THE ROLE OF MICRORNAs IN HIV-1 LATENCY

by

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The Role of MicroRNAs in HIV-1 Latency

Debbie Sophia Ruelas

Abstract

Despite significant advances in our understanding of HIV, a cure has not been realized for the more than 34 million infected with this virus. HIV is incurable because infected individuals harbor cells where the HIV provirus is integrated into the host’s DNA but is not actively replicating and thus is not inhibited by antiviral drugs. Similarly, these latently infected cells are not detected by the immune system. Intermittently, these latently infected cells produce a burst of virus before retreating back into latency. These small bursts of virus contribute both to the low viral load detected in treated subjects and to rapid reseeding of HIV infection following ART withdrawal. We show that miR-155 helps reestablish viral latency by interfering with the HIV-activating effects of TRIM32. TRIM32, an E3 ubiquitin ligase, activates the HIV LTR by an unexpected mechanism involving direct ubiquitination of IκBα leading to NF-κB nuclear translocation and engagement of the duplicated κB enhancers within the LTR. These findings highlight a latency-promoting role for one cellular miRNA that acts by suppressing an HIV activator. Understanding the precise mechanisms governing HIV latency will be pivotal for development of a safe, effective, and scalable HIV cure.
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Chapter One:

Introduction
1.1 HIV-1 Latency in CD4\(^+\) T cells

AIDS was first recognized in the summer 1981 (Friedman-Kien, 1981; Gottlieb et al., 1981; Siegal et al., 1981) and HIV was first isolated two years later (Barre-Sinoussi et al., 1983) and proven to be the cause of AIDS in 1984 (Gallo et al., 1984; Popovic et al., 1984; Sarngadharan et al., 1984; Schupbach et al., 1984). More than 35 million people have died of AIDS. The virus continues to hit the hardest in Sub-Saharan Africa, where 1 in every 20 adults is infected. Although there is no cure for HIV, more than 30 different anti-HIV drugs have now been approved for clinical use targeting different steps in the viral life cycle. While not eradicating HIV, combinations of these agents can routinely drive viral loads down to undetectable levels (Dahl et al., 2010). Indeed, HIV is now managed as a chronic rather than acute disease (Pomerantz and Horn, 2003). Nevertheless, for every 10 people started on antiretroviral therapy in the developing world, 16 people are newly infected. We clearly do not yet have a winning HIV/AIDS strategy for the developing world plus the escalating cost for treatment will become increasingly difficult for developed countries to meet (John-Stewart, 2013). Strategies for either eradicating the virus from infected individuals or boosting their immune response so that antiviral drugs can be discontinued—a functional cure—are urgently needed.

Post-integration HIV latency refers to the rare but extremely stable proviral reservoir formed within resting memory CD4\(^+\) T cells (and other less well characterized reservoirs—described further down in detail). Latency is
established early during acute infection, likely within days of initial infection (Chun et al., 1998a). Although transcriptionally silent, this reservoir is fully capable of producing infectious virus when the host cell is reactivated by recall antigen or various cytokines or when ART is discontinued.

Naïve CD4$^+$ T cells exist in a resting state until they encounter an antigen, after which they undergo activation and proliferation to generate effector cells that clear the associated pathogen from the body. The majority of these activated cells die within a few weeks. However, some of these cells revert back to a resting state and persist as memory T cells that are capable of responding to the same antigen in the future. It is precisely these cells that form a primary reservoir for latent HIV proviruses. It is possible that latent infection reflects infection just as these cells retreat to a resting state. Because these cells can persist in a quiescent state for long periods of time, they represent an ideal cellular reservoir for the maintenance of latent virus. Antigen or cytokine activation of these cells leads to the induction of transcription factors, like NF-κB and NFAT, that, in turn, promote reactivation of the latent HIV proviruses (Colin and Van Lint, 2009). Following activation, cytopathic effects or immune responses cause the rapid death of most HIV-infected cells (Figure 1.1).

Importantly, it was recently shown that antigen-specific stimulation of patient cytolytic T lymphocytes (CTLs) prior to virus reactivation from latently infected cells is essential for effective killing of HIV-1 infected cells in vitro (Shan et al., 2012). This suggests that boosting the CTL response in infected patients
may be necessary to deplete the HIV reservoir. Alternatively, a strategy could be employed to boost NK cell activity, or to treat with drug cocktails that induce the death of reactivated cells.

1.2 Cellular Reservoirs

The introduction of combination antiretroviral therapy (ART) in 1996 was a major advance that revolutionized the care of HIV-infected individuals (Harrington, 2005). These drugs also provided new insights into the dynamics of HIV-1 replication in vivo. Specifically, a four-phase decline in virus was detected following ART administration. The initial rapid phase of decay occurs within the first two weeks of treatment and is accompanied by a 99% drop in HIV levels in the blood. This is largely due to the rapid decline of free virus ($t_{1/2} \leq 6$ hours) and productively infected cells ($t_{1/2} \leq 1.6$ days) (Ho et al., 1995; Wei et al., 1995).

The second phase of decay proceeds more slowly and results mainly from the death of infected macrophages, partially activated CD4$^+$ T cells and dendritic cells. The half-life of macrophages can be several weeks long, depending on the type of tissue in which they reside. Macrophages are susceptible to infection with HIV, but are more resistant to the cytopathic effects of the virus compared to CD4$^+$ T cells (Ho et al., 1986). Partially activated CD4$^+$ T cells are also described as having a longer turnover rate than fully activated CD4$^+$ T cells, and are therefore likely to contribute to the slower decay of the second phase. In general, the dendritic cell lifespan is highly dependent on anatomical location
and cellular subtype (Shortman and Naik, 2007). Following HIV infection in vitro, myeloid dendritic cells are capable of surviving for over 45 days (Popov et al., 2005). However, in vivo it has been shown that peripheral blood myeloid dendritic cells do not contain detectable HIV DNA following a 6-week ART regimen (Otero et al., 2003). Langerhans cells have been shown to resist HIV infection unless stressed by skin abrasion or co-exposed to other sexually transmitted organisms (reviewed by (Coleman and Wu, 2009)), and when infected have a half-life of about 15 days. For these reasons, HIV-infected Langerhans cells are not thought to contribute significantly to the latent reservoir. In contrast, follicular dendritic cells, found in the lymphoid tissues, are capable of trapping and retaining HIV virions on their surface for several months following infection (Smith et al., 2001). These cells may in fact provide a mechanism for a longer-term persistence and subsequent transfer of virus to CD4+ T cells by a process termed trans infection.

Taking into account this second phase of decay, it was initially predicted that 2.3–3.1 years of treatment would be sufficient to eliminate HIV-1-infected cells, including latently infected lymphocytes (Perelson et al., 1997). However, this calculation was made with the acknowledgement that additional longitudinal studies were necessary to exclude the presence of very long-lived infected cells.

In 1995, the first in vivo evidence of the presence of a latent HIV reservoir was revealed. This was demonstrated by isolating a highly purified population of resting CD4+ T cells from HIV-1-infected patients and performing inverse PCR to
detect integrated HIV-1 DNA (Chun et al., 1995). At the time, a critical area of investigation was to determine the frequency of latently infected cells as well as the replication competency of the virus in patients on ART. Because resting memory CD4\(^+\) T cells can persist for months to years, these cells could represent a long-term viral reservoir in patients on ART. Indeed, several laboratories demonstrated that replication-competent virus could persist in the resting CD4\(^+\) T cells of patients on ART regimens and that these infected cells were present at about 1 in every 10\(^6\) resting CD4\(^+\) T cells (Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997). Using direct longitudinal analysis of the decay rate of the latent reservoir, it was demonstrated that latent cells capable of producing replication-competent virus had a very long half-life (43.9 months). Based on this turnover rate, it was estimated to take 73 years of therapy to eradicate the latent reservoir (Finzi et al., 1999; Siliciano et al., 2003). Recent reports from the Siliciano lab suggest that the number of latently infected cells may be 40–50 times higher than originally estimated (unpublished), proving that eradication of the viral reservoir will be even more challenging than previously predicted.

The exact cellular subsets responsible for the third and fourth phases of decline are not yet clear, but are likely to include the well-defined latent reservoir of resting memory CD4\(^+\) T cells. In support of this idea, Chomont et al. identified two viral reservoirs within the memory CD4\(^+\) T-cell pool. Known as central memory (T\(_{CM}\)) and transitional memory (T\(_{TM}\)) CD4\(^+\) T cells, these reservoirs are...
characterized by differing decay rates in ART-treated patients and persist by two distinct mechanisms. HIV-infected patients who have normal CD4 counts and who have begun an early treatment regimen carry a viral reservoir of limited size that consists mainly of $T_{CM}$ cells. $T_{CM}$ cells proliferate at extremely low levels and are able to survive for decades, although this reservoir may be partially depleted at a slow rate due to CTL killing and the virus' intrinsic cytopathic effects. In contrast, HIV-infected patients with low CD4 counts have a viral reservoir consisting mainly of $T_{TM}$ cells. This is accompanied by continuous immune activation in the majority of these patients, including increased levels of IL-7. IL-7 has been shown to induce proliferation of memory CD4$^+$ T cells, and may induce the homeostatic proliferation and survival of $T_{TM}$ cells in ART-treated individuals with low CD4 counts (Chomont et al., 2009). This could be an additional mechanism by which this latently infected reservoir persists for long periods of time. This study also raises the possibility that earlier therapeutic intervention could more effectively limit the size of this proliferating reservoir.

In addition to peripheral blood, two important anatomical sanctuary sites for HIV are the central nervous system (CNS) and gut-associated lymphoid tissue (GALT). Monocytes are able to differentiate into macrophages and microglial cells after crossing the blood-brain barrier, thus providing a mechanism for viral persistence in the CNS (Gras and Kaul, 2010). In the GALT, there are higher levels of viral replication compared with PBMCs (Chun et al., 2008). ART is also found to be less effective in the GALT, and ongoing viral
replication in this anatomical site may lead to further infection of nearby CD4+ T cells.

Although ART is capable of decreasing the viral load to levels below the limit of detection, the persistence of a latent reservoir of replication-competent provirus remains a major obstacle to achieving a cure. It is well known that latently infected CD4+ T cells can be activated to produce infectious virus in vitro as well as in vivo. Therefore, stimulation of this latent reservoir and forcing virus to enter into a productive viral lifecycle may be an essential step in eradicating HIV-1 in infected patients.

1.3 Viral Persistence

The mechanism by which virus persists in the presence of ART is uncertain and may actually involve multiple mechanisms. The most widely accepted school of thought is that the long-lived reservoirs of memory CD4+ T cells establish latency early on and persist for an extremely long time. Another school suggests that ongoing rounds of replication are occurring and that this serves to maintain the latent reservoir. In support of this latter model, recent studies demonstrated that intensification of a suppressive ART regimen by the addition of raltegravir (an HIV integration inhibitor) results in a temporary increase in episomal DNA in some HIV-infected patients. In addition, integrase inhibitors are known to increase the level of episomal cDNA when active replication is blocked, suggesting that a low level of replication may be occurring
in ART-suppressed patients (Buzon et al., 2010). However, it has also been shown that therapy intensification has no effect on reservoir size (Dinoso et al., 2009; Gandhi et al., 2010). Furthermore, longitudinal clonal genotypic analyses demonstrated that ART-suppressed patients do not develop drug resistance (Kieffer et al., 2004), suggesting that the latent reservoir is not evolving during treatment. It should be noted that blips in viral load do not represent replication of drug-resistant virus, but instead simply reflect biological and statistical variations in levels of the prevailing virus (Nettles et al., 2005). Even in patients that respond well to ART (e.g. patients exhibiting undetectable plasma viral loads below 40-50 copies of HIV-1 RNA in standard assays), a low level of free virus can be detected by sensitive assays (Dornadula et al., 1999). When considered together, the balance of evidence favors the conclusion that residual viremia most likely reflects the intermittent production of virus from stable long-lived reservoirs rather than ongoing, low-level productive infection.

1.4. Mechanisms of Post-integration Latency

A better understanding of the mechanisms of post-integration latency will be necessary to uncover novel targets and methods for attacking and eradicating the latent reservoir. In particular, it will be important to find new ways to reactivate the latent provirus within each and every cell in the reservoir where it resides. It is also essential that the cellular host be induced to die either as a result of viral reactivation or a subsequent immune response against the virus.
In the next sections, we discuss the various mechanisms that likely underlie HIV latency. However, it is important to point out that latency is likely a multifactorial process. This fact could complicate development of an effective anti-latency therapy.

1.4.1. Site and Orientation of Integration

In most instances, HIV-1 proviral cDNA integrates into regions of the host genome that are actively transcribed (Schroder et al., 2002). Following integration, the provirus is replicated and transcribed along with the cellular DNA that surrounds it. Some of these viral RNAs are then translated into viral proteins, while two copies of the full-length viral RNA are incorporated as the genetic material for new infectious virions. Along with viral RNA, a number of viral proteins are incorporated into these virions including the Gag, Pol and Env structural proteins. Additionally, the viral integrase protein, which associates with viral DNA and the preintegration complex (PIC) (Farnet and Haseltine, 1991) is incorporated as is Vpr. Various host proteins are included as well. For example, the cellular LEDGF/p75 is incorporated due to its tightly binding to integrase (Cherepanov et al., 2003). LEDGF/p75 is believed to guide the protein complex and viral DNA to intronic regions of actively transcribed genes (Lewinski et al., 2006). Most latent proviruses from virally suppressed patients on ART reside in host genes that are actively transcribed (Han et al., 2004). Although it may seem contradictory that latent provirus exists in actively transcribed regions, this
phenomenon points to the potential involvement of transcriptional interference as a mechanism for suppressing expression of the integrated provirus.

One mechanism of transcriptional interference involves promoter occlusion, where the provirus integrates downstream of the host gene in the same transcriptional orientation or polarity (Figure 1.2). This may result in “read-through” by the host RNA Pol II, which displaces constitutively expressed transcription factors like Sp1 that bind to the HIV-1 LTR and are essential for viral gene expression (Greger et al., 1998). Furthermore, it has been shown in a Jurkat CD4+ T-cell model (J-Lat cells) that transcriptional interference can be reversed by inhibiting transcription of the upstream gene or by cooperatively activating viral transcription initiation and elongation through viral Tat or TNFα-mediated NF-κB activation (Lenasi et al., 2008). Specifically, NF-κB can bind the HIV LTR with exceptionally high affinity and block the upstream elongating RNA Pol II, thus overcoming transcriptional interference leading to HIV reactivation.

Another mechanism of transcriptional interference, referred to as convergent transcription, occurs when the provirus integrates in the opposite orientation or polarity relative to the host gene (Figure 1.2). This leads to collision of the RNA Pol II complexes from the host and viral promoters and early arrest of transcription from both promoters or the weaker of the two (Lewinski et al., 2005). It is also possible that convergent transcription results in double-stranded RNA when both strands of viral DNA are elongated. This may lead to silencing of viral transcription or translation through RNA interference (Hu et al., 2004),
RNA-directed DNA methylation (Morris et al., 2004), or the generation of antisense RNA (Scherer and Rossi, 2003).

More recently it has been demonstrated that HIV integrated into cancer genes can contribute to persistence and clonal expansion of infected cells (Maldarelli et al., 2014; Wagner et al., 2014). These studies suggest that proliferation of latently infected cells may be a major factor in sustaining the viral reservoir. In both studies, DNA was harvested from the blood cells of HIV-infected patients on successful ART for over a decade. Subsequently, the sites of proviral integration in the human genome were analyzed. Both groups found expanded proviral clones that were enriched for proviruses in a particular set of cellular genes that encode factors involved in controlling cell division or cancer. This supports the idea that proviral insertion into these genes results in unregulated growth or persistence of the host cell. Specifically, a gene encoding the basic leucine zipper transcription factor 2 (BACH2) was identified as a frequent site of HIV integration. BACH2 normally functions as a transcriptional regulator and has been shown to control CD4 T cell senescence and cytokine homeostasis (Kuwahara et al., 2014). Therefore, this finding provides evidence that the latent reservoir may persist through proviral integration into genes related to cell proliferation and cancer. However, further experiments must be performed to provide molecular proof that these integrations indeed promote the proliferation of latently infected cells. Furthermore, these studies only amplified a very small portion of the HIV genome, and it is possible that there are
inactivating mutations in other parts of the provirus. Therefore, it is necessary to provide evidence that these proviruses encode replication-competent HIV genomes. If proviral integration into cancer genes does indeed contribute to persistent infection, it may then be necessary to block this proliferation, and may complicate efforts to clear the latent reservoir.

1.4.2. Initiation of Transcription

The 5’LTR of HIV contains multiple sites for the binding of cellular transcription factors, including NF-κB, NFAT, Sp1, and AP1 (Figure 1.3). These key cellular factors may be activated by external stimuli in order to enhance HIV-1 transcription. In resting cells, NF-κB and NFAT are sequestered in the cytoplasm (and are unable to promote HIV-1 transcription in the nucleus), but undergo nuclear translocation following appropriate cellular activation. Both NFAT and NF-κB are able to bind to κB sites in the HIV-1 LTR (Kinoshita et al., 1998). However, it is likely that NF-κB plays a larger role in HIV transcription (Chan et al., 2013; Kim et al., 2011). Specifically, we have shown that none of the NFATs play an essential role in the transcriptional activation of latent HIV. Rather RelA is an important antagonist of HIV latency and maximal NF-κB induction involves the action of calcineurin following T cell activation (Chan et al., 2013). It has been shown that binding of NF-κB is not sufficient to induce HIV gene expression, while Sp1 is necessary (Perkins et al., 1993).
In the cytoplasm of resting cells, NF-κB exists as a p50/RelA heterodimer bound in an inactive form through interactions with IκBα. In the nucleus of HIV-infected resting cells, NF-κB p50/p50 homodimers are bound to the 5’LTR. The p50/p50 homodimer lacks the essential transactivation domain present in the p50/RelA heterodimer (the prototypical NF-κB complex). In addition, p50 homodimers can actively inhibit transcription by recruiting HDAC-1 to the LTR and promoting latency through histone deacetylation and chromatin condensation (Williams et al., 2006). The NF-κB heterodimer is activated by IKK-mediated phosphorylation of serine-32 and -36 on IκBα followed by βTrCP-mediated ubiquitylation and degradation of IκBα by the 26S proteasome. These events enable NF-κB translocation to the nucleus and displacement of p50/p50 NF-κB homodimers. The p50/RelA heterodimers recruit histone acetyltransferases (HATs), like p300/CBP, resulting in acetylation of histone tails and transcriptional activation (Zhong et al., 2002). To initiate transcription, RelA interacts with the Cdk7 kinase subunit of TFIIH to stimulate phosphorylation of serine-5 within the CTD of RNA Pol II (Kim et al., 2006). Similarly, interaction of RelA with p-TEFb results in phosphorylation of the RNA Pol II CTD at serine-2 and subsequent elongation (Barboric et al., 2001). P300 also mediates acetylation of RelA at lysines 218, 221 and 310. Acetylation at lysine-310 enhances the transcriptional activity of RelA while acetylation at lysine-218 and -221 enhance DNA binding and make RelA resistant to IκBα binding.
Following Ca$^{2+}$ release via the PKC pathway, calcineurin dephosphorylates and induces nuclear localization of the cytoplasmic components of NFAT (Crabtree and Olson, 2002). NFAT binds to the 5'LTR at two sets of NFAT binding sites in addition to the κB sites. Furthermore, it has been shown that CsA, which is a potent inhibitor of NFAT, is capable of inhibiting promoter activity in primary cells (Cron et al., 2000). Like NF-κB, NFAT can also bind p300/CBP and recruit HATs from nuclear extracts of cell lines (Garcia-Rodriguez and Rao, 1998).

Our understanding of the key signaling pathways that promote transcription in active versus latent cells has helped to identify points of intervention that could be used to overcome latency and purge the latent reservoir. The non-tumor-promoting phorbol ester, prostratin, promotes HIV transcription by PKC-mediated activation of NF-κB, which thereby induces RelA to bind the latent HIV-1 promoter (Williams et al., 2004). Activation of the PKC pathway by prostratin also results in down-regulation of the CD4 receptor and the CXCR4 and CCR5 coreceptors on the host cell, decreasing the likelihood of new infections (Hezareh et al., 2004). Although NF-κB-inducing agents are strong activators of HIV-1 transcription, they also cause global cellular activation and may induce a toxic inflammatory response in patients in the form of a cytokine storm.
In addition to PKC activation, there have been attempts to purge the latent reservoir through global cellular activation using cytokines. For example, an array of cytokines, including IL-6, TNFα, and IL-2, are capable of being potent inducers of viral replication in the resting CD4⁺ T cells of ART-treated patients (Chun et al., 1998b). In clinical trials of ART-suppressed patients, researchers observed no difference in the amount of proviral DNA and determined that IL-2 had little effect on viral latency (Dybul et al., 2002; Stellbrink et al., 2002). In another study, HIV-infected patients were co-treated with ART and a combination of IL-2 and IFN-γ. At the end of the trial patients rebounded with high plasma HIV-1 RNA levels when ART was stopped (Lafeuillade et al., 2001). In patients with near-complete suppression of plasma viremia (below 5 copies/mL), treatment with anti-CD3 antibodies and IL-2 triggered activation and proliferation of T cells along with stimulation of HIV replication (up to 1500 copies/mL). However, the anti-CD3/IL-2 therapy was toxic, and resulted in long-lasting depletion of CD4⁺ T cells in the peripheral blood and lymph nodes (Prins et al., 1999; van Praag et al., 2001). IL-7 is another cytokine that is capable of activating viral gene expression. For example, in latently infected cells from SCID-hu (Thy/Liv) mice, ex vivo treatment with IL-7 was sufficient to reactivate latent virus while causing minimal cell cycle progression (Brooks et al., 2003). However, Chomont et al. showed that IL-7 mediates homeostatic proliferation and subsequent viral persistence in patients with low CD4⁺ T cell levels through effects on transitional memory T cells (Chomont et al., 2009). Although IL-7, and
possibly other cytokines, can influence the stability of the HIV reservoir, these findings suggest that global immune activation strategies may do more harm than good, and that limiting immune activation in combination with agents like IL-7 might be more effective.

1.4.3. Transcriptional Elongation

The HIV transactivator Tat plays a major role in the elongation phase of transcription (Figure 1.3). During the earlier (Tat-independent) initiation phase, cellular transcription factors (like NF-κB) are recruited to the HIV LTR promoter region. This is followed by the Tat-dependent elongation phase, which promotes much higher levels of HIV-1 transcription compared to the initiation phase. When Tat is present, 99% of the transcripts are transcribed to their full length. However, in the absence of Tat, 87% of the initiated transcripts terminate prematurely at positions +55 to +59 (Kao et al., 1987). This block is due to the activity of negative transcription elongation factor (N-TEF), which is composed of the negative elongation factor (NELF) and DRB-sensitive inducing factor (DSIF) that are known to restrict the transcription of other cellular genes (Yamaguchi et al., 2013). Although these short transcripts predominate in the absence of Tat, a small amount of full-length transcript is produced, including Tat, thereafter promoting full-length HIV transcription by positive feedback. To overcome the transcription block caused by N-TEF, Tat binds to the transactivation-responsive element (TAR), a short nucleotide stem-loop located at the 5’end of all HIV
transcripts. Following TAR binding, Tat recruits the PTEF-b complex, composed of CyclinT1 and Cdk9 (Wei et al., 1998). As part of the PTEF-b complex, Cdk9 phosphorylates the CTD of RNA Pol II, primarily at serine-2, promoting enhanced processivity of RNA Pol II (Kim et al., 2002). In addition, PTEF-b mediates phosphorylation of DSIF and NELF, which results in removal of NELF from the LTR and allows productive elongation to begin (Fujinaga et al., 2004; Ivanov et al., 2000).

Transcriptional activation also depends on the acetylation state of Tat. Tat can be acetylated at lysines 50 and 51 by CBP/p300 and GCN5, respectively, within the TAR-binding domain. This leads to the dissociation of Tat from TAR, thus promoting the switch between the early and late phases in HIV transcriptional elongation (Ott et al., 2004). Also, the bromodomain of p300/CBP-associated factor (PCAF) can bind to acetylated Tat at lysine-50, thereby competing with the Tat/TAR interaction and promoting the dissociation of Tat from TAR (Mujtaba et al., 2002). Disruption of the Tat/CyclinT1/TAR complex via p300-mediated Tat acetylation facilitates the transfer of Tat to the elongating RNA Pol II complex (Kaehlcke et al., 2003). Furthermore, both in vitro and in vivo, Tat is deacetylated by human sirtuin 1 (SIRT1), a NAD⁺-dependent class-II protein deacetylase. SIRT1-mediated deacetylation allows Tat to return to its unacetylated form, thereby enabling the initiation of a new transcription cycle (Pagans et al., 2005). In addition, experimental and computational studies performed by Weinberger et al. (2005) demonstrate how stochastic fluctuations...
(noise) in the activity of the LTR governed by Tat can influence the viral latency decision (Weinberger et al., 2005). This provides further evidence for the importance of Tat in regulating HIV-1 latency.

Levels of active PTEF-b are key in promoting the switch to productive elongation, and therefore play a large role in controlling the expression levels of viral genes. PTEF-b is inactive when its components CyclinT1 and Cdk9 are bound to the 7SK small nuclear ribonucleoprotein complex containing the inhibitory molecule HEXIM1 (Yik et al., 2003). In resting CD4+ T cells which are nonpermissive for HIV-1 replication, the levels of Cyclin T1 and T-loop-phosphorylated CDK9 are very low but increase upon cellular activation (Budhiraja et al., 2013). The release of PTEF-b from HEXIM1 is mediated through stress-inducing agents, including the small molecule hexamethylenebiacetamide (HMBA) (Contreras et al., 2007). Choudhary et al. showed that HMBA-treated resting memory CD4+ T cells from ART-suppressed patients were reactivated through phosphorylation of the CTD of RNA Pol II in a Cdk9-dependent manner. HMBA increases the nuclear expression level of Cdk9, which is then recruited to the LTR by Sp1 (Choudhary et al., 2008).

There has been great interest in a novel group of transcriptional regulators shown to control viral gene expression. Recent studies demonstrated the effectiveness of JQ1 and other bromodomain (BET family) inhibitors in inducing latent reactivation in cell lines and primary cell models of HIV latency (Banerjee et al., 2012; Bartholomeeusen et al., 2012; Li et al., 2013) Specifically, it was
shown that bromodomain-containing protein 4 (Brd4) could compete with HEXIM1 for PTEF-b binding. Therefore, inhibiting the binding of Brd4 to PTEF-b could promote increased binding of Tat to PTEF-b. In contrast, Boehm et al. recently showed that BET inhibitors, like JQ1, are dependent on PTEF-b, but independent from Tat. In addition, Brd2 was identified as a new Tat-independent suppressor of HIV transcription (Boehm et al., 2013). However, this mechanism of action has not been fully worked out. Interestingly, it is also known that HMBA increases the level of active PTEF-b-Brd4 complexes (He et al., 2006). The BET family therefore represents a promising new target for therapies aimed at overcoming HIV-1 latency.

1.4.4. Chromatin Modification

The expression of genes, including those of integrated HIV-1, is dependent on chromatin structure, which can be altered by epigenetic modifications. The main structural units of chromatin are nucleosomes, which consist of pairs of four core histones (H2A, H2B, H3, and H4) that form an octamer and become enwrapped by 1.7 turns of DNA (147 base pairs). When formed around promoters, these structures can epigenetically determine whether the associated genes are expressed or not. For example, a genome in a compact structure bound tightly by nucleosomes is transcriptionally repressed due to blockade of the promoter region from the transcriptional machinery; DNA in this state is called heterochromatin. In contrast, euchromatin refers to the
relaxed and transiently open state of chromatin, which encourages transcription. It is known that changes in chromatin condensation status can be mediated through chromatin remodeling complexes that use ATP to disrupt nucleosome-DNA contacts, move nucleosomes along DNA, and remove or exchange nucleosomes (Hargreaves and Crabtree, 2011). However, changes in chromatin structure are also brought about by various posttranslational modifications, including acetylation and methylation.

Histone acetyl transferases (HATs) catalyze the addition of acetyl groups to target histones, whereas histone deacetylases (HDACs) remove these acetyl groups. These opposing functions of HATs and HDACs allow for switching between different acetylation states, and determine whether genes are transcriptionally activated or repressed, respectively. In terms of HIV-1 therapy, there has been great interest in using HDAC inhibitors to block HDAC deacetylase activity, which would create a less repressive chromatin state and allow for HIV transcription. Early experiments showed that, independent of the viral integration site, the 5’LTR of HIV contains two nucleosomes, nuc-0 and nuc-1 (Verdin et al., 1993). Nuc-1 is rapidly and specifically disrupted following treatment with HDAC inhibitors, thus allowing for increased HIV-1 transcription via chromatin modification (Van Lint et al., 1996). More recent studies revealed that multiple DNA-binding complexes could be responsible for the recruitment of HDACs to initiator and enhancer regions of the HIV-1 promoter, thereby inducing repressive effects on chromatin structure. HDAC1 is recruited by a
number of transcription factors to the 5'LTR, including (but not limited to) Late SV40 Factor (LSF) (He and Margolis, 2002), Activating Protein-4 (AP-4) (He and Margolis, 2002), NF-κB p50/p50 homodimers (Williams, Chen et al. 2006), C-promoter Binding Factor-1 (CBF-1) (Tyagi and Karn, 2007), and Sp1 (Jiang et al., 2007). Although four different classes of HDACs exist, class-I HDACs (HDAC1, 2, and 3) dominate at the HIV LTR in CD4+ cell line models (Keedy et al., 2009).

As previously noted, increasing HIV-1 transcription represents a possible strategy for eradicating latent provirus from HIV-1 infected patients. Initially, HDAC inhibitors held promise as good candidates for viral purging because they failed to promote global T cell activation yet could activate the virus. These cells could also act on a broad range of cellular types, not just T cells. However, a prominent concern is their ability to affect multiple biological processes, as reflected by cancer studies showing that HDAC inhibitors affect transcription of about 10% of cellular genes (Glaser et al., 2003).

Initial studies in patients using valproic acid (VPA), an HDAC1 inhibitor, were encouraging. When given to patients on intensified ART, VPA was reported to accelerate the reduction of HIV-infected resting T cells (Lehrman et al., 2005). Unfortunately, later reports did not demonstrate the same decay of infected resting T cells following VPA treatment (Archin et al., 2008; Sagot-Lerolle et al., 2008; Siliciano et al., 2007; Steel et al., 2006). Another more potent class-I HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), can
induce HIV-1 transcription in resting CD4\(^+\) T cells from patients on ART (Archin et al., 2009). In other studies, treatment with either VPA or SAHA results in synergistic activation of latent HIV in patient PBMCs when combined with the PKC agonist, prostratin (Reuse et al., 2009). In addition to prostratin, another PKC agonist, bryostatin, also had synergistic effects when used in combination with HDAC inhibitors (Perez et al., 2010). Combinatorial strategies, in which multiple pathways are targeted, are likely to be a more effective strategy for clearing the latent reservoir to levels that are necessary for achieving a cure.

### 1.4.5. DNA Methylation

Transcriptional regulation by DNA methylation is catalyzed by DNA methyltransferases and occurs largely at CpG dinucleotides. DNA methylation is associated with gene silencing and is present at high levels within heterochromatic regions of mammalian DNA (Razin and Cedar, 1977). CpG methylation occurs at the 5'LTR of HIV-1, and likely acts by preventing the binding of essential transcription factors such as NF-κB and Sp1 (Bednarik et al., 1991). In a study by Kauder et al., it was demonstrated that during latency the HIV-1 promoter is hypermethylated at two CpG islands surrounding the transcriptional start site in both J-Lat cell lines and in primary CD4\(^+\) T cells. One of the two methylated CpG islands is occupied by methyl-CpG binding protein 2 (MBD2) and HDAC2, thereby promoting a transcriptionally repressive state. Inhibition of DNA methylation with 5-aza-2'deoxyctydine (aza-CdR) prevents the
recruitment of MBD2 and HDAC2 and synergizes with NF-κB activators to promote a dramatic increase in viral gene expression (Kauder et al., 2009). Furthermore, in another study by Blazkova et al., CpG methylation of the HIV-1 5′LTR was shown to prevent reactivation in an in vitro model of latency and in memory CD4+ T cells from HIV-infected patients. This methylation pattern was responsible for the maintenance but not the establishment of HIV-1 latency (Blazkova et al., 2009). In addition, the combination of 5-aza-CdR and prostratin had a synergistic effect on HIV-1 reactivation. The use of DNA methylation inhibitors with PKC agonists or other drugs could therefore be an efficient strategy for eradicating the virus from HIV-1-infected patients.

1.4.6. MicroRNAs

MicroRNAs play an important role in antiviral defense and also have the potential to maximize viral gene expression. MicroRNAs are expressed by all multicellular eukaryotes and comprise 1-3% of expressed genes (Bartel, 2004). They represent a class of 20-25 nucleotide-long non-coding RNAs that modulate gene expression through base pairing between the seed sequence of the miRNA and its complementary seed match sequence (usually at the 3′ UTR of target mRNA) (Bartel, 2009). The biogenesis of miRNAs is well characterized (Figure 1.4). The miRNAs are transcribed by RNA polymerase II (Pol II) or RNA polymerase III (Pol III) as a long primary miRNA transcript (pri-miRNA) that forms distinctive hairpin structures. Then they are cleaved in the nucleus by the
microprocessor complex, Drosha-DGCR8, which generates a precursor hairpin (pre-miRNA) that ranges in length from 60 to 110 nucleotides. The pre-miRNA is transported to the cytoplasm by exportin-5-Ran-GTP, where Dicer, in complex with TRBP, removes its terminal loop to generate the miRNA duplex intermediate. The duplex is incorporated with argonaute (Ago2) into the RNA-induced silencing complex (RISC), allowing preferential strand separation of the mature miRNA. RISC is guided to the mRNA target to repress mRNA translation or destabilize mRNA transcripts through cleavage or deadenylation (Brodersen and Voinnet, 2009; Kim et al., 2009).

The miRNAs are highly conserved among species and act as key regulators in a wide variety of biological processes, including immune regulation and the response to viral infection. A number of miRNAs are known to be involved in modulating TCR signaling, thereby influencing protective immunity (Curtale et al., 2010; Li et al., 2007). T cell activation is a central feature of the adaptive immune response and it has been demonstrated that resting CD4+ T cells have a distinct miRNA profile following activation by anti-CD3 and anti-CD28 antibodies in vitro. Analysis by miRNA microarray showed that expression levels of several miRNAs were up-regulated in activated T cells, while other miRNAs were suppressed (Cobb et al., 2006). In addition, stimulation of naïve T cells resulted in major modifications to gene expression at the mRNA and protein level. Although more research is necessary to investigate the role of
miRNAs in this process, it is likely that induced or suppressed miRNAs regulate key factors involved in T cell activation.

Through cellular regulation, miRNAs can play important roles in virus-host cell interactions and have been shown to be involved in regulation of HIV-1. Studies performed by Triboulet et al. (Triboulet et al., 2007) demonstrated that knock down of Dicer and Drosha resulted in increased viral replication in PBMCs and Jurkat cells, suggesting that miRNAs may be involved in regulation of HIV-1. Based on this observation, they performed a microarray comparing infected and noninfected Jurkat cells, and found several miRNAs that were upregulated following infection. The authors focused on a poly cistronic miRNA cluster, miR-17~92, that was substantially decreased upon HIV-1 infection, and demonstrated that two miRNAs in this cluster (miR-20a and miR-17-5p) target the 3’ UTR of PCAF (cofactor for Tat transactivation of integrated HIV-1). This work provided the first evidence for the role of cellular miRNAs in the regulation of HIV-1 through host factors.

In 2007, Huang et al. (Huang et al., 2007) found that cellular miRNAs can regulate HIV-1 by directly targeting the 3’ UTR of HIV-1 mRNA in resting CD4+ T cells. These studies were carried out by transfecting distinct fragments of the HIV 3’ UTR (containing a GFP reporter) into resting CD4+ T cells. Following the transfection of a few of the fragments, GFP expression was substantially decreased. Bioinformatic analysis revealed putative miRNA-binding sites for these fragments. The authors also compared miRNA levels in resting and
activated CD4+ T cells, and determined which miRNAs were present in resting T cells that could target specific fragments of the HIV 3’ UTR based on seed sequence. Specific inhibitors of these miRNAs increased viral production in resting CD4+ T cells isolated from HIV-1-infected individuals on suppressive HAART. This suggests that manipulation of cellular miRNAs could be used to purge the HIV-1 reservoir.

More recently it has been shown that miR-155 exerts an anti-HIV-1 effect by targeting several HIV-1 dependency factors involved in post-entry, pre-integration events, leading to severely diminished HIV-1 infection in monocyte-derived macrophages (MDMs) (Swaminathan et al., 2012). In these macrophages, miR-155 expression is induced by polyinosine-polycytidylic acid (poly(I:C); a synthetic analog of dsRNA) and bacterial lipopolysaccharide (LPS), the ligands for Toll-like receptors (TLR) TLR3 and TLR4, which are known to decrease HIV-1 infection in MDMs. The use of a miR-155 specific inhibitor (but not a scrambled control) restored infectivity in poly(I:C)-stimulated MDMs. Our findings also find that miR-155 exerts an anti-HIV-1 effect, however we find that this block also occurs at the transcriptional level, by targeting the viral activator, TRIM32 in CD4 T cells thereby promoting HIV-1 latency.

MicroRNA-155 is a well-studied miRNA and is a known to play a role in the regulation of immune responses. It is derived from the non-coding transcript of the proto-oncogene bic (B-cell integration cluster) gene and represents the only evolutionarily conserved sequence of this gene, suggesting that miR-155 plays
an important role in bic function. Studies have shown that mir-155 controls differentiation of CD4 T cells into T helper type 1 (Th1), Th2, and Th17 subsets (O'Connell et al., 2010; Vigorito et al., 2007) and regulates the development of regulatory T (Treg) cells (Kohlhaas et al., 2009; Lu et al., 2009). miR-155 also regulates CD8 T cells and is necessary for normal B-cell differentiation (Lind et al., 2013) and antibody production (Vigorito et al., 2007). MicroRNA-155 expression is transiently induced following the activation of macrophages, dendritic cells, B cells, and T cells (Haasch et al., 2002; O'Connell et al., 2007; Thai et al., 2007), through the AP-1 and NF-κB transcription factors. Interestingly, miR-155 is induced upon EBV infection in primary B cells as part of the latency III program, resulting in the growth and transformation of B cells (Linnstaedt et al., 2010; Yin et al., 2008). While EBV induces the expression of miR-155, Kaposi’s Sarcoma-associated herpesvirus (KSHV) and Marek’s disease virus type 1 (MDV-1) encode viral mimics of miR-155 (Cullen, 2013). KSHV mir-K11 and MDV-1 miR-M4 both share the first eight nucleotides with miR-155, including the entire seed sequence critical for target regulation. Therefore, both miR-K11 and miR-M4 regulate seed targets of miR-155 (Gottwein et al., 2007). KSHV K-11 has been shown to phenocopy miR-155-induced B cell hyper-proliferation (Boss et al., 2011; Dahlke et al., 2012), while deletion of MDV-1 miR-4 abolished the induction of T cell lymphomas by MDV-1 in chickens(Zhao et al., 2011). Together these data demonstrate that mir-155 plays a critical role in viral regulation in several different disease contexts.
1.5. Functional Cure

Thirty years ago, the term “HIV cure” referred to the complete eradication of virus from an HIV-infected patient. Now, there is mounting evidence that a “cure” can be achieved that does not completely abolish the virus, but results in sustained remission in the absence of ART. Timothy Ray Brown, also known as “the Berlin patient”, is the first patient to be either completely or functionally cured of HIV. Brown was an HIV-positive patient who developed acute myeloid leukemia, and was treated for his cancer by stem cell transplantation from an individual homozygous for the CCR5 gene variant Δ32 (Allers et al., 2011). Individuals with this mutation express a nonfunctional truncated variant of the CCR5 coreceptor, and are therefore resistant to infection with CCR5-tropic viral strains. This treatment resulted in eradication of virus from his body without the need for any further ART in the 6 years since the therapy. Although there are reports that trace amounts of virus have been found in his blood (Yukl et al., 2013), it appears he has at least been functionally cured. This unique case proves the concept that HIV infection can be effectively controlled by a boosted immune response, and the likelihood of transmission diminished without the necessity of lifelong antiretroviral therapy. Along these lines, recent studies have identified 14 HIV patients (Visconti cohort) who maintained long-lasting control of viremia for several years following the interruption of ART (Saez-Cirion et al., 2013). These 14 patients were distinct from elite controllers in that they lacked
protective HLA alleles and did not develop strong HIV-specific T-cell responses. However, these patients initiated ART during the very early primary stage of HIV infection. In contrast, most patients begin treatment regimens during the later chronic stage of HIV infection. Follow-up studies showed that following the interruption of therapy in these individuals, long-lived resting CD4⁺ T cells represented only a minor population within the total viral reservoir compared to patients who initiated therapy later. These individuals provide additional evidence that a functional cure is possible, and that treatment during the early stage of infection may significantly decrease the formation of viral reservoirs in longer-lived memory cells.

Another case that was originally thought to support the concept of very early treatment was the report of a baby girl from Mississippi infected with HIV either in utero or during birth. She received three antiretroviral drugs within 31 hours of birth and then continued therapy for the first 15 months of her life, until her mother stopped administering drugs for several months. Surprisingly, for 27 months, the child did not exhibit a viral rebound nor any detectable virus in her blood. However, after 27 months, doctors discovered that the virus had begun to replicate and the child was once again put on ARTs. The disappointing outcome of this case has dampened hopes that early and aggressive treatment might lead to a cure. Even so, this case remains important because it demonstrates that early and aggressive treatment does indeed prevent the virus from replicating. Although this case is less relevant in developed countries where
expectant mothers with HIV routinely receive treatment to prevent transmission, it is highly relevant for developing regions like Sub-Saharan Africa where it is more common for untreated HIV-positive mothers to give birth. This particular study casts doubts on the idea that a “cure” may not require a complete viral purge, but instead may be achieved functionally by minimizing the size of the latent reservoir via early and aggressive antiretroviral treatment. It is possible that a cure may only be attained through elimination of the entire population of the latent proviruses. If this is the case, it is possible that we are in fact further to a cure than previously thought.
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Figure 1.1: HIV-1 Infection and Reactivation of CD4+ T Cells

HIV-1 infects activated CD4+ T cells, which have increased nucleotide pools, cytokines, and transcription factors compared to non-activated cells. Most of these infected cells die due to cytopathic effects of the virus or lysis by HIV-specific CTLs. Cells that revert back to a resting memory T cell survive and may undergo homeostatic proliferation. Upon initiation of ART these latently infected cells persist. However, treatment of these cells with reactivating agents causes the cells to actively produce virus and ultimately leads to either spontaneous cell death or death through immune system clearance.
Sequestration of essential transcription factors, like NFAT and NF-κB, in the cytoplasm leads to silencing of viral gene expression. Two major mechanisms of transcriptional interference occur in latently infected cells. In promoter occlusion, the host promoter is positioned upstream of the provirus and the host RNA Pol II reads through the HIV-1 LTR, causing displacement of necessary transcription factors. In convergent transcription, provirus and the host gene are in the opposite orientation, leading to collision of the RNA Pol II complexes. Levels of CyclinT1, which forms P-TEFb and is important for HIV-1 transcription and Tat transactivation, are low in resting CD4+ T cells. DNA methylation and restrictive chromatin structures contribute to transcriptional silencing leading to HIV-1 latency.
Following cellular activation or drug treatment NFAT and NF-κB translocate to the nucleus and bind sites at the HIV-1 LTR. NFAT and NF-κB recruit p300/CBP to the LTR, resulting in acetylation of histone tails and transcriptional activation. In the case of NF-κB, proteosomal degradation of IκBα permits NF-κB translocation and displacement of the p50 homodimers. This is followed by Tat-dependent elongation, in which Tat recruits the P-TEFb complex to TAR. Cdk9 phosphorylates the CTD of RNA Pol II, resulting in increased processivity. P-TEFb phosphorylates DSIF and NELF, resulting in removal of NELF from Pol II, converting DSIF into a positive elongation factor, thereby promoting productive elongation.
Figure 1.4: Biogenesis of miRNAs in mammals

Pri-miRNAs are transcribed by Pol II and cleaved by the microprocessor, Drosha-DGCR8, to produce a hairpin structure called the pre-miRNA. Following translocation to the cytoplasm by exportin, the pre-miRNA is further processed by Dicer. This results in a duplex of two ~22-nt RNAs. The weaker 5’-base pairing is preferentially loads as the mature miRNA into Ago2, while the miRNA* strand is degraded. RISC complexes are guided to the target mRNA for destabilization and/or repression of translation.
Chapter Two:

microRNA-155 Reinforces HIV Latency by Downregulating the TRIM32 Viral Activator
2.1. Introduction

2.1.1. The challenge of HIV eradication

With the advent of combination ART, hopes were high that these new and potent agents might cure HIV-infected subjects (Perelson et al., 1997). However, despite consistently reducing viral loads to undetectable levels, a cure was not achieved (Chun et al., 1995) because of the persistence of latent but integrated HIV proviruses in a drug-insensitive reservoir formed at least in part by memory CD4 T cells. Considerable effort is now focused on devising ways to attack this latent reservoir. One approach termed “shock and kill” involves the identification of a cocktail of inducing agents used in conjunction with ART to activate the latent virus. Virus activation will either lead to the spontaneous death of these cells due to cytopathic effects of the virus or these cells will be cleared by the immune system. One clear challenge is to effectively induce the activation of latent proviruses without also activating the host cell to levels that produce a toxic cytokine storm.

The elimination of HIV latency is in its simplest terms a transcriptional problem. HIV transcription is controlled by the long terminal repeat (LTR) that contains multiple binding sites for cellular transcription factors, such as NF-κB, NFAT, Sp1, and AP1 (Colin and Van Lint, 2009). NF-κB-inducing agents like TNFα and prostratin are known to be strong activators of HIV-1 transcription (Williams et al., 2004) but in keeping with the pleiotropic effects of NF-κB, these agents alter the expression of many cellular genes.

The study of HIV latency in vivo is complicated by the rarity of latently infected cells and a current inability to purify such cells. Many studies have relied heavily on
cell-line models of chronic HIV infection. One such model is the J-Lat model of post-integration latency developed by infecting Jurkat cells using recombinant 

envelope-deleted virus containing a GFP reporter (Jordan et al., 2003). Infected cells were then screened for latently infected cells that were transcriptionally inactive in the absence of cellular stimulation. Transcription of the HIV genome could be induced by a number of biological and chemical activating agents including NF-κB activators like PMA, prostratin, PHA, and TNFα. However, more physiological activation via the TCR complex with anti-CD3 antibodies were ineffective, likely because of downregulation of TCR complex components. We recently developed an additional clonal cell line, J-Lat 5A8, that retains surface CD3 expression and responds to TCR stimulation (Chan et al., 2013). Of note, when responsiveness of a variety of latency cell models including those formed in primary CD4 T cells were compared to resting CD4 T cells from aviremic patients, J-Lat 5A8 cells along with a primary CD4 T cell model developed by Dr. Sharon Lewin’s laboratory displayed strong similarity to the activation profile exhibited by patient cells (Spina et al., 2013). These data also suggest that the cell signaling pathways within J-Lat 5A8 cells are similar to ex vivo cells from patients. In the current study we have employed J-Lat 5A8 cells and primary CD4 T cells to study select aspects of the regulation of HIV latency including how CD4 T cells retreat back into latency after a transient burst of virus production.

2.1.2. Role of miRNAs in regulation of HIV

MicroRNAs (miRNAs) are evolutionarily conserved RNAs that regulate a wide
variety of biological processes including immune regulation and the response to viral infection. These miRNAs correspond to 20-25 nucleotide-long non-coding RNAs that modulate gene expression through base pairing of the miRNA seed sequence to its target mRNA (usually located within the 3' UTR). This interaction leads to either translational repression or mRNA cleavage thereby reducing the final amount of target protein produced. In the context of HIV-1 infection, host miRNAs have been shown to inhibit HIV through cellular regulation of PCAF (Triboulet et al., 2007), cyclin T1 (Chiang et al., 2012), as well as other HIV-1 factors involved in trafficking and/or importing pre-integration complexes into the nucleus (Swaminathan et al., 2012). Cellular miRNAs also regulate HIV-1 by directly targeting the 3' UTR of HIV-1 mRNA (Huang et al., 2007; Nathans et al., 2009). Whether miRNAs regulate viral latency is unknown. In this study, we identify multiple miRNAs that inhibit HIV-1 reactivation and uncover a novel miRNA-target interaction that reinforces latency in infected cells.

2.1.3. TRIM32

Tripartite motif-containing (TRIM) proteins are E3 ubiquitin ligases containing a RING finger domain, one or two B-box domains, and a coiled coil region. TRIM32, a member of the TRIM-NHL family (named after the NCL-1, HT2A, and LIN-41 proteins), contains a C-terminal domain believed to mediate protein binding. Specifically, the NHL domain of TRIM32 binds to Ago1, which activates certain miRNAs required for neural differentiation (Schwamborn et al., 2009). In addition, TRIM32 regulates the induction of type I IFNs and the cellular antiviral response by activating STING via K63-linked ubiquitination (Zhang et al., 2012). Interestingly, TRIM32 expression also activates NF-
κB (Albor et al., 2006). A more recent report demonstrates that certain TRIM proteins (including TRIM32) that induce NF-κB also promote HIV-1 LTR expression (Uchil et al., 2013). These studies highlight the importance of TRIM32 in NF-κB-mediated transcriptional activation of HIV-1. However, it is unknown whether TRIM32 plays a role in NF-κB signaling in a manner that antagonizes HIV latency. In this study we demonstrate that miR-155 reinforces HIV latency by inhibiting the expression TRIM32, which normally induces NF-κB via a novel mechanism. This effect likely contributes to the reestablishment of viral latency in transiently activated CD4 T cells.

2.2 Results

2.2.1 Knockdown of Enzymes Involved in miRNA Biogenesis Results in Increased Activation of Latent HIV

Because various miRNAs block HIV replication, we hypothesized that select miRNAs promote HIV latency in infected CD4 T cells. To test this possibility, miRNA-deficient J-Lat 5A8 cells were prepared by knocking down the expression of two enzymes required for miRNA biogenesis, DGCR8 and Dicer. Knockdown was achieved by introduction of an shRNA lentiviral vector (pSicoR-MS1) containing an mCherry reporter into the J-Lat 5A8 cells. The mCherry-positive cells, which were successfully infected with the lentivirus were isolated by fluorescent cell sorting followed by qRT-PCR to assess target gene expression. Knockdown of both DGCR8- and Dicer exceeded 90% in the mCherry positive 5A8 cells compared to levels obtained in cells receiving a negative control (5A8 shScramble) (Figure 2.1A). Additionally, DGCR8 and Dicer levels were much lower in the knockout cells than in cells receiving no shRNA.
and further each knockdown appeared specific as expression of the counterpart microRNA biogenic enzyme was not affected nor was the expression of GAPDH altered (Figure 2.1B). To assess the effects of diminished miRNAs on HIV reactivation, the infected cells were cultured alone or stimulated with anti-CD3/CD28 or TNFα. Both DGCR8- and Dicer-knockdown cells exhibited significantly greater increases in HIV-1 reactivation with both agonists as demonstrated by increased GFP expression, compared to the shRNA scramble control knockdown (Figure 2.1C). We also tested several different shRNA controls in addition to the shRNA scramble negative control (Figure 2.7). These findings indicate that taken as whole, microRNAs generally act to reinforce HIV latency.

We next sought to identify the specific miRNAs that support HIV latency using a TaqMan Low Density Array (TLDA) for profiling of 754 unique human miRNAs. We identified miRNAs that were differentially expressed between activated or latent cells (Table 2.1) and validated top candidates in individual TaqMan assays (Figure 2.8).

2.2.2. Introducing miR-155 into Dicer-deficient Cells Decreases the Level of Reactivation

To determine whether candidate miRNAs inhibited HIV-1 reactivation, each candidate miRNA was reintroduced into Dicer-deficient cells. Whereas knocking down Dicer doubled the level of reactivation in stimulated cells (Figure 2.1C), introducing the top four candidate miRNAs from both GFP-positive and GFP-negative cells countered this effect restoring a lower level of reactivation compared to cells receiving a negative control miRNA mimic (Figure 2.2A). Introducing miR-155 produced the greatest decline
in HIV-1 reactivation (78.3% rescue) (Figure 2.2A), occurring in a dose-related manner (Figure 2.2B). Two of the miRNAs tested—miR-1290 and miR-505*—did not significantly affect reactivation levels, suggesting that this effect is specific and does not occur with introduction of every miRNA. In addition, we observed the same effect when we introduced some of these candidate miRNAs into the shScramble control, excluding the possibility that this effect is specific to cells with decreased levels of Dicer (Figure 2.9). To further evaluate miR-155 levels when cells were stimulated, we examined miR-155 expression in individual TaqMan assays. MiR-155 was expressed at 50-fold higher levels in GFP-positive cells (~50-fold more) compared to unstimulated cells. MiR-155 levels were also increased in GFP-negative cells following stimulation of the culture, but to a lesser extent (Figure 2.2C). Together, these findings suggest that specific miRNAs that are abundant in reactivated cells serve to counter viral activation and to perhaps promote a return to latency in cells undergoing transient production of virus. Conversely, in resting cells, miR-155 helps maintain the latent state.

To determine the levels of miR-155 in a primary CD4 T lymphocyte model of HIV-1 latency, we employed a dual-fluorescence model, HIV-DuoFluo, recently reported by the Verdin laboratory (Calvanese et al., 2013). In this model, HIV-DuoFluo (R7GEmC) expresses GFP under control of the HIV promoter and mCherry under control of the constitutive promoter EF1α. Upon integration of this virus into CD4 T cells, mCherry is constitutively expressed and marks all cells that became infected by the reporter virus. Active viral expression occurs in a smaller subset of cells leading to expression of GFP. Latently infected cells are identified by the expression of mCherry coupled with the absence of GFP expression. CD4 T cells from two blood donors
(Donor 9600, and Donor 9601) were infected with R7GEmC and sorted for uninfected cells, latently infected cells (mCherry-positive, GFP-negative), and reactivated cells (mCherry-positive, GFP-positive). Both donors exhibited high levels of miR-155 in cells that were stimulated but uninfected. This finding is consistent with previous observations indicating that the activation of both lymphoid and myeloid cells leads to upregulation of miR-155 (Haasch et al., 2002; O'Connell et al., 2007). Levels of miR-155 were much higher in reactivated cells (mCherry-positive, GFP-positive) than in latently infected cells (mCherry-positive, GFP-negative) (Figure 2.2D). These findings suggest that miR-155 may principally act within reactivated cells while also reinforcing viral latency in unstimulated cells harboring virus. Interestingly, miR-29b levels did not change in any of the tested cell populations. This microRNA has been implicated in the regulation of cyclin T1 (Chiang et al., 2012).

2.2.3. MicroRNA-155 Directly Targets TRIM32

Next, we sought to identify the key host genes whose action was suppressed by miR-155 using the TargetScan prediction algorithms. These analyses identified TRIM32 as a high-value hit because it had a favorable aggregate P_CT and context+ score. In addition, the 3’UTR of TRIM32 contains an 8mer site that precisely matches the seed region (positions 1-8) of miR-155 (Figure 2.3A). Furthermore, TRIM32 represented an intriguing potential target because of a prior report of its direct binding to the activation domain of Tat, a transactivator of HIV transcription (Fridell et al., 1995). To study whether miR-155 directly binds to the 3’UTR of TRIM32, the predicted target site was cloned downstream of a Renilla luciferase reporter construct. In this
assay, a decrease in luciferase activity in the presence of miR-155 demonstrates binding of the miRNA and subsequent inhibition of luciferase expression. To further assess the specificity of this response, Renilla luciferase reporters containing either a mutated version of the highly conserved TRIM32 seed region or a perfect match over the entire miR-155 binding region were prepared and tested in parallel in HeLa cells (Figure 2.3A). As expected, miR-155 inhibited luciferase expression of the reporter containing a precise binding site match for miR-155 but did not inhibit luciferase expression when either the mutant TRIM32 seed region or empty vector was tested. When the construct containing the predicted TRIM32 binding site was transfected with miR-155, luciferase levels dropped significantly (Figure 2.3B). These results confirm that the 3’UTR of TRIM32 is a direct target of miR-155.

Studies were next performed to assess miR-155 effects on endogenous TRIM32 levels in J-Lat 5A8 cells. The introduction of miR-155 decreased levels of TRIM32 mRNA in these cells by ~70%, compared to a negative-control miRNA (Figure 2.3C). Conversely, TRIM32 mRNA levels increased 4-5 fold in J-Lat 5A8 cells following stimulation with anti-CD3/CD28 antibodies or TNFα (Figure 2.10). The endogenous targets of miR-155 within the entire murine transcriptome have been recently analyzed using AGO differential HITS-CLIP (dCLIP) (Loeb et al., 2012). These studies revealed that the putative miR-155 binding site in the TRIM32 3’UTR is in fact bound by the miR-155-AGO complex in wild-type CD4 T cells, but not in miR-155−/− cells (Figure 2.3D; image obtained from CLIP Base http://cbio.mskcc.org/~aakhan/clipseq/). Together, these findings provide strong evidence supporting TRIM32 as a bona fide cellular target of miR-155.
2.2.4. TRIM32 Regulates HIV-1 Latency

Next we assessed the effects of TRIM32 shRNA knockdown in latently infected J-Lat 5A8 cells. After infecting cells with a lentivirus encoding TRIM32 shRNA, TRIM32 mRNA and protein levels were both reduced by ~40% (Figure 2.4A and 2.4B). Conversely, TRIM32 mRNA and protein levels were not reduced in cells receiving a scrambled control shRNA (Figure 4A and 4B). In agreement with one or more miRNAs postranscriptionally regulating TRIM32 expression, TRIM32 protein, but not mRNA, was increased when Dicer was knocked down (Figure 2.4A and 2.4B). Activation of J-Lat 5A8 cells with anti-CD3 and anti-CD28 antibodies resulted in decreased GFP expression when TRIM32 expression was knocked down but increased expression following Dicer knockdown (Figure 2.4C). These findings suggest that TRIM32 promotes reactivation of latent HIV-1.

Next, we examined the effects of lentiviral-mediated overexpression of TRIM32 (pCDH-TRIM32) in J-Lat 5A8 cells. By both qRT-PCR and immunoblotting TRIM32 levels were significantly higher in pCDH-TRIM32 cells compared to cells infected with the empty lentiviral vector (pCDH) (Figure 2.4D and 2.4E). Stimulation with anti-CD3/CD28 and TNFα resulted in increased levels of latent virus reactivation in cells expressing TRIM32 (Figure 2.4F). TRIM32 also stimulated HIV-1 reactivation in the absence of anti-CD3/CD28. Together, these findings confirm TRIM32 as an effective host antagonist of HIV latency.
2.2.5. TRIM32 Promotes Reactivation of Latent HIV-1 by Stimulating NF-κB Signaling

TRIM32 has been reported to interact with the activation domain of HIV-1, HIV-2 and EIAV Tat proteins (Fridell et al., 1995). To assess whether the activating effects of TRIM32 on latent HIV required the presence Tat, TRIM32 was expressed in J-Lat A72 cells that lack Tat (Jordan et al., 2001). TRIM32 effectively induced latent provirus expression alone or in combination with anti-CD3/CD28 antibodies or TNFα (Figure 2.5A). These results indicate the TRIM32 is able to function in a Tat-independent manner. Recent studies have shown that TRIM32 can induce NF-κB in 293 cells (Uchil et al., 2013; Zhang et al., 2012). We examined whether TRIM32 activates NF-κB using Jurkat κB-dsRed cells, which contains a basal promoter and five κB enhancer elements controlling expression of a dsRed reporter (Chan et al., 2013). When TRIM32 was expressed in these cells, the level of dsRed expression increased compared to the empty vector control in both the absence and presence of stimulation (Figure 2.5B). These findings confirm the NF-κB-inducing activity of TRIM32.

Next, nuclear and cytoplasmic extracts from TRIM32-expressing cells were immunoblotted. In the absence of concomitant PMA and ionomycin stimulation, TRIM32 expression was associated with an increase in nuclear RelA expression and a decrease in cytoplasmic IκBα levels (Figure 2.5C). Based on the pattern of nuclear Sp1 and cytoplasmic IκBα, nuclear and cytoplasmic fractions were of high quality (Figure 2.5C). To confirm TRIM32-induced nuclear translocation of NF-κB, electrophoretic mobility shift assays (EMSA) were performed with nuclear extracts and ³²P-labeled κB and Oct1 DNA probes. Unstimulated cells expressing TRIM32 displayed
increased binding activity in the form of a more slowly migrating complex (p50/RelA heterodimer) that was fully competed with unlabeled wild type κB probe but not by a mutant κB probe. (Figure 2.5D). In contrast, Oct1 DNA binding did not change in the presence or absence of TRIM32. Together, these results indicate that TRIM32 expression induces NF-κB activation in CD4 T cells.

To further investigate the mechanism through which TRIM32 induces NF-κB, an IκB kinase (IKK) assay was performed (Figure 2.5E). Unexpectedly, the same level of IKK activity was found in cells expressing TRIM32 versus the negative control. However, brief stimulation with PMA and ionomycin induced increased IKK activity. Furthermore, there was no evidence of phosphorylated IκBα in the lysate of cells expressing TRIM32 (Figure 2.5E). These findings suggest that TRIM32 acts downstream of the IKKs in the NF-κB pathway. Since our prior experiments indicated that TRIM32 expression promotes degradation of IκBα, we hypothesized that TRIM32 directly binds to and ubiquitinates IκBα. To test this possibility, TRIM32 and IκBα interaction was tested in cells expressing HA-tagged IκBα and Flag-tagged full-length TRIM32 or Flag-tagged mutant TRIM32 containing a deletion in the RING domain (TRIM32ΔRING), thereby removing its E3 ubiquitin ligase activity. When anti-TRIM32 antibodies were used in the immunoprecipitations, both IκBα and the IκBα superrepressor containing Ser32/36Ala mutations corresponding to the key IKK phosphoacceptor sites (Kwon et al., 1998) were coimmunoprecipitated. However, when anti-IκBα antibodies were used, lower amounts of the TRIM32ΔRING protein were coimmunoprecipitated compared to wild type TRIM32 (Figure 2.5F). The ability of TRIM32 to ubiquitinate IκBα was next tested. In the presence of the β-lactone
proteasome inhibitor, full-length TRIM32 promoted ubiquitination of IκBα while TRIM32ΔRING did not. In addition, TRIM32 also promoted ubiquitination of the IκBα super-repressor, but at slightly lower levels compared to wild type IκBα (Figure 2.5G). To confirm these findings, we assayed the ability of recombinant TRIM32 (wild type versus catalytic-dead mutant) to ubiquitinate radiolabeled IκBα. Indeed, only wild type TRIM32 enabled ubiquitination of IκBα and not the I22E mutant (Figure 2.5H). Together, these results suggest that TRIM32 bypasses many of the usual upstream NF-κB signaling steps by directly binding to and ubiquitinating IκBα.

2.3 Discussion

Achieving a cure for HIV will require both the complete suppression of active viral replication in vivo and the clearance of the transcriptionally silent proviral latent reservoir. Current antiviral drugs effectively target the active virus but have no effect on the latent reservoir. One potential approach for attacking the latent reservoir is to identify combinations of agents that activate latent proviral transcription without inducing full T cell activation. These agents would promote viral replication under the cover of ART without inducing a toxic cytokine storm (Archin and Margolis, 2014). Attacking the latent reservoir is further complicated by the fact that only a fraction of the latent proviruses appear to respond to these inducing agents (Ho et al., 2013). The variegated nature of the response suggests that repeating cycles of induction will be required. The half-life of productively infected cells is usually quite short often measuring less than 24 hours (Ho et al., 1995). However, recent studies suggest that induction of virus production in the latent reservoir does not result in the death of the
virus producing memory T cells (Shan et al., 2012). The production of viral proteins however does render this population of cells transiently visible to the immune system and thus potentially vulnerable to elimination by cytotoxic T cells (Descours et al., 2012; Shan et al., 2012).

Latently infected cells do not appear to remain continuously latent. Rather, these cells are intermittently stimulated by cytokines or other uncharacterized signals to produce a burst of virus sometimes culminating in a visible “blip” in the viral load (Nettles et al., 2005). Furthermore, low levels of virus persist in subjects despite ART (Dornadula et al., 1999) apparently reflecting intermittent production of virus by cells within the latent reservoir. However, after producing virus, these cells appear able to retreat back to a quiescent state with reestablishment of viral latency.

We have explored the potential involvement of miRNAs as key regulators of HIV latency. This notion was reinforced when knockdown of two essential enzymes required for miRNA biogenesis—DGCR8 and Dicer—was shown to antagonize HIV latency. These findings are also consistent with prior reports describing an overall increase in HIV replication following knockdown of Drosha and Dicer in multiple cell lines and PBMCs (Nathans et al., 2009; Triboulet et al., 2007). Our findings suggest that miRNAs predominantly promote inhibition HIV-1 replication and thus function as pro-latency factors. However, some individual miRNAs may promote viral reactivation by blocking the action of inhibitory transcription factors.

We now describe a cellular pathway involving miR-155 and one of its many cellular targets, TRIM32, that appears to promote a return to latency in reservoir cells transiently producing virus. MiR-155, which is expressed at high levels in activated
cells, impairs the expression of TRIM32, which normally serves as an HIV-activating agent. TRIM32 activates latent HIV by stimulating nuclear translocation of NF-κB. However, our studies reveal that TRIM32 activates NF-κB in a novel manner involving direct ubiquitination of IκBα. Specifically, TRIM32 induction of NF-κB proceeds independently of IκB kinase (IKK) activation within signalosomes. Our studies of the potential role of microRNAs in the regulation of HIV latency have led to the identification of miR-155 and its inhibition of TRIM32 activation of NF-κB as events that promote the reestablishment of HIV latency in reservoir cells undergoing transient viral production.

We focused on miR-155 in this study because, compared with other miRNAs, miR-155 most potently blocked HIV-1 reactivation in J-Lat 5A8 cells. As noted previously, amongst all of the continuous T cells lines, J-Lat 5A8 cells most closely mirrors the pattern of responsiveness found in latently infected cells from patients suppressed with ART (Spina et al., 2013). MiR-155 levels are higher in activated than in resting T cells (Banerjee et al., 2010; Haasch et al., 2002; Rodriguez et al., 2007; Thai et al., 2007). Prior studies have implicated miR-155 in the regulation of cellular proteins involved in trafficking and/or nuclear import of HIV pre-integration complexes including ADAM10, TNFO3, Nup153, and LEDGF/p75 (Swaminathan et al., 2012). Our findings now extend the effects of miR-155 to a post-integration level where it acts to promote maintenance of the latent reservoir by promoting a reestablishment of latency.

MiR-155 is known to play a key role in the regulation of various viruses. For example, EBV strongly induces the expression of miR-155 leading to increased growth and transformation of B cells (Linnstaedt et al., 2010; Lu et al., 2008). In addition,
Kaposi’s Sarcoma-associated herpesvirus (KSHV) and Marek’s disease virus type1 (MDV-1) encode viral mimics of miR-155, which are thought to be involved in oncogenic transformation of infected cells (Gottwein, 2013). KSHV miR-K11 and MDV-1 miR-M4 both contain the full identical seed sequence of miR-155, and therefore regulate many of the same cellular targets (Gottwein et al., 2007; Skalsky et al., 2007). Furthermore, miR-155 regulates a variety of targets involved in mammalian immunity (So et al., 2013). For example, during thymic differentiation, increases in miR-155 promote T regulatory cell fitness and proliferative potential by targeting suppressor of cytokine signaling 1 (Socs1) (Lu et al., 2009). Also, miR-155 is required for the development of inflammatory Th17 cells in experimental autoimmune encephalomyelitis (EAE) (Murugaiyan et al., 2011; O'Connell et al., 2010). However, the precise role of miR-155 in immune processes is not well understood because miR-155, like many miRNAs, interacts with a large number targets, some of which exert opposing actions.

Although cellular miRNAs may be capable of directly targeting HIV (Nathans et al., 2009), our focus was on uncovering host gene products that participate in the regulation of HIV-1 latency. In general, miRNAs are regarded as fine-tuners of gene expression (Bartel and Chen, 2004). Having identified miR-155 as the most HIV-repressive microRNA that was induced in our analysis, we used TargetScan (Lewis et al., 2003) for in silico prediction of its cellular targets. Through this process we identified TRIM32 as a potential miR-155 target and experimentally confirmed its ability to interact with 3’UTR sequences in the TRIM32 mRNA and suppress its expression. Of note, the Rudensky lab performed experiments with 12 miR-155 wild-type mice and
12 miR-155 knockout mice identifying miR-155 target genes in CD4 T cells (Loeb et al., 2012). When we searched for TRIM32 on their CLIP base database, we found that the evolutionarily conserved TRIM32 binding sequence did bind to an AGO-miR-155 complex in vivo. Taken together, these findings confirm TRIM32 as a bona fide miR-155 host gene target.

As we predicted, when levels of TRIM32 were reduced with shRNA, lower reactivation of latent HIV was observed. Conversely, when TRIM32 was overexpressed in cells harboring latent provirus, increased viral activation was detected. Notably, TRIM32 expression was sufficient to induce reactivation, even in the absence of other stimuli. These findings may be relevant in the context of aviremic patients on HAART who commonly experience intermittent biological and statistical fluctuations in levels of viremia, termed blips (Nettles et al., 2005). It is possible that the brief episodes of viral release are related to changes in the levels of miR-155 expression, which in turn could result in decreased TRIM32 expression and resiencing of the virus. Along these lines, elevated miR-155 levels during “shock and kill” may also be responsible for suppressing latent reactivation, thereby decreasing viral production and allowing the cell to remain undetected by the immune system.

A prior study indicated that TRIM32 interacts with Tat but did not identify any functional consequences of this interaction. While we certainly do not exclude a role for TRIM32-Tat interaction in HIV biology, our findings indicate that TRIM32 functions independently of Tat. Specifically, we have shown in biochemical and functional experiments that TRIM32 expression promotes nuclear NF-κB translocation and engagement of the cognate κB enhancer sites present in the HIV-1 LTR. Consistent
with our findings is a recent report demonstrating that a number of TRIM proteins including TRIM32 are capable of inducing the expression of NF-κB, AP-1, and activating the HIV-1 LTR in HEK293 cells (Uchil et al., 2013). But contrary to our findings, in that study knocking down TAK1 (upstream of IKK) decreased the level of NF-κB induction produced by eight different TRIMs (including TRIM32). Surprisingly, we observed that TRIM32 expression did not activate the IKK complex. Instead, TRIM32 appeared to activate NF-κB by direct ubiquitination of IκBα promoting release and nuclear translocation of the NF-κB heterodimer. We identified IκBα as a novel target of the E3 ligase TRIM32.

We found that TRIM32 can directly ubiquitinate IκBα *in vitro*, whereas a single point mutation (I22E) that abrogates the activity of ubiquitin transfer without destabilizing the overall fold of the RING domain prevents IκBα ubiquitination. Although there are over 600 human RING-based E3 ligases that control many cellular processes, we have only a rudimentary understanding of their functions, substrates, and mechanism of action (Deshaiies and Joazeiro, 2009). In the canonical NF-κB pathway ubiquitination of IκB is carried out by the SCF-βTrCP E3 ligase (SKP1-CUL1-F-box ligase containing the F-box protein βTrCP) (Spencer et al., 1999; Winston et al., 1999; Yaron et al., 1998). We propose a novel form of NF-κB activation, independent of IKK activation, in which TRIM32 (and perhaps other TRIMs) directly binds to and ubiquitinates IκBα.

It is important to note that TRIM32 targets a number of substrates involved in diverse pathways (Albor et al., 2006; Ryu et al., 2011; Schwamborn et al., 2009; Zhang et al., 2012), and modulation of these pathways may also promote NF-κB signaling.
For example, TRIM32 targets STING for K63-linked ubiquitination, thereby promoting its activation and subsequent induction of NF-κB and production of IFN-β in response to Sendai virus and Herpes Simplex Virus 1 infection (Zhang et al., 2012). This suggests that TRIM32 (via STING) plays an important role in the innate immune response against these viruses. However, in the context of HIV-1, activation of the NF-κB pathway by TRIM32 leads to the production of virus. It seems likely that the suppressive interplay of the inducible \( \text{miR-155} \) with the similarly inducible TRIM32 was designed as a means to control the overall cell stimulatory effects of TRIM32. In the case of HIV, inhibition of TRIM32 by \( \text{miR-155} \) has the untoward effect of promoting a return of viral latency thereby providing a mechanism for HIV persistence in infected hosts (Figure 2.6).

It is clear that a better understanding of the mechanisms governing both the maintenance of latency and the return to latency in transiently activated reservoir cells is needed if we are to mount a successful attack on the latent reservoir. Indeed, it might be easier to prevent a reservoir cell from returning to latency than to rouse a truly latent provirus from its transcriptional torpor. For example, if we sustain the action of TRIM32 either by antagonizing \( \text{miR-155} \) or by other means, these cells would be unable to retreat back to a latent state. It is possible that they would spontaneously die. However, in the absence of such death, they would certainly be visible to the immune system and thus vulnerable to CTL attack. Assuming that the entire latent reservoir participates in the production of intermittent bursts of virus production, an intervention aimed at preventing the subsequent retreat of these cells back into latency could over time lead to degradation of the latent reservoir.
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Table 2.1: TLDA Identifies miRNAs Involved in HIV-1 Latency

J-Lat 5A8 cells were stimulated with αCD3/CD28 and sorted for either GFP-positive (reactivated) or GFP-negative cells (latent) cells. TLDA was performed, and fold-changes were calculated using the ΔΔCt method. Differences in expression between latent and activated cells were analyzed using moderated t-statistics. Samples in bold indicate the top candidates that were individually validated and carried into future experiments. RNU6 was used as a housekeeping control. Data represent four independent experiments.

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Figure 2.1: Stable Knockdown of DGCR8 and Dicer Promotes HIV-1 Reactivation
A. Knockdown of DGCR8 and Dicer mRNA in J-Lat 5A8 cells that were infected with lentiviruses expressing shRNAs as measured by qRT-PCR. Values represent +SEM, **p<0.01; *p<0.05 (two-tailed t-test), n=3.
B. Knockdown as described in (A) measured by immunoblotting.
C. Increased reactivation of stable DGCR8 and Dicer knockdowns assessed by FACS 24h post-stimulation. +SEM, ***p<0.001 (two-way ANOVA), n=3.
Figure 2.2: Introduction of miR-155 into Dicer-deficient Cells Reduces the Level of HIV-1 Reactivation

A. J-Lat 5A8 shDicer cells were nucleofected with candidate miRNA mimics (or a negative-control mimic) and stimulated with αCD3/CD28 24h-post nucleofection. Cells were then analyzed for GFP by FACS ~16h post-stimulation. J-Lat 5A8 shScramble cells were nucleofected with a negative-control mimic. Data shown as percentage decrease compared with shDicer negative-control mimic. +SEM, *p<0.05; ***p<0.001 (one-way ANOVA), n=5—20.

B. Introduction of increasing concentrations of mir-155 mimic increases latent reactivation. J-Lat shDicer cells were nucleofected with either a negative control miRNA mimic or with a mir-155 mimic at various concentrations. Cells were stimulated with with αCD3/CD28 24h-post nucleofection, and analyzed for GFP by FACS ~16h post-stimulation. +SEM, ***p<0.001 (Two-way ANOVA), n=3.

C. J-Lat 5A8 cells were stimulated with αCD3/CD28 and sorted for GFP at 24h. Unstimulated, GFP-negative, and GFP-positive cells were analyzed for miR-155 levels by qRT-PCR. +SEM (ANOVA), n=3.

D. miR-155 is increased in non-infected and infected/reactivated cells, but not in latently infected cells. CD4 T cells from two donors were activated, and latent and reactivated cells were sorted using a dual-reporter virus, then analyzed by qRT-PCR.
A) miRNAs Upregulated in GFP+ Cells

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B) Negative Control miRNA

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<th>miRNA Mimic</th>
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C) Fold Increase in miR-155

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D) miRNA Levels for Donor 9600

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Figure 2.3: miR-155 Directly Targets TRIM32

A. Prediction of miR-155's binding site. The TRIM32 3'UTR site is predicted to bind miR-155 perfectly at the seed region. This site is well conserved among many mammals. The TRIM32 mutant 3'UTR contains a mutated seed region.

B. Luciferase binding assay shows miR-155 specifically binds to TRIM32 3'UTR. TRIM32 sites (and positive-control sites complimentary to miR-155) were cloned into psi-Check2, transfected into HeLa cells (with or without miR-155), and analyzed for luciferase levels 24h-post transfection. Renilla luciferase was normalized to that of firefly luciferase for each sample. +SEM, ***p<0.001 (two-way ANOVA), n=3.

C. miR-155 decreases endogenous TRIM32 levels. Negative-control miRNA and miR-155 mimics were nucleofected into 5A8 shDicer cells. Cells were collected 24h-post nucleofection, and analyzed by qRT-PCR. ***p<0.001 (two-tailed t-test), n=3.

D. Map demonstrates that miR-155 binds to TRIM32 3'UTR in wild-type miR-155 mice, but not to miR-155 knockout mice. Figure adapted from Rudensky lab database (CLIP Base).
A

TRIM32 3' UTR 5' UCCAUUUCUCAUCAAGCAUUAU 3'
miR-155 3' UGGGGAUAGGCUAAUCGUAAUU 5'
TRIM32-mut 3' UTR 5' UCCAUUUCUCAUCAAUUGGCUAU 3'

Human Hsa CUUUCAUUUCUCAUCAAGCAUUAU-------AAUGAGAA
Chimp Ptr CUUUCAUUUCUCAUCAAGCAUUAU-------AAUGAGAA
Rhesus Mnl CUUUCAUUUCUCAUCAAGCAUUAU-------AAUCGAGAA
Mouse Mmu AGCUCUG-------UUCUAUCAGCAUUAACUAUUAAAAGUAAUAA
Rat Rno CUUUCUG-------UUCUAUGAAGCAUUAACUAUUAAAACUAGUAA

B

% Inhibition of Luciferase Reporter Activity in HeLa Cells

Empty Vector  miR-155 Control  TRIM32  Mutated TRIM32

Binding Sites

C

% Inhibition of TRIM32 in 5A8 Cells

Negative Control miRNA

D

CLIP Base
TRIM32 I Snap Out

Total Read Count

mir-342  mir-18  mir-378  mir-31  mir-142-3p

mir-23  mir-342  mir-132  mir-155

62
Figure 2.4: TRIM32 Regulates Reactivation of Latent HIV-1

A. Lentivirus expressing shRNAs targeting TRIM32, Dicer, and a Scramble control were introduced into 5A8 cells. TRIM32 levels were measured by qRT-PCR. +SEM, ***p< 0.001 (one-way ANOVA), n=3.

B. Knockdown of TRIM32 as described in (A) measured by immunoblotting.

C. Knockdown of TRIM32 results in less reactivation following stimulation. 5A8 cells were infected with lentivirus expressing shRNAs, stimulated with αCD3/CD28 for 24h, and analyzed by FACS. +SEM, **p<0.01 (one-way ANOVA), n=3.

D. Increased TRIM32 expression in J-Lat 5A8 cells. Cells were infected with virus expressing TRIM32 (pCDH-TRIM32) or empty vector (pCDH), and TRIM32 expression was analyzed by qRT-PCR. +SEM, ***p<0.001 (One-way ANOVA), n=3.

E. Increased expression of TRIM32 as described in (D) was measured by immunoblotting.

F. TRIM32 expression promotes HIV-1 reactivation. 5A8 pCDH-TRIM32 or pCDH cells were stimulated with either αCD3/CD28 or TNFα. +SEM, ***p<0.001 (two-way ANOVA), n=3.
Figure 4

A. % Inhibition of TRIM32 in 5A8 Cells

B. Western blots showing TRIM32 and GAPDH levels in 5A8 cells infected with different shRNAs.

C. % GFP+ 5A8 Cells

D. Induction of TRIM32 mRNA

E. Western blots showing TRIM32 and Hsp90 levels in 5A8 cells infected with pCDH-TRIM32.

F. % GFP+ 5A8 Cells with different stimuli.
Figure 2.5: Expression of TRIM32 Promotes HIV-1 Reactivation by Inducing NF-κB Activation

A. J-Lat A72 cells, which lack Tat, showed increased reactivation in the presence of pCDH-TRIM32, but not pCDH (empty vector). These stable cell lines were unstimulated or stimulated with either αCD3/CD28 or TNFα, and analyzed by FACS. +SEM, ***p<0.001 (two-way ANOVA), n=3.

B. TRIM32 promotes NF-κB activation in Jurkat κB-dsRed cells. Cells were infected with pCDH-TRIM32 or pCDH lentivirus and stimulated with either αCD3/CD28 or TNFα, and analyzed by FACS. +SEM, **p<0.001 (two-way ANOVA), n=3.

C. TRIM32 expression increases RelA levels in the nucleus and decreases IκBα levels in the cytoplasm. Nuclear and cytoplasmic extracts were isolated for unstimulated 5A8 cells, and 5A8 cells treated with PMA and Ionomycin for 30 min.

D. TRIM32 specifically promotes binding at κB sites and not at mutant κB sites. 5A8 cells were infected with pCDH or pCDH-TRIM32 lentivirus and nuclear extracts were isolated. An EMSA was preformed with 32P-labeled NF-κB probes, or negative-control 32P-labeled Oct1 probes. In addition, nuclear lysates were pre-incubated with wild-type κB cold probes or with mutated κB cold probes.

E. IKK kinase activity is not increased in unstimulated TRIM32-expressing 5A8 cells compared with pCDH controls. Positive controls show that IKK kinase activity is increased following 30-min treatment with PMA and Ionomycin.

F. Immunoprecipitations of Flag-tagged TRIM32 and HA-tagged IκBα in 293T cells show that they are able to form a complex with each other. Wildtype TRIM32, but not TRIM32ΔRING, binds to both wildtype IκBα and the IκBα super-repressor (SS/AA).

G. In the presence of a proteasome inhibitor immunoprecipitation of IκBα followed by blotting for His-tagged ubiquitin shows that wildtype TRIM32 is capable of ubiquitinating IκBα (wildtype and SS/AA) in 293T cells.

H. IκBα is ubiquitinated by wild type TRIM32 in vitro, but not by the I22E mutant deficient in E2 binding. Recombinant TRIM32 (wild type or I22E) was incubated with 35S-labeled IκBα for 0 or 1 h. Ubiquitination reactions were visualized by autoradiography.
**Figure 5**

**A**

Uninfected A72 pCDH A72 pCDH-TRIM32

% GFP in A72 Cells

No Stim α-CD3/CD28 TNFα

% dsRed in KB Reporter Cells

No Stim α-CD3/CD28 TNFα

**C**

5A8 pCDH-TRIM32 5A8 pCDH-TRIM32

RelA IκBα TRIM32 Sp1 β-Actin

Nuclear Extract

Cytoplasmic Extract

**D**

NF-κB Oct1

NF-κB/Rel DNA Binding Motif

AGTGGAGCTTTCCAGGCT

CCCTGAAAGG

Mutation in NF-κB/Rel DNA Binding Motif

AGTGGAGCTTTCCAGGCT

CCCTGAAAGG

**E**

pCDH TRIM32

TRIM32

IKK IP

IKK Kinase Assay

**F**

pCMV + IκBα WT IκBα SS/AA TRIM32 WT TRIM32 6Ring

α-IκBα IP α-TRIM32 Lysate

**G**

IkBa WT IkBa SS/AA pCMV + TRIM32 WT TRIM32 6Ring Ubiquitin + β-Lactone

α-IκBα IP α-TRIM32 Lysate

**H**

TRIM32

WT I22E

Time (h): 0 1 0 1

α-IκBα Lysate α-TRIM32
Figure 2.6: Schematic Representation of miR-155 effects on TRIM32
Activation of the canonical NF-κB pathway promotes the degradation of IκBα and the production of miR-155 and TRIM32. TRIM32 is independently capable of binding to and ubiquitinating IκBα to promote its degradation. MiR-155 is able to block HIV-1 proviral reactivation by blocking the activity of TRIM32.
Figure 2.7: Comparison of Negative Controls

J-Lat 5A8 cells lacking Dicer show higher levels of GFP compared to negative controls. Cells were stimulated for 24h, then examined for GFP expression by flow cytometry. shEmpty cells are infected with virus produced from the empty pSicoR backbone; shLuc cells express shRNA targeting Luciferase; shScramble cells express a negative-control sequence used in the majority of experiments described in this paper; shA3Bmm cells express a mismatched negative-control shRNA sequence for A3B.
Figure 2.8: Validation of TLDA

RNA from Unstimulated, GFP positive, and GFP negative J-Lat 5A8 cells were examined by qRT-PCR for levels of mature miR-146a, -885-5p, -221, -155, -518b, -1290, -505*, and -29b. RNU6b was used as a housekeeping control and cells were normalized to Unstimulated.
Figure 2.9: Introduction of Candidate miRNA Mimics in J-Lat 5A8 Cells

J-Lat 5A8 cells were nucleofected with candidate miRNA mimics miR-146a and -518b (or a negative-control mimic) and stimulated with αCD3/CD28 24h-post nucleofection. Cells were then analyzed for GFP by FACS ~16h post-stimulation.
Figure 2.10: Increase in TRIM32 Following Cellular Activation

TRIM32 mRNA increases in J-Lat 5A8 cells following stimulation with αCD3CD28 and TNFα.
Chapter Three:

General Discussion and Future Directions
3.1 Implications of this research and potential future directions.

HIV, the virus that causes AIDS, has become a serious global challenge. Since the first cases were reported in 1981, tens of millions of people have died of AIDS-related causes. While HIV occurs in all regions of the world, 68% of cases are in sub-Saharan Africa and 7 out of every 10 infections in this region involve young women. Most of the people currently infected with HIV do not have access to prevention, care, and treatment, and there is still no available cure. Therefore, it is imperative that future treatments are not only more safe and effective than current therapies, but that they are accessible to populations living in the hardest hit regions of the world.

A deeper understanding of the molecular mechanisms that underlie HIV-1 latency are necessary in order to achieve our goal of developing an HIV cure. The cellular processes that regulate the transcriptional activity of HIV involve an intricate system of cellular factors that often interact with and regulate one another. Advances over the past few decades have identified important HIV-1 transcriptional activators such as NF-κB, AP-1, Sp1, and NFAT (Colin and Van Lint, 2009). These studies have enabled the strategic design of therapeutic approaches to promote viral eradication. Our studies have identified two host factors that play a role in directing the transcriptional activation of HIV: miR-155 and TRIM32. Our research provides new insights into the pathways and functions of these two important viral regulators, and advances our
understanding of the sophisticated molecular mechanisms that control HIV-1 latency.

miR-155 has been shown to be an important regulator of the immune system, and is known to be involved in mediating viral gene expression. Specifically, it has been demonstrated that miR-155 plays an anti-HIV role at the level of entry by targeting host proteins that facilitate entry and nuclear import of HIV (Swaminathan et al., 2012). Now we demonstrate that miR-155 also regulates HIV transcription, and reinforces HIV latency in CD4 T cells. It is well known that miR-155 is highly induced in lymphocytes following cellular activation. We find that miR-155 is highly upregulated in CD4 T cells following HIV-1 reactivation compared to cells that remain latently infected. The results of our TLDA also identified other miRNAs that were specifically induced in either reactivated or latent cells following TCR activation. When we reintroduced these candidate miRNAs into Dicer-deficient cells they reduced the level of reactivation following activation compared to negative control miRNAs. We chose to pursue the study of miR-155 since its introduction resulted in the greatest decrease in HIV-1 reactivation. However, it would be interesting to characterize the role of the other miRNAs that we identified as candidates. The first step in selection of candidate miRNAs would be to determine whether these miRNAs are differentially expressed in reactivated versus latently infected cells using a dual reporter virus in CD4 T cells. When we validated the expression of miR-155, we also determined that the levels of miR-29b were unchanged
between latently infected and reactivated cells, suggesting that miR-29b may not play a substantial role in regulating HIV latency. This is pertinent information due to the fact that miR-29b is thought to regulate the expression of CycT1, thereby regulating latency (Chiang et al., 2012). However, since miR-29b is not expressed in either latent or reactivated primary CD4 T cells it is unlikely to be playing a role in this process.

There are many known targets of miR-155, including BACH1, FADD, IKBKE, RIPK1, and SOCS. Many of these gene targets participate in immune regulation. Targeting of these genes by miR-155 may have opposing outcomes depending on cell type. Therefore, it is difficult to predict the overall effect of knocking down miR-155 in humans as a form of therapy. In any case, there has been great interest in antagonizing the levels of miR-155 in vivo to inhibit chronic inflammatory diseases (van Rooij et al., 2012), malignant growth (Babar et al., 2012), viral infections (Wang et al., 2011), and cardiovascular diseases (Corsten et al., 2012). In addition, miR-155 has also recently been shown to have an anti-HIV effect in monocyte-derived macrophages (MDMs) (Swaminathan et al., 2012). Levels of miR-155 increase following TLR3/4-stimulation and resulted in an accumulation of late RT products and in low levels of pro-viruses and 2-LTR circles. miR-155 exerts an anti-HIV-1 effect by targeting several HIV-1 dependency factors (ADAM10, TNPO3, Nup153, LEDGF/p75) involved in post-entry, pre-integration events, leading to diminished HIV-1 infection. It would be interesting to examine whether miR-155 also plays an inhibitory role at pre-
integration steps in different subsets of primary CD4 T cells. Here we find that TRIM32 is a novel target of miR-155. We demonstrate that introduction of miR-155 into J-Lat 5A8 cells decreases the mRNA level of TRIM32 to 70% compared to a negative control miRNA. We also perform luciferase reporter assays that result in significantly less reporter activity following introduction of miR-155, compared to a negative control miRNA. Furthermore, a dCLIP study by the Rudensky lab has shown that a miR-155-Ago complex specifically binds to TRIM32 in activated CD4 T cells from mice (Loeb et al., 2012). Future experiments would investigate the role of miR-155 and additional cellular targets in primary CD4 T cells. Using data from existing biochemical assays (Loeb et al., 2012) or conducting a microarray study could be used to uncover additional targets of miR-155 that play a role in HIV-1 latency.

TRIM32 belongs to the tripartite motif (TRIM) protein family, has a RING finger, B-box, and coiled-coil domain structure as well as an NHL domain at the C terminus. TRIM32 functions as an E3 ligase for actin, a protein inhibitor of activated STATy (PIASy), dysbindin, and c-Myc. In addition, our research has identified IκBα as a novel target for TRIM32. We have found that TRIM32 ubiquitinates IκBα independently of canonical NF-κB signaling and activation of the IKK signalosome. It would be interesting to determine whether other TRIMs are also capable of directly ubiquitinating IκBα. Future studies could explore the role of TRIM32 in NF-κB signaling in other biological contexts. Perhaps other
viruses alter the production of miR-155 as a way to regulate TRIM32 and ultimately control NF-κB signaling.

The interactions we have uncovered between miR-155 and TRIM32 are relevant in terms of blips, transient elevations in virus, that occur with HIV-infected patients on ART. Little is known about these blips, but it is thought that the cells which release virus exhibit a high level of activation. Therefore these virus-producing cells have elevated miR-155 levels. This may act to reinforce latency in cells that are responsible for producing blips. If we antagonize miR-155 we may be able to block the return back to latency, and subsequently increase viral production and ultimately cause cell death through cytopathic effects of the virus or immune detection. This could represent a novel approach to eradication of the viral reservoir that does not rely on reactivating cells via shock-and-kill, but exploits the cells that are already undergoing low levels of viral replication. Future studies would require a better understanding of blips, and whether they arise from a large population of cells or a small number of cells. In general, a better understanding of the mechanisms of HIV-1 latency is necessary if we are to develop new viral eradication strategies. Research into HIV and HIV-associated diseases must therefore increase, and global awareness and education must become priorities.
Chapter Four:

Materials and Methods
4.1 qRT-PCR

Total J-Lat cell RNA was purified using TRIzol reagent (Invitrogen), and the RNA concentration was determined with an ND-1000 spectrophotometer (NanoDrop). TaqMan Low Density Array (TLDA) human miRNA assays (version 2.1 and 3.0; Applied Biosystems) were used to calculate changes in miRNA expression between latent cells and reactivated cells collected from four independent experiments. Briefly, cDNA was generated using TaqMan reverse transcription kit and Megaplex primer pools A (v2.1) and B (v3.0). TLDA cards A and B were loaded with reverse transcription products and PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Fold changes in the expression of each miRNA were calculated (ΔΔCt method), and differences in expression between latent and activated cells were analyzed using moderated t-statistics. Linear contrasts were used to make all pairwise comparisons between groups. Follow-up analyses of specific miRNAs were performed using TaqMan microRNA assays. RNU6 was used as an endogenous control. TaqMan gene expression assays (Applied Biosystems) were used to quantify the expression of mRNA transcripts. The following primers and probes were used in gene expression assays: DGCR8 (Hs00256062_m1), Dicer (Hs00229023_m1), TRIM32 (Hs00705875_s1). GAPDH or β-Actin was used as an endogenous control for ΔΔCt calculations.

4.2 Lentiviral Infection

Lentiviral particles were produced as described (Naldini et al., 1996). For J-Lat infections, 100,000 cells were incubated with 4μg/ml polybrene (Sigma), RPMI, and
viral suspension for ~2h at 37°C. After 24h the cells were washed and cultured in RPMI. Infectious titers were determined by transducing J-Lat cells with serial dilutions of viral stocks. Sequences and cloning of lentiviral vectors are detailed in the Extended Experimental Procedures.

4.3 Antibodies

Protein levels were analyzed by immunoblotting using the following antibodies: DGCR8 (ab37272, AbCam), Dicer (ab13502, AbCam), TRIM32 (ab96612, AbCam), IκBα (sc-371, Santa Cruz), RelA (sc-109, Santa Cruz), β-actin (A5316, Sigma), Sp1 (sc-59, Santa Cruz), GAPDH (ab8245, AbCam), HSP90 (sc7947, Santa Cruz), and His (sc-803, Santa Cruz). Immunoprecipitations were carried out using Protein G Dynabeads (BD protocol) and the following antibodies: FLAG (F1804, Sigma), and HA (sc-7392, Santa Cruz). For ubiquitin assays, 5A8 cells were transfected then pre-treated with 1.5µM β-lactone overnight, and cells were lysed in NP-40 buffer supplemented with protease inhibitor and N-ethylmaleimide (NEM).

4.4 Transfections

J-Lat 5A8 cells were infected with lentivirus expressing shRNA targeting Dicer and sorted by the mCherry-positive population. These sorted shDicer-infected J-Lat cells were cultured for ~1wk and were nucleofected (Lonza) using Solution R and program O-028 with 2uM miRIDIAN microRNA mimics (Dharmacon), unless otherwise noted. After 24h, cells were stimulated with either anti-CD3 and anti-CD28 or TNFα. Cells were analyzed by FACS following overnight or 24h stimulation. For
immunoprecipitation experiments, 4 million 293T cells were transfected with 3.5µg of TRIM32, IκBα, pCMV constructs and 0.7µg of His-Ubiquitin using FUGENE HD for 24h. For dual-luciferase assays, the indicated luciferase plasmid (150 ng) was transfected into HeLa cells in a 24-well plate with or without miR-155 mimic (10 pmol) using Lipofectamine 2000 (Invitrogen). The Dual-Luciferase Reporter Assay System (Promega) was used to measure the levels of both firefly and Renilla luciferase 24h post-transfection. Cloning procedures of the luciferase reporter constructs are provided in the Extended Experimental Procedures.

4.5 In Vitro Kinase Assay

5A8 cells were unstimulated or treated (30min) with PMA (20nM) and ionomycin (2µM). A total of 15 million cells were lysed in whole cell lysis buffer and the assay was performed as previously described (Williams et al., 2004).

4.6 EMSA

Nuclear extracts were prepared (4µg/sample) and were incubated with poly(dl-dC) and salmon sperm DNA for 15 min, followed by the addition of [γ-32P]ATP-labeled consensus κB or Oct-1 oligonucleotides (Promega). For cold-probe competition 100X cold κB probes were added 10 min prior to labeled probes. Samples were separated on non-denaturing gels and analyzed by autoradiography.
4.7 Infection of CD4 T cells with Dual Reporter Virus

Primary CD4 T cells and PBMCs were purified from healthy donor blood (Blood Centers of the Pacific, San Francisco, CA, USA and Stanford Blood Center). CD4 T cells were isolated by negative selection using the RosetteSep Human CD4 T Cell Enrichment Cocktail (StemCell Technologies). PBMCs were purified by Histopaque®-1077 density gradient. Purified CD4 T cells from peripheral blood were cultured in RPMI 1640 medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 mg/ml). Purified CD4 T cells isolated from peripheral blood were stimulated with αCD3/αCD28 activating beads (Life Technologies) at a concentration of 1 bead/cell in the presence of 30U/ml IL-2 (PeproTech) for 3 days. All cells were spinoculated with HIV Duo-Fluo I at a concentration of 100 ng of p24 per 1x10^6 cells for 2hr at 1,200 X g at 37°C. After spinoculation all cells were returned to culture in the presence of 30 U/ml IL-2. Infected CD4 T cells were sorted based on GFP and mCherry fluorescence 4 days post infection using a FACS AriaII (BD Biosciences), and RNA was extracted immediately using The Ambion® PARIS™Kit (Life Technologies).

4.8 Lentiviral Vectors

shRNAs were cloned into the pSicoR lentiviral vector, which encodes an mCherry reporter driven by an EF-1α promoter (pSicoR-MS1). shRNAs against human DGCR8, Dicer, TRIM32, and negative control Scramble were cloned into pSicoR-MS1 using the following oligonucleotide sequences: shScramble forward (TGT CAA GTC TCA CTT GCG TCT TCA AGA GAG ACG CAA GTG AGA CTT GAC TTT TTT C), shScramble
reverse (TCG AGA AAA AAG TCA AGT CTC ACT TGC GTC TCT CTT GAA GAC GCA AGT GAG ACT TGA CA); shDGCR8 forward (TGA AAG AGT TTG TTA TTA ACT TCA AGA GAG TTA ATA ACA AAC TCT TTC TTT TTT C), shDGCR8 reverse (TCG AGA AAA AAG AAA GAG TTT GTT ATT AAC TCT CTT GAA GGT AAT AAC AAA CTC TTT CA); shDicer forward (TGC AGC TCT GGA TCA TAA TAT TCA AGA GAT ATT ATG ATC CAG AGC TGC TTC TTT TTT C), shDicer reverse (TCG AGA AAA AAG CAG CTC TGG ATC ATA ATA TCT CTT GAA TAT TAT GAT CCA GAG CTG CA); shTRIM32 forward (TGC AAA CAA ATG CTG ATA TAT TCA AGA GAT ATA TCA GCA TTT GTT TGC TTT TTT C), shTRIM32 reverse (TCG AGA AAA AAG CAA ACA AAT GCT GAT ATA TCT CTT GAA TAT ATC AGC ATT TGT TTG CA).

The pCDH-TRIM32 lentiviral expression vector was constructed by performing PCR on the MGC human TRIM32 cDNA clone (Accession: BC003154, CloneID: 2906024; Thermo Scientific) using the following primer sequences: TRIM32 (EcoRI) forward (GTT TCT GAA TTC GAT GGC TGC AGC AGC AGC TTC TC), TRIM32 (BamHI) reverse (GTT TCT GGA TCC CTA TGG GGT GGA ATA TCT TCT CAG AT). PCR products were digested with EcoRI and BamHI and ligated into the pCDH-EF1α-MCS-IRES-Puro lentiviral vector.

4.9 Plasmids for Luciferase Assay

Luciferase reporter constructs were generated using the psiCHECK2 vector (Promega). Each 3'UTR contained two miRNA binding sites separated by an EcoRI restriction site. Oligonucleotide duplexes surrounding the TargetScan-predicted miR-155 binding site for TRIM32 (25nt) were ligated into the XhoI/NotI sites of psiCHECK2.
The construct containing mutations for the predicted miR-155 binding site of TRIM32 was encoded in the oligonucleotide duplexes, and was cloned in the same manner. Positive-control binding sites were composed of sequences with inverse complementarity to the miRNA sequence. Sequencing was performed to confirm that all constructs contained the expected DNA sequence.

4.10 Plasmids in 293T experiments

TRIM32 plasmids were a kind gift of Dr. Ki-Sun Kwon (Ryu et al., 2011). IκBα plasmids were constructed as previously described (Sun et al., 1996).

4.11 Cloning, expression, and purification of TRIM32

TRIM32 cDNA was cloned between the KasI and XhoI restriction sites of pPROEX-HTa. The I22E mutant was cloned by site-directed mutagenesis. A saturated culture of Arctic Express (DE3) cells (Agilent) harboring pPROEX-HTa-TRIM32 was diluted to an Od600 of ~0.2 and grown at 30°C for 3 hours. Cells were subsequently induced with 0.1 mM IPTG (supplemented with 0.1 mM ZnSO₄) and grown for an additional 24 hours at 12°C. Cells were resuspended in lysis buffer (50 mM Tris 8.0, 150 mM NaCl, 0.1 mM ZnSO₄, 10 mM imidazole, 10% glycerol, and 0.5 mM PMSF) and lysed by sonication. Recombinant Trim32 was batch purified using Ni-NTA resin, eluted with elution buffer (50 mM Tris 8.0, 150 mM NaCl, 0.1 mM ZnSO₄, and 200 mM imidazole, 10% glycerol), dialyzed overnight in dialysis buffer (50 mM Tris 8.0, 150 mM NaCl, 0.1 mM ZnSO₄, 10% glycerol, 1 mM DTT), and concentrated.
4.12 Cloning and in vitro synthesis of (NFKBIA) IκBα

*NFKBIA* cDNA was cloned between the FseI and Ascl restriction sites of pCS2. 40 µl of rabbit reticulocyte lysate (Promega), 12 µl of $^{35}$S-labeled amino acids (MP Biomedicals, catalog no. 0151006), and 12 µl of pCS2-NFKBIA (at a stock concentration of 500 ng/µl) were mixed and incubated at 30°C for 2 hours. In vitro transcribed/translated (IVT/T) IκBα substrate was purified with anti-HA agarose resin, eluted with 0.2 ml of 0.4 mg/ml 3xHA peptide, and concentrated five-fold in volume.

4.13 Ubiquitylation assay

In a 15-µl reaction volume, 0.3 µl of 10 µM E1 (200 nM final), 0.3 µl of 235 µM UbcH5 (5 µM final), 1.5 µl of 10 mg/ml ubiquitin (1 mg/ml final), 1.5 µl of 10 x ubiquitylation assay buffer (250 mM Tris 7.5, 500 mM NaCl, and 100 mM MgCl$_2$), 2.3 µl of energy mix (150 mM creatine phosphate, 20 mM ATP, 20 mM MgCl$_2$, 2 mM EGTA, pH to 7.5 with KOH), and 2 µl of $^{35}$S-labeled IκBα were mixed with 1.5 µl of 20 µM Trim32 or Trim32 I22E (2 µM final) at 30°C for 1 hr. Reactions were stopped by the addition of SDS sample loading buffer, resolved on a 10% SDS-acrylamide gel, and exposed on an autoradiography screen.
Chapter 5

Abbreviations and References
5.1 Abbreviations Used

Ago: Argonaute
AIDS: acquired immune deficiency syndrome
ART: antiretroviral therapy
Aza-CdR: 5-aza-2’dexocytidine
Brd4: bromodomain-containing protein 4
CCR5: C-C chemokine receptor type 5
CD4: cluster of differentiation 4
CNS: central nervous system
CTD: C-terminal repeat domain
CTL: cytotoxic T lymphocyte
CXCR4: C-X-C chemokine receptor type 4
DSIF: DRB-sensitive inducing factor
EBV: Epstein-Barr virus
GALT: gut-associated lymphoid tissue
HAT: histone acetyl transferase
HDAC: histone deacetylase
HIV: human immunodeficiency virus
HMBA: hexamethylene biacetamide
IFN-γ: interferon gamma
IKK: I-kappa-B kinase
IVT/T: In vitro transcribed/translated
IKBα: nuclear factor-kappa-B inhibitor alpha
KSHV: Kaposi’s Sarcoma-associated herpesvirus
LPS: lipopolysaccharide
LTR: long terminal repeat
MBD2: methyl-CpG binding protein 2
MDM: monocyte-derived macrophage
MDV: Marek’s disease virus
miRNA: microRNA
N-TEF: negative transcription elongation factor
NELF: negative elongation factor
NEM: N-ethylmaleimide
NF-κB: nuclear factor-kappa-B
NFAT: nuclear factor of activated T-cells
PBMC: peripheral blood mononuclear cell
PCAF: p300/CREB-associated factor
PHA: phytohemagglutinin
PIASy: protein inhibitor of activated STATy
PIC: preintegration complex
PKC: protein kinase C
PMA: phorbol myristate acetate
Pol II: RNA polymerase II
poly (I:C): polyinosine-polysytdylic acid
PTEF-b: positive transcription elongation factor b
RISC: RNA-induced silencing complex
SAHA: suberoylanilide hydroxamic acid
SCF-βTrCP: SKP1-CUL1-F-box ligase containing the F-box protein
SCID: severe combined immunodeficiency
SIRT1: sirtuin 1
TAK1: (MAP3K7) mitogen-activated protein kinase kinase kinase 7
TAR: transactivation-responsive element
T_{CM}: central memory T lymphocyte
TCR: T-cell receptor
TLDA: TaqMan Low Density Array
TLR: Toll-like receptor
TRBP: TAR RNA binding protein
TRIM: tripartite motif
T_{TM}: transitional memory T lymphocyte
UTR: untranslated region
VPA: valproic acid
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