into cells and while ART suppresses active virus replication, a pool of cells which have integrated provirus but are not actively transcribing viral genes or producing virions make up the "latent reservoir." The latent reservoir reactivates – i.e. begins to express viral genes and produce virions upon cessation of antiretroviral therapy. Thus, it is key to understand the mechanisms of HIV latency; how it is established, the factors that maintain latency, and factors that contribute to reactivation from latency.

1.1 Dynamics of the Latent Reservoir

CD4+ T cell lymphocytes are the primary target of HIV-1 infection and it is thought that subsets of these cells largely contribute to the expansion of the latent reservoir. The ability to prevent the latent reservoir from forming seems unlikely as the latent reservoir forms regardless of early intervention of ART therapy [8]. There are several mechanisms that are postulated to be involved in establishment and expansion of the latent reservoir. When CD4+ T cells are infected,

a portion of the cells are activated, and a portion of those cells are cleared by the immune system. A subpopulation of those cells will become memory T cells and harbor latent provirus until a stimulus provokes transcription of proviral genes or the cell dies [1, 9, 10].

CD4+ T cells are a component of the adaptive immune system that play a key role in the recognition of foreign antigen by their T-cell receptors, upon which T cells undergo expansion (rapid proliferation) and secrete cytokines which further mount an immune response. T cells that have not yet found cognate antigen are located in the thymus and known as naïve T cells. T cells are stem-like and differentiate over time, with proliferation and self-renewal decreasing as differentiation occurs. The differentiation of memory T cells (T_M) occurs in the following order: naïve T cells (T_N), stem cell-like memory T cells (T_{SCM}), central memory T cells (T_{CM}), and effector memory T cells (T_{EM}) (depicted in **Figure 1.1**). The latent HIV reservoir largely resides in T_{CM} and T_{EM} cells – and thus exists in multiple states of T cell proliferation potential. On encountering antigen that the T cell receptors recognize, naïve T cells differentiate into T_{SCM} and subsequently into T_{CM}. Both of these T cell subtypes maintain self-renewal properties. What is key about these subtypes, especially in the context of HIV-1 infection, is their ability to recognize antigen and activate on response thereby dividing and expanding the pool of cells to respond to foreign antigen on recurrent infection [5].

Antigen-driven proliferation of T cells is one of the major processes that contributes to clonal expansion of the latent reservoir [5]. When the T cell receptor of an HIV-1 infected memory T cell sees its cognate antigen, the T cell will undergo rapid proliferation and expansion. As the T cells proliferate, cells harboring latent provirus also expand and replicate the integrated genome, thus enlarging the latent reservoir. It is important to note that this can occur without actively producing virus [11]. It has been shown that some T cells from PLHIV who underwent

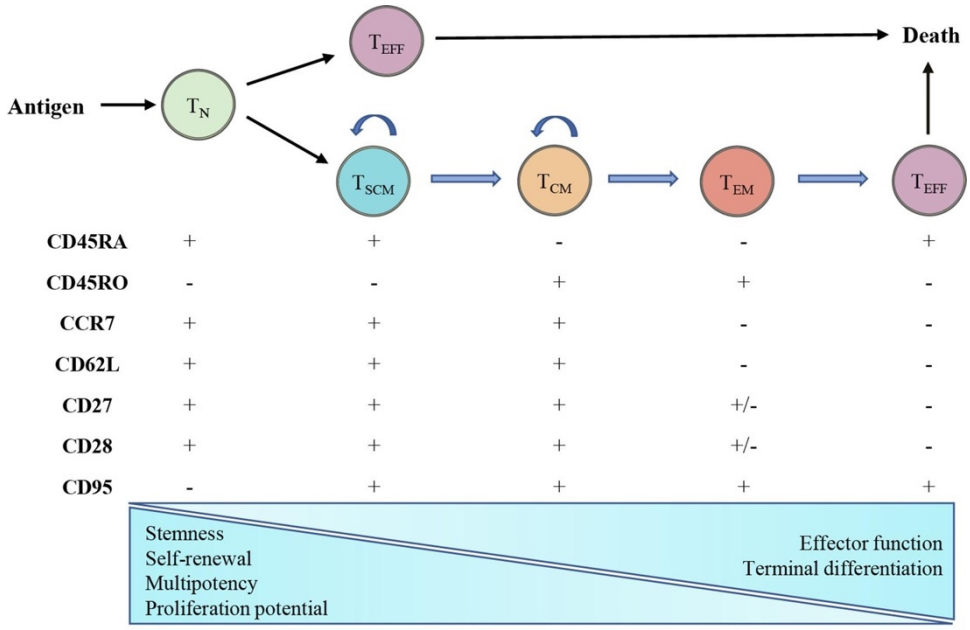

Figure 1.1: The T cell lymphocyte subtypes are depicted above in the chronological order of their differentiation; naïve T cells (T_N), stem cell-like memory T cells (T_{SCM}), central memory T cells (T_{CM}), and effector memory T cells (T_{EM}). Each T cell subtype expresses a unique set of cell surface markers. The T_{SCM} and T_{CM} subtypes exhibit self-renewal potential. As stem cells differentiate from subtype to subtype (as indicated by arrows), they lose their stemness and self-renewal potential and eventually become terminally differentiated. Reproduced with permission, Elsevier license # 5621660382224 [3].

chemotherapy are responsive to cytomegalovirus (CMV) and Epstein-Barr virus (EBV) and those CMV and EBV-responding T cells harbor HIV-1 at a higher frequency than other CD4+ T cells [12]. CMV commonly affects PLHIV and drives inflammation. Thus, CMV can be a source of expansion of the HIV latent reservoir through antigen-driven proliferation. It also has been shown that those who have more viral shedding of CMV and EBV tend to have higher diversity of HIV DNA, suggesting that indeed CMV+ or EBV+ T cells actually comprise of a significant population that contribute to the reservoir [13].

While antigen-driven proliferation is one of the major components that contributes to the expansion of HIV-1 infected cells, homeostatic proliferation of T cells which is characteristic of T_{SCM} and T_{CM} cells also contributes to clonal expansion of HIV-1 infected cells. For example, treatment of HIV-1 infected CD4+ T cells from PLHIV with the cytokine IL-7 led to a dramatic increase in the number of CD4+ T cells that harbored HIV DNA [14]. Moreover, memory T cells

have a half-life of approximately 3.7 years [1] and thus their longevity constitutes a major obstacle to the elimination of latently infected cells.

HIV-1 integration site can also impact proliferation of T cells. It is known that HIV-1 integrates into more actively transcribed genes [15], and therefore HIV-1 integration sites near genes involved with cell growth may promote expansion of those latent cells [16]. Host genome HIV-1 integration sites that have been shown to be over-represented include *BACH2, MKL2, NFATC3* and *STAT5B* [5]. For example, genes that have HIV-1 integration upstream of a proliferation gene such as *BACH2* can lead to increased expression of that proliferation gene, and subsequently lead to clonal expansion. In a similar manner, integration of HIV-1 downstream of a proto-oncogene, such as *VAV1,* can lead to aberrant splicing and its truncated expression leads to increased cell proliferation and expansion of that clonally integrated site [17].

Collectively, it is estimated that over 50% of cells in the latent reservoir are maintained through clonal expansion [5]. The dynamic nature of the various mechanisms of HIV-1 latency is depicted in **Figure 1.2** [5]. While the expanded cells contain a mix of cells that harbor replication-competent and replication incompetent virus, replication-competent virus nevertheless can reactivate upon immune stimulation. Critically, antiretroviral therapy does not stop the expansion of cells that harbor the latent provirus. Thus, the expansion of HIV-1 clonally infected cells poses a major challenge towards elimination of the latent reservoir.

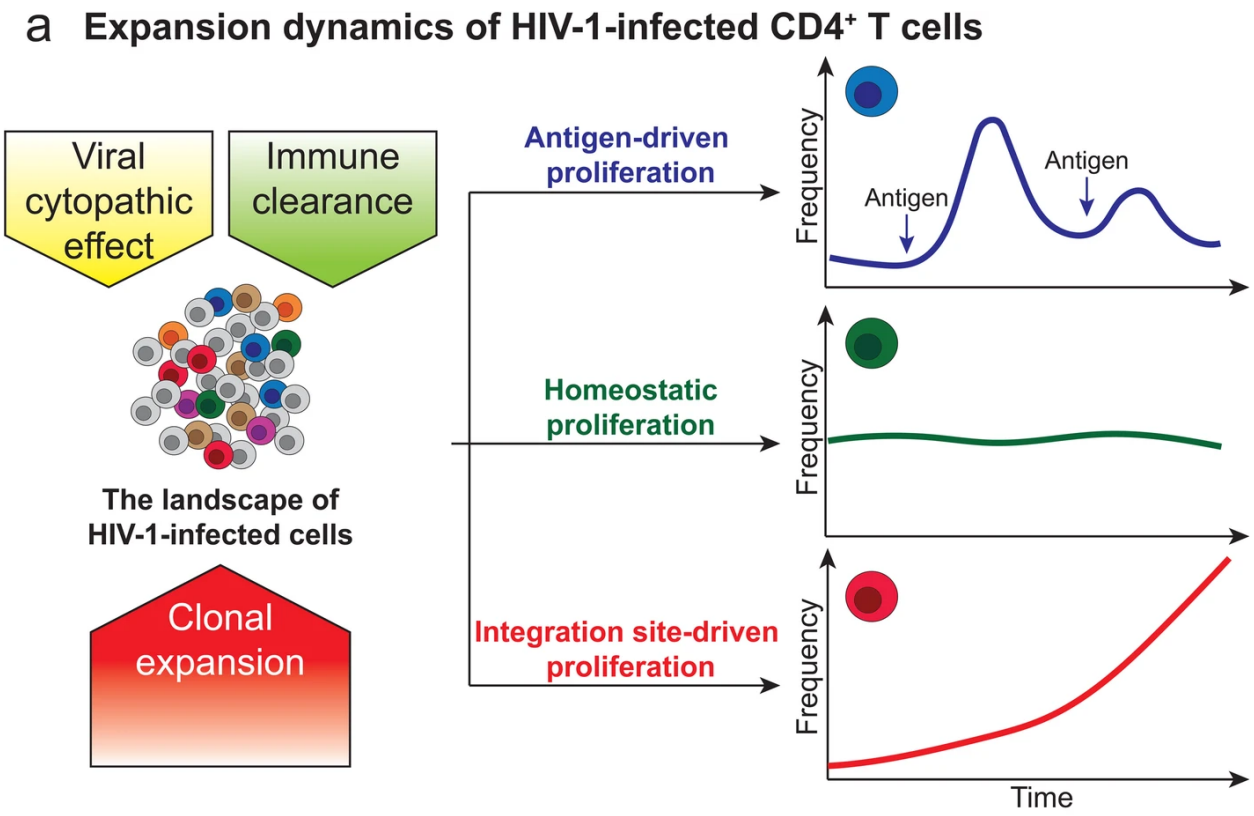

Figure 1.2: A model describing the dynamics of the latent reservoir. Several factors contribute to the decrease and increase of HIV-1 infected cells and establishment of the latent reservoir. On left, while viral cytopathic effects and immune clearance of HIV-infected cells decreased the overall pool of cells which are infected, this is counterbalanced with the clonal expansion of latently infected cells. On right, the various mechanisms contributing to the frequency of HIV infected cells is shown. Image is reproduced with permission under the Creative Commons CC BY license [5].

Prior to ART initiation, cells enter but do not persist in the latent reservoir, and slowly decline over time. Decline of the reservoir in the pre-ART period may occur through several mechanisms, including immune clearance of cells which are infected and as a result of the natural half-life of infected cells. While studies in non-human primates have shown that the reservoir can be established during the initial stages of lentiviral infection [18], more recent work has shown that the latent reservoir that persists over time is actually established at the time of ART initiation. One study examined the viral sequences that were circulating in nine women living with HIV, before and during antiretroviral treatment. Remarkably, about 71% of the

sequences that were present post-ART initiation were also present prior to ART initation [19]. This suggests that ART largely stabilizes the latent reservoir. To that end, initiation of ART can change the rate at which cells enter the latent reservoir [19, 20]; when ART is initiated, new HIV infections are blocked through the action of administered integrase inhibitors and nucleoside reverse transcriptase inhibitors [21]. Changes in the latent reservoir are thought to occur in part by changes in cell signaling which lead integrated provirus into deeper latency, the gradual increase of epigenetic marks over time, and viral suppression increasing the half-life of CD4+ T cells, including HIV+ T cells [1, 19, 22].

There are several implications of these observations. First, ART therapy when administered may contribute to a more homogenous latent reservoir, and it may be easier to target and eliminate a reservoir that is less diverse and comes from a limited number of clones. Second, the latent reservoir likely differs for those who have administered ART early after infection versus later in infection, and thus targeting latent cell populations may be different for these two groups. Thus, the amount of time that lapses between infection and ART treatment poses a major challenge for eliminating the latent reservoir and achieving a functional cure for HIV, especially for those whom it is harder to obtain and maintain consistent administration of ART.

1.2 The Tissues of the Latent Reservoir

Although HIV latency is most conveniently measured in CD4+ T-cells in the peripheral blood, these are a minority of the infected cells in the body. In fact, HIV-1 infects and resides in a wide range of tissues and poses a major challenge for the functional cure of HIV. During initial stages of infection, one of the major targets of HIV-1 is the gut-associated lymphoid tissue, also

sometimes referred to as GALT. The lymphoid tissue of the gut lines the intestinal epithelium and provides a barrier that protects the human body from bacterial invasion from the microbiota of the intestinal tract. When these cells are infected and die, bacteria from the intestine can penetrate the epithelium. Elevated lipopolysaccharides (LPS) of these bacteria in the blood leads to systemic immune activation [23-25]. The GALT is a major component of the latent reservoir and that this tissue is replication-competent reservoir poses a unique challenge for eradication of the latent reservoir.

HIV-1 is not limited to infecting only CD4+ T cells, but also infects cells of the myeloid lineage and may maintain a reservoir of latent infection in macrophages. That is, HIV-1 typically exhibits tropism for cells which express CCR5 or CXCR4 receptors (R5 T-cell tropic and X4 T-cell tropic, respectively) and have high CD4 receptor expression, but is also able to adapt to infect low CD4 expressing T-cells (M-tropism). M-tropic viruses also can target macrophages [26]. The ubiquitous presence of macrophages throughout the body poses an additional challenge for elimination of the latent reservoir, especially in compartments which are notably harder to access such as the central nervous system. Recently, it has been shown that microglia – resident macrophages of the central nervous system – harbor replication-competent HIV; when microglial cells were isolated from an individual with HIV, virus from these cells was able to infect other microglial cells and peripheral blood mononuclear cells (PBMCs) [27]. HIV-1 nucleic acid has been detected in other tissues aside from the peripheral blood – including in the brain and cerebrospinal fluid (CSF), lymphatic tissue, and other non-lymphoid tissues including the liver, kidney, adipose tissue and reproductive tract even in those who are on antiretroviral therapy **(Figure 1.3) [1].** However, the presence of HIV-1 DNA in a tissue is not necessarily indicative of replication-competent virus, as there are many barriers to viral production. Most of the latent

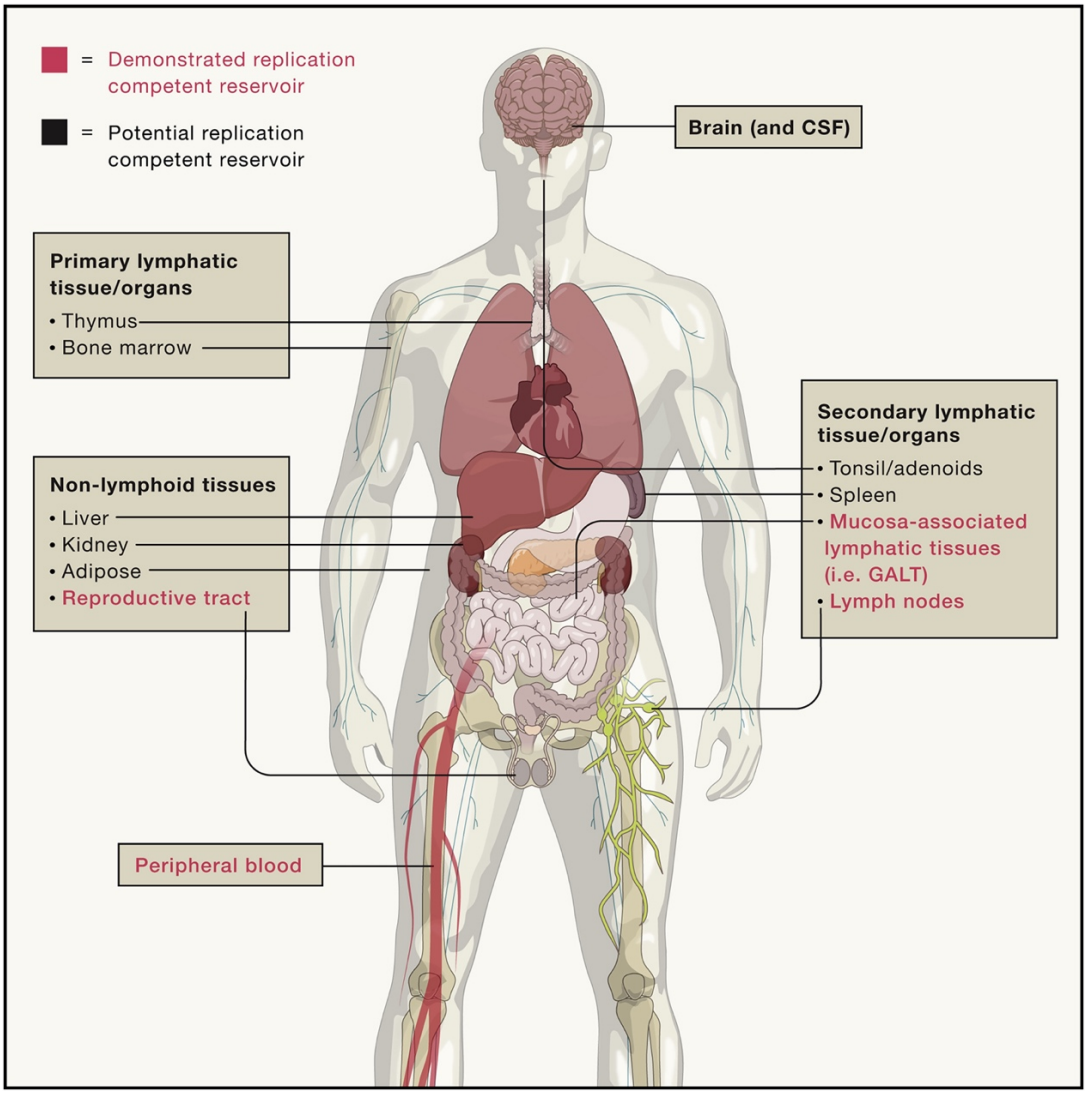

Figure 1.3: Tissues and organs of the latent reservoir. The various tissues in the human body where it is known that HIV establishes replication competent reservoirs is shown in **red text**, whereas the tissues where HIV nucleic acid has been detected but virus has not been recovered is shown in **black text**. Reproduced with permission (Elsevier license **5621650622248) [1].**

reservoir consists of defective HIV-1 proviral DNA sequences which result in part from the host protein APOBEC3G which induces G-to-A hypermutation in the viral genome [1, 28, 29]. It is important to note that the lack of detection of a replication-reservoir in a particular tissue does not rule out the possibility that a replication reservoir does not exist in these tissues. Moreover, detection of HIV-1 nucleic acid in various tissues poses a challenge in cure approaches that rely on elimination of the reservoir latent reservoir, in that drug therapies need to be able to access

and target a variety of cell types and tissues. The diversity of the latent reservoir and the tissues it resides in therefore poses a major barrier to a cure for HIV.

1.3 HIV-1 viral replication and transcriptional mechanisms

While a description of the entire HIV lifecycle is outside the scope of this thesis introduction, two important aspects of HIV replication, integration into the host cell genome, and transcriptional regulation of the HIV LTRs are critical elements of latency establishment, maintenance, and release. Shortly after viral entry, the HIV RNA genome is transported via the capsid through the cytoplasm and nuclear envelope, where capsid uncoating and viral replication occurs. HIV is a positive-sense RNA genome that undergoes reverse transcription to generate cDNA. It subsequently undergoes integration into the DNA of host cells, by way of a viral enzyme known as Integrase. Integrase itself does not have preference for any particular DNA content, but rather is driven to integration site preferences by virtue of binding a host protein called LEDGF/p75. LEDGF has a binding domain for integrase as well as a chromatin-binding domain which thereby links integrase to DNA through LEDGF. HIV DNA integration into host cells by LEDGF largely favors transcriptionally active regions, and thus integration favors a chromatin state that is conducive to transcription of HIV viral genes for initial rounds of replication [15, 30, 31]. Additional host factors, including CPSF6 which bind to the capsid protein, are also critical for the integration of HIV into active gene regions. It has been shown for example that when Capsid and CPSF6 interactions are abolished, HIV integrates into transcriptionally silenced regions at the nuclear lamina as opposed to more active gene-dense regions in the nuclear interior [32]. Thus, CPSF6 facilitates and directs the pre-integration complex away from the nuclear periphery to allow for active gene expression [32]. CPSF6 and

LEDGF together are two examples of host factors that HIV hijacks in order to facilitate successful integration and expression of viral genes to enable viral fitness.

After integration, transcriptional mechanisms of HIV-1 play a key role in the formation of the latent reservoir as well as the maintenance of the reservoir over a sustained period of time, even in the presence of ART. HIV-1 transcription relies on several components of viral machinery but also uses host machinery in order to allow for promote transcription of host genes. The 5' Long-Terminal Repeat (LTR) of the integrated HIV provirus serves as the region for recruitment of viral and host factors that enable transcription to occur. There are host transcription factor binding sites present in the 5' LTR, including those for NF-κB, Sp1, NFAT, IRF, C/EBP, AP-1, and several others [33, 34]. Some of these TFs seem to be essential for activation of HIV-1 transcription, including NF-κB and Sp1 [35], whereas others are not essential but do have an effect on levels of HIV-1 transcription, including LEF-1 and YY1 [34, 36, 37]. Collectively these transcription factors aid in the recruitment of RNA polymerase II to the HIV promoter to allow for initiation of transcription.

As is the case with most host genes, transcription of HIV-1 is stalled shortly after transcription initiation. HIV-1 needs to overcome the stalled RNA polymerase II in order to allow for productive elongation and transcription of viral genes. Host genes relieve paused RNA polymerase II using a variety of factors, such as those associated with the Super Elongation Complex (SEC). The SEC includes the positive Transcription Elongation Factor b (P-TEFb) complex, ELL, AFF family proteins 1-4, and EAF [6]. P-TEFb is made up of a complex of Cyclin-Dependent Kinase 9 and either Cyclin T1, Cyclin T2, or Cyclin K [38]. P-TEFb can also be associated with BRD4 or the 7SK small nuclear ribonucleoprotein (snRNP) complex **(Figure 1.4)** [6]. The HEXIM1 protein and 7SK snRNP serve as modulators of P-TEFb activity; when

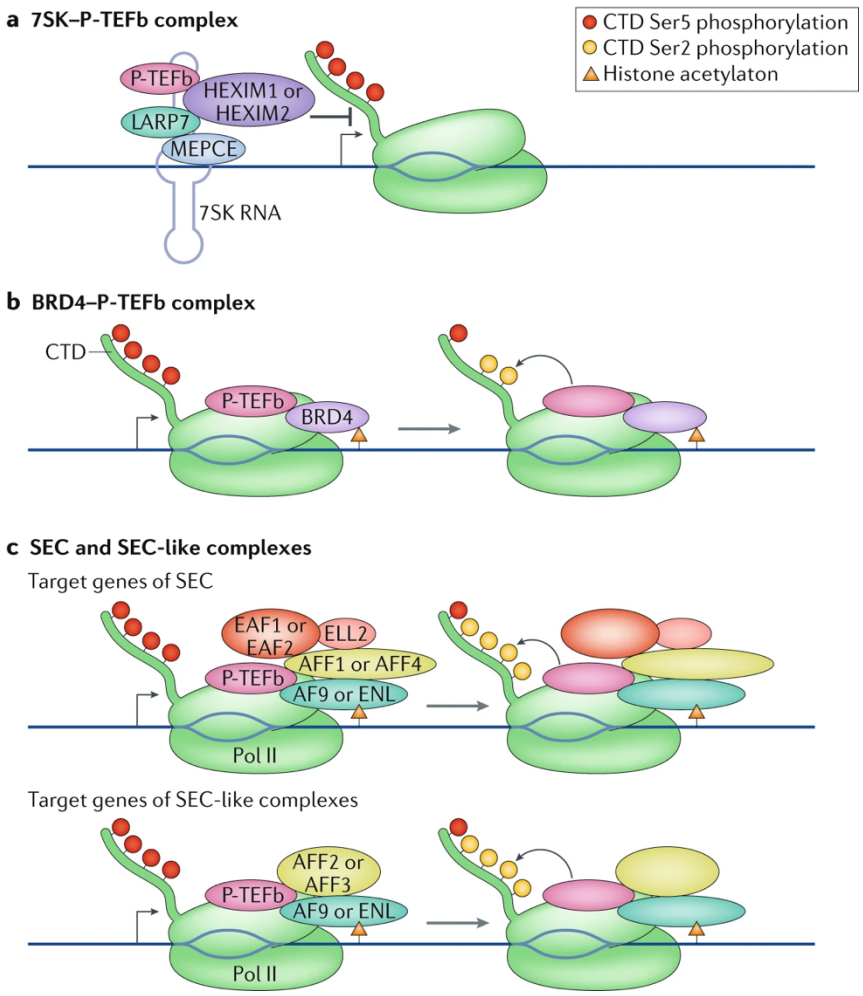

Figure 1.4: The various complexes of P-TEFb and regulation of activity. **A.** RNA Pol II shown in green is phosphorylated at Ser5 (marker of transcription initiation), but does not progress when P-TEFb is in an inactivated complex with the 7SK RNA and HEXIM1/2. Thus, RNA PolII transcription is stalled in this example. **B.** The BRD4 complex can recognize acetylated histones and recruit the P-TEFb complex to RNA PolII to allow for phosphorylation of Ser2 and productive elongation of host genes. **C.** The super elongation complex (SEC) o SEC-like complex respond to acetylated histones. The SEC comprises of several different components but includes the P-TEFb complex, and thus P-TEFb can promote Ser2 phosphorylation and allow for productive transcription elongation of host genes. Reproduced with permission under Springer Nature License #: 5621661476525 [6].

bound to the 7SK snRNP and HEXIM1, P-TEFb is inactive [6]. Upon release of P-TEFb from HEXIM1 and 7SK, P-TEFb is active and can phosphorylate RNA polymerase II to relieve the stalled RNA polymerase and allow for productive transcription elongation and processivity.

Because host genes and HIV-1 proviral genes alike rely on transcription elongation factors to relieve paused RNA polymerase II, HIV-1 has evolved a mechanism that allows it to compete with host genes for viral fitness. Early in its transcription, HIV-1 transcribes an RNA hairpin known as the trans-activation response (TAR) element. This hairpin serves as a binding

site for the viral Tat protein, and together the viral Tat and TAR elements recruit the P-TEFb complex to the 5' LTR and relieve stalled RNA polymerase II. With elongation of HIV-1 genes, transcription of the structural proteins and the enzymes essential for synthesis and integration of HIV-1 into the host genome can occur. Encoded in the viral genome are the structural proteins – collectively *gag* – which encode for the Matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins. The *pol* gene encodes for the reverse transcriptase enzyme, integrase, and a protease [39]. The *env* gene encodes for the HIV-1 envelope protein, a glycoprotein that is required for viral entry in host cells [40]. Genomic RNA is packaged along with the structural proteins and the envelope glycoproteins in order to infect new cells. Collectively, HIV-1 genes have evolved to hijack host cell machinery for productive transcription of viral genes.

1.4 Molecular Mechanisms of HIV Latency Establishment and Maintenance

There are several molecular mechanisms that help to establish and maintain the HIV-1 latent reservoir, but the common theme is that each mechanism leads transcriptional silencing of proviral genes. These can broadly be classified into three categories; epigenetic silencing mechanisms, transcription initiation blocks, and transcription elongation blocks. HIV-1 preferentially integrates into regions of actively transcribed genes, and this is key for the transcription of genes prior to latency establishment [15]. In the scenario that HIV-1 integrates into a more heterochromatic region, or that integration occurs in a euchromatic region that is later silenced, these factors can lead to reduction of viral transcripts. The latter is thought to occur in part on initiation of ART; when ART is initiated, cells that harbor latent virus can undergo epigenetic silencing that leads to downregulation of the HIV-1 proviral genes. The 5' LTR has several nucleosomes which can be subjected to epigenetic histone modifications much

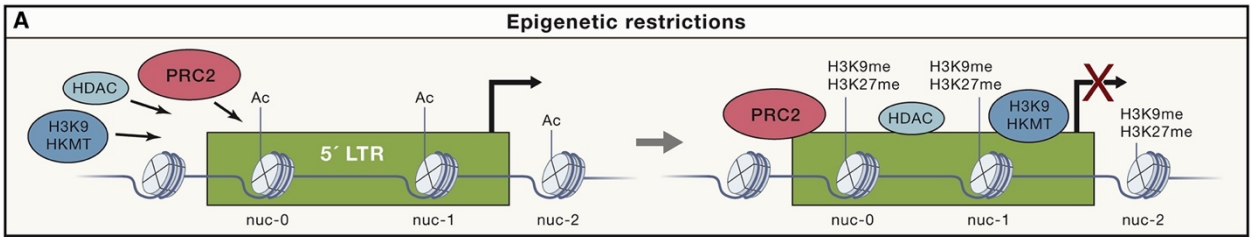

Figure 1.5: Epigenetic restrictions that contribute to HIV latency. Histone deacetylases (HDACs), the Polycomb Repressive Complex 2 (PRC2), and the H3K9 histone lysine methyltransferase (HKMT) all contribute to the epigenetic silencing of nucleosomes that are present at the LTR Reproduced with permission under Elsevier license # 5621650622248 [1].

like those surrounding host genes, including H3K27 methylation by the histone methyltransferase EZH2 of polycomb repressive complex 2 (PRC2), and H3K9 methylation. In contrast, there are also acetylation marks at the nucleosomes, which are signatures of active transcription. Host histone deacetylases (HDACs) can remove these active epigenetic marks, thus leading to a more transcriptionally silent provirus. Moreover, it is well understood that covalent epigenetic modifications are intact through cell division. This leaves room for the epigenetic marks of the HIV LTR – whether repressive or active – to expand in the latent reservoir. Collectively, epigenetic marks can contribute to the establishment and maintenance of the latent reservoir **(Figure 1.5)** [1].

In addition to these epigenetic mechanisms that control HIV latency, another contributing factor that facilitates entry into the latent reservoir is the availability of transcription factors which are essential for binding the LTR and initiating transcription. NF-κB is predominantly present in the cytoplasm where it is bound to inhibitors of NF-κB (IκBs). In the canonical NF-κB signaling pathway, upstream signals – including TNFα and interleukin-1β (IL-1β) trigger the phosphorylation and degradation of IκB, and the resulting NF-κB can translocate to the nucleus [41]. Thus, in the absence of inflammatory signals, NF-κB is in the cytoplasm and not active [42]. In memory T cells, it is expected that there is relatively low NF-κB signaling, unless these T cells are activated, in which case NF-kB signaling resumes. An additional transcription factor

NFAT acts at the LTR, but NFAT levels have been shown to be low in resting CD4+ T cells [43, 44]. The lack of host transcription factors via blocked signaling or abundance therefore is a major contributing factor to HIV latency.

Abundance of P-TEFb for transcription elongation is considered another major factor that contributes to viral latency. Despite the presence of the viral Tat protein and TAR element, the relative availability of free P-TEFb and competition between HIV-1 transcription elongation and host transcription poses a challenge for viral fitness. This may in part be exacerbated by low levels of the viral Tat protein. It has also been proposed that overall levels of P-TEFb in resting CD4+ T cells is low thereby leading to blocks of transcription elongation in resting T cells [45]. Cyclin T1 (CCNT1) of the P-TEFb complex is phosphorylated at two amino acid residues – Thr143 and Thr149 – by protein kinase C (PKC) and this promotes binding to CDK9. Without this phosphorylation, CCNT1 is degraded by the proteasome, thus leading to less free P-TEFb for transcription elongation of both host genes and viral genes [46]. The regulatory mechanisms of P-TEFb in part may contribute to HIV latency in memory T cells.

1.5 Strategies and approaches to targeting the latent reservoir

The approaches to targeting the latent reservoir can be broadly classified into two approaches – “shock and kill” (also sometimes described as “shock and suicide”) and “block and lock.” The shock and kill approach seeks to target latently infected cells – preferably for a short period of time – using small molecule drugs to reactivate the latent cell population. These small molecule drugs are called latency reversal agents (LRAs). As previously described, the latent cell population has multiple barriers that lead to the transcriptional silencing of HIV-1. These LRAs largely seek to upregulate transcription of proviral genes, by targeting the mechanisms that

facilitate the entry and maintenance of HIV latent cell populations – the epigenetic silencing of provirus, as well as transcription initiation and elongation. Variations of the shock and kill approach also rely on antigen-driven activation of latent proviruses and enhanced immune control such as administration of long-acting broadly neutralizing antibodies [47]. However, in this thesis, I will concentrate on LRAs as a means of activating the latent provirus.

One of the challenges of evaluating different LRAs is that their effectiveness in latency reactivation is dependent largely on the model of latency that is being employed. A model commonly used for HIV latency are Jurkat T cell lymphocytes with a single latent provirus in their genome (called J-Lat cells for Jurkat Latent). The ease of manipulation and scalability of this model provides an excellent tool for identifying host genes important for latency and have also been useful in the context of identifying the host genes that are important throughout the viral life cycle for establishing infection [48]. To generate J-Lat models, Jurkat cells are often infected with a GFP reporter virus, cultured over time, and eventually a subset of these previously GFP+ cells will naturally go into latency. The GFP- cells can be used in bulk, but also individual clones can be isolated. The advantage of the former is that these cells represent a wide variety of integration sites and contexts, whereas the advantage of J-Lat clones is that the integration site can be mapped and can be used to characterize in depth the chromatin landscape that facilitates transcriptional silencing. A common theme is that LRAs tend to be more potent in Jurkat T cell lymphocytes than in more pertinent and relevant models, and thus J-Lat models serve as an excellent way to screen and identify critical components that underly HIV latency but require further validation using more relevant models.

A more relevant model of HIV latency are primary CD4+ T cells. Cells are isolated from blood from healthy donors and then infected with an HIV reporter virus, and cultured into

latency over a span of several weeks [49, 50]. The drawback of primary T models are that cell quantities are often a limiting factor, the cells have a limited lifespan in culture, and primary T cells cultured in vitro as not the same as those in vivo. Primary CD4+ T cells can also be isolated from PLHIV – typically from aviremic individuals – and viral reactivation can be detected using a quantitative viral outgrowth assay (QVOA) [51, 52]. However, these studies are invasive since they require large quantities of blood and human subject approvals, and therefore would most often be done only after validation by other approaches.

Animal models of latency have also been used for characterizing LRAs, but at the expense of scalability, time and cost. It is known that HIV does not infect mice, due to several barriers including differences in key host genes including CCNT1 and differences in the immune system [53-55]. Bone marrow/liver/thymus (BLT) humanized mice have provided a good model for characterizing LRAs as these mice respond to HIV-1 infection as well as ART therapy. BLT mice are generated in backgrounds of immunodeficient mice, and fragments from human fetal liver and thymus are transplanted into these mice [56]. Finally, aside from directly testing LRAs in PLHIV, non-human primate models are excellent for characterizing the effectiveness of LRAs. Studies performed on rhesus macaques serve as one of the best and most accepted models of HIV and SIV infection (simian immunodeficiency virus)[57]. Primary CD4+ T cells isolated from healthy donors and J-Lat models are the models used in this dissertation.

1.6 Latency Reversal Agents (LRAs) and Latency Promoting Agents (LPAs)

Small molecules that either activate latent proviruses (LRAs), or do the opposite and enhance HIV latency (LPAs) have been developed. The most potent LRAs to date have dramatic effects on cell viability or broadly affect pathways and thus are not feasible for use as LRAs in a

clinical setting. As one example, Phorbol 12-myristate 13-acetate and ionomycin (PMAi) are typically used as positive controls as latency reversal agents but drastically affect cell viability of peripheral blood mononuclear cells (PBMCs) [58]. Additionally, stimulation of the CD3/CD28 co-receptors or TNFalpha and T cell activation can serve as a mechanism to upregulate NF-KB signaling, but these signaling mechanisms act broadly and can have dramatic effects on cells.

Thus, a major goal in the search for an HIV cure is to identify those LRAs which can be used at low doses and have minimal effects on cells but have a long-lasting impact on HIV replication. Most recently, AZD5582 has been shown to reactivate latency in HIV-infected humanized mice models, and in SIV-infected rhesus macaques [59]. AZD5582 is a non-canonical NF-KB activator that is a mimetic of a second mitochondrial-derived activator of caspase (SMAC). Under normal conditions the cellular inhibitor of apoptosis protein (cIAP) is active and contributes to silencing of the NF-KB pathway by degrading an NF-KB inducing kinase (NIK). AZD5582 inhibits the cIAP proteins and thereby promotes NF-KB signaling by enabling downstream processing of p100 to p52 which can translocate to the nucleus and activate transcription of target genes with RelB [59, 60]. Importantly, AZD5582 can be used at relatively low concentrations and for the most part had minimal effects on the animals tested; in animals that experienced side effects, these were alleviated upon stopping AZD5582 treatment [59]. AZD5582 may be a good option to stimulate reactivation in HIV-1 infected cells which have a block to transcription initiation that maintains their latency.

While AZD5582 is a promising candidate, the consensus in the HIV field is that targeting the latent reservoir will likely involve targeting multiple arms of HIV latency [49, 61]. There are many additional drugs that have been developed to target the epigenetic mechanisms and transcriptional blocks to latency. One of the earliest LRAs – SAHA/Vorinostat – is a histone

deacetylase inhibitor. Acetylation marks serve as active markers of transcription on histones. In normal cell biological conditions, the histone deacetylase enzymes remove this active chromatin marks that are recognized by transcriptional enhancers. Thus, inhibiting these enzymes can promote active transcription of proviral genes in contexts where latency contributions come from silenced, heterochromatic regions. In addition to removing acetylation marks from histones, HDACs can also regulate histone crotonylation [62], and crotonylation has been shown to sensitize AZD5582-induced latency reversal [63]. SAHA/Vorinostat has shown some promising results in people living with HIV; individuals treated with Vorinostat had an increase in cell-associated HIV RNA, but ultimately the pool of latently infected cells did not decrease in these people [64].

Additional latency reversal approaches have targeted the transcription elongation arm of latency. To date, the transcription elongation arm largely involves the use of bromodomain inhibitors, including I-BET151 and JQ1 [65, 66]. These bromodomain inhibitors target several BRD proteins, including BRD2, BRD3 and BRD4 [67, 68]. BRD4 binds to acetylated histones and recruits the P-TEFb complex to allow for transcription elongation of host genes. The mechanism of action of these bromodomain inhibitors and their role in HIV reactivation is not entirely clear. Many have argued that BRD4 is antagonistic to HIV transcription as HIV transcription relies on the P-TEFb complex for transcription elongation in addition to host genes and thus HIV transcription competes with BRD4 and host genes for free P-TEFb . Two isoforms of BRD4 exist, a short and long isoform. The long isoform of BRD4 contains a P-TEFb binding region, whereas the short isoform does not. In fact, the overexpression of the C-terminal domain of the long isoform of BRD4 is sufficient to inhibit Tat-dependent transactivation [69]. Thus, bromodomain inhibitors like JQ1 and I-BET151 can promote the reactivation of HIV by

inhibiting bromodomain proteins from binding P-TEFb and thus allowing for P-TEFb to be recruited by Tat and TAR to the LTR for transcription elongation [66]. BRD2 and BRD3 have separate functions as well, and thus the mechanism of latency reactivation can be difficult to decipher. Recent studies have suggested that the combined treatment of latently infected cells with an inhibitor of apoptosis protein inhibitor (IAPi) such as AZD5582, and with a pan-bromodomain inhibitor drug I-BET151 showed latency reversal. Bromodomain inhibitors specific for BRD1 or BRD2 also have transcription elongation of proviral genes and latency reversal, but transcription is more pronounced with the pan-BETi. Therefore, there is likely some degree of HIV latency reactivation from the BRD3 and BRD4 proteins which are also targeted by the pan-BETi drugs I-BET151 and JQ1 [61].

The combined use of LRAs targeting multiple mechanisms of latency is increasingly becoming an approach to target the latent reservoir in *in vitro* studies. One such example is the combination of AZD5582 and I-BET151, which together have been used to lead to improved reactivation over either LRA alone. Moreover, the use of these two LRAs can also lead to "synergistic" reactivation from latency, in that the use of two LRAs leads to more viral production than either LRA used alone [61]. Synergistic combinations of drugs are advantageous as they can be used at lower doses to achieve a desired affect and thereby reduce the toxicity and side effects which often happens at higher dosages. The challenge with AZD5582 and I-BET151 is that while there is synergistic latency reversal in latently infected Jurkat T cell models, this synergistic drug combination does not lead to reversal CD4+ T cells isolated from aviremic donors. Ultimately, it was found that there remain additional blocks to transcription elongation as there are reduced transcripts of full length proviral sequences relative to more potent (but toxic) LRA combinations [61].

In a parallel manner as LRAs function in a "shock and kill" approach, latency promoting agents (LPAs) function in a "block and lock" approach. A block and lock approach may be key for targeting the persistent reservoir in those cells which cannot be eradicated by the shock and kill approach. LPAs seek to lock the HIV promoter into a permanently silenced state by targeting factors required for HIV replication in order to prevent viral reactivation from latency. Some of these LPAs are targeted at the viral Tat-TAR interaction and thus prevent transcription of the provirus. For instance, didehydro-Cortistatin A (dCA) inhibits Tat/TAR interaction and therefore "promotes" latency by inhibiting Tat transactivation [70]. Other approaches have relied on siRNA in order to target the LTR and prevent transcription of proviral genes which can lead to epigenetic silencing on recruitment of histone modifying complexes to the LTR region [71, 72]. The only potentially successful block-and-lock drug to date is ruxolitinib, a JAK/STAT inhibitor which has made it to a clinical Phase 2a study [73].

Collectively, both shock and kill and block and lock therapeutic approaches will likely involve manipulation of multiple arms of HIV latency for a desired outcome. Either approach alone may not be sufficient for elimination or silencing of the reservoir. The shock and kill approach relies on reactivating integrated provirus from a large range of tissue contexts and integration sites, and therefore the block and lock approach may be essential for permanently silencing viral replication in those cells which are not eliminated by shock and kill. Thus, block and lock drugs need to be further explored in a therapeutic context.

1.7 An HIV Dependency Factor Screen

HIV dependency factors are host factors that HIV requires for its viral replication cycle. At each stage of the viral replication cycle, there is at least one host factor that facilitates HIV

viral replication. Briefly, as examples; CD4 and CXCR4 receptors are host proteins that facilitate the binding and entry of HIV viral particles into cells. Integration into actively transcribing gene regions away from the nuclear lamina requires the host genes LEDGF/p75 and CPSF6. The transcription of HIV relies extensively on host genes, including the transcription factor NF-KB and the P-TEFb complex, comprising of CCNT1 and CDK9. These dependency factors may serve as excellent targets for silencing the latent reservoir, especially if their inhibition has greater effects on HIV replication than on host cell function. Previous work in our lab has examined the role of dependency factors in HIV replication by performing a CRISPR screen. This CRISPR screen was able to identify novel host dependency factors across multiple strains of HIV [48]. In this CRISPR screen, a library called the HIV-Dependency factor gene library (HIV-Dep) was developed by selecting for the top hits from a genome-wide screen, along with genes that were top hits from another screen using the Human Epigenome Library (HuEpi) [49]. The HIV-Dep library consists of 525 genes, with 8 guides targeting each gene and 210 non-targeting controls. Altogether, this HIV-Dep library contains 4401 guide RNAs. The basis of the screen was to generate CRISPR knockout pools using the HIV-Dep gene library in Jurkat T cells, and subsequently infecting these knockout cells with different strains of HIV. The screen was able to robustly identify host genes that were required for HIV replication regardless of the strain of HIV used, but also was sensitive enough to differentiate tropism of different viruses. For example, the LAI strain of HIV requires CXCR4 and CD4 receptors for binding and entry of viral particles, but not CCR5; this was reflected in the CRISPR screen results. Beyond the host cell receptors, the screen was able to identify gene hits across a wide variety of pathways, including transcription, rRNA processing, chromatin remodeling, protein modification and translation. When a subset of gene hits across these different pathways were selected for

validation, over 90% of the selected hits were validated to be required for HIV replication. Moreover, several of these gene hits were novel and not previously described to be involved as dependency factors [48].

When I started my project, I saw the potential for the HIV-Dependency factor gene set to inform several aspects of HIV latency. First, HIV transcription is one of the major facets that contributes to latency, and thus I hypothesized that there would provide key information on potential novel transcription factors required for reactivation from latency. Several transcriptional genes that one would anticipate as important for latency reactivation were in the library, including NFKB1 and Sp1 [48]. Second, I saw the potential to understand the pathways that are critical for HIV replication but not for HIV latency or are unique as latency factors. This characterization has heretofore not been examined in depth and may help to identify factors that are important specifically for latency reactivation. Moreover, there are likely factors required both as dependency factors and as latency factors, and thus may help to identify novel gene targets for preventing latency reactivation. Finally, these dependency factors comprise of factors across a wide variety of pathways, and thus while it is anticipated that transcription-associated factors are critical for reactivation from latency, I saw the potential to identify other factors, including protein modifying enzymes, factors involved in translation, and rRNA processing.

1.8 Thesis Rationale

The underlying hypothesis of this thesis work was that a subset of HIV Dependency Factors would also be critical in HIV latency reversal. Thus, the goal of this thesis work was to identify dependency factors using the HIV Dependency CRISPR library to identify factors that may be used in a block and lock model of HIV latency. We were able to identify several putative

host dependency factors that are required for reactivation from latency. Moreover, we identified a gene – CCNT1 – that is a well-established regulator of transcription elongation of both host and viral genes, that is required for reactivation from latency. Contrary to expectations based on the described functions of CCNT1 in transcription elongation in normal cell biology, I found that knockout of *CCNT1* did not affect the growth and proliferation of Jurkat T cells. This led me to investigate the role that *CCNT1* may have in host gene regulation. I found that knockout of *CCNT1* whether in Jurkat T cells or in CD4+ T cell lymphocytes had very little effect on host cell function; the number of genes and magnitude of their differential expression in CCNT1 knockouts versus wild-type was minimal. Moreover, I found that CCNT1 regulates HIV-1 genes to a greater magnitude than any other host gene, suggesting that P-TEFb for host transcription elongation may rely on a paralog of CCNT1 for function. Thus, because HIV-1 but not host T cells rely on CCNT1, this protein may be a good target for specifically silencing HIV-1 proviruses without affecting host T cell biology.

Chapter 2: A CRISPR Screen of HIV Dependency Factors Reveals That *CCNT1* Is Non-Essential in T Cells but Required for HIV-1 Reactivation from Latency

2.1 Abstract

We sought to explore the hypothesis that host factors required for HIV-1 replication also play a role in latency reversal. Using a CRISPR gene library of putative HIV dependency factors, we performed a screen to identify genes required for latency reactivation. We identified several HIV-1 dependency factors that play a key role in HIV-1 latency reactivation including *ELL*, *UBE2M*, *TBL1XR1*, *HDAC3*, *AMBRA1*, and *ALYREF*. Knockout of Cyclin T1 (*CCNT1*), a component of the P-TEFb complex important for transcription elongation, was the top hit in the screen and had the largest effect on HIV latency reversal with a wide variety of latency reversal agents. Moreover, *CCNT1* knockout prevents latency reactivation in a primary CD4+ T cell model of HIV latency without affecting activation of these cells. RNA sequencing data showed that CCNT1 regulates HIV-1 proviral genes to a larger extent than any other host gene and had no significant effects on RNA transcripts in primary T cells after activation. We conclude that CCNT1 function is non-essential in T cells but is absolutely required for HIV latency reversal.

2.2 Introduction

The existence of an activatable latent reservoir is a key barrier to virus elimination in people living with HIV as cells which harbor an integrated latent proviral genome persist in the presence of antiretroviral treatment. The multifaceted nature of HIV latency suggests a combination of methods and approaches will need to be used to effectively reduce this reservoir.

Factors that ultimately block HIV-1 transcription including host epigenetic silencing mechanisms, blocks to transcription initiation and transcription elongation all contribute to a silent, or nearly silent, HIV reservoir.

The "shock and kill" approach to reservoir reduction involves using latency reversal agents (LRAs) to promote viral transcription and viral reactivation in the latent reservoir and then eliminating those reactivated cells using immunological approaches or methods that rely on recognition of newly synthesized viral proteins [1, 74, 75]. The shock and kill approach is attractive in that it seeks to eliminate the latent reservoir by killing cells harboring transcriptionally-competent proviral sequences. However, these LRAs must target a broad range of proviruses with highly-variable epigenetic and gene expression contexts in different cells and tissues [76, 77]. Another strategy, called "block and lock", involves targeting factors that are required for HIV replication in order to prevent viral reactivation [78, 79]. Such approaches rely on molecules called Latency Promoting Agents (LPAs) that seek to lock the HIV promoter into a permanently silenced state. For instance, didehydro-Cortistatin A (dCA) inhibits Tat/TAR interaction and therefore enforces latency by inhibiting Tat transactivation [70]. Other approaches have used siRNAs to target the LTR and prevent transcription of proviral genes which can lead to epigenetic silencing on recruitment of histone modifying complexes to the LTR region [71, 72]. Thus far, only one block-and-lock drug, ruxolitinib – a JAK/STAT inhibitor, has made it to a clinical Phase 2a study [73]. Both "shock and kill" and "block and lock" therapeutic approaches will likely involve manipulation of multiple arms of HIV latency for a desired outcome, and therefore a more comprehensive understanding of these mechanisms is an important consideration for approaches to eliminate the latent reservoir and achieve a functional HIV cure.

We previously performed a CRISPR screen using a novel system called Latency HIV-CRISPR to identify host genes involved in epigenetic control that maintain latency [49]. In this screen, knockout of genes promotes reactivation from latency, suggesting that these host genes normally function to repress HIV-1 transcriptional activation. In the present study, we modified this system to identify host genes that are required for HIV-1 to reactivate from latency, i.e. are necessary for HIV-1 to come out latency. We hypothesized that a subset of host genes that HIV requires for replication, called HIV dependency factors, would also be required for reactivation from latency. Our goal was to identify proteins whose function is more important for HIV-1 reactivation than for normal T cell biology.

Transcription of HIV-1 is dependent on several host mechanisms, with the P-TEFb complex being a key component that interacts with a viral protein, Tat, and a viral RNA element, TAR, to allow for transcription elongation. Both HIV-1 and host genes use CCNT1 and CDK9 in the P-TEFb complex in order to enable transcription elongation [80]. CCNT1 has a paralog – CCNT2 – which also forms the P-TEFb complex [81] and *in vitro* studies have shown that another host protein CCNK, can also interact with CDK9 to form the P-TEFb complex [38]. However, while HIV-1 Tat viral protein binding sites are conserved in CCNT1 and CCNT2 only the CCNT1-Tat complex can bind with the viral TAR RNA in order to recruit P-TEFb to the LTR [82].

Here, we performed a CRISPR-Cas9 screen using the Latency HIV-CRISPR technique [49] for factors necessary for HIV-1 to be released from latency in the presence of a combination of LRAs. We used a custom CRISPR guide library, called the HIV dependency factor gene library (HIV-Dep), that had been previously used to identify novel host dependency factors across multiple HIV strains[48]. We identified and validated factors important in latency

reactivation including *ELL1*, *TBL1XR1*, *UBE2M*, *HDAC3*, *AMBRA1*, and ALYREF. Cyclin T1 (*CCNT1*), which forms the P-TEFb transcriptional elongation complex with Cyclin-dependent Kinase 9 (*CDK9*) was the top gene hit in two J-Lat models in our screen. We found that Cyclin T1 is essential for reactivation from latency in J-Lat cells as well as in a primary T cell model of HIV latency using a broad range of LRAs. *CCNT1* knockout had no effect on cell proliferation in the J-Lat model, and did not affect activation through the T cell receptor in primary CD4+ T cells. Moreover, we performed bulk RNA sequencing on *CCNT1* knockouts and found HIV-1 genes were the most depleted relative to wild-type *CCNT1* over any host gene in J-Lat cells, whether or not they were treated with an LRA. RNA sequencing in uninfected primary T cells knocked out for *CCNT1* showed very few changes in host cell transcript expression. Together, our findings show that some HIV-1 dependency factors are more important for HIV replication and reactivation than for host cell biology and suggest that *CCNT1* could be a promising therapeutic target for silencing HIV-1 into deeper latency. To that end, other genes uncovered in our screen may also be worth exploring further as factors for a block and lock mechanism for HIV.

2.3 Methods

2.3.1 Cell Culture and Maintenance

HEK293T cells were cultured in DMEM (ThermoFisher, 11965092) along with Penicillin/Streptomycin (Pen/Strep) and 10% Fetal Bovine Serum (FBS). J-Lat cells were cultured in RPMI 1640 media (ThermoFisher, 11875093) supplemented with Pen/Strep, 10% Fetal Bovine Serum (FBS), and 10 mM HEPES (ThermoFisher, 15630080). Cells were maintained at 37ºC with 5% CO_2. Cells were routinely tested and found to be free of

mycoplasma contamination. Primary CD4+ T cell media used was RPMI 1640 + 1x Anti-Anti (Gibco, 15240096), 1x GlutaMAX (ThermoFisher Scientific; 35050061), 10 mM HEPES, and 10% FBS.

2.3.2 HIV-CRISPR Library Transduction and Virus-Encapsidated CRISPR Guide Screening

The HIV-Dep library containing 525 genes (4191 sgRNAs) was previously described [48]. For transduction of J-Lat cells, HEK293T cells were seeded in 20x6 well cell culture plates, transfected with the HIV-DEP plasmid (667 ng), psPax2 (GagPol, 500 ng), and MD2.G (VSVG, 333 ng) per well in 200 uL of serum-free DMEM (Thermo Fisher Scientific) along with 4.5 uL of TransIT-LT1 reagent (Mirus Bio LLC; MIR2305). VSVG pseudotyped lentivirus was harvested and filtered through a 0.22 um filter (Sigma-Aldrich, SE1M179M6). Virus was titered using TZM-bl (NIH AIDS Reagent Program; ARP-8129) cells. J-Lat 10.6 and J-Lat 5A8 previously knocked out for *ZAP* [49] were transduced with HIV-CRISPR library lentivirus with DEAE-Dextran (final concentration 20 ug/mL, Sigma-Aldrich; D9885) at a multiplicity of infection (MOI) of 0.5. After 24 hours, puromycin (Sigma, P8833) at a final concentration of 0.4 ug/mL was added to the culture to select for cells that received the vector. The screen was performed 11 days after transduction, by treating the HIV-Dep library transduced J-Lat cells with latency reversal agents AZD5582 1 nM (MedChemExpress, HY-12600) and I-BET151 2.5 uM (SelleckChem, S2780) or DMSO (Sigma, 472301) control. After 24 hours (day 12), the supernatants were harvested, filtered (Millipore Sigma, SE1M179M6), and loaded over a 20% sterile sucrose solution (20% sucrose, 1 mM EDTA, 20 mM HEPES, 100 mM NaCl, distilled water) placed on a prechilled SW32Ti rotor. The viral pellets were then concentrated at *70,000 x g* for 1 hour at 4ºC and gently resuspended in 140 ul of DPBS (Gibco; 14190144) and allowed to

resuspend overnight at 4ºC. Simultaneously, transduced cells were harvested to isolate genomic DNA (gDNA). Cells were centrifuged and resuspended in DPBS. Cells were then spun down, supernatant removed, and cell pellets were frozen until ready for gDNA extraction.

2.3.3 Latency HIV-CRISPR Screen

Viral RNA (vRNA) and gDNA was isolated as previously described [83]. Briefly, vRNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen, 52904). Reverse transcription of vRNA was performed using SuperScript Reverse Transcriptase Kit (ThermoFisher, 18064014). gDNA was isolated using the QIAamp DNA Blood Midi Kit (Qiagen, 51183). vRNA and gDNA were both amplified by PCR using R1_forward primer and R1_Reverse primer using Herculase II Fusion DNA Polymerase (Agilent, 600677). PCR products were cleaned up using the QIAquick PCR clean up kit (Qiagen, 28104) and a second round of PCR was performed using R2_reverse primer and R2_IndexX primer (see supplementary file). The 230bp band was verified to be present and the amplified PCR products were cleaned up using double-sided SPRI via AMPure Beads (Beckman Coulter, A63880). Purified samples were normalized to a concentration of 10 nM using Qubit dsDNA HS Assay Kit (Invitrogen, Q32854) before sequencing.

Adapter sequences were computationally trimmed from sequencing results and the viral sequencing was compared relative to genomic knockout pool to determine the relative enrichment or depletion of each guide. An artificial NTC sgRNA gene set was generated that is equivalent to the number of genes present in the HIV-Dep library “synNTCs” by iteratively binning the NTC sgRNA sequences. MAGEcK and MAGEcK Flute statistical [4, 84] analyses were used to analyze the depletion of guides/genes in the RNA viral supernatent relative to their

abundance in the cell DNA. Z-scores were determined as previously described [48, 85]. For each HIV-Dep LAI replicate, and for each replicate of J-Lat CRISPR screen, z-scores were calculated. An average of the z-scores from each replicate was used to generate a heatmap. Heatmaps were generated using Morpheus (https://software.broadinstitute.org/morpheus). Code for z-score analysis of CRISPR screen data can be found at https://github.com/amcolash/hiv-crispr-zscore-analysis.

2.3.4 Validation of Screen Hits

Genes identified in the HIV-Latency screen that were depleted after LRA treatment were validated either by lentiviral knockout or by electroporation of RNA guides and Cas9. For genes validated by lentiviral knockout, a forward and reverse primer corresponding with 2 individual guides targeting each gene were cloned into pLCV2 and cells were transduced as described above. Puromycin selection continued for 10-14 days until treated with LRAs. For pooled electroporation knockout experiments, CRISPR/Cas9-mediated knockout was performed against genes of interest using Gene Knockout Kit v2 (Synthego). Guides targeting genes of interest with 1 uL of 20 uM Cas9-NLS (UC Berkeley Macro Lab) and RNP complexes were made with SE Cell Line 96-well Nucleofector Kit (Lonza, V4SC-1096). Complexes were incubated at room temperature for ten minutes, and 2E5 cells of J-Lat 10.6 were centrifuged at *100 x g* for 10 minutes at 25ºC, and were resuspended in Cas9-RNP complexes and electroporated on Lonza 4D-Nucleofector using code CL-120. Cells were recovered with RPMI media pre-warmed to 37ºC. Knockout pools were maintained for 10-14 days to allow for expansion and subsequently treated with LRAs. In both cases, reactivation was measured by RT activity as described [86] 24 hours after LRA treatment and genomic DNA analyzed to assess the degree of gene knockouts.

For *CCNT1* knockout clones, CRISPR/Cas9-mediated knockout was performed using Gene Knockout Kit v2 (Synthego). Guides targeting *CCNT1* were complexed with 1 uL of 20 uM Cas9-NLS (UC Berkeley Macro Lab) and RNP complexes were made with SE Cell Line 96-well Nucleofector Kit (Lonza, V4SC-1096). Complexes were incubated at room temperature for ten minutes, and 2E5 cells of J-Lat 10.6 were centrifuged at *100 x g* for 10 minutes at 25ºC, and were resuspended in Cas9-RNP complexes and electroporated on Lonza 4D-Nucleofector using code CL-120. Cells were recovered with media pre-warmed to 37ºC. Five days post-electroporation, single cells were sorted into a 96-well U-bottom plate filled with 100 uL RPMI media (20% FBS).

To assess the growth of *CCNT1* knockout J-Lat 10.6 relative to wild-type, three individual flasks of either wild-type, *CCNT1* Knockout 1 or *CCNT1* Knockout 2 J-Lat 10.6 were maintained for each line. Cells were resuspended at a concentration of 2E5 cells/mL in a total of 10 mL RPMI media. Cells were monitored and split approximately every two days. Cell counts prior to splitting were taken, the volume of cell suspension removed (the same volume was removed for each line) was tracked, and subtracted from overall cell count. These values were tracked over a span of nine days.

2.3.5 Protein Isolation and Western Blotting

Cell pellets (1.5E6-3E6 cells) from pooled lentiviral knockout experiments (NTC10 and *CCNT1* sg1 and sg2) and clonal knockout experiments (J-Lat 10.6 *CCNT1* KO clone 1 and 2) were isolated from each respective experiment. Supernatant was removed and cells were resuspended in 500 uL of cold (4ºC) 1x PBS. Cells were pelleted, resuspended in 100 uL of RIPA buffer (150 mM NaCl (Sigma, S3014), 50mM Tris pH 8.0, 1% NP-40 (Calbiochem, 492016),

0.5% Sodium Deoxycholate (Sigma-Aldrich, D6750), and 0.1% SDS (Sigma-Aldrich, L4509), Benzonase 1 uL/mL (Millipore, 70664), and cOmplete Protease Inhibitor Cocktail (Roche; 11697498001), and incubated on ice for 10 minutes with repeated vortexing. Cell lysate was pelleted at *20,000 x g* for 20 minutes at 4ºC. Clarified supernatant was transferred to a new tube and quantified by BCA. Samples were prepared by adding 4x NuPAGE LDS Sample Buffer (ThermoFisher, NP0007) with 5% 2-Mercaptoethanol (Sigma-Aldrich, M3148) and denatured at 95ºC for 5 minutes. Lysates were run on a NuPAGE 4-12% Bis-Tris pre-cast gel (ThermoFisher Scientific; NP0336) and transferred to a nitrocellulose membrane (Biorad; 1620115). After transfer, nitrocellulose membrane was blocked in 0.1% Tween/5% Milk in 1XPBS solution for 30 minutes at room temperature. Primary antibodies used for western blotting were mouse α-CCNT1 (Santa Cruz Biotechnology, sc-271348, 1:500), mouse α-CCNT2 (Santa Cruz Biotechnology, sc-81243, 1:500), and rabbit α-actin (Sigma-Aldrich, A2066 1:5000). Antibodies were diluted in 1x PBS-Tween 0.1% (PBST) and rocked on nitrocellulose membrane overnight at 4ºC. Membrane was washed with PBST 3-5 times, for 5 minutes each wash. The following secondary antibody dilutions were made 1:2000 in PBST: goat α-mouse IgG-HRP (R&D Systems; HAF007) and goat α-rabbit IgG-HRP (R&D Systems; HAF008). SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher; 34095) was used for CCNT1 and CCNT2, and SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher, 34580) was used for Actin. Visualization was done on a BioRad Chemidoc MP Imaging System.

2.3.6 Genomic Editing Analysis

Cells for each knockout were pelleted, washed with 1X PBS, supernatant removed, and cell pellets frozen at -80ºC until ready for DNA isolation. Genomic DNA was isolated using

QIAamp DNA Blood Mini Kit (Qiagen; 51104). The gene of interest was amplified using primers described using either Q5 High-Fidelity DNA polymerase (NEB; M0491S) or Platinum Taq DNA polymerase High Fidelity (ThermoFisher Scientific; 11304011). PCR products were purified using AMPure beads (Beckman Coulter, A63880) or QIAquick PCR clean up kit (Qiagen, 28104) and submitted to Fred Hutch Genomics shared resource for sequencing. Analysis was performed using Inference of CRISPR Edits (ICE) [87]. Briefly, ICE analysis compares Sanger sequencing from wild-type and CRISPR edited sequences, determining the insertion and deletions from these sequences, and generating knockout scores along with a correlation value as assessments of the knockout.

2.3.7 LRA Treatments

For J-Lat 10.6 or J-Lat 5A8 cells, LRAs were used at the following concentrations: TNFα (Peprotech, 300-01A) 10 ng/mL; AZD5582 (MedChemExpress, HY-12600) 1 nM; I-BET151 (SelleckChem, S2780) 2.5 uM; Prostratin (Sigma-Aldrich, P0077) 0.1 uM; SAHA/Vorinostat (SelleckChem, S1047), 2.5 uM. For CD3/CD28 antibody stimulation Anti-CD3 clone UCHT1 (Tonbo, 40-0038-U500) was plated on 96-well flat bottom plate at 10 ug/mL in 1x PBS, incubated overnight at 4ºC, aspirated, and CD28 clone 28.2 antibody (Tonbo, 40-0289-U500) was added to RPMI media at a concentration of 4 ug/mL for cell resuspension. Cells for each experiment were resuspended at a concentration of 5E5 cells/mL in appropriate LRA media, and 200 uL was aliquoted into 96-well flat bottom TC plate. For Primary CD4+ T Cell LRA treatment, PMA (Sigma-Aldrich, P1585) was used at a concentration of 10 nM, in combination with ionomycin (Sigma-Aldrich, I0634) was used at a concentration of 1 uM. For primary cell experiments, CD3 antibody (Tonbo, 40-0038-U500) was used at a concentration of 10 ug/mL

and CD28 antibody (Tonbo, 40-0289-U500) at a concentration of 5 ug/mL. All LRA treatments were performed for 24 hours unless otherwise indicated.

2.3.8 Primary CD4+ Cell Isolation and Latency Model

All centrifugation steps of Primary CD4+ T cells were performed at *300 x g* for 10 minutes at 25ºC unless otherwise noted. PBMCs were isolated from used leukocyte filters (Bloodworks Northwest) over a Ficoll gradient (Millipore Sigma, GE17-1440-02), cryofrozen at a concentration of 10-20E6 cells/mL in 90% FBS/10% DMSO, and stored in liquid nitrogen until ready to use. On thawing, PBMCs were washed dropwise with pre-warmed RPMI-1640 media (Thermo Fisher) and treated with benzonase (25 U/mL) (Sigma-Aldrich, E1014) for 15 minutes at room temperature. PBMCs were maintained at a concentration of 2E6 cells/mL overnight at 37ºC. The following day, CD4+ T cells were isolated using the EasySep Human CD4+ T cell Isolation Kit (Stemcell Technologies, 17952) and subsequently activated using the T Cell Activation/Expansion Kit (Miltenyi Biotec, 130-091-441). From this point forward, CD4+ T cells were cultured in RPMI + IL-2 (final concentration 100 U/mL, Roche, 10799068001), IL-7 (final conc. 2 ng/mL, Peprotech, 200-07) and IL-15 (final conc. 2 ng/mL, Peprotech, 200-15) unless otherwise noted. Cells were activated continually for two days prior to infection.

Lentivirus for infection of primary CD4+ T cells was generated by transfecting HEK293T cells with *Δ6-dGFP-Thy1.2-Gagpol+* Plasmid (900 ng, gift from Ed Browne Lab), psPax2 plasmid (450 ng), and MD2.Cocal plasmid (150 ng, gift from Hans-Peter Kiem Lab [88]. After two days, virus was filtered using a Millipore filter (Millipore Sigma, SE1M179M6).

On day of infection, activation beads were first magnetically removed. Infection of CD4+ T cells was performed by aliquoting 5E6 CD4+ T cells iteratively into 50 mL falcon tubes, and

resuspending in virus + polybrene (final conc 8 ug/mL, Sigma-Aldrich, TR-1003) or RPMI media + polybrene for the uninfected control at a concentration of 1E6 cells/mL. Spinoculation was performed for *1100 x g* for 2 hours at 30ºC. Cells were maintained at a concentration of 1E6 cells/mL.

Three days post-infection, a small portion of cells were taken to assess infection by staining with CD90-AF700 antibody (Biolegend, 140323) for 20 minutes (1:1000 dilution in FACS Buffer), fixing with 4% paraformaldehyde and sorting by AF700 and GFP on SP Celesta 2 Cell Analysis Machine (Flow Cytometry Core, Fred Hutch). CD90+ cells were then isolated using the CD90.2 Cell Isolation Kit (Stemcell Technologies, 18951). Two days after CD90+ cells were purified, cells then were electroporated using electroporation code EH-100 and using the P3 Primary Cell 96-well Nucleofector Kit (Lonza, V4SP-3096). Knockout pools were maintained for an additional nine days prior to coculturing with H80 feeder cell line with IL-2 (Final conc 20 U/mL) in RPMI (no longer cultured with IL-7 and IL-15). Four days later, the cells were treated with PMAi or CD3/CD28 antibody co-stimulation (or unstimulated control) and analyzed on SP Celesta 2 (Core Facility) to evaluate reactivation potential by assessing Thy1.2, CD90+ and GFP+ cells. An early activation marker of T cells was also monitored using PE-Conjugated CD-69 antibody (Biolegend, 310906). Analysis was performed on FlowJo software. Genomic DNA was isolated at the end of experiment from uninfected and knockout cells to assess for genomic ICE analysis.

2.3.9 Primary CD4+ T cell activation Test

CD4+ cells were isolated from healthy donors and activated as described above. After two days of activation, beads were magnetically removed. Three days later, cells electroporated

following the protocol above, and treated with CD3/CD28 antibody after cells were allowed to recover for two additional days. Activation was monitored using PE-Conjugated CD69 antibody (Biolegend, 310906) on SP Celesta 2. Genomic DNA was isolated for analysis.

2.3.10 RNA-seq analysis of CCNT1 knockout cells.

For RNA isolated from J-Lat 10.6 cells, cells first were passaged and split equally three times prior to isolation. J-Lat 10.6 either wild-type for *CCNT1* or knocked out for *CCNT1* were each treated with TNFα (Peprotech, 300-01A) at 10 ng/mL or unstimulated in triplicate. For primary cell experiments, knockouts were performed similarly as described in "Primary CD4+ T cell activation Test," and RNA was isolated after LRA treatment. In both J-Lat and primary CD4+ T cell isolation experiments, 0.1-2E6 cells were isolated and resuspended in 350 uL of RLT Plus (Qiagen, 1053393) + 1% 2-mercaptoethanol (Millipore Sigma, M3148). Cells were frozen in buffer RLT plus until ready to continue with isolation. Thawed RLT lysates were then run over a QIAshredder column (Qiagen, 79654) and subsequently over a gDNA eliminator column. Qiagen RNeasy Plus Mini Kit was then used in order to obtain purified total RNA. RNA was submitted for TapeStation RNA assay or HighSense RNA assay (Fred Hutch Core Facilities) and RINe scores were all found to be ≥ 9.6.

2.3.11 RNAseq Analysis Methods

Quality assessment of the raw sequencing data, in Fastq format, was performed with fastp v0.20.0 [89] to ensure that data had high base call quality, expected GC content for RNA-seq, and no overrepresented contaminating sequences. No reads or individual bases were removed during this assessment step. The fastq files were aligned to the UCSC human hg38 reference

assembly using STAR v2.7.7 [90]. STAR was run with the parameter "--quantMode GeneCounts" to produce a table of raw gene-level counts with respect to annotations from human GENCODE build v38. To account for unstranded library preparation, only unstranded counts from the table were retained for further analysis. The quality of the alignments was evaluated using RSeQC v3.0.0 [91] including assessment of bam statistics, read-pair inner distance, and read distribution. Differential expression analysis was performed with edgeR v3.36.0 [92] to identify the differences between knockout stimulated and stimulated for with *CCNT1* and *AAVS1* genes, as well as differences between the two genes in knockout and knockout stimulated conditions. Genes with very low expression across all samples were flagged for removal by filterbyExpr, and TMM normalization was applied with calcNormFactors to account for differences in library composition and sequencing depth. We constructed a design matrix to incorporate potential batch effects related to donor information, after which the dispersion of expression values was estimated using estimateDisp. Testing for each gene was then performed with the QL F-test framework using glmQLFTest which outputs for each gene a p-value, a log2(fold change) value, and a Benjamini-Hochberg corrected false discovery rate (FDR) to control for multiple-testing. The results were plotted using ggplot2 v3.3.5 [93]. For analysis of J-Lat 10.6 RNA sequencing data, we used the reference genome previously assembled and described for J-Lat 10.6 [49]. Using this reference, we masked the 5' LTR of the integrated provirus. All splice variants as well as genomic RNA that terminate at a polyA site in the 3' LTR are similarly named "HIV-1."

Chapter 2.3 Results

2.4.1 A Latency HIV-CRISPR Screen of HIV Dependency Factors to Identify Latency Reversal Factors

We recently developed and validated a CRISPR sublibrary of guide RNAs targeting host genes important for HIV replication across multiple strains (the HIV dependency factor or HIV-Dep library). The HIV-Dep library has guides targeting 525 genes represented by 8 guides targeting each gene and 210 non-targeting controls (NTCs) [48]. A MetaScape analysis [94] of the HIV-Dep library shows the most enriched gene ontology is chromatin organization, followed by several processes involving gene expression, DNA metabolism, and viral infection pathways **(Figure 2.1A).** Genes in many of these categories were previously validated to be important in acute HIV-1 infections [48]. We hypothesized that a subset of these HIV dependency factors are also necessary for activation of HIV from latency. Thus, to investigate host genes that are required for reversal of HIV-1 latency, we performed a CRIPSR screen using a modification of the HIV-CRISPR system [2, 49, 83] **(Figure 2.1B).** Briefly, this screen in the context of latency reversal relies on transducing latently infected Jurkat T cells (J-Lats) with an HIV-CRISPR lentiviral vector containing a library of sgRNAs. The sgRNAs are flanked by a Ψ-packaging signal, allowing the guides to be packaged into budding virions. We employed this modified latency HIV-CRISPR assay to identify factors important for latency reactivation using two different J-Lat models that contain independently-derived integration sites; J-Lat 10.6 and J-Lat 5A8. The goal for this screen was to treat the cells with activating doses of LRAs, deep sequence the supernatant containing the guides compared with the gDNA knockout pool. In contrast to a previous HIV-CRISPR screen where we examined epigenetic factors whose knockout would activate HIV from latency by analyzing guides enriched in the viral supernatant **(Figure 2.1B,** scenario 1) [49], in the present screen the expectation is that genes required for reactivation from

latency would be depleted in the viral supernatant relative to the genomic knockout pool **(Figure 2.1B, scenario 2).**

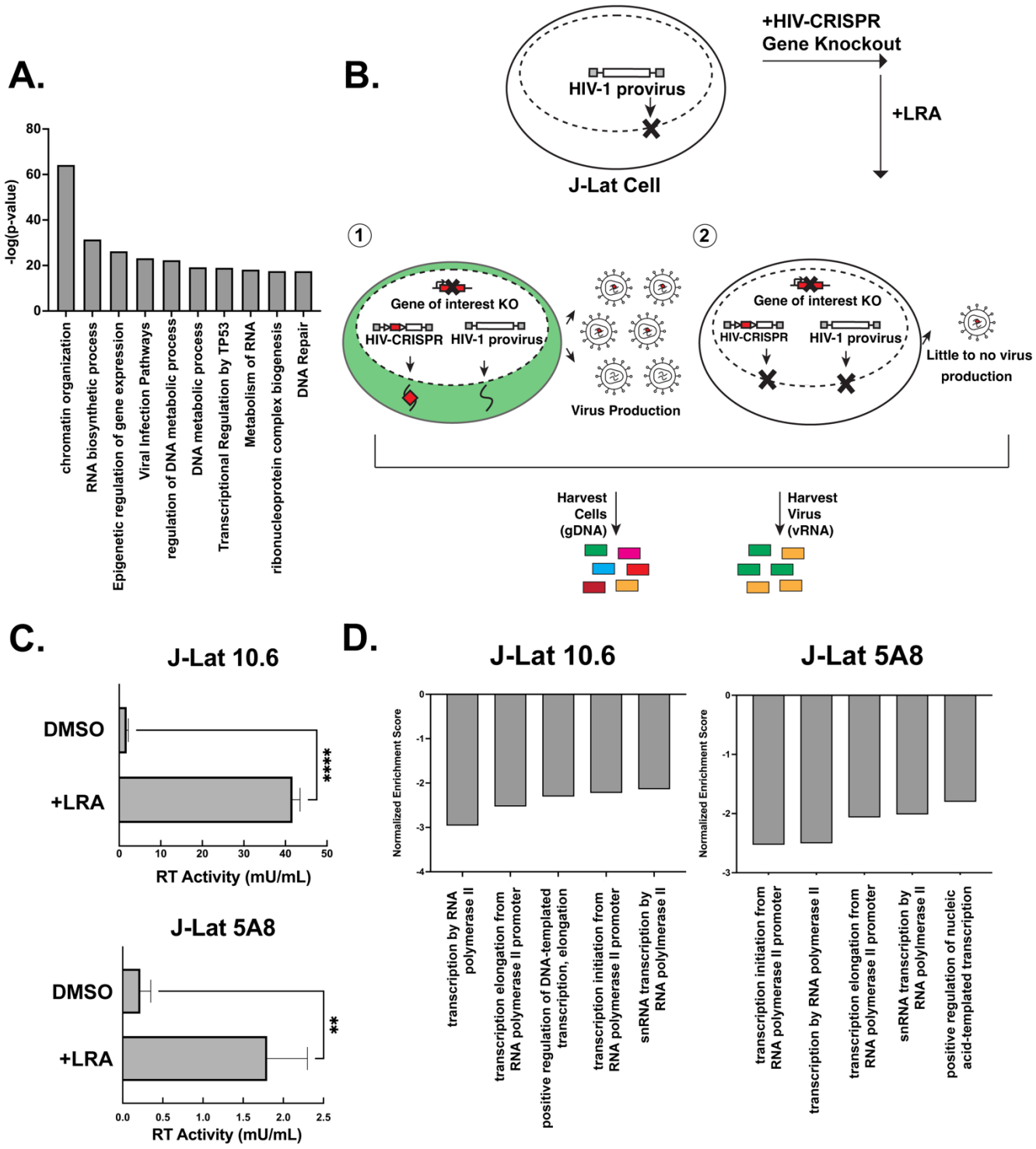

Figure 2.1 A Latency HIV-CRISPR Screen to identify factors required for latency reversal. **(A)** A Metascape analysis of the genes in the HIV-Dep gene library is shown, with enriched pathways on the x-axis and statistical significance on the y-axis. **(B)** Overview of latency HIV-CRISPR screen of HIV Dependency Factors. The HIV-CRISPR vector has intact 5' and 3' LTRs and can be packaged by HIV-1 after integration [2] J-Lat cells were transduced with an HIV-CRISPR library of genes of HIV-1 dependency factors, selected for integration by puromycin selection, and treated with a latency reversal agent (LRA). Viral RNA (vRNA) and genomic DNA (gDNA) are harvested at the end of the experiment. Guides corresponding with genes that do not affect reactivation from latency are packaged in virions and enriched in the supernatant relative to the genomic DNA pool (scenario 1, left). For genes that are important for latency reactivation after treatment of cells with an LRA, these guides will be depleted in the viral supernatant relative to the genomic DNA knockout library (scenario 2, right). **(C)** Supernatant from J-Lat cells transduced with the HIV-DEP gene library were measured for Reverse Transcriptase (RT) activity after treatment with the LRA combination AZD5582 (1 nM) and I-BET151 (2.5 uM). Error bars represent technical triplicates, unpaired t-test was used for statistical analysis. p-value < 0.01 = **, < 0.0001 = **** **(D)** MAGEcKFlute [4] was used to analyze screen results of the depleted genes. The normalized enrichment score is on the y-axis (negative because guides to these genes are depleted from the viral supernatant) and the x-axis is the biological processes.

supernatant and genomic DNA pool, we used MAGEcK analysis in order to compare the guides enriched or depleted in the supernatant with the genomic knockout pool to identify those genes depleted in the supernatant. We generated a gene set enrichment analysis [4] of our most depleted hits and found the top five enriched pathways in both J-Lat 10.6 and J-Lat 5A8 were related to transcription **(Figure 2.1D).** Furthermore, we also saw pathways for RNA splicing and polyadenylation. This is consistent with transcriptional regulation being one of the major axes of host control that underly release of HIV-1 from latency. We conclude that our screen can identify and enrich for gene pathways that are relevant for release of the HIV-1 provirus from latency in the presence of AZD5582 and I-BET151 combination treatment.

To understand the role that HIV dependency factors play in terms of latency reactivation, we compared our screens with previous HIV-CRISPR screens that were aimed at identifying factors required for HIV replication in Jurkat cells [48]. A z-score analysis was used as a measure of how depleted genes were in each of the screens and to allow for a cross-comparison regardless of the magnitude of depletion of each guide. Sorting the mean z-score for HIV-1 replication (marked as LAI in Figure 2.2A) shows that the most depleted genes are *CXCR4* and *CD4* which are essential for HIV replication but not for latency reactivation **(Figure 2.2A, left).** This is

expected since J-Lat cells are already infected with HIV-1. Other factors that scored highly in the HIV-1 replication screen, but not in the present HIV latency screen include genes of unknown function in the HIV lifecycle such as *ATP2A2* and *SS18L2* **(Figure 2.2A, left).** In contrast, nearly all of the most depleted factors in the HIV latency screens were also highly depleted in the HIV replication screen **(Figure 2.2A**, right, sorted by most depleted in the HIV latency screens). We conclude that a subset of HIV dependency factors are required for reactivation from latency.

We chose to validate a subset of the hits in the HIV latency screen that were among the top twenty ranking hits and were shared hits in both J-Lat 10.6 and J-Lat 5A8 cells **(Figure 2.2B)** by electroporating Cas9 ribonucleoprotein complex (RNP) complex containing 3 unique guides against each gene or by lentiviral transduction of single guide RNAs. We tested *CCNT1*, *ELL*, *UBE2M, TBL1XR1, HDAC3*, *AMBRA1, ALYREF, and SBDS* **(Figure 2.2C).** As a negative control we included guides targeting the adeno-associated virus integration site 1 (*AAVS1*) "safe harbor" locus, a gene whose disruption does not adversely affect the cell [95], or a non-targeting control (NTC). Pooled knockouts were validated by genomic sequencing and Inference of CRISPR Edits (ICE) analysis. In addition, the CCNT1 pooled knockout was also validated by Western blotting.

In the J-Lat 10.6 line we found that there is reduced reactivation in *CCNT1*, *ELL*, *UBE2M, TBL1XR1, HDAC3*, *AMBRA1,* and *ALYREF* knockouts relative to non-targeting controls and guides targeting a safe harbor locus, *AAVS1* **(Figure 2.2C).** We did not see a significant effect in the *SBDS* knockout cells, but interestingly *AMBRA1* and *ALYREF* which were less depleted than *SBDS* in the J-Lat screens did show a phenotype. However, the strongest effect on preventing HIV latency reversal was the knockout of *CCNT1* which was also the top hit

in our screen. We conclude that the screen is able to identify genes that are key for latency reactivation in the J-Lat models.

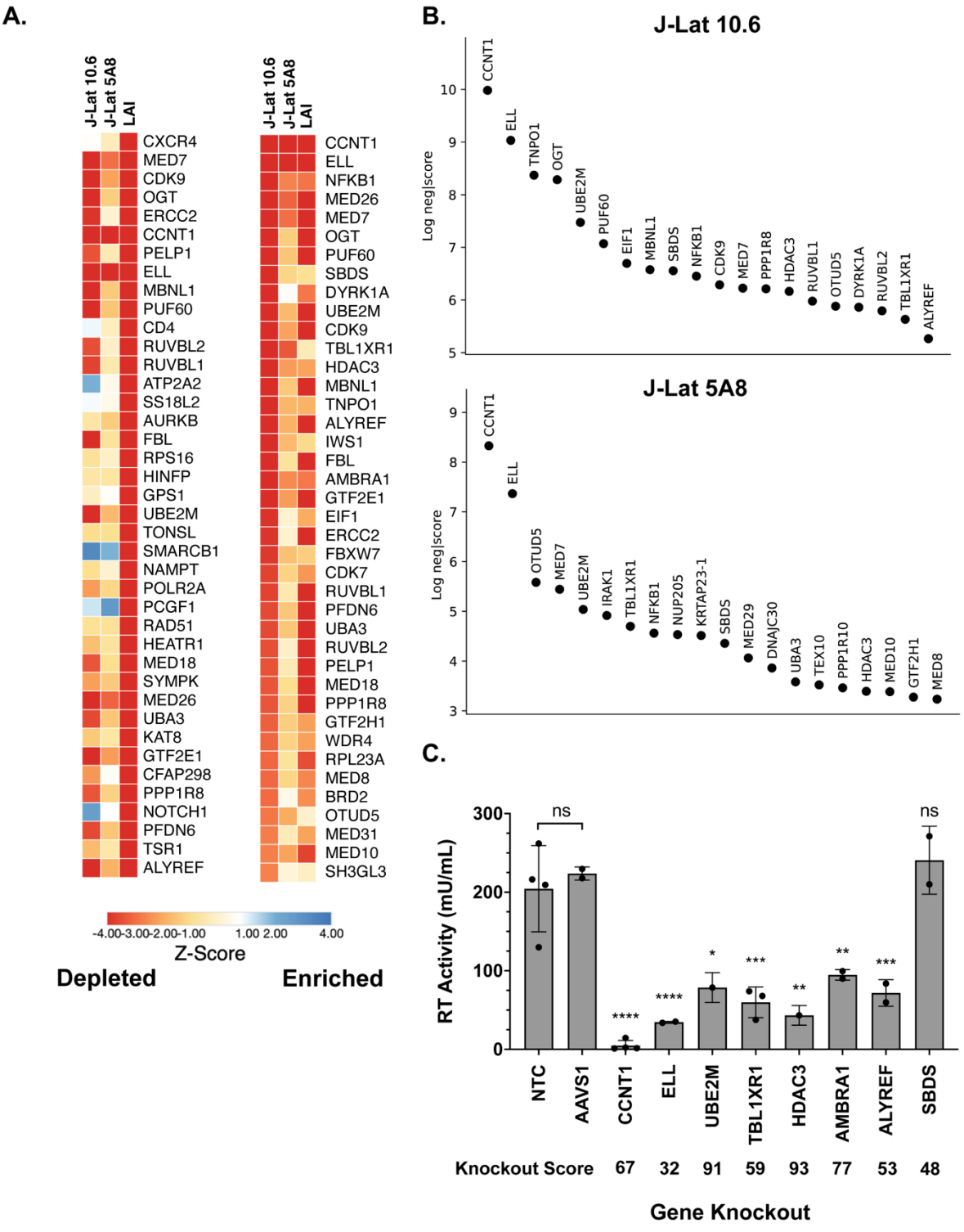

Figure 2.2. Analysis and Validation of Top Hits from HIV-CRISPR screen. **(A).** Z-score analysis of the depleted versus enriched guides across multiple screens. J-Lat 10.6 and J-Lat 5A8 are screens from this study, whereas LAI represents Jurkat cells infected with an LAI strain of HIV-1 from previous screen performed using the same gene library in Jurkat cells to identify HIV Dependency Factors [48]. Z-scores are sorted by the most depleted genes in the LAI screen (left panel) and by the most depleted genes in the J-Lat 10.6 line from this study (right panel). The mean z-score of two replicates each of J-Lat 10.6 and J-Lat 5A8, and of four replicates of the LAI screen is shown. Most depleted genes are red and most enriched genes are blue. Z-scores were that were less than -4 were capped at -4 in the heat map. **(B)**. The top 20 most depleted hits from each J-Lat line in ranked order are shown. **(C)** Selected hits from the screen were tested by performing gene knockouts (x-axis), treating with the LRA combination AZD5582/I-BET151, and assayed for reverse transcriptase activity. Gene knockouts were performed using a lentiviral knockout approach and/or an electroporation with Cas9 and RNPs. Each point represents a single lentiviral or electroporation knockout experiment done in triplicate. An average of RT activity from two guides targeting each gene was taken for lentiviral knockouts, and the electroporation knockouts included three individual guides

2.4.2 Cyclin T1 is essential for reactivation from latency in both J-Lat and primary T cells

Cyclin T1 (CCNT1) is a well characterized regulator of HIV transcription that binds to the viral protein Tat and TAR [96-98] and was the top hit for both J-Lat models. Additionally, CDK9 which binds to Cyclin T1 in order to form the positive transcription elongation factor complex (P-TEFb) is substantially depleted in the CRISPR screen of both cell lines. In order to explore this hit further across a broader range of LRAs, we generated clonal knockout lines of CCNT1 in the J-Lat 10.6 cell line. The clonal knockouts are completely abrogated of CCNT1 expression as shown by Western blotting and by sequencing of genomic DNA **(Figure 2.3A, left**). Moreover, we did not see an upregulation of CCNT2, a paralog of CCNT1 that also binds CDK9 as part of the host P-TEFb complex [81, 82] **(Figure 2.3A, right).**

HIV latency is a result of a combination of blocks that prevent transcription initiation and elongation, and LRAs target a broad range of these different facets of proviral gene expression. We explored a range of LRAs in the CCNT1 clonal knockout lines. We found that CCNT1 is necessary for latency reversal with both CD3/CD28 activation and with Tumor Necrosis Factor Alpha (TNFα) cytokine. Reactivating with CD3/CD28 and TNFα are mechanisms that result in the upregulation of NF -κB signaling, a facet that emphasized the transcription initiation component of latency. We therefore explored additional means of reactivation including

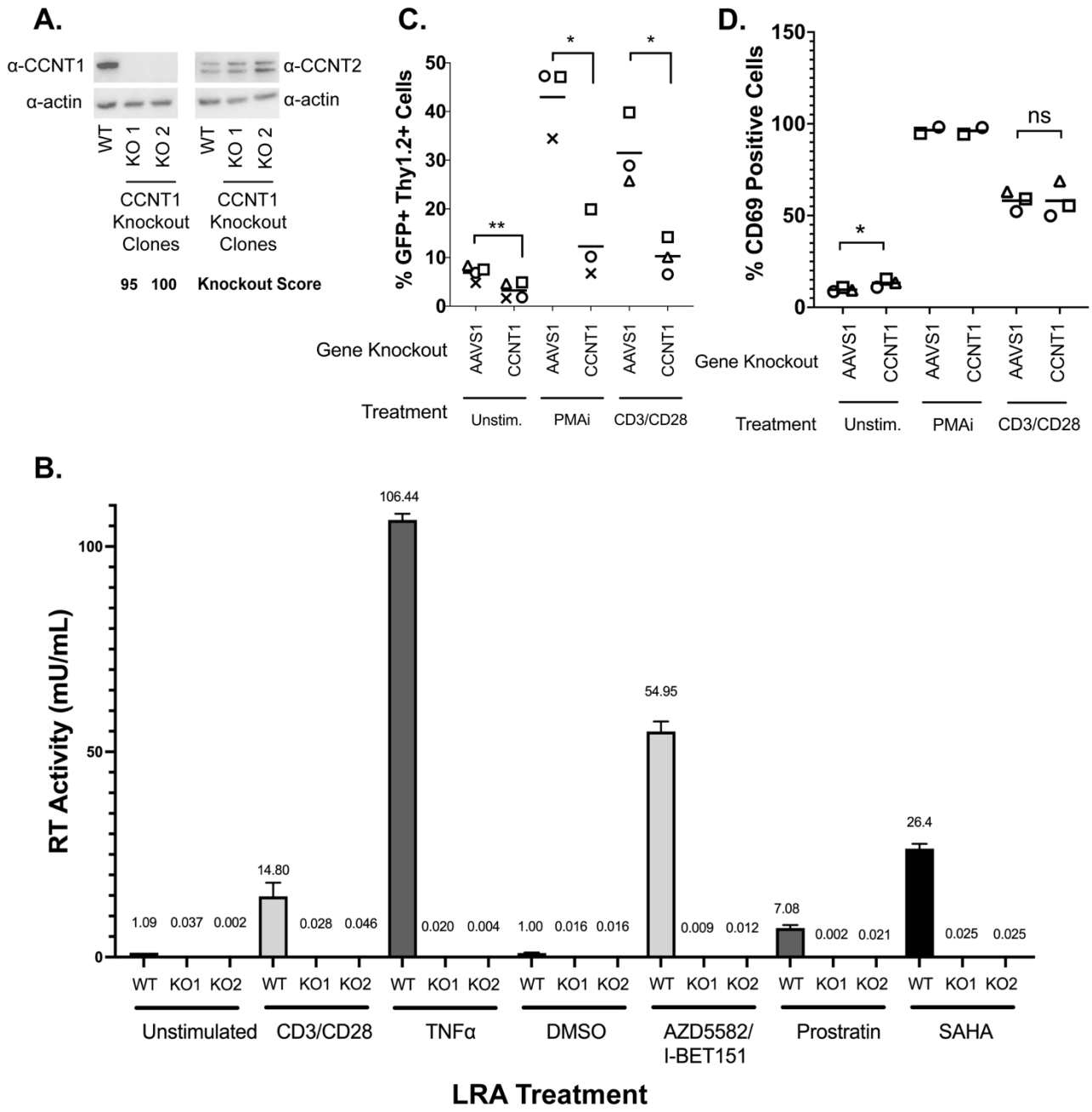

Figure 2.3 *CCNT1* is required for reactivation of HIV-1 from latency in Jurkat T cells and primary CD4+ T cells from healthy donors. (A). Western blot of cell lysates of J-Lat 10.6 either wild-type or clonally knocked out for CCNT1 is shown, with two separate knockout clones. Actin was used as loading control. Left: CCNT1 antibody is shown, Right: CCNT2 antibody is shown. ICE Knockout scores are shown for each knockout clone of *CCNT1* (B). J-Lat 10.6 cells wild-type for CCNT1 and the two clones knocked out for *CCNT1* were treated with the LRAs shown on the bottom. The mean of RT activity in the supernatant 24 hrs after LRA treatment is shown on the Y axis and above each bar. Averages and standard deviation of the experiment done in triplicate is represented (C). Primary CD4+ T cells from three different healthy donors were infected with a dual-reporter virus that monitors cells active and latent infection (Thy 1.2, CD90 marker) and actively transcribing provirus (GFP marker). Cells were either knocked out for *AAVS1* control or *CCNT1* and either untreated, stimulated with PMAi, or stimulated with anti-CD3/anti-CD28 antibodies at the end of latency establishment. Each shape represents an individual donor. (D) CD69 expression was monitored with the different LRA treatments. *CCNT1* ICE knockout scores were: 80, 76, 53, and 37 for each of four donors for CD3/CD28 and two donors for PMAi. A paired t-test was used for comparison of *AAVS1* knockout vs *CCNT1* knockout between donors. p-value ≥ 0.05 = ns, < 0.05 = *, < 0.01 = **.

AZD5582 and I-BET151 together, Prostratin – an activator of PKC and known inducer of P-TEFb activity [45, 99] – and SAHA/Vorinostat [100], the histone deacetylase inhibitor (HDACi) **(Figure 2.3B**). In all treatments, cells wild-type for CCNT1 were able to reactivate, but CCNT1 knockout prevented latency reactivation with each LRA. We conclude that CCNT1 is essential for reactivation from latency for multiple diverse mechanisms of latency reversal in J-Lat cells.

We also investigated the role of CCNT1 in latency reactivation in primary CD4+ T cell lymphocytes isolated from healthy donors. We first activated and infected peripheral blood CD4+ T cell lymphocytes with an HIV-1 dual-reporter virus previously described [49]; the first marker is a destabilized GFP reporter is a marker of active provirus expression. The destabilized GFP has a short half-life and thus is indicative of active expression of the provirus. The second marker, Thy1.2 (mouse CD90) viral reporter is a cell surface marker that allows for us determine cells that have, at one point, been infected. This cell surface marker has a slow turnover and persists over the latency establishment period, and thus marks cells that have been infected with the dual-reporter virus, but may not be actively producing virus. After infection with dual reporter virus, infected cells were knocked out by electroporation with Cas9 and gRNA for CCNT1 or control AAVS1. Cells were cultured for an additional two weeks to enter latency, and then measured for the capability for latency reactivation after LRA treatment as determined by flow cytometry for dual positive GFP and CD90 expression **(Figure 2.3C).**

We tested knockouts from three independent donors with the potent LRA combination phorbol 12-myristate 13-acetate (PMA) and ionomycin as well as with CD3/CD28 antibody co-stimulation **(Figure 2.3C).** In control AAVS1 knockout we found that there is an increase in the percentage of total cells that are both Thy1.2+ and GFP+ on treatment with PMAi or CD3/CD28 co-stimulation indicating an increase in cells that have active transcription of viral genes (5.46%

without LRA, 39.7% with LRA) **(Figure 2.3C).** In contrast, the CCNT1 knockouts had a stark reduction in Thy1.2+ and GFP+ cells on treatment with PMAi and CD3/CD28 co-stimulation relative to AAVS1 knockout **(Figure 2.3C).** We also noted that there is a modest reduction of Thy 1.2+ GFP+ cells in the CCNT1 knockout that have not been treated with PMAi or CD3/CD28 co-stimulation. This is consistent with our previous result in clonal knockouts in J-Lat cells suggesting that minimal levels of HIV-1 transcription that occur in latent cell populations are lower in CCNT1 knockouts. We conclude that Cyclin T1 is an essential gene for latency reactivation.

To exclude the possibility that Cyclin T1 blocks the ability for CD4+ T cells to activate, as well as ensure T cell activation is occurring properly in our experiments, we simultaneously stained cells for the early activation marker CD69. PMAi and CD3/CD28 co-stimulation both show a significant degree of activation over unstimulated cells. We saw no significant change between AAVS1 and CCNT1 knockout in any of the conditions **(Figure 2.3D**). We conclude that CCNT1 is key for latency reactivation in primary CD+4 T cells but does not affect the ability of these cells to be activated upon stimulation.

2.4.3. Cyclin T1 is non-essential in T cells and regulates host genes to a much lesser extent than it regulates HIV-1

Given that P-TEFb has been reported to be required for transcription elongation of many host genes [43], we were initially surprised that knockout of *CCNT1* is viable. However, we did

not see a drastic change in cell growth measured over a span of nine days **(Figure 2.4A).** This led us to broadly investigate the role of Cyclin T1 in transcription in T cells by performing bulk

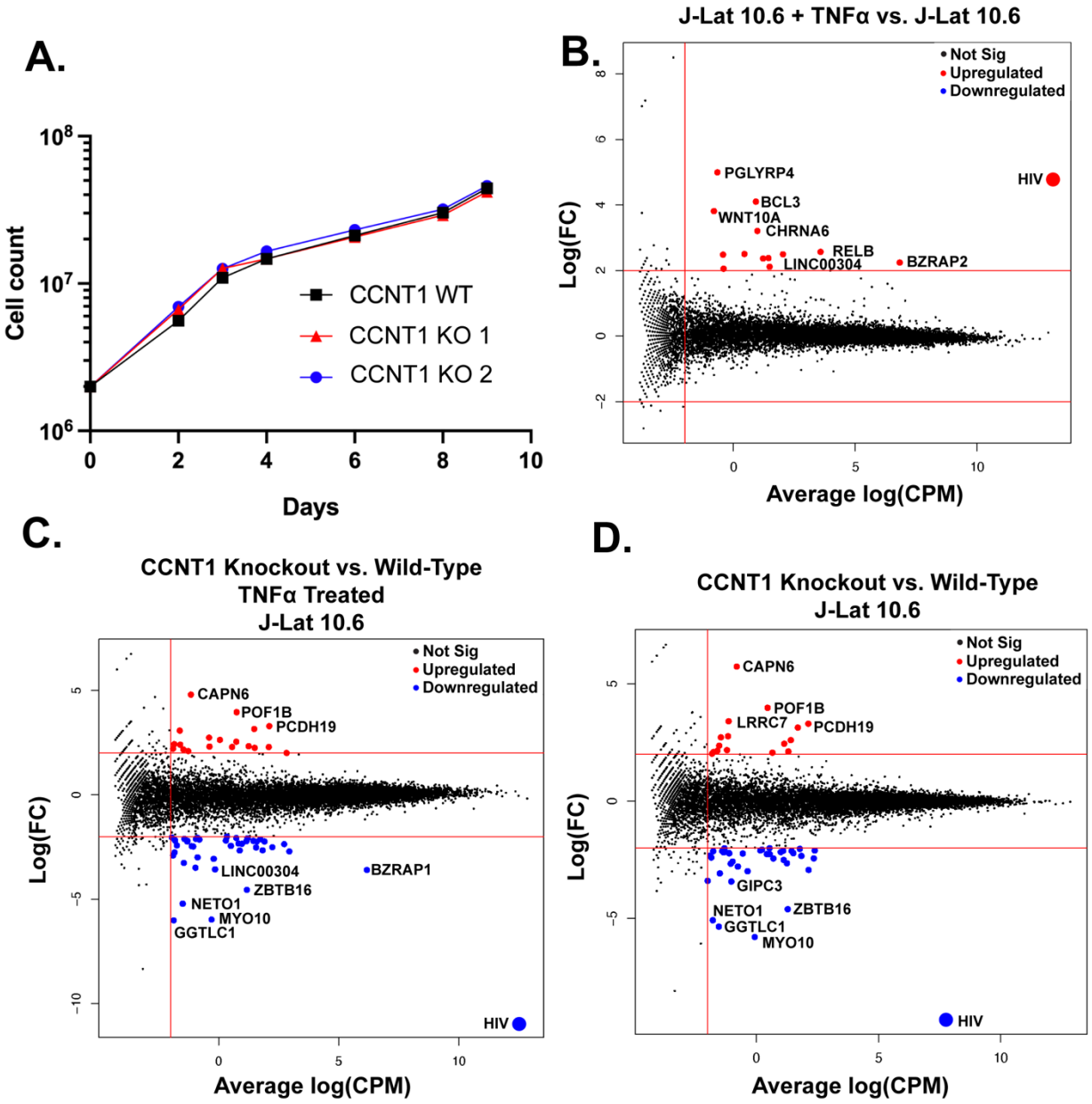

Figure 2.4 Cell proliferation and RNA sequencing analysis of CCNT1 knockouts in J-Lat 10.6 cells. **(A).** Cell counts were monitored over a span of nine days in J-Lat 10.6 cells in WT or clonally knocked out CCNT1 cells. The average of three experimental replicates are shown with standard deviation. **(B-D).** Log_2 FC (fold-change) is plotted on y-axis with the average Log_2 CPM (counts per million) across technical replicates on the x-axis. Red lines on the signify genes that have an average Log_2 CPM > -1, and a $|Log_2 FC| > 2$. Red dots signify upregulated genes whereas blue genes signify downregulated genes for each comparison. **B)** Differential gene expression of J-Lat 10.6 with TNFα treatment versus J-Lat 10.6 (untreated) is shown. **C).** J-Lat 10.6 CCNT1 KO cells (two independent clones each tested in technical triplicate and averaged) versus the J-Lat 10.6 wild-type cells – both were treated with the LRA TNFα and gene expression comparison is shown. **D).** J-Lat 10.6 CCNT1 KO cells versus wild-type CCNT1 differential gene expression is shown – neither cell line was treated with an LRA.

RNA sequencing of J-Lat 10.6 cells and two independent clonal knockouts of *CCNT1* in the J-Lat 10.6 cells either without an LRA, or treated with TNFα. As a control, we first compared the RNA sequencing data from wild-type J-Lat 10.6 line that has been treated with TNFα, versus the J-Lat 10.6 line (*CCNT1* is wild-type in both cases). HIV-1 transcripts are among the most significantly upregulated genes in the TNFα treatment for wild-type **(Figure 2.4B).** We also see upregulation of *PGLYRP4, RELB, and BCL3*, which are genes related to NF-κB signaling or otherwise known to be upregulated by TNFα **(Figure 2.4B)** [101-103]. We next examined how HIV-1 and host gene transcripts are affected in TNFα treated cells that have *CCNT1* knocked out relative to TNFα treated J-Lat 10.6 cells that are wild-type for *CCNT1* **(Figure 2.4C).** Strikingly, RNA transcripts related to HIV-1 genes in *CCNT1* knockout are the most depleted transcripts over any host gene, relative to wild-type *CCNT1* (Log_2(FC) = -10.92) **(Figure 2.4C)**. Even in the absence of LRA, we find that HIV-1 transcripts are the most depleted relative to other host genes (Log_2(FC) = -9.29) when comparing *CCNT1* knockout versus wild-type **(Figure 2.4D).** Thus, basal transcription of HIV-1 transcripts that occur in J-Lat lines are highly dependent on Cyclin T1. Regardless of TNFα treatment, the host genes that were highly depleted in *CCNT1* knockout included *FAM222A-AS*, *GGTLC1*, *MYO10, NETO1*, and *ZBTB16*. Notably, we did not find significant upregulation of *CCNT2* transcripts in the CCNT1 knockout versus wild-type (Log_2(FC) = 0.078) or in the LRA treated cells (Log_2(FC) = 0.139). Nonetheless, *CCNT1* knockout affects the HIV-1 provirus far more than any other transcriptional unit in the J-Lat cells.

We further investigated the effect of *CCNT1* knockout on uninfected primary CD4+ T cells. *CCNT1* was knocked out by electroporation of *CCNT1* guides complexed with Cas9 in three independent donors and the knockout was validated to be over 90% by sequence analysis.

The *AAVS1* locus was knocked out in parallel as a control. Similar to the primary cell latency model **(Figure 2.3C),** we found that the *CCNT1* knockout did not affect expression of the CD69 activation marker after treatment with anti-CD3/anti-CD28 beads **(Figure 2.5A).** As expected, comparison of RNA sequencing on primary cells stimulated with anti-CD3 and anti-CD28 antibodies versus unstimulated cells shows dramatic upregulation and downregulation of genes **(Figure 2.5B);** for example, there is upregulation of IL31 which is a cytokine known to be upregulated by activated T cells [104]. However, the same RNA-seq analysis of *AAVS1* knockout cells compared to *CCNT1* knockout cells upon stimulation with anti-CD3/anti-CD28 beads shows that *CCNT1* knockout cells have the same expression profile as the control knockout cells, i.e. there are no significant differences in upregulated or downregulated genes in the comparison **(Figure 2.5C)** when *CCNT1* is knocked out. We also compared RNA expression profiles of the *CCNT1* knockout cells with the controls *AAVS1* knockout cells in the absence of anti-CD3 and anti-CD28 stimulation, and again find very few genes which are upregulated or downregulated **(Figure 2.5D).** In addition, the magnitude of these gene expression changes was minimal. As an example, the most enriched gene for *CCNT1* knockout compared to *AAVS1* knockout has a $-\log_2$FC less than 2, and the most depleted gene has a $-\log_2$FC greater than -2 **(Figure 2.5D).** Thus, we conclude that there are minimal changes in gene expression when *CCNT1* is knocked out in primary CD4+ T cells with and without T cell receptor stimulation. Together, we conclude that *CCNT1* does not play an essential role in peripheral primary CD4+ T cells.

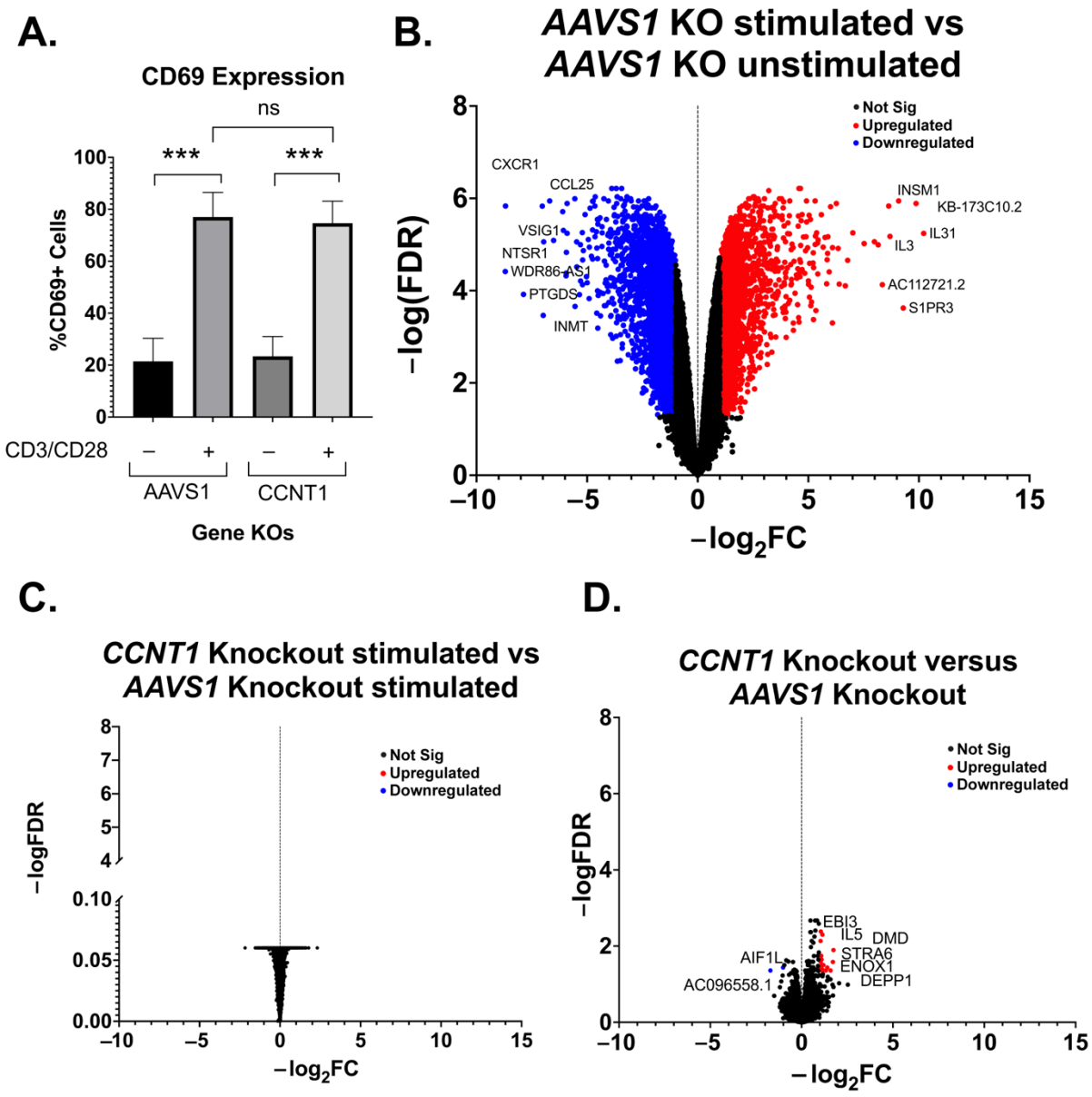

Figure 2.5 Primary T cells transcripts are largely unaffected by *CCNT1* knockout. **(A)** Uninfected CD4+ T cells from three donors were knocked out for *AAVS1* or *CCNT1* and then treated with CD3/CD28 antibody co-stimulation. Cells were analyzed by flow cytometry to measure CD69 expression. One-way ANOVA was used for analysis with Dunnett's multiple comparison tests. **(B–D)** Volcano plots of primary CD4+ T cell RNA sequencing data are shown, with $-\log_2$FC shown on the x-axis and −log(FDR) on the y-axis. RNA was isolated from three biological replicates. An FDR = 0.05 was used as a cutoff for significance, and the cutoff for significant gene expression was |Fold-Change| > 1. A subset of genes for each condition are marked that have significance. **(B)** Differential gene expression between *AAVS1* knockout stimulated with CD3/CD28 versus unstimulated is shown. **(C)** A comparison of *CCNT1* versus *AAVS1* knockout is shown, and both were stimulated with anti-CD3/anti-CD28 antibodies. **(D)** *CCNT1* versus *AAVS1* knockout is shown, and neither of these are stimulated with anti-CD3/anti-CD28 antibodies. *p*-value ≥ 0.05 = ns (not significant), <0.001 = ***.

2.5 Discussion

We used an HIV-CRISPR screening approach to identify host genes required for activation of HIV from latency starting from the hypothesis that a subset of host genes previously identified as being necessary for HIV replication are also necessary for HIV reactivation from latency. Among the genes identified include many genes involved in transcription elongation, transcription initiation and protein degradation. The top hit in our screens was Cyclin T1 (*CCNT1*) which we show is essential for reactivation from latency across a wide range of latency reversal agents of different mechanisms of action, as well as in primary T cells. In contrast, *CCNT1* appears to be redundant with other host genes for normal transcriptional regulation in T cells and is therefore an attractive target for specifically silencing integrated HIV-1 proviruses.

2.5.1 Cyclin T1 is much more important for HIV latency reversal than for T cell biology in vitro

Despite the described role of Cyclin T1 and the P-TEFb complex in host gene transcription, we were able to generate knockout clones of *CCNT1* without affecting cell growth and viability. We also did not see a significant upregulation of CCNT2 protein expression. Collectively, we interpret our results to mean that *CCNT1* is dispensable in T cells and that *CCNT2* or *CCNK* may compensate for the loss of *CCNT1*. One model is that there are redundant mechanisms that govern transcription elongation of host genes. Previous work on *CCNT1* and *CCNT2* knockouts in mice illustrated unique phenotypes, initially suggesting the possibility that these two genes have separate functions despite both being able to form the P-TEFb complex [105, 106]. RNA sequencing of CCNT1 and CCNT2 knockdowns by another group using shRNA in HeLa cells also suggested these two proteins are regulating different sets of genes

[107]. However, while CCNT1 had very large effects on HIV-1 transcripts, we found that CCNT1 has minimal effects on host gene transcription in Jurkat cells. We did observe a modest downregulation of several host genes including *GGTLC1*, *MYO10*, *NETO1*, *ZBTB16*, and *BZRAP1*. GGTLC1 is a metabolic enzyme and member of the gamma-glutamyl transpeptidase family, of which there are several paralogs [108]. Myo10 is an unconventional myosin that associates with actin and filopodia. This gene has ubiquitous but low expression across tissues [109], but has been reported to promote HIV-1 infection in human monocyte derived macrophages [110]. *ZBTB16* (also known as *PLZF*) is a transcription factor and is known to be important for natural killer T cells, but repressed in non-innate T cells and not upregulated in T cell activation [111]. Collectively, we see slight changes in gene expression in J-Lat cells on *CCNT1* knockout that lead to drastic changes in HIV-1 gene expression, but few host genes seem to be affected on knockout.

On the other hand, there were no significant changes in gene expression of *CCNT1* knockout versus *AAVS1* knockout in primary CD4+ T cells activated with CD3/CD28 co-stimulation. Knockouts of *CCNT1* in primary CD4+ T cells also had little effect on cell viability and cell surface expression of an activation marker, CD69. In the unstimulated condition we see some low magnitude gene expression changes; *AIF1L* is a mildly downregulated gene, and to date there is no clear known function of this gene in T cell biology. In human podocytes, this gene is known to function in actomyosin contractility and thus cells which lack this gene have increased filopodia [112]. Upregulated genes include *IL5*, *DMD*, *STRA6*, *ENOX1*, and *DEPP1*. None of these genes are particularly implicated in T cell biology. Mutations in the *DMD* (Dystrophin) gene are implicated in Duchenne's Muscular Dystrophy, an X-linked recessive disorder. We also saw upregulation of *MYOF* (Myoferlin), a gene whose mutations are associated

with muscle weakness [113, 114]. An interesting possibility is that *CCNT1* positively and negatively regulates genes associated with muscle function, given we saw an upregulation of these genes implicated in muscle disease, and a downregulation of *MYO10* in the J-Lat 10.6 RNA sequencing data on *CCNT1* knockout.

We reason that while CCNT1 and CCNT2 gene regulation may have tissue-specific contexts, CCNT1 is likely non-essential in CD4+ T cells. Data from DepMap indicate that *CCNT1* is classified "strongly selective" indicating there are cell lines in which this gene is more essential, but that *CCNK* is it is considered widely essential in most CRISPR screens [115]. Previous work suggests CCNT1 is targeted by proteasomal degradation in resting CD4+ T cells, and thus CCNT1 protein expression in resting CD4+ T cells is low [46, 116, 117], but our data suggests that it is not necessary for T cell activation. While we saw little effect of *CCNT1* knockout on host RNA transcripts in a relevant target cell type for HIV-1 infection, we cannot rule out the possibility that *CCNT1* does play a key role in host biology in more differentiated T cell functions or in other HIV-1 prone cell types including macrophages and glial cells. We interpret this to mean that the role of *CCNT1* may be redundant in T cells for host gene expression but not for HIV-1 activation.

CCNT1 binds to the viral Tat protein which subsequently binds to the TAR region and enables transcription initiation of proviral genes. Previous work suggests that CCNT2 can bind to the viral Tat protein, but this complex does not bind to TAR and thus cannot initiate viral transcription. Mutation of a single amino acid residue asparagine 260 to cysteine in CCNT2 was sufficient to rescue function of Viral Tat and TAR interaction [82]. Similarly, mutation of a tyrosine residue at 261 to cysteine in mouse Cyclin T1 protein rescues Tat /TAR function [54,

118]. Thus, the level of specificity is not at the Tat interface, but rather the TAR interface which might be an evolutionary advantage for the virus to resist host escape.

2.5.2. Other hits in the HIV-CRISPR screen

Several genes involved in transcription are among our most depleted genes. Notably, NFKB1 – the transcription factor that binds to 5' LTR to allow for transcription initiation of proviral genes, is among our top hits. We also see other transcription-related genes depleted in both cell lines. ELL – an elongation factor for RNA polymerase II and component of the super elongation complex – is the second most-depleted hit. We also note that there are several post-translational modifying enzymes that are novel in terms of latency reactivation. The Ubiquitin Conjugating Enzyme E2 M (UBE2M) is highly depleted and is known to be involved in the neddylation pathway, which uses a ubiquitin-like conjugation process. UBA3, which makes up the E1 enzyme of the neddylation conjugation pathway, also is depleted but to a lesser degree. Both of these neddylation genes were also depleted in our previous CRISPR screen on Jurkat T cells to identify dependency factors, and *UBE2M* validated for several strains of HIV [48]. Histone Deacetylase 3 (HDAC3) forms a complex with TBL1XR1 as part of the SMRT N-CoR (nuclear coreceptor complex), which regulates modification of histones and gene regulation [119-121]. siRNA studies of TBL1XR1 have found redundancy with its paralog TBL1X, whereas HDAC3 was found to be essential. Vorinostat, a commonly used LRA targets HDAC3 along with Class I and Class II HDACs [122]. It is unclear why *HDAC3* knockout may prevent latency reactivation, but we reason latency reactivation depends in part on a noncatalytic activity of *HDAC3*.

A genome wide CRISPR screen was previously performed that identified factors important for latency reversal [123]. In that study, the authors generated a pool of latently infected cells and performed a whole genome CRISPR knockout screen, treated with a panel of different LRAs, sorted for GFP- cells and identified genes specific for latency reversal as well as common genes required regardless of reactivation approach. In comparing our screens, we find many of our hits are shared with the "common" cluster of genes where they tested TCR cross-linking, TNF-a, PMAi, and AZD5582 as LRAs and identified the common genes required for reactivation: *CCNT1, HDAC3, NFKB1, MBNL1, UBE2M, TBL1XR1, UBA3, AMBRA1, SBDS,* and *MED7*. Thus, despite only screening with AZD5582 and I-BET151, we are able to identify several hits that promote latency reactivation regardless of LRA used. *UBA3* and *UBE2M* are of interest as they are both components of the neddylation pathway [124], and while *NEDD8* is not in our HIV-DEP gene library – the whole genome screen identified *NEDD8* as a hit in their AZD5582 screen [123]. In contrast, there are several hits that are depleted and validated in our more targeted screens but not the whole genome screen such as *ELL* and *ALYREF* **(Figure 2.2).** Nonetheless, there is overall good agreement between screens, validating the approach of searching for host factors involved in latency through CRISPR screens combined with LRAs.

2.5.3. HIV Dependency Factors versus host genes necessary for latency reversal

Our initial hypothesis was that HIV-1 dependency factors may play a role in latency reactivation given the importance of transcription in establishing infection and that transcription is a major facet that contributes to latency. Consistent with our hypothesis, we find that a large proportion of genes are important as both HIV dependency factors and as HIV latency reversal factors **(Figure 2.2A).** While transcription is the major category of genes in our screens **(Figure**

2.1C and D), the factors however span beyond transcription; we find factors involved that are key for reactivation, including *UBA3*, *UBE2M*, *AMBRA1* and *ALYREF*. In contrast, we also observe factors that are important as HIV-1 dependency factors but not in latency reactivation including *ATP2A2*, *SS18L2*, *SMARCB1* and *PCGF1* that were depleted in Jurkat T cells screens but not in J-Lat screens. *ATP2A2* is a Calcium Transporting ATPase that was found to be upregulated during G1/S phase of the cycle by Tat, but its role in the viral life cycle is otherwise unknown [125]. Similarly, *SS18L2* was found to be upregulated in HIV-1 in early infection, as found from RNA profiling of CD4+ and CD8+ T cells in people living with HIV-1 versus those who were either nonprogressors or control HIV-1 negative groups [126]. SMARCB1 is a component of the SWI/SNF chromatin remodeling complex along with INI1 (Integrase Interactor-1) and is known to play many roles in HIV-1 replication, including integration, transcription and particle maturation [127]. *PCGF1* (Polycomb group RING finger protein 1) was also depleted in HIV-1 dependency factor screens, but not in J-Lat screens in this study. Polycomb Group Proteins largely lead to transcriptional repression through methylation of histones, and thus are thought to contribute to HIV-1 latency. This might contribute to the opposite phenotype we see in this study versus infection screens; *PCGF1* may play a role in maintaining latency but is required for establishing infection. An interesting possibility is that PCGF1 is required for infection as it helps to establish a chromatin landscape that leads to either productive transcription at the integrated provirus, or even transcriptional silencing which may ultimately contribute to HIV-1 latency. Collectively, the latency HIV-CRISPR screens can help to narrow down the stage of the viral life cycle dependency factors are playing a role in, but also can give insight into novel latency reversal factors.

2.5.4 Gene Paralogs in a "Block and Lock" Latency Approach

Our Latency HIV-CRISPR screen in this study revealed our top hit *CCNT1* was able to be knocked out with little effect on cell biology, likely due in part to its paralogs *CCNT2* and *CCNK*. Cyclin T1 and T2 are paralogs that have sequence similarities at the amino acid level in the N-terminal region (81% identity), but the C-terminal domain is more divergent and far less similar (~46% amino acid identity between CCNT1 and CCNT2 [38, 81]. Cyclin K also is similar in N-terminal domain but has a shorter C-terminal region and therefore is a much smaller protein. One possibility is that there is a sequence in this C-terminal domain that adds specificity for gene regulation in CCNT2 or CCNK that allows for regulation of host genes and recruitment to different cellular promoters. Collectively, our findings suggest that CCNT1's paralogs are sufficient for transcription elongation of host genes but that CCNT1 is required for transcription of HIV-1 genes.

This approach to "block and lock," whereby a factor is required for viral replication but not for host function, may be a good path forward in further identifying gene targets to inhibit HIV-1 viral reactivation. Separate but parallel approaches have been used in cancer contexts, whereby synthetic lethality is exploited to promote death of cancer cells. A recent study has led to identification of paralogs with redundant function that lead to cell death when a pair of gene paralogs are knocked out [128]. From this study, 12% of paralogs tested lead to cell death in their context. We interpret this to mean that there is a great deal of gene paralogs which may serve redundant functions. Ongoing work will seek to identify factors that are like *CCNT1* in that when targeted, have drastic effects on viral replication, and minimal effects on the host – by focusing on top hits that have gene paralogs and thus may have redundancy. Other screen hits had Gene Effect scores similar to *CCNT1* – including *TBL1XR1*, *OTUD5*, and *AMBRA1* on the DepMap

Portal [115], suggesting that these may either have paralogs or dispensable functions for cell biology.

While LPAs have been developed in a block and lock approach, this approach still remains a challenge. In the case of dCA – for instance –HIV confers resistance to this drug through mutations in the LTR, Nef and Vpr [129, 130]. Targeting CCNT1 – or additional gene paralogs with redundant functions – may prove to be a strong complement to these LPAs, given how drastic an affect *CCNT1* Knockouts have on HIV-1 replication. Although the shock and kill approach and discovery of LRAs has been a large area of focus in recent years, there may be a role for both approaches in permanently silencing the latent reservoirs in those tissue reservoirs which are resistant to LRAs. Further investigation of *CCNT1* knockout in macrophages, microglial cells and other resident tissues, as well as other genes which have redundancy in a similar regard as *CCNT1*, will provide a good path forward to identify additional block and lock mechanisms that may supplement other approaches to an HIV functional cure.

Chapter 3: Perspectives and Future Directions

3.1 *CCNT1* in a block and lock model of latency

The most significant finding of my thesis work is the ability to genetically knockout *CCNT1* to prevent HIV reactivation from latency without having a dramatic effect on cell biology in Jurkat T cells or in primary CD4+ T cell lymphocytes. In latently infected Jurkat T cells, CCNT1 regulates HIV more than any host cellular gene. A natural extension of this work would be to understand whether this gene expression pattern holds true in other subsets of primary T cells that harbor latent proviruses. Another interesting follow-up would be to specifically investigate HIV-1 RNA transcripts to understand the degree of transcription that occurs in the *CCNT1* knockout background. One of the challenges in the RNA sequencing experiments I have described here was having long enough reads to differentiate between the spliced transcripts of viral genes, which all share a similar 3' end, as well as distinguishing genomic RNA from the RNA corresponding with each gene. An approach resolve this would be to perform digital droplet PCR on *CCNT1* knockout cells, and to use fluorescent probes targeting each viral gene; these methods have been developed and used by several groups [61, 131, 132]. These experiments would provide insight into whether there is a complete block of RNA transcription elongation or whether there is some degree of RNA transcripts present in the *CCNT1* knockout background.

Other key experiments would involve isolating CD4+ T cells from PLHIV to understand the role of CCNT1 in other CD4+ T cell subtypes. We observed minimal changes in RNA gene expression profiles from healthy donors after CCNT1 depletion, but instead of infecting with a dual reporter virus as in this thesis work, it would be interesting to measure how knockout of *CCNT1* affects gene expression in various CD4 T cell subtypes – whether naïve T cells, stem cell

memory T cells, central memory T cells or effector memory T cells. There are various cell surface markers that identify the different T cell subtypes **(Figure 1.1),** and so one approach would be to knockout *CCNT1* and sort the T cell subtypes either through flow cytometry or magnetic bead separation, and perform RNA sequencing and the quantitative viral outgrowth assay (QVOA) to understand the implications for HIV and host cell gene expression [133]. In our primary latency model, one of the main challenges is that we need to activate the T cells prior to knockout in order to expand the knockout cells. This would be a challenge in this experiment as well, as electroporation knockouts drastically affect viability of cells. One solution would be to develop a proteolysis targeting chimera (PROTAC) targeting CCNT1, or identify drugs that can target CCNT1 and inhibit its binding with CDK9. PROTACs are molecules that bind proteins of interest with a linker and target them for ubiquitination via an E3 ubiquitin ligase domain [134]. While these PROTACs take time to develop, it would be fruitful to identify an approach to degrade *CCNT1* and largely eliminate the expression of HIV-1. The Dep-Map portal and canSAR AI platform also identifies CCNT1 as a protein that has a druggable structure [115, 135]. Thus it may be possible to develop a molecule or ligand that binds and inhibits CCNT1 binding with CDK9 and Tat.

Studies in mice have suggested that *CCNT2* knockout is embryonic lethal. This same study found *CCNT1* knockouts were not embryonic lethal, but had developmental defects [105]. There are differences in mouse *CCNT1* and human *CCNT1* genes, which in part explains why HIV-1 is unable to infect mice; that is, there is a cysteine residue in human CCNT1 that is not present in the mouse CCNT1 protein which is required for interaction with Tat [54]. Thus there may be differences in how *CCNT1* knockout affects humans versus mice. Regardless, in a therapeutic context, it may be useful to isolate CD4+ T cells to perform knockouts specifically in

this cell type, whether by lentiviral knockout or other gene therapy approach. Initial tests might involve taking a small percentage of total CD4+ T cells from PLHIV to assess the outcomes of *CCNT1* knockout on host cell function and viral production. This approach could potentially eliminate the expression of HIV-1 genes in this cell type which is the primary target of HIV-1 infection. It would be more challenging to target other tissues and the implications of *CCNT1* knockout in the brain, reproductive tract, bone marrow and GALT for example would require more investigation in cell models and in organoids or tissue samples.

Questions regarding the tractability of *CCNT1* knockout for may be addressed using the humanized mouse BLT (bone-marrow, liver, thymus) model. The approach would be to infect BLT mice with a strain of HIV, and subsequently following up with a *CCNT1* knockout selectively in different tissues or in CD4+ T cells by using a tamoxifen-inducible Cre system [136, 137]. The consequences of *CCNT1* knockout can be addressed by examining the effects on the immune system, gut, bone-marrow, and other viral reservoirs by taking biopsy samples. BLT mice exhibit several trademarks of HIV infection including T cell depletion and immune activation [138] and thus would be a good stepping stone for understanding the feasibility of *CCNT1* knockout in human cell types. Alternatively, if a drug or PROTAC is developed to inhibit CCNT1 function, these could be used as a method to understand the pharmacological outcomes of these approaches.

In the longer-term, once the effect of CCNT1 knockout or inhibition is better established in a mouse model, non-human primate models would be a gold standard for understanding the relevance of these manipulations in humans. A major barrier for the success of LRAs is to induce reactivation in all latently infected cell populations to eliminate them with a shock and kill approach. Targeting CCNT1 may be an excellent complement to transcriptionally silence latently

infected cells which are recalcitrant to reactivation by LRAs. There are several additional questions to address; notably, does the timing of *CCNT1* intervention matter in the course of infection and in combination with ART? Does time of ART intervention after infection affect the ability for CCNT1 inhibition to take place? It will also be important to investigate the long-term ability for a CCNT1 target to inhibit viral expression and viral reactivation; this would expand possible outcomes for PLHIV where it is more difficult to regularly administer treatment.

3.2 Additional screen hits and follow-up

Several of our screen hits validated, including *ELL*, *ALYREF*, *UBE2M, TBL1XR1, HDAC3,* and *AMBRA1*. What is striking about these hits is that they represent such a broad spectrum of cellular pathways. As brief examples, ELL is a member of the super elongation complex (SEC), whereas AMBRA1 is an autophagy protein, and UBE2M is a protein that is a member of the neddylation pathway. Results from the DepMap portal suggest that *TBL1XR1* and *AMBRA1* have gene effect scores similar to *CCNT1*, and thus these genes potentially can be knocked out without dramatic effect on cell biology. Presumably, these genes have redundancy with other host genes and can be knocked out in cells with little effect on cell biology. It would be interesting to characterize these various genes for their impact on cell viability and ability to prevent latency reactivation in primary cells. Moreover, it would be interesting to see whether knockout of more than one gene or combined inhibition of CCNT1 and these other gene hits leads to prolonged suppressed viral transcription.

I find that HIV-1 dependency factors comprise many genes, some of which are also required for latency reactivation in Jurkat T cells. While the HIV-Dep gene library has over 500 genes, there are likely other genes that could be targeted to prevent latency reactivation. One

future direction would be to perform a CRISPR screen with other gene libraries, including pgPEN gene paralog library [128] to potentially identify other gene knockouts that do not affect cell viability but drastically prevent HIV-1 replication. An opposite phenotype would also be a fruitful outcome; if there is a host gene similar to *CCNT1* that can be targeted but instead of preventing latency reactivation it promotes reactivation, perhaps we could target these genes in a latency reversal approach without drastically affecting host cells. Another approach might be to perform a CRISPR screen using a kinase gene library ("kinome") [139, 140] or to cross-reference both the kinase and paralog gene library, as kinases are the second most targeted group for drug targets [141].

3.3 Primary CD4+ T cell CRISPR approaches

While Jurkat T cells provide a convenient and tractable model for understanding genes important for HIV latency, this is an immortalized cancer cell line and is subjected to much different conditions than *in vivo* primary CD4+ T cells. A major gap in the field of HIV latency is the ability to directly screen primary T cell subsets for genetic factors that are important for various aspects of HIV latency. Multiple hurdles inhibit the ability to perform our CRISPR screen in CD4+ T cells; the use of CRISPR/Cas9 lentiviral vectors has largely been unsuccessful in primary CD4+ T cells. More recent advances including the T cell optimized for packaging (TOP) vector for delivering guide RNAs and transgenes into primary T cells may provide a solution for delivering Cas9 and guide RNAs as in our other CRISPR Screens [2, 48, 49, 142]. The other major challenge is the loss of cell quantities that occurs during the latency establishment period in our primary CD4+ T cell models. If given the tools and means to do so, an approach where CD4+ T cells were isolated from PLHIV, and our CRISPR screening

approach were used to understand genes required for latency reactivation or promote reactivation on knockout, this would provide powerful and useful information on further potential targets for an HIV cure.

3.4 – Cyclin Paralogs in the P-TEFb Complex

What are the mechanisms that govern transcription elongation in host cells? In this thesis work, I found little effect on cell biology in *CCNT1* knockout and far more pronounced effects on HIV transcription. Thus, a major question to address is which cyclin – whether CCNK or CCNT2 – is active in P-TEFb complexes and participates with CDK9 for phosphorylation of RNA polymerase II in T cells. Although CCNT1 is not necessary for T cell growth, its binding partner, CDK9, is essential. This is consistent with general consensus that CDK9 inhibitors are toxic; while there is one FDA-approved CDK9 inhibitor, flavopiridol, this drug has significant toxicity. Screens of CDK9 knockout in most cancer lines show this gene is essential for function [115]. Additional CDK9 inhibitors are being developed by pharmaceutical companies in a cancer context [143]. While CDK9 inhibitors are known to interact with several different proteins aside from those in the P-TEFb complex, one explanation of CDK9's essentiality in cells is that CDK9 is an absolutely required component of the P-TEFb complex, whereas the cyclin component is interchangeable with CCNT1, CCNT2 or CCNK. Further characterization of these various cyclin components and their effect on cell biology would be imperative to address these questions.

Another major question regarding these paralogs is the specificity they have for separate genes. Previous work suggests that CCNT1 and CCNT2 are regulating different sets of genes, however this work was performed in HeLa cells and thus the broad relevance remains in question [107]. Moreover, the findings of *CCNT1* and *CCNT2* knockout in mouse models

suggest that these paralogs regulate different processes; *CCNT2* knockouts were lethal whereas *CCNT1* knockouts were viable but with developmental defects [105]. If these gene paralogs are indeed regulating different sets of host genes, the mechanisms that govern the specificity of each of these Cyclins for different genes could potentially be harnessed in a therapeutic context to block transcription elongation of specific genes. Moreover, targets against P-TEFb are actively being developed for a broad range of diseases, including cancer, cardiac hypertrophy, and inflammation [144]. In the case that these different gene paralogs are functioning redundantly or do not have gene specificity, perhaps these different Cyclin paralogs could be targeted to lead to a modest and tempered reduction of P-TEFb activity, unlike the CDK9 inhibitors which altogether prevent P-TEFb activity by inhibiting the kinase domain of this complex.

3.5 Final thoughts and remarks

Collectively, my thesis work has shown the value in investigating HIV dependency factors in a block and lock model of latency. Whole genome screens have been performed to identify factors involved in latency, however we have found that whole genome screens often are unable to detect genes that are known to be important for HIV replication [48]. Using the latency HIV-CRISPR system [49], we can incrementally test how various LRAs and gene sets contribute to or are resistant to latency reactivation by examining guide RNAs that are either enriched or depleted in the screens. Moreover, the CRISPR screen is sensitive in that gene knockouts which are lethal are generally removed from the screen, as genomic DNA from dead cells is washed away and excluded from genomic DNA analysis in the CRISPR screen. I saw this in my thesis work, where gRNAs corresponding to *CCNT1* were the most depleted, but in fact not depleted as a consequence of cellular toxicity or lack of gene knockout. These latency CRISPR screens, if

developed to be performed in primary CD4+ T cells, will be an extraordinarily powerful tool to understand potential gene targets in working towards an HIV cure.

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