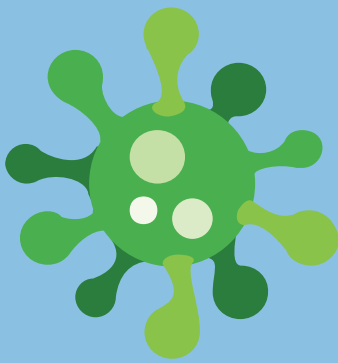


# Unbiased Approaches to Unravel the Molecular Mechanisms Driving HIV Latency



Michael Röling



*One of history's few iron laws is that luxuries tend to become necessities and to spawn new obligations – Yuval Harari, Sapiens*

*Waterloo was won on the playing fields of Eton – Arthur Wellesley,  
1st Duke of Wellington (apocryphal)*

*Even his griefs are a joy long after to one that remembers all that he  
wrought and endured – Homer, The Odyssey*



Unbiased Approaches to Unravel the Molecular  
Mechanisms Driving HIV Latency

Onbevooroordeelde onderzoeksbenaderingen om de moleculaire mechanismen van  
latent HIV te bestuderen

Thesis

to obtain the degree of Doctor from the  
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Prof. dr. F.A. van der Duijn Schouten

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## Table of contents

Chapter 1	General Introduction Introduction Scope of thesis
Chapter 2	Allele-specific long-distance regulation dictates IL-32 iso- form switching and mediates susceptibility to HIV-1
Chapter 3	A two-color haploid genetic screen identifies novel and druggable host factors in HIV latency reversal
Chapter 4	Gliotoxin, identified from a screen of fungal metabolites, disrupts 7SK snRNP, releases P-TEFb, and reverses HIV-1 latency
Chapter 5	Summary and general discussion
List of Abbreviations	
Curriculum Vitae	
Portfolio	
Acknowledgements	

Release

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NtRTIs  
NRTIs  
NNRTIs

Integration

Integras  
inhibito



# Introduction & scope of thesis

Revised from:

**Molecular mechanisms controlling HIV transcription and latency; implications for therapeutic viral reactivation**

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Translation

RNA export

## Introduction

### Abstract:

Persistence of transcriptionally silent replication competent HIV-1 is a major barrier to clearance of the virus from patients; current combinatorial antiretroviral therapies are successful in abrogating active viral replication, but are unable to eradicate latent HIV-1. A “shock and kill” strategy has been proposed as a curative approach in which latent virus is activated and infected cells are removed by immune clearance, while new rounds of infection are prevented by antiretroviral therapy. Much effort has been put toward understanding the molecular mechanisms maintaining HIV latency and the nature of reservoirs, to provide novel therapeutic targets. This has led to the development of latency reversal agents (LRAs), some of which are undergoing clinical trials. Targeting multiple mechanisms underlying HIV latency via a combination of LRAs is likely to result in more potent activation of the latent reservoir. Therefore, novel as well as synergistic combinations of therapeutic molecules are required to accomplish more potent latency reversal.

**Keywords:** HIV-1 latency, Latency reversal agents (LRAs), Combinatorial antiretroviral therapy

### 1. Introduction

Human immunodeficiency virus-1 (HIV-1) is a lentivirus, a subgroup of Retroviridae. Like all retroviruses, HIV-1 virions consist of an RNA genome with viral proteins encapsulated in a viral envelope. The viral proteins execute key steps to establish a productive infection by stably integrating in the host genome. Unlike most retroviruses, HIV-1 can also directly infect nondividing cells. HIV-1 preferably infects a subset of T-lymphocytes (CD4+ T-cells) that play a crucial role in the immune response. HIV-1 infection causes exhaustion and ultimately depletion of the host immune system, a syndrome termed acquired immuno-deficiency syndrome (AIDS). HIV-1 came into prominence with the outbreak of the AIDS epidemic in the 1980s. Major steps have been taken toward treating this viral infection. In particular, combinatorial antiretroviral therapy (cART) successfully abrogated HIV-1 replication. Thus, for compliant patients with access to c-ART, HIV infection has become a chronic rather than a lethal disease. However, cessation of antiretroviral therapy results in viral rebound in infected patients, even after years of cART(1, 2). This is because in a small fraction of infected cells, HIV persists in a latent but replication-competent state. Latent HIV is unaffected by cART, but infection can rebound upon cART interruption. Therefore, HIV latency is the main challenge in developing a curative therapy for HIV.

The quest for an HIV-1 cure involves the development of either a sterilizing or a functional cure. A sterilizing cure would require complete removal of viral genetic

material from the infected patient and thus the stable depletion of latently HIV-infected cells. A functional cure, on the other hand, requires the patient's immune system to suppress HIV-1 replication life-long in the absence of cART without disease progression, loss of CD4+ T cells and HIV transmission. The functional cure does not aim to eradicate the virus entirely from the patient. Both the sterilizing and functional cure strategies are currently the subject of major research efforts.

## **2. Clinical picture of HIV**

The AIDS epidemic in the 1980s led to the identification of HIV as the causative agent. AIDS is a condition in which depletion of CD4+ T-cells overtime leads to the loss of the host immune system's ability to fight infections and cancers, eventually leading to death. As HIV was identified as the causative agent, cure efforts focused on disrupting the viral lifecycle. In the early 1990s, the first antiretroviral therapies – monotherapies – had limited success as they resulted in rebound of viremia due to the appearance of resistant viral strains. Resistant HIV required novel therapeutic strategies. Therefore, a combination of anti-retrovirals, targeting distinct steps of the viral life cycle was developed in so-called combinatorial antiretroviral therapy (cART). cART has proven to be extremely successful in lowering the amount of viral RNA in plasma below the limits of detection by standard laboratory techniques. Unfortunately, the therapy does not eradicate the virus as cessation of medication invariable causes re-emergence of viral replication(1–6). Thus, a fraction of the virus escapes the effects of cART, as treatment intensification does not affect the reservoir(7). So despite undetectable HIV-1 DNA levels, plasma viremia can rebound. However, patients that start cART early during the acute infection phase of HIV have a smaller reservoir than those that start during the chronic phase (8, 9). The source for this recurring viral replication is a small pool of latently infected cells that harbor integrated proviruses which, while silent, are not recognized by either the immune system nor are they subject to cART. Moreover, HIV can persist in the presence of cART in certain anatomical sites if drug penetrance is incomplete. According to the World Health Organization (WHO), the number of HIV-infected individuals worldwide in 2019 was estimated to be approximately 38 million (10). The vast majority of infected people live in sub-Saharan Africa, where access to appropriate diagnostic centers and cART is limited. Estimates put new infections at 5,600 a day in 2014.

## **Genetic variation and HIV**

In the late 1980s HIV-1-resistant individuals, individuals who were repeatedly exposed to HIV yet remained uninfected were observed, indicating a clear role for genetics in HIV-1 susceptibility. This led to the identification of the protective capacity of the deletion in chemokine receptor CCR5(11). No other gene variant was identified that conferred such protection. The advent of microarray and later high-throughput sequencing technology allowed for genome-wide association

studies (GWASs) to look for association of genetic variants with a phenotype or outcome. Yet GWAS to identify genetic variants that confer protection against HIV-1 infection found no candidates(12). Similarly, progression of HIV to AIDS was also variable. Indeed, specific HLA alleles are predictors of disease progression to AIDS(13–15). But other variants are harder to find using GWAS either due to their weak effect size or relative rarity causing them to fall below the strict threshold for genome-wide significance ( $P < 5 \times 10^{-8}$ ). Many studies are underpowered because they lack the cohort size needed to detect variants associated with a phenotype. Although several GWASs have identified single-nucleotide polymorphisms (SNPs) associated with HIV disease acquisition or progression, the functional significance of these genetic variants is unclear because the SNP is located in noncoding regions of the genome of which the function is unknown(16, 17). This can be elucidated when GWAS data is combined functional genomics data. Genome wide high throughput methods like ChIP-seq annotate the genome and help to predict regulatory elements such as enhancers. This approach has the potential to decipher GWAS noncoding SNP data and assign biological functions to regulatory elements.

### **HIV-1 replication cycle and state-of-the-art antiretroviral therapy**

HIV-1, as all viruses, is a parasite of the host cell and hijacks key cellular processes to establish a productive infection. To produce new virions, the virus goes through a viral replication cycle. HIV-1 infects cells expressing the cell surface CD4 and either of the co-receptors CCR5 or CXCR4. These cells include T-cells, monocytes, macrophages and dendritic cells. *In vivo*, HIV infects mostly activated CD4+ T-cells as quiescent and resting CD4+ T-cells are less permissive to infection due to low expression of CD4 and CCR5, and minimal metabolism(18–21). The low metabolism is characterized by low levels of available dNTPs for reverse transcription and lack of energy sources(21–26). Additionally, the cortical actin barrier in resting cells is thought to inhibit virus entry, reverse transcription and nuclear import(27, 28). So the first step in HIV's replication cycle consists of entering the cell by docking at the cell surface receptor CD4 and co-receptors CCR5/CXCR5 and fusing to the cell, un-packaging of the genome, reverse transcription of the viral RNA genome into double-stranded DNA, which is the main component of the pre-integration complex, followed by integration of the double-stranded DNA genome into the host genome, transcription of the provirus, translation of viral proteins and genome, and ultimately virion biogenesis followed by budding from host cell and maturation. Modern cART targets most steps in the HIV viral replication cycle (Figure 1). There are currently 28 approved agents for the treatment of HIV infection(29). They fall into six mechanistic major classes, which act at different stages in the HIV replication cycle:

1. Fusion inhibitors: enfuvirtide (ENF, T-20), the only currently available fusion inhibitor, binds to the gp41 receptor site, preventing the fusion of

the virus with the target cell.

2. C-C chemokine receptor type 5 (CCR5) antagonists: maraviroc (MVC) is currently the only available CCR5 antagonist. This drug is an entry inhibitor, specifically blocking the human chemokine receptor CCR5.
3. Nucleoside (nucleotide) reverse transcriptase inhibitors (NRTIs) block the addition of nucleosides to the DNA chain during reverse transcription of RNA.
4. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) bind to and inhibit the enzyme reverse transcriptase (RT), preventing conversion of viral RNA to DNA during infection.
5. Integrase inhibitors (INIs): raltegravir (RAL), elvitegravir (EVG) and Dolutegravir (DTG) are the only currently available drugs in this class. They target the HIV enzyme integrase (IN) that is required for insertion of viral genetic material into human DNA.
6. Protease inhibitors (PIs) bind to the catalytic site of HIV aspartic protease, blocking the processing of viral proteins.

These antivirals comprise the various current cART regimens that are used in the clinic. cART has proven to be extremely successful in suppressing viral replication in compliant patients. In fact, it has been argued that the theoretical potential of cART has already been reached(1). Therefore, in the developed world with access to medication, HIV has become a chronic and not a lethal disease.

### **The burden of lifelong cART**

Due to the long half-life of a latently infected resting memory CD4+ T-cells (estimated at 44 months), cART would take over 70 years in order to eradicate HIV from the infected patient(1, 2, 30). Implementation of cART has provided long-term suppression of viral replication, improving the life expectancy and life quality of infected patients. Unfortunately, the economic burden of cART is debilitating. According to the Centers for Disease Control and Prevention (CDC), lifetime costs of treating HIV infection is estimated to be \$379,668 per infected individual in the United States(31). Under cART, residual viral replication causes chronic immune activation and inflammation(32). Additionally, patients on cART overtime can experience several side effects of cART such as: cardiovascular diseases (e.g., myocardial infarction); microbial translocation, co-infections, metabolic disorders, regulatory T cell (Treg) deficiency, and decreased thymic function non-AIDS cancers (e.g., anal cancer, liver cancer, Hodgkin's disease); liver, kidney, and bone disease as well as neurologic complications, such as dementia(33–35).

Interestingly, most of these conditions are associated with the ageing process. Hence, it is thought, that HIV infection controlled by cART accelerates ageing. And importantly, as HIV persists in a latent state that is not targeted by cART, cART is a therapeutic management of the disease as opposed to a curative treatment. Thus, there is much need to develop a curative therapy for HIV.

### Defining the reservoir

Latency is established before the symptomatic primary infection phase occurs. Start of cART as early as 10 days after the onset of symptoms of primary HIV-1 infection did not prevent the latent reservoir from forming(8). Another paper pinpoints the establishment of latency somewhere in the first two weeks of infection (Fiebig I) and showed viral rebound after a median of 2.8 cART treatment(9). Similarly, perinatally infected children experience viral rebound after treatment cessation, despite cART initiation within 30 hours of birth(36–39). In laboratory controlled experiments on rhesus monkeys with SIV, a well-established model of HIV research, the latent reservoir was established within the first 3 days of infection, as initiation of cART 3 days after intrarectal SIV infection did not prevent viral rebound after 24 weeks of treatment(40). In the first days (1-3 days) after SIV infection, few infected cells are actively producing virus(41). Surprisingly, resting CD4+ T cells are the primary productively infected cells at the portal site of entry(39). In the same paper, this was also observed but in lymph node and tonsil tissue samples of patient in the acute stage of HIV infection.

So, it is clear the latent viral reservoir is seeded early and is long-lived, but a clear definition of the viral reservoir is difficult due its heterogeneous and dynamic nature. A broad definition would be the persistence of viral replication over time in a defined cell type and/or anatomical site(s). Some definitions emphasize the transcriptional silence of the replication competent virus, as replication during cART is not detectable but returns upon nonadherence to or cessation of treatment(42, 43). To complicate matters, a majority of latently infected cells contain defective virus(44–47) and these defective proviruses are more stable than intact proviruses(48). This is in part due homologous recombination during reverse transcription of viral RNA(44, 49) and inactivating C to U nucleotide substitutions due to deamination by APOBEC3G(50). Although defective provirus cannot replicate, it might still elicit an immune reaction through the production of viral proteins(45, 51) and thus should be included in the definition of HIV-1 reservoir because of its clinical relevance. The active reservoir is due to residual viral replication caused by suboptimal diffusion of antiretroviral drugs in lymphoid tissues(52). Viral evolution in the presence of cART(53) and treatment intensification leading to increased episomal viral DNA in cART suppressed individuals(54, 55) suggest cART is inefficient in blocking replication. However, other studies did not find that treatment intensification decreased residual viremia(7, 56–58). In a study that used single-cell RNA fluorescence in situ hybridization-flow cytometry (FISH-flow) to measure viral transcription in unfractionated peripheral blood mononuclear cells (PBMCs) from treated and untreated HIV-infected patients, effector memory CD4+ T cells were the main active reservoir(59). Another study, using similar methods found the reservoir in central/transitional memory CD4 T cell and effector memory cells depending on

the conditions used(60). A study using in vitro infected resting CD4+ T cells and cells from cART suppressed patients found Gag protein expression from integrated proviruses in a subset of CD4/CD8 double-negative T cells(61). The latent reservoir is due to inactivity of viral replication of an otherwise replication competent virus. Although the focus is on transcriptional blockade, in a wider context posttranscriptional blockades can be included as well, such as blocks in (alternative) splicing, nuclear export of viral RNA(62–64), translation and viral particle assembly and maturation(64). Also, the blocks in viral replication can change over time, as for example, the 5'LTR of HIV is progressively methylated during cART(65). Over time, infected cells are cleared because of immune surveillance or viral cytotoxicity(66). The remaining cells seem to exhibit signs of deeper latency, such as enrichment of intergenic integrations of the provirus, antisense integrations and either relative proximity to or increased distance from active transcriptional start sites and to accessible chromatin regions(67). Clonal expansion of the latent reservoir is the homeostatic proliferation of latently HIV infected cells. In particular resting memory CD4+ T cells can undergo clonal expansion(68). Clonal expansion can be antigen mediated(69) or integration site driven(70, 71), but was also observed in Th1 cells(72). Two states of latency can be defined based on the integration state of HIV: pre-integration latency and post-integration latency. Defects in integration or in a prior phase of the viral replication cycle (e.g., incomplete reverse transcription) might result in unintegrated viral DNA. The half-life of the linear pre-integration complex is approximately 1 day(73). The linear unintegrated viral DNA can also be circularized, resulting in slightly extended half-life of the virus(74). In quiescent cells, the pre-integrated virus can reside near the centromere for week(75). Unintegrated virus can replicate, albeit very inefficiently(76). The half-life of both forms of unintegrated virus is too short and replication inefficient to serve as the source required for the long-term persistence of latent HIV making pre-integration latency less clinically relevant. Post-integration latency occurs when the HIV virus is stably integrated into the host genome, but a productive infection is not achieved. The site of integration and the abundance of transcription factors are crucial for determining whether an infection will be latent or productive. The site of integration will determine the chromatin environment (such as histone modifications), relative position to other genes (intronic insertion vs gene desert), and position within the nucleus of the provirus. Identifying and understanding the anatomical sites and cell types that harbor the latent reservoir is key in developing therapeutic strategies to eradicate HIV. Studies on cART suppressed HIV patients identified integrated HIV in different subsets of CD4+ T cells such as naïve CD4+ T cells(77), central memory (TCM) and transitional memory (TTM) CD4+ T cells(78), memory CD4+ T cells(79), CD4+ T memory stem cells (TSCM cells)(80) and Th17 cells(81). While all these cells harbor latent reservoir, the characteristic of the reservoir differ. Naïve CD4+ T cells contain an inducible reservoir(82, 83). Effector memory cells contain the largest

fraction of intact provirus(47, 59). Th1/17 cells harbored high levels of provirus in patients on cART(79, 84).

### **Defining the reservoir by anatomical compartment**

Among the anatomical compartments affected by HIV-1, the gut and the lymph nodes (LN) are the primary anatomical sites of the latent reservoir. Although the latent reservoir resides in other tissues and organs as well. Latent infection was found in the central nervous system (CNS), testis, adipose tissue and gut-associated lymphoid tissues (GALT)(85–88). In addition to T cells, monocytes, astrocytes and tissue resident macrophages may contribute to viral persistence in these tissues(89–92). Approximately 5-10 times more HIV-1 RNA can be obtained from GALT than from blood cells in patients receiving cART(93, 94), potentially indicative of lower penetrance of cART in cells within this reservoir. However, the contribution of these compartments to rebound of viremia after cART cessation remains controversial(95, 96). The source of infection in the CNS is most likely infected monocytes, which are able to cross the blood–brain barrier as the virus itself cannot(97–100). Some tissues such as the germinal center of second lymphoid organs(101, 102) and testis(88) may be inaccessible to HIV-specific CD8+ T cells, leaving space for the viral reservoir. In addition, these HIV-specific T lymphocytes have limited cytotoxicity(103, 104), due to persistent exhaustion(105) and/or immunosuppressive environments(106).

### **Defining the reservoir by cell type**

The biggest pool of latently infected cells comprises resting memory CD4+ T-cells. It is thought that these latent infections are predominantly generated while activated infected cells revert back to a resting memory state(107, 108). During this process, as the genome of the (partially) activated cell condenses and is silenced in transition to a memory state, so does the HIV genome(23, 24, 109). There is also evidence for direct infection of resting cells by HIV, resulting in the generation of a latent infection(110). Studying these cells in patients is challenging as the frequency of latently infected cells in suppressed patients is very low, estimated to be 1 latent cell per 1 million of uninfected cells(93, 111). Naive T-cells are also found to be latently infected; however, the frequency of such cells is even smaller than resting memory cells(78). Interestingly, the naive T-cell reservoir may increase over time in suppressed individuals due to high proliferation of these cells compared to resting memory cells(77). HIV is found also in cells of monocyte/macrophage lineage such as macrophages in brain and lung sections of infected individuals on anti-retroviral therapy(112, 113). However, proviral transcription occurs in these cells at low levels; therefore, it is debatable whether these cells are part of the latent reservoir(114, 115).

### **Defining the reservoir cell markers**



To provide a clear definition of the reservoir and a possible therapeutic target, cell markers of the latent reservoir are invaluable. Thus far a definitive marker or set of markers has proven elusive, but some have been proposed. The use of cell surface markers provides an obvious avenue to easily obtain cell populations without using invasive techniques that alter cells. Expression data from latently infected primary CD4+ T-cells identified CD2 as a marker for cells with enriched provirus and reactivatability(116). In patients on cART, CD30 co-localized with viral transcription in gut-associated lymphoid tissues(117). Treatment with brentuximab vedotin, an anti-CD30 antibody drug conjugate used for the treatment of Hodgkin and other aggressive lymphomas, caused a transient reduction of CD4+ T cells expressing CD30 and subsequently reductions in cell-associated HIV RNA and HIV DNA levels and plasma viremia(117). Although the contribution is limited, CD4+ T cells positive for CD20 were enriched for HIV RNA(118). Treatment with Rituximab, an anti-CD20 monoclonal antibody, and latency reversal agents reduced the pool of HIV-expressing cells in samples from patients on cART(118). CD32a is a promising marker, because of its specificity, but difficulties in obtaining pure samples had to be overcome(119, 120).

T cells exhaustion is the progressive loss of function during chronic infection, as is the case with HIV(121). Because of constant stimulation by high antigen loads, inhibitory immune checkpoint (ICs) proteins are up-regulated, resulting in suppressed immune response despite chronic infection. CD4+ T cells that were also positive for PD-1, LAG-3 and TIGIT, alone or in combination, were enriched for integrated HIV DNA and memory CD4+ T cells with at least one of these markers were enriched for reactivatability of latent HIV(122, 123). Also, CTLA-4+ PD-1- memory CD4+ T cells outside the B-cell follicle were enriched for viral DNA in cART treated patients(124).

### **Clinical proof-of-concepts for HIV-1 eradication**

Because the latent reservoir is seeded so early after infection, a sterilizing cure has proven difficult. Thus far, only two patients, the so-called Berlin patient and London patient, were cured from HIV-1 after receiving hematopoietic stem cell transplantations as part of their cancer treatments(125–127). The hematopoietic stem cells used for transplantation came from donors bearing a homozygous thirty-two base pair deletion in the CCR5 co-receptor gene (CCR5 $\Delta$ 32). The mutant CCR5 impedes viral entry in the first phase of the infection by R5 tropic viruses(128–131). It is estimated that between 1% and 15% of the European Caucasian population harbor this mutation, while it occurs less frequently in African and Asian populations(129, 130). In this patient, cART was ceased a day before the first transplant and after 7 years, no viremia or other indications of viral replication have been detectable(131). Unfortunately, due to onerous treatment the success of these cases has proven to be difficult to replicate. Two HIV-1-positive patients, the “Boston patients”, received HSCs transplants after

developing Hodgkin's lymphoma(132). Both patients carried heterozygous CCR5Δ32 mutation. While still under cART regimen, no viral production was observed which led to cessation of therapy. Unfortunately, after several months, strong viral rebound occurred in these patients. Follow-up analysis pointed to the likely presence of a small refractory source of cells, which is thought to have seeded the viral rebound; phylogenetic studies revealed that only a few latent proviruses contributed to the viral rebound(132). Similarly, in the "Essen" patient a rapid viral rebound of a pre-existing CXCR4 tropic virus was observed(133). Several other similar studies have been conducted with infected patients suffering from either leukemias or lymphomas who received autologous or allogenic HSC transplantation alongside cART as a strategy to deplete the latent pool of cells. However, in most of these studies, viral rebound was detected following therapy interruption(134). Encouragingly, a recent allogenic HSCT that was less aggressive and toxic compared to previous patients was performed on another patient(127). 16 months after transplantation cART was interrupted and the patient has been in remission ever since. Due to the burden of treatment, HSCT is currently not a viable sterilizing cure. In another case, the Mississippi baby, an infant presumably infected *in utero*, received cART 30 h after birth. As newborns do not have resting memory CD4+ T-cells, it was reasoned that cART will prevent establishment of the latent reservoir – the main impediment in eradication strategies. One month after therapy, viremia reached undetectable levels and cART stopped after 18 months. Unfortunately, 2 years post therapy interruption, rebound of viremia was detected(131, 135).

The immune system of rare "elite controllers" maintains low HIV-1 plasma levels, without the need of medication for many years. Although the capability of these patient to control viral replication is not completely understood, their circulating myeloid dendritic cells and CD8+ T-cells are more effective in depletion of infected CD4 T-cells(136–142). Interestingly, the ARNS VISCONTI cohort showed that cessation of long-term cART, started during the acute phase of HIV-1 infection, resulted in post-treatment control (PST) of infection. Fourteen of the studied individuals were able to keep or even further reduce the viral reservoir. Furthermore, these individuals were able to maintain long-lasting, low level of viremia(142). Recently, a perinatally infected baby displayed more than 11 years of HIV-1 remission. At 3 months of age, plasma HIV-RNA reached  $2.1 \times 10^6$  copies/ml, and cART was administered for about 5–6 years. At 6.8 years of age, no HIV-1 RNA was detectable and cART was discontinued. After more than 12 years, plasma viremia still remains undetectable(143). Therefore, this case provides the first evidence that early initiated, long-term cART can result in stable and durable HIV-1 remission. Data from the Berlin and Boston patients provided a rationale for the creation of HIV-resistant cells. Since the CCR5Δ32 homozygous mutation is not lethal and not associated with abnormal immune functions(131), many approaches to silence the CCR5 gene have been or are under investigation(144–

147). These studies all employ genome editing technologies such as transcription activator-like effector nuclease (TALEN), clustered regularly interspaced short palindromic repeats (CRISPRs) or zinc-finger nucleases (ZNFs), which target the genome with high specificity and introduce deletions in the sequence of interest, in this case in the DNA sequence of CCR5 or/and CXCR4 co-receptors(144, 145, 148). The rationale for this approach is based on the notion that cells bearing mutated CCR5 protein are not permissive to infection with R5 HIV-1 viruses, while cells with a mutated CXCR4 are resistant to C4 viruses. The double knock-out of both CCR5 and CXCR4 would allow resistance to infection regardless of viral tropism. However, the safety of such an approach remains to be elucidated. Uninfected HSCs isolated from infected individuals are engineered with either technology and then transfused back into patients. The ZNF approach targeting CCR5 has shown some promising results, although the sizes of cohorts used have been small. Gene-modified cells persisted in patients over 9 months, and cells seemed to expand and undergo trafficking to other tissues(146). An increase in CD4+ T-cell counts was observed in all individuals. Importantly HIV-1 DNA in the blood decreased. The encouraging outcome of this study has resulted in phase II clinical trials. Another gene therapy-based approach is the introduction of HIV-1 expression-dependent suicide genes encoding either toxic or pro-apoptotic proteins such as members of the Bcl-2 protein family. Constructs that are responsive to Tat and Rev viral proteins were tested(149). While obtaining encouraging results, activity of such suicide genes only affects cells that are actively producing viruses, thus the latent pool of cells would still be unaffected. Despite many attempts at HIV-1 cure, thus far only two cases, the "Berlin patient" and the early treated infant have resulted in eradication(125, 126, 143). Due to safety and economic issues associated with transplantation and gene therapy approaches, broad use of such a therapeutic approach is not feasible for HIV cure. Moreover, the gene therapy approach provides a functional rather than sterilizing cure. Nevertheless, all these studies provided valuable insights into the biology of the latent reservoirs. They constitute a proof-of-concept for HIV-1 cure. Moreover, it seems that immediate initiation of cART contributes to restricting the establishment of the latent pool. These studies highlight the need for more robust, cheaper, and feasible treatments in order to achieve HIV-1 eradication among all infected individuals. In 2004, the concept of so-called "shock and kill" or "kick and kill" therapy was proposed(150–152). The aim is to specifically reactivate proviruses in latently infected cells (shock) and eliminate the infected cells via viral cytopathic effects or/and render the cells susceptible to immune clearance (kill). New rounds of infection would be prevented by cART. "Shock and kill" therapy relies on the identification of potent and specific latency reversal agents (LRAs) alongside induction of an effective immune response against the reactivated latent pool of cells. The LRAs currently under investigation do not result in sufficient reactivation of latent HIV *in vivo*. Therefore, novel molecules that

specifically reactivate latent HIV-1 are urgently needed.

### 3. Model systems and assays to detect and study HIV-1

To study the complex nature of HIV-1 latency, reliable model systems are required that recapitulate the nature and dynamics of the latent reservoir *in vivo*. Several cell lines of lymphocytic or monocytic lineage, primary-cell models, as well as animal models, are used to study HIV latency(153).

#### Cell lines

Immortalized cell lines of T-cell and monocytic origins are cost-effective and easy to use in the study of latent HIV. They allow fast read-outs in large scale for mechanistic molecular characterization of HIV gene expression. Therefore, cell lines are an attractive platform for screening and mechanistic characterization of LRAs. To generate a latent cell line, cells must first be latently infected with a HIV derived virus. Several different HIV derived viruses are used ranging from full length to minimal virus and can make use of a wide range of reporter constructs (e.g. GFP or luciferase). The viral Tat/TAR axis is of vital importance for the transcriptional regulation of HIV and can be included or excluded from the viral construct used. Latent infection of relevant cell lines derived from T-cells or monocytic lineage, depending on reservoir of interest generate cell lines that can be used to study the molecular mechanisms of HIV latency(154–157).

Ach-2 and U1 cells are characterized by low expression of HIV-1, which can be strongly upregulated upon TNF $\alpha$  or mitogens stimulation(154, 155). However, in these cell lines, latency results from mutations in Tat protein (U1 cell-line) or in RNA stem loop TAR (Ach-2)(156, 157). Therefore, these cell lines do not represent complexity of latency found *in vivo*, however, they do allow Tat/TAR-independent HIV-1 reactivation investigation. A more appropriate system to study latency are J-Lat cell lines derived from Jurkat cells of T-lymphocytic origin(158–160). These cells have integrated replication-competent full-length or minimal proviral constructs with an intact promoter and Tat-TAR axis, a GFP reporter gene replaces the Nef sequence in full-length proviruses or is located downstream of Tat in minimal proviruses(158). More recently this system was adapted to have pre-selected integration sites using CRISPR-Cas9(161).

These cell lines have been extremely useful to delineate the molecular requirements of HIV transcription activation and silencing. Although useful for molecular analysis and screening platforms, the cell line model systems of HIV latency also present some limitations; first, clonal cell lines are derived from a single integration event, and therefore do not reflect the diverse distribution of integration sites in the host chromatin(162, 163). Consistently, results vary depending on the cell lines used, indicating possible clonal cell line effects(164). Due to the above mentioned limitations and the considerable difference between cell line models and primary cells in terms of proliferative capacity, genomic

stability and mechanisms involved in establishing and maintaining latency, generally latency models based on primary cells are preferable.

### **Primary cells**

To more closely resemble infection *in vivo* and validate putative LRAs more accurately, several primary cell models have been developed. Depending on the cell status at infection, these models can be divided into two groups. The first group relies on purification of CD4+ T-cells from healthy donors, that are then activated and subsequently infected. Depending on the method, CD4+T-cells are purified and stimulated with  $\alpha$ -CD3/IL-2(165),  $\alpha$ -CD3/ $\alpha$ CD-28(166),  $\alpha$ -CD3/ $\alpha$ CD-28/IL-2(167), or Ag-MDDC (antigen-loaded monocyte-derived dendritic cells)(168), and infected with virus. Productively infected cells die due to virus-induced apoptosis or become latent by reverting back to a resting state. To limit infection to only one replication cycle, replication-defective viruses or antiretroviral drugs are also used. The rationale for these systems rely on the notion that a portion of activated, infected CD4+ T-cells transition to a quiescent state, shutting down general transcription and slowing down metabolism, resulting in latency(1, 3, 4, 78, 169, 170). Depending on the method used, different populations of latently infected cells are generated for use in reactivation studies. Phenotype of the cells in methods suggested by Sahu and Marini central memory T ( $T_{CM}$ ) cells remain in culture, in Yang's protocol mainly effector memory T ( $T_{EM}$ ) cells are produced, in Bosque and Planelles's method cells phenotype resembles central memory-like ( $T_{CM}$ ). The main disadvantage of these methods is the time needed to obtain results, which varies from 1 to 4 months. Furthermore, they are labor-intensive and technically challenging. The second group uses direct infection of resting memory CD4+ T-cells, which immediately after integration become latent. Cells are infected after purification and can be used after several days for reactivation studies(169, 170). Stimulation of CCR7, CXCR3, or CCR6 receptors increases the susceptibility of resting memory CD4+ T-cells to infection without T-cell activation. In the methods of Swiggard and Lassen, central memory T ( $T_{CM}$ ) and effector memory T ( $T_{EM}$ ) cells are the source of latent HIV-1; in Saleh's method naïve resting memory T-cells, in addition to  $T_{CM}$  and  $T_{EM}$  cells, constitute the latent pool. The main advantage of these methods is the time needed to evaluate the potency of putative LRA, as results can be obtained within one week. Depending on the protocol used, the amounts of cells that become latent differ from as little as 1% to up to 40%. In models where cells are activated, on average more latently infected cells are generated. Using these models, we can quantify the level of reactivation of HIV-1 in a reliable manner by measuring the production of the viral protein p24 by enzyme-linked immunosorbent assay (ELISA) or quantification of viral transcription by quantitative RT-PCR, or by detection of GFP/luciferase in case of reporter-based constructs. A novel detection method distinguishes uninfected, productively infected, and latently infected cells using a dual reporter system. A

modified HIV-1 derived genome containing GFP as a reporter of viral transcriptional activity and mCherry under an EF1a promoter as a reporter of infection (latent or productive) allows easy isolation of the different cell populations(171). Ultimately, the golden standard for testing activity of LRAs are primary cells from infected individuals under cART obtained by leukaphoresis, a process in which white blood cells are specifically isolated while other blood components are reverted back to the patients' circulatory system. The isolated cells are uninfected, latently infected, and infected with defective viruses. Large amounts of CD4+ T-cells are required and isolated from patients for testing LRAs. The development of primary cell models greatly improved the quest for LRAs, yet results differ between each model system(164). No *in vitro* models completely recapitulate the full range of latent cells *in vivo*; instead, only a small sub-fraction of latently infected cells is represented. Hence, the validation process of putative LRAs requires testing on cells derived from infected individuals(172).

### **Animal models of HIV-1 infection**

The number of animal models available to study latency is limited. The toxicity of putative LRAs can be assessed with use of mouse and non-human primate (NHP) models(173). Two mouse models have been developed and used in HIV latency studies: the humanized SCID (SCID-hu) mouse, transplanted with human thymus and liver fragments, and the humanized blood, liver, and thymus (BLT) mouse which has a human immune system with full mucosal immunity(174–176). Unfortunately, SCID-hu mice do not express human proteins involved in the viral replication cycle; therefore, the study of HIV-1 in these mice is restricted to events taking place within organs of human origin in this model. In addition, HIV-1 is not responsive to cART in these animals. BLT mice are a better model of HIV-1 infection, as they produce resting memory CD4+ T-cells of human origin. However, some components of cART do not repress replication in BLT mice(85). NHP models employ the Simian immunodeficiency virus (SIV) infection in rhesus and pig tailed macaques to recapitulate HIV-1 infection in humans(177, 178). NHP models allow the monitoring of the spread of infection. Moreover, infection in this model can be controlled by antiretroviral therapy. NHP models are helpful in studying the first stages of latency establishment, as investigating this part of HIV-1 infection is extremely challenging in patients because the pool of latently infected cells is established early during infection(40, 41, 179). One caveat to the use of SIV-based NHP models of HIV latency is that the viral 5'LTR or promoter of SIV is substantially different in sequence from HIV-1(180) and therefore latent SIV response to LRAs, which is a direct consequence of promoter-mediated transcription activation may vary substantially from latent HIV-1. In addition, animal models are far more expensive than cell-based systems. Nor do they fully reflect human infection or metabolism. Finally, ethical concerns are inherent to the use of NHP models of HIV latency.

## Detection of the latent reservoir

The study of latent HIV infection requires accurate measurement of the size of the latent reservoir and the extent of reactivation following LRA treatment. This aim is complicated by frequent occurrence of defective virus. Depending on the experimental aim, different detection methods can be employed. These methods generally rely on PCR, protein quantification, or reporter detection. The quantitative viral outgrowth assay (QVOA) is a well-established method to estimate the latent pool. The assay relies on the use of serial dilutions of cells obtained from an infected individual in co-culture with uninfected cells that are permissive to infection. Viral proteins are detected by ELISA. Unfortunately, QVOA is time-consuming, costly, and might generate false-negative results as not all replication-competent proviruses are reactivated, and thus not detected(163). Attempts have been made to improve sensitivity, reproducibility and ease of use(181–183). The HIV reservoir can be quantitated by detecting the number of viral DNA copies present in the cells. The recently introduced digital droplet PCR (ddPCR) improves on classic and nested qRT-PCR by simultaneously amplifying thousands of nanoliter reactions in combination with very sensitive detection system based on flow cytometry(173, 184, 185). ddPCR is therefore superior to nested qRT-PCR in its ability to resolve rare events such as latent HIV-1. Although PCR based methods provide increased sensitivity for the detection of viral genetic material, these approaches also detect defective proviruses, which results in false-positive results. Another recent PCR-based method for reservoir detection evades false positive results from defective proviruses. The *Tat/rev* induced limiting diluting assay (TILDA) relies on PCR amplification of multiply spliced RNA (msRNA) of *tat/rev* transcripts that are present in productively infected cells and absent in latent infection(186). Small amounts of cells isolated from patients are divided into two equal parts and distributed in limiting dilution. One half is left unstimulated while the other is activated with PMA/Ionomycin. After 12 hours, cells are lysed and subjected to ultrasensitive nested RT-PCR. By employing statistical modeling, the frequency of cells that are expressing msRNA in both groups is estimated and based on the unstimulated group a threshold of activation can be set. Using the TILDA assay, the size of the reservoir is estimated at 24 cells per million, which is more than measured by QVOA but less than measured by PCR methods(111, 163, 186). The assay more accurately estimates the true size of the latent reservoir, is highly sensitive, reproducible, fast, relatively inexpensive, and requires only 10 mL of patients' blood. However, a limitation on the TILDA assay is that it detects the presence of viral transcripts but not the production or release of infectious viral particles; therefore, it may still overestimate the true size of the reservoir, yet to a smaller extent than other PCR-based methods. Additionally, signal detection relies on amplification of highly variable region of the HIV-1 DNA; therefore, detection of all subspecies of HIV-1 might be challenging and require

extra optimization steps. While PCR techniques can distinguish different viral forms focusing on the LTR such as integrated HIV-1 DNA, non-integrated HIV-1 DNA or both(187, 188) and Alu-based techniques are used to detect integrated virus(189, 190), it is more challenging to distinguish between replication competent and defective virus. As a result, they tend to overestimate the reservoir. Different techniques try to accurately assess replication competence probing multiple sites in the virus a more(191) or focus on regions frequently mutated in the viral genome(192). Another way to circumvent the inability to distinguish defective or intact virus is use a measure of active replication in the form of cell free or cell associated RNA(183) or detecting transcripts rarely produced by defective provirus(193, 194). Thanks to the developments in high-throughput sequencing, it is now possible to identify genetically intact provirus and understand proliferation dynamics of the latent HIV-1 reservoir(47, 72). More recently, intact proviral DNA assay (IPDA) use digital droplet PCR to quantify intact proviruses and distinguish from defective provirus by checking for hypermutations and large deletions(195).

Unfortunately, all current methods to detect latent HIV-1 have limitations. First, the pool of latently infected cells in patients is extremely low, resulting often in a high noise-to-signal ratio. Furthermore, defective or hyper-mutated proviruses are detectable by PCR-based techniques, yet irrelevant for eradication strategies. Moreover, not all replication-competent proviruses are inducible in the first round of treatment, yet get reactivated upon subsequent rounds of stimulation(163). Thus, assays to measure latency reversal are overestimating – in the case of PCR-based methods – or underestimating – in the case of QVOA – the latent pool. This poses a main problem in measuring efficiency of the reactivation of HIV-1.

#### **4. Molecular mechanisms of latency**

Although replication-competent, latent HIV is transcriptionally silenced but susceptible to reactivation upon certain stimuli. Following integration into the host genome, transcription from the HIV provirus is controlled by key cellular host factors, and subject to host cell gene regulation similar to endogenous genes. Although transcription initiation is the first step at which latency can arise, following steps in transcription such as, blocks in proximal elongation, distal transcription/polyadenylation (completion) and multiple splicing can also cause latency(64). Since viral transcription initiation, elongation, and termination are tightly regulated by host proteins, HIV is also widely used as a model system to study gene regulation.

#### **Host antiretroviral mechanisms thwart infection**

Upon entering the cell, HIV's RNA genome is reverse transcribed into double-stranded DNA (dsDNA). This process requires freely available deoxynucleotide triphosphates (dNTPs). By limiting the pool of freely available dNTPs, the



nucleotide scavenger SAMHD1 restricts viral replication in non-cycling myeloid cells and quiescent CD4+ T-cells(196–199). Additionally, SAMHD1 has 3′–5′ exonucleases (RNAse) activity that specifically cleaves single-stranded RNA(200, 201). Interestingly, Vpx, encoded by HIV-2 and Simian immunodeficiency virus, is an accessory protein packaged into the virion, which induces SAMHD1 degradation(202). Additionally, APOBEC3G limits viral replication by catalyzing the deamination of cytidine to uridine in the viral single-stranded DNA (ssDNA) genome during reverse transcription(203). Interestingly, APOBEC3G is inactive in memory CD4+T-cells, which helps to explain why this cell type is more permissive to HIV-1 infection. Therefore, activated CD4+ T-cells are the main target cell type of HIV infection and of the main source of the latent reservoir.

### **Integration of HIV into the host genome requires host factors**

The reverse-transcribed viral DNA genome is incorporated in the pre-integration complex (PIC). The PIC is imported into the nucleus. Chromatin organization at the nuclear pore determines integration(204–207). Host factors identified so far that affect viral integration are lens epithelium-derived growth factor (LEDGF/p75/PSIP1) and hepatoma-derived growth factor related protein 2 (HRP-2/ HDGFRP2), through an integrase binding domain. In the absence of LEDGF, provirus integration is decreased 10-fold and HIV's pattern of integration is altered(208–210). Disruption of LEDGF/p75-integrase interaction inhibits integration and residual integration is away from active genes, leading to reactivation resistant latency(211). Simultaneous LEDGF and HRP-2 knockdown further decreases viral replication(212). Nevertheless, knockdown of both factors does not completely abolish HIV-1 integration, indicating that IN alone and/or in cooperation with other host factors can still integrate(213). PIC nuclear import stimulates export to the cytoplasm of INI-1 and PML, disrupting this effect greatly improves integration efficiency(214–216). Upon knockdown of transportin-3/TNPO3 and nuclear pore protein, RanBP2/Nup35 HIV-1 integrates randomly(217). Therefore, nuclear import affects the site of integration with a preference for open chromatin.

### **Integration site determines transcriptional activity of the provirus**

HIV preferentially integrates into active genes both in patient material and transformed cell lines(162, 218–221). In T-cells provirus integrates close to super-enhancer genomic elements that cluster in specific nuclear regions(222, 223). Moreover, HIV-1 integrates in regions of genome that are in close proximity to nuclear envelope(206). Latent integrations are in or close to aliphoid repeat elements in heterochromatin, whereas productive integrations avoid insertion in or near heterochromatin(158). Integration is associated with transcription-inducing histone modifications (i.e., H3 & H4 acetylation and H3K4 methylation) but not transcription-inhibiting modifications (i.e., H3K27 trimethylation and DNA

CpG methylation)(224). A comparison of integration sites in resting and activated CD4+ T-cells showed that in both cell types HIV integrates in active genes. However, in activated cells, insertions were enriched for gene dense, CpG island-rich and high G/C-content regions(225). Latency in infected Jurkat cell lines correlated with integrations in gene deserts, centromeric heterochromatin, and highly expressed cellular genes(220). Within the nucleus, HIV-1 is located mostly in decondensed chromatin at the nuclear periphery, while it disfavors heterochromatic regions(226). Interestingly, latent proviruses were found to interact with a pericentromeric region of chromosome 12 in quiescent cells(227). Also, latent proviruses are typically found far away from promoters and enhancers(223) and tend to be more associated with the nuclear lamina(228). In a study of viremic progressors and viremic controllers, integration was enriched into, or in close proximity to, Alu repeats, local hotspots, and silent regions of the genome(229). In addition, close proximity of the provirus to PML bodies is associated with latency, an association that is lost upon reactivation(230). Sense and antisense integration relative to host genes can greatly affect the transcriptional state of HIV. Integration in sense orientation can lead to promoter occlusion, whereas integration in antisense orientation can lead to collision of the transcriptional machinery. Promoter occlusion occurs when the transcriptional machinery is depleted from the viral promoter by a dominant host promoter that is transcribed and negatively affects proviral expression. Indeed, chimeric transcripts of the host gene and in sense viral integrations were observed(231, 232). Additionally, Han et al. compared the effect of sense and antisense insertions of HIV relative to the active HPRT gene(233). In this setting, sense integration enhanced viral expression whereas antisense integration (transcriptional collision) led to suppression. Sense integrations were shown to be modestly preferred in latent cells, a preference that was not present in productively infected cells(234). However, integration of HIV can also upregulate host gene transcription, this only affects genes downstream of the integration, but not upstream(235). Transcriptional interference and transcriptional collision are examples of host genes interference with viral expression. On the other hand, reactivation of HIV may lead to suppression of host gene expression(231). In a cell model with a latent integration into the HMBOX1 gene, the host gene was repressed upon reactivation(236). Analysis of the transcriptome of latently infected cells reveals how latent infection affects gene expression. Reactivated infected cells derived from virally suppressed, HIV-1 patients show enrichment of nonsense-mediated RNA decay and viral transcription pathways. In addition, cellular factors, IMPDH1 and JAK1, that support HIV-1 transcription and cellular survival factors, IL2 and IKBKB, were up-regulated in latently infected cells entering that are starting to be reactivated(235). In a similar approach single reactivated latent cells from HIV-1-infected individuals on cART revealed upregulation of genes implicated in silencing

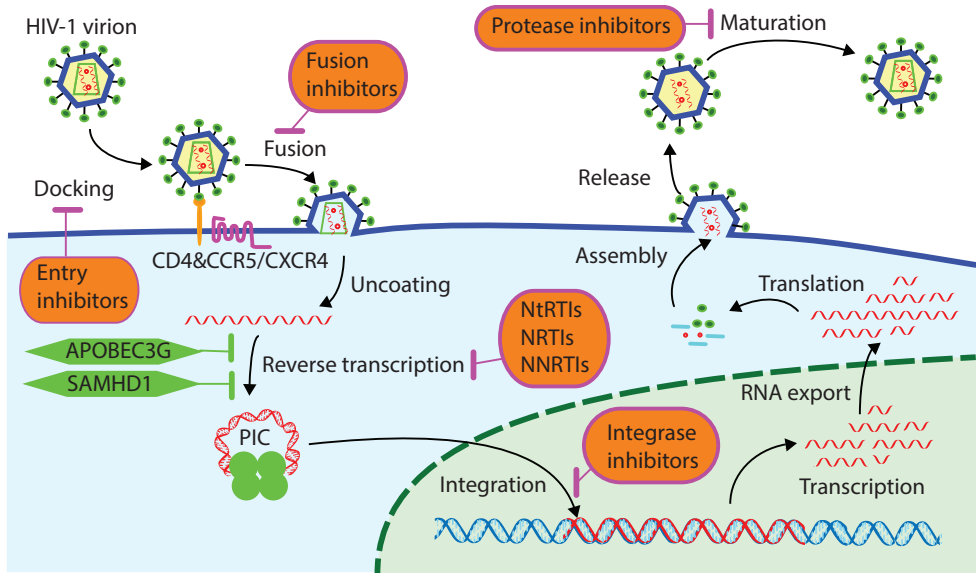


Figure 1: The viral replication cycle can be targeted pharmacologically at different stages

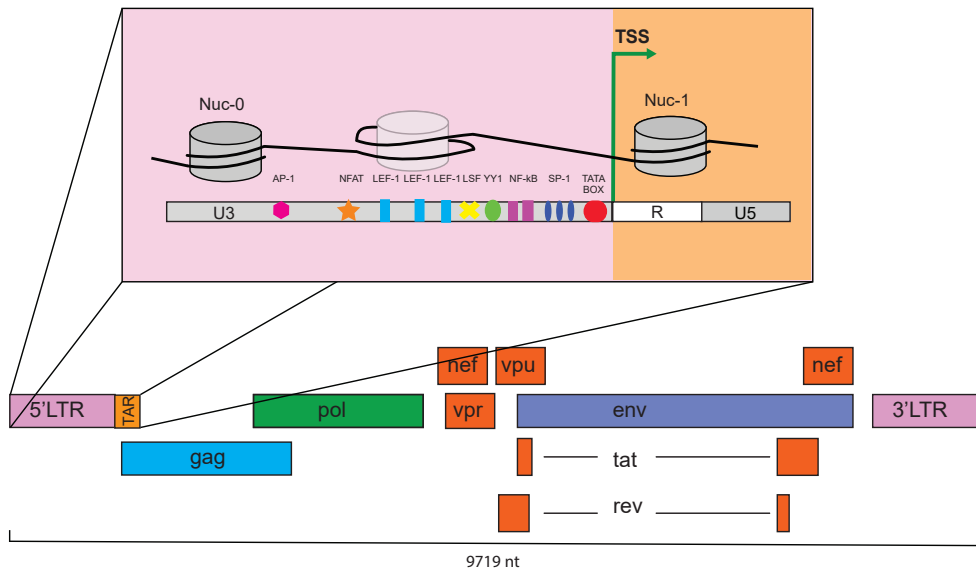


Figure 2: The genome of HIV-1

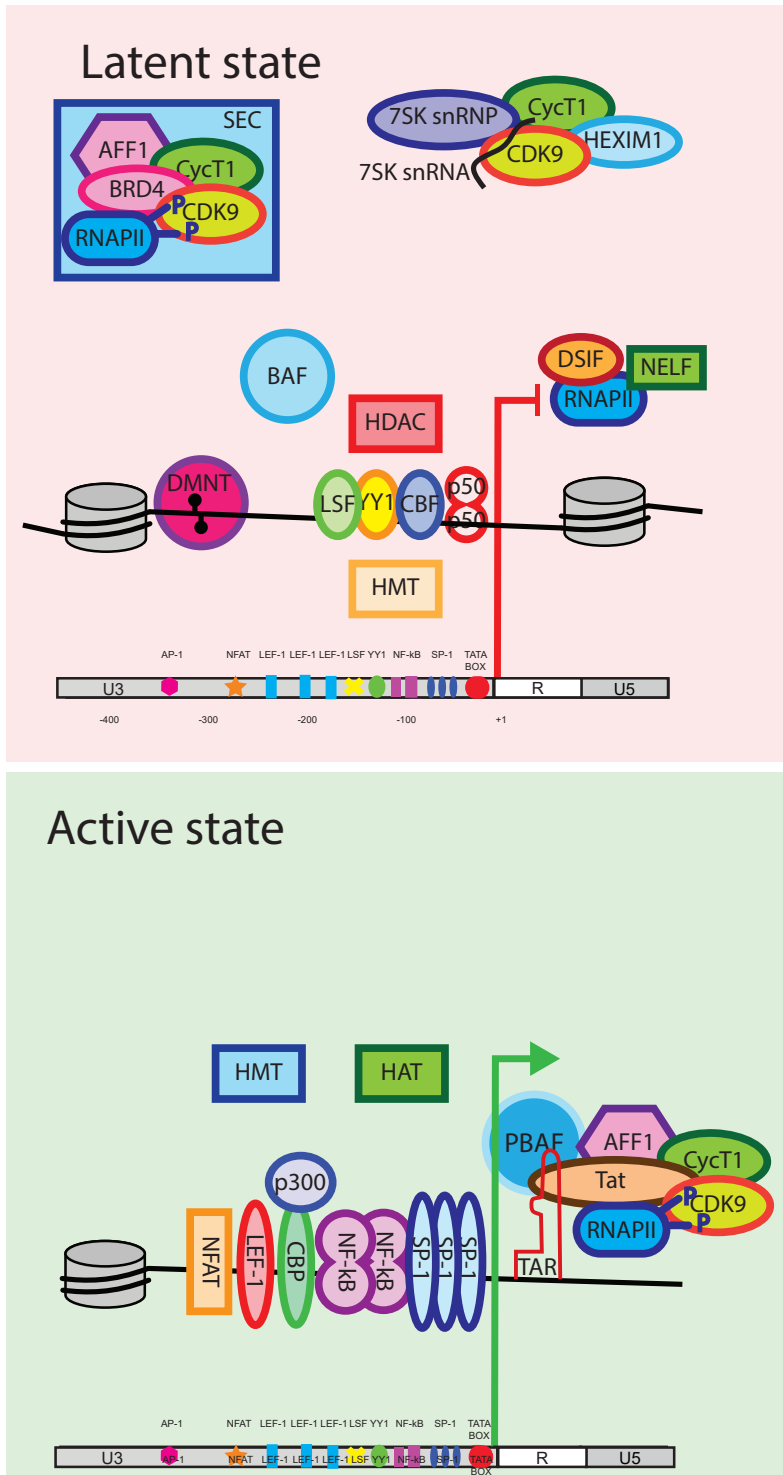


Figure 3: Molecular mechanisms in latent and productive HIV-1 infection

the virus and cell division without activation of the cell death pathways(237). An important observation with profound therapeutic consequences is that the integration site also affects the response to LRAs(223, 228).

### **The 5' LTR contains numerous putative transcription factor binding sites**

The provirus is flanked by a 5' and 3' long terminal repeats (LTRs). While transcription can be initiated from both LTRs, the 5' LTR is dominant and serves as the HIV promoter, although 3' transcription is activated when the 5' LTR is defective(238). Transcriptional interference has been proposed as the mechanism by which the 5' LTR exerts its dominance over the 3'(239). Interestingly, low-level antisense transcription takes place at the 3' LTR, a mechanism by which latency can be maintained(240–244). Sense transcription results in 40 to 50 physiologically relevant transcripts due to alternative splicing of the HIV-1 genome(245, 246). Finally, both LTRs also act as a source of negative sense transcription, which could potentially affect the expression of neighboring genes(247, 248). The 5' LTR contains three regions – U3, R, and U5 (Figure 2)(249). The R region, immediately next to the transcription start site (TSS), contains the trans-activation response (TAR) element, an important regulator of HIV expression. The U3 region contains the core promoter (nucleotides 78 to 111 downstream of TSS), a core enhancer (nucleotides 105 to 179), and a modulator region (nucleotides 145 to 1104)(250, 251). The core promoter contains three Sp1 binding sites in tandem, a TATA box, and an initiator element at the transcription start site. The core enhancer contains two NF-κB-binding sites. The modulator region – so-called because early experiments with deletion upstream of the LTR caused increased activity of the LTR, was shown by later experiments to contain binding sites for both repressive and activating factors including nuclear factor of activated T-cells NFAT, STAT5, NF-κB p65/p50 heterodimers, lymphocyte enhancer factor (LEF-1), CCAAT/enhancer binding protein (C/EBP) factors, AP-1, and activating transcription factor/cyclic AMP response element binding (ATF/CREB) factors (Figure 2)(250, 252–260). It is well established that these transcription factors have binding sites within HIV-1 sequence. Moreover, they are strong activators of HIV-1 transcription of which NF-κB is considered the most critical(261–264). In addition to the presence of these sites, bioinformatic tools indicate that this region of the HIV LTR contains a tightly clustered distribution of multiple transcription factor consensus binding elements(265).

### **Nucleosome positioning at the 5' LTR controls viral expression**

Regardless of integration position, the latent 5' LTR typically contains two nucleosomes, Nuc-0 and Nuc-1, at fixed positions(266). Nuc-1 blocks transcription elongation as it is positioned just downstream of the TSS. Nuc-1 is displaced upon virus reactivation(266–268). Nucleosomes can be altered by chromatin

remodeling complexes. A third unstable or loosely positioned nucleosome is located in between nuc-0 and nuc-1(269) (Figures 2 and 3A). BCL11B, together with the chromatin remodeling complex NuRD, strongly represses HIV-1 transcription(270). BCL11B is specifically expressed in T-cells and neurons. Interestingly, the NuRD complex consists of several proteins with histone deacetylase activities – i.e., HDAC1 and HDAC2(271).

The ATP-dependent chromatin remodeler BAF (SWI/SNF-A) was discovered by our group to be essential to both the establishment and maintenance of HIV latency (Figure 3). The BAF complex utilizes energy from ATP to push Nuc-1 from an energetically favorable position upstream of the TSS to a suboptimal region, downstream of TSS, resulting in a transcriptional block(269). siRNA depletion of the BAF complex de-repressed proviral transcription. Furthermore, in siRNA-mediated BAF knockdown, latency establishment occurred less frequently than in the presence of the functional complex. The PIC through LEDGF interacts with INI-1 a subunit of BAF, allowing nucleosomes to be deposited at the provirus, contributing to latency establishment(214).

### **Positive host factors bind to the 5' LTR**

Initial transcription of HIV-1 is entirely dependent on host factors. The 5'LTR contains numerous transcription factor binding sites(252). One of those transcription factors is a master regulator of the immune response, NF- $\kappa$ B. NF- $\kappa$ B acts as a transcription factor and is a potent activator of HIV-1 transcription initiation and elongation. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) is a hetero dimer comprised of p50 and p65 subunits involved in T-cell activation. It interacts and functions cooperatively with numerous proteins. Independent of Tat, NF- $\kappa$ B can reactivate HIV to high expression levels(272). Mutated NF- $\kappa$ B-binding sites on the LTR inhibit basal transcription and Tat transactivation(273). NF- $\kappa$ B, Sp1, and other factors (LEF-1, Ets1, and TFE-3) bind to sites near NF- $\kappa$ B sites and synergistically activate HIV transcription, even in the presence of repressive chromatin structures(274, 275). NF- $\kappa$ B and AP-1, a heterodimer of proteins from the c-Fos, c-Jun, ATF, and JDP families, cooperatively trans-activated LTR activity through the ERK1/ERK2 mitogen-activated protein kinase (MAPK) pathway(259). Acetylation of Lys310 in NF- $\kappa$ B p65 subunit is an activating mark that is removed by NAD<sup>+</sup>-dependent protein deacetylases SIRT1 and SIRT2(276). Tat positively affects NF- $\kappa$ B by inhibiting SIRT1 and stimulating degradation of I $\kappa$ B, a protein that sequesters NF- $\kappa$ B in the cytoplasm(273, 277). The viral nucleocapsid (NC) protein enhances NF- $\kappa$ B-mediated activity by interacting with the LTR(278). p65 recruits TFIIH which is part of the preinitiation complex and its subunit CDK7 with kinase activity activates CDK9, resulting in increased HIV transcription(279–281). The cell surface receptor OX40, bound by its ligand gp34, activates transcription from 5' LTR, in a manner dependent on the presence of NF- $\kappa$ B-binding sites on the LTR(282). The transcription factor E2F-1, a

regulator of S-phase gene expression, inhibits LTR transcription through the recruitment of p50 at the NF- $\kappa$ B-binding sites on the LTR(283). Members of the SV40-promoter (Sp) specific transcription factor family regulates LTR activity. Sp1 and Sp4 are activators of HIV-1(284). Expression of Sp transcription factors changes during monocytic maturation, suggesting differences in susceptibility to LTR activation during differentiation(285). Nuclear factor of activated T-cells (NFAT) can induce LTR activity in T-cells(253). NFAT recruits HATs through CBP/p300, which results in reactivation of HIV-1 transcription(286). The Janus kinase (JAK)/signal transducers and activators of transcription (STAT5) can stimulate or inhibit HIV transcription. STAT5 binds to its binding sites in the U3 enhancer region on the LTR where it promotes transcription(254). In response to a broad range of cytokines (e.g., IL-2, IL-7, IL-15) and granulocyte-macrophage colony-stimulating factor (GM-CSF) JAK-mediated phosphorylation of a C-terminal tyrosine residue activates STAT5A and STAT5B. Homodimers or heterodimers of activated STAT5A and STAT5B translocate to the nucleus to stimulate HIV expression (287, 288). Interestingly, STAT5 $\Delta$ , an isoform of STAT5 truncated on the C-terminus, acts as a repressor of LTR activity(289). Indeed, in the promonocytic cell line U1 high levels of STAT5 $\Delta$  are present. Upon stimulation with GM-CSF, STAT5 $\Delta$  blocks RNAPII from binding to LTR U3 region, inhibiting activity of HIV promoter(290). STAT5 $\Delta$  promotes p50 homodimers binding to the LTR, contributing to latency maintenance(291). In monocytes and macrophages, CCAAT/enhancer binding protein (C/EBP) factors are crucial for activation of HIV-1(258, 292–294). C/EBP, a member of the bZIP superfamily, contains a DNA-binding domain and a leucine zipper for homo- and heterodimerizations. Similar to SP1, levels of C/EBP vary during myeloid development(295). Interestingly, the HIV-1 LTR contains several C/EBP binding sites(257). Some studies employing mutagenesis of binding sites for activator protein-1 (AP-1) within proviral genome showed that AP-1 transcription factor is the crucial activator of proviral transcription, as proviruses with altered AP-1-binding sites were less prone to reactivation even if treated with strong activator such as phorbol 12-myristate 13-acetate – PMA(296). Furthermore, the latent pool was bigger in cells infected with a virus carrying a deletion in AP-1 sites, implicating that the AP-1 protein is necessary for successful provirus transcription(297). Heterodimeric protein AP-1 is formed upon phosphorylation of c-Jun N-terminal kinase (JNK) in JNK/MAPK pathway(298). It is well established that activation of TLR signaling induces nuclear localization of NF- $\kappa$ B and AP-1 mediated via JNK pathway(299–301).

In addition to the already mentioned potent viral trans-activating protein Tat, to a lesser extent the multifunctional viral protein, viral protein R (Vpr), positively affect viral transcription. Vpr is a multifunctional HIV-1 protein that plays a role in nuclear import of the PIC and cell cycle arrest in proliferating cells. Vpr also activates LTR activity through multiple mechanisms. Vpr recruits p300 to the 5' LTR increasing acetylation, resulting in HIV-1 transcription(302). Moreover, Vpr

interacts with Sp1 and TFIIB, part of the transcription initiation complex, stimulating proviral transcription(302–304). Vpr induces proteasomal degradation of HDAC1 and 3 which activated latent HIV-1 in J-Lat cell lines, PBMCs from HIV-1 infected patients and macrophages(305, 306)). In microglial cells and CD4+ T cells, Vpr associates with the Cul4A-DDB1-DCAF1 ubiquitin ligase complex, which results in the degradation of CTIP2, a negative regulator of P-TEFb(307). The viral protein Vpr also affects DNA Damage Response (DDR). Vpr causes DNA damage and DNA replication stalling, but inhibits double-strand breaks (DSB) repair via homologous recombination (HR) and nonhomologous end joining (NHEJ)(308). Two J-Lat cell lines have an increased (DDR) in reaction to double strand DNA breaks (DSBs), despite detectable viral expression(309). A comparison of the transcriptome of uninfected and latently infected cells identified 826 differentially expressed genes enriched for p53 signaling(310). This altered DDR might offer an opportunity for therapeutic intervention.

### **Repressive host factors at the 5' LTR**

Not all host transcription factors have an activating effect on LTR activity (Figure 3). YY1 and LSF recognize binding sequences in the LTR and repress transcription through epigenetic modification(311). The subunits SUPT16H and SSRP1 of the FACT (facilitates chromatin transcription) complex are recruited to LTR, where they repress viral transcription(312). SUPT16H disrupts Cyclin T1 association with Tat. C-promoter binding factor-1 (CBF-1) also represses HIV through epigenetic silencing(313, 314). c-Myc recruits an epigenetic silencing factor to repress HIV-1(315). Estrogen receptor-1 (ESR-1) was also identified as a repressive host-factor, which means reversing latency in women might prove more difficult(316). Transcription factors initiate LTR activity, but full-length transcripts are not produced because transcription elongation is inhibited. DRB sensitivity-inducing factor (DSIF), a heterodimer composed of hSpt4 and hSpt5 proteins, induces capping of RNA from newly initiated transcription complexes(317). The subunit hSpt5 interacts directly with nascent RNA as it appears from the RNAPII exit site and recruits negative elongation factor (NELF) (Figure 3)(318–320). Paused Pol II is stabilized by DSIF and NELF. NELF prevents binding of the anti-pausing transcription elongation factor IIS (TFIIS) and binds DSIF and exiting RNA(321). Escape of transcripts from the promoter proximal pause site is prevented by NELF, which induces termination of transcription over several hundred bases(322). NELF plays a role in early elongation complexes distinct from RNA Pol II pause-release(323). Moreover, the binding sequence of NELF subunit E recognizes a homologous sequence on TAR, increasing association of NELF with the LTR, which results in transcription silencing. Indeed, experiments where NELF is knocked down show higher basal HIV transcription and reactivation from latency(324–326). A novel, RNA interference independent, mechanism mediated by microprocessor and termination factors causes transcriptional silencing and chromatin remodeling



at the HIV-1 promoter(327). Microprocessor binds to TAR, which is then cleaved by Drosha into two RNAs, a 5'-end and 3'-end product. The 5' is further processed in an Rrp6-dependent manner into a transcription repressing RNA species. The 3' RNA recruits termination factor Xrn2 and Setx, which induces RNAPII pausing and premature termination of transcription(327).

### **The viral protein Tat transactivates viral transcription through recruitment of P-TEFb**

While some host transcription factors recruit RNAPII, transcription elongation does not occur resulting in the generation of short abortive transcripts by promoter proximal pausing(328, 329). These ~60nt transcripts include TAR, which has a stem-loop structure and binds near the HIV 5'LTR Transcription Start Site (TSS), inhibiting RNA-polymerase. However, due to T-cell activation or leaky transcription HIV's potent trans-activating protein Tat is produced. This initiates the viral phase of transcription, in which TAR directly binds to Tat, which recruits the positive transcription elongation factor b (P-TEFb) to the LTR(330). P-TEFb consists of CDK9, a serine/threonine kinase, and CyclinT1. The N-terminal cysteine-rich region of Tat (Cy22-Cy37) binds to Cyt1 through Zn<sup>2+</sup>-mediated interactions(331–333). Tat binding to P-TEFb induces significant conformational changes in P-TEFb, allowing Tat and Cyt1 to cooperatively recognize and stably bind TAR(334, 335). Active P-TEFb shifts RNAPII promoter proximal pausing to transcriptional elongation leading to a productive infection(336). Tat-P-TEFb also phosphorylates NELF-E resulting in the dissociation of NELF from TAR and the paused RNAPII complex(320, 324, 326, 337). CDK9 phosphorylates RNAPII at the carboxyl terminal domain (CTD) at Ser2 and Ser5 residues of the 52 heptad YSPTSPS repeats, which regulates progression to the elongation phase of transcription(338–340). The phosphorylation status determines regular and alternative RNA splicing and the 3' end recruitment of polyadenylation factors(341, 342). Ser2 phosphorylation of the RNAPII CTD recruits splicing-associated c-Ski-interacting protein, SKIP, and stimulates elongation transcription and alternative splicing of the Tat-specific splice site through interactions with U5 snRNP proteins and tri-snRNP110K(343). CDK9 also phosphorylates Thr4 on the C-terminal repeats (GS(Q/R)TP) of hSpt5, a subunit of DSIF. This converts DSIF into a positive elongation factor that prevents nascent RNA from breaking off from the transcription complex prematurely and inhibits pausing of RNAPII at arrest sites(344, 345). By removing several blocks Tat-P-TEFb induces transcriptional elongation as well as co-transcriptional processing. During active transcription elongation, increased recruitment of RNAPII to TSS maintains a stable level of RNAPII at the promoter proximal region(326). Throughout transcription, Tat-P-TEFb remains associated with the elongating transcription machinery(344, 346, 347). Productive infection requires the presence of Tat. Exogenous expression of Tat rescues HIV from latency(348). Mutations in Tat affect how it interacts with

binding partners such as P-TEFb and NFκB which results in a propensity for latency(334). Several host factors have also been described to interfere with tat transactivation function; The nucleolar protein NOP2/NSUN1, a m5C RNA methyltransferase, binds to the 5'LTR and competes with Tat for TAR RNA binding, knockdown of NOP2 causes reactivation of latency(349). Forkhead box transcription factors, FoxO1 and FoxO4, destabilize Tat mRNA and suppress Tat-mediated transcription(350). Thus, the balance between positive and negatively acting host transcription factors as well as Tat expression is crucial in determining whether productive transcription will occur at the HIV 5'LTR or if it will become latent.

### **Availability of P-TEFb is tightly regulated**

In activated T-cells, inactive P-TEFb predominantly resides in the 7SK small nuclear ribonucleoprotein (snRNP) complex (Figure 3)(351–353). Phosphorylation of CDK9 on Ser175 by CDK7 is a mark of free, activated P-TEFb(281, 354). And in the nucleus, the 7SK snRNA complex localizes near actively transcribed genomic region(355, 356). The 7SK snRNP complex consists of 7SK snRNA, HEXIM1 (or its homolog HEXIM2), the La-related protein 7 (LARP7), and the 7SK-specific 5' methylphosphate capping enzyme (MePCE). The snRNA functions as a scaffold: it binds two units of P-TEFb and one HEXIM1/2 homo-/heterodimers(357, 358). MePCE and LARP7 protect the 7SK RNA from nuclease degradation, MePCE binds the 5' end, LARP7 the polyuridine 3' end(359–361). Tat disrupts the interaction between pTEFb and HEXIM1/7SK snRNA and recruits P-TEFb to 5' LTR, resulting in active transcription(339). Additionally, through the binding of nascent RNA, SRSF2 and P-TEFb are released from the 7SK complex and induce transcription elongation in a manner similar to TAR/Tat-mediated recruitment of P-TEFb(362). Tat recruits UBE2O to ubiquitinate HEXIM1 which causes P-TEFb in the cytoplasm to be released from the 7SK complex(363). Phosphorylation of HEXIM1 at Tyr271 and Tyr 274 decreases retention of P-TEFb in the 7SK RNP(364). CTIP2 associates with HEXIM1 whereby it inhibits P-TEFb by repressing the CDK9 kinase activity of P-TEFb(365). However, levels of HEXIM1 are low as well, so sequestration of P-TEFb in the 7SK RNP complex might not be the restriction factor of reactivation(366). BRD4 interacts with P-TEFFb and can recruit it from 7SK snRNP complex to promote transcription(360, 367). Due to similarities in their C-terminal P-TEFb interacting domains(367), Tat and BRD4 compete for P-TEFb(368, 369). In a latent model, knockdown of BRD4 results in Tat-dependent reactivation of HIV-1(370). BRD4 binds to LTRs with low Ach3 but high Ach4 content, where it disrupts Tat's interaction with super elongation complex (SEC). Disruption of KAT5, the lysine acetyltransferase responsible for acetylating H4, causes reactivation in both a primary cell I model and cells from patients on cART(371). Bromodomain and extra-terminal domain family of proteins (BET) play an important role in repression of the HIV-1 transcription. BET proteins are responsible for the

recruitment of P-TEFb to transcribed genes(369, 372). BRD4 competes with viral protein Tat for binding site on pTEFb, and it represses HIV-1 transcription(368, 369). BRD4 degradation leads to a global disruption of productive transcription because of defective transcription elongation complexes resembling CDK9 inhibition(373). The short isoform of BRD4 recruits the BAF complex and as a result promotes HIV-1 latency(374). Knockdown of BRD2 indicates this protein contributes to the maintenance of latency. These results are consistent with the notion that BRD2 is the binding repressor complex such as HDACs(375, 376). In resting CD4<sup>+</sup> T cells levels of Cyclin T1 and active CDK9 are low, but increase upon activation(314, 366, 377, 378). The Hsp90/Cdc37 complex binds and stabilizes CDK9 in the cytoplasm in resting CD4<sup>+</sup> T cells(281). Phosphorylation status of Thr186 on CD9 directs association with CycT1 and 7SK snRNP (phosphorylated) or Hsp90/Cdc37 complex (unphosphorylated)(281).

### **P-TEFb activates HIV-1 transcription elongation as a component of the SEC**

P-TEFb is required for activation of HIV transcription but does not explain the maximum observed viral expression; therefore, additional factors are necessary(379, 380). P-TEFb is an integral part of the super elongation complex (SEC) (Figure 3), which is a potent activator of transcriptional elongation of host genes(347, 381). It is composed of one of two scaffold proteins, AF4/FMR2 proteins AFF1 or AFF4. Translocations of AFF1 and AFF4 resulting in fusion proteins are commonly found in mixed lineage leukemia (MLL)(382–384). The resultant fusion proteins cause aberrant recruitment of SEC to MML-specific genes(385). AFF1 and AFF4 recruit many other proteins to the SEC(386), such as ELL family of elongation stimulatory factors ELL1 and ELL2, which inhibit RNAPII pausing and synergistically improve Tat-transactivation with P-TEFb(381). Moreover, knockdown of ELL2 strongly suppresses viral expression(315, 333, 343, 364). Tat and AFF4 inhibit the polyubiquitination-mediated degradation of ELL2, increasing available levels of SEC(364, 387). Knock out of ELL2, AFF1, or AFF4 by CRISPR-Cas9 strongly reduces reactivation of latent HIV by LRAs(388).

### **Tat can be extensively post-translationally modified – “Tat code”**

Modifications on numerous amino residues of Tat regulate the interaction with a wide variety of host proteins. In comparison to the histone code which is used to explain the multiple modification on histone tails and their function, a “Tat-code” has been proposed(85). Tat is phosphorylated on Ser16 and Ser 46 by CDK2, modifications which result in transcription inhibition(389). Acetylation of Lys28 increases affinity for P-TEFb binding and is removed by HDAC6(390–392). Tat dissociates from TAR and binds acetyltransferase PCAF which acetylates Tat at Lys50 and Lys51(390, 391, 393–396). Acetylated Lys50 allows recruitment of the PBAF (SWI/SNF B) chromatin remodeling complex to the LTR(393, 397–399). SIRT1 deacetylates Tat at Lys50 as part of a late phase of transcriptional regulation,

stripping Tat of acetyl groups allowing its reuse in subsequent rounds of transcriptional cycles(400). Monomethyl-transferase Set7/9 and LSD1, respectively, methylate and demethylate Lys51. Demethylated Lys51 of Tat enhances HIV-1 transcription(400, 401). Hdm2 polyubiquitinates Lys71, activating Tat(402).

### **Epigenetic modifications regulate latency**

Epigenetic modifications of nucleosomes such as histone-acetylation and -methylation and of DNA such as DNA-methylation play an important role in regulating the proviral transcription. Nucleosomes are the basic units of organization of chromatin and consist of a combination of histone subunits. Histones have an amino acids tail that can be extensively modified. Two extensively studied modifications that regulate expression effects are histone-acetylation and histone-methylation. Histone-acetylation by histone acetyl transferases (HATs) induces chromatin loosening, while histone deacetylases (HDACs) reverse the effect by removing the acetyl group (Figure 3). HATs such as p300/CREB-binding protein (p300/CBP) and p300/CBP-associated factor (P/CAF) can be recruited to activate the HIV LTR(256, 403). HDAC1, HDAC2, HDAC3, and HDAC6 repress HIV(404–407). Numerous host factors recruit HDACs to the LTR. A negative regulator of P-TEFb, CTIP2 in cooperation with COUP-TF and Sp1 also recruits HDAC1 and HDAC2 to the HIV LTR in microglial cells(408, 409). Host factors LSF and YY1 co-operatively bind to the LTR, where YY1 recruits HDAC1 to deacetylate Nuc-1(311). CBF-1 and c-Myc also repress HIV through the recruitment of HDAC1(313–315).

Methylation of histones by histone methyltransferases (HMT) can act as an activating or repressing mark depending on the histone tail residue modified (e.g., methylation of lysine 4 on histone 3 (H3K4) is activating whereas H3K9, H3K27, and H4K20 methylation is repressive). HMTs modify specific histone residues, e.g., EZH2 (H3K27me3), SUV39H1 (H3K9me3), G9a (H3K9me2), and G9a like protein, GLP (H3K9me2). The repressive methyl groups deposited by these HMTs contribute to the maintenance of latency(410–414). Moreover, EZH2 is suspected to recruit additional repressive proteins such as HDACs and other HMTs(412). SMYD2, that mono-methylates lysine 20 at histone H4 (H4K20me1) was found to reactivate latent HIV in an RNAi screen in T cell lines and in primary CD4+ T cells(415). In a primary cell model of latency marks of heterochromatin H3K9me3 or H3K27me3 gradually stabilized 4 months after infection. At the same time, despite prolonged proviral silencing, a subset of cells maintained active chromatin marks (H3K27ac) and elongating RNAPII at the latent provirus(416). A novel histone modification mark of latency was proposed when histone lysine crotonylation was linked to latency reactivation. Over-expression of the crotonyl-CoA-producing enzyme acyl-CoA synthetase short-chain family member 2 (ACSS2), leads to increased histone lysine crotonylation and in turn to a propensity for

reactivation(417).

DNA methylation at CpG dinucleotides represses transcription by disrupting the binding of transcription activators to their binding sites or indirectly through the binding methyl-CpG binding proteins (MeCPs). In cell line models of latency, the HIV-1 LTR contains two CpG islands that are hypermethylated (Figure 3)(418). Methyl-CpG binding domain protein 2 (MDB2) and HDAC-2 bind to the second CpG island on the HIV LTR and are displaced from there when cells are treated with cytosine-methylation inhibitor 5-aza-2'-deoxycytidine(418). In memory CD4+ T-cells from long-term aviremic and viremic patients, an increase in HIV LTR DNA methylation was observed in the aviremic patients(419). The methylation of the HIV LTR in long-term non-progressors and elite controllers is increased compared to the LTR of aviremic patients on cART(420). In contrast, this difference was not found in the first CpG island of resting memory CD4+ T-cells from aviremic patients, indicating that the mechanism by which DNA-methylation regulates latency deserves further exploration. In another study low level of promoter methylation and higher levels of intragenic methylation were observed in HIV-1 patient cohort(421).

### **Post-transcriptional control regulates viral expression**

Only a small fraction of cells reactivated by LRAs actually produce viral proteins(59). The lack of viral proteins, but presence of viral transcripts suggests that post-transcriptional regulation plays a role in latency. Indeed, pharmacological inhibition of splicing blocks HIV replication(422). But splicing factors can positively and negatively affect viral replication: Depletion of Up Frameshift Protein 1 (UPF1) inhibited reactivation and viral RNA expression and overexpression modestly reactivated, its interacting partners UPF2 and SMG6 negatively affected reactivation(423). Inhibition of splicing factor 3B subunit 1 (SF3B1) disrupts Tat-mediated HIV transcription and reactivation from latency by different LRAs(424). While depletion of matrix-associated RNA binding protein Matrin 3 (MATR3) did not affect transcriptional reactivation of latent provirus, production of Gag was inhibited. Although MATR3 was nearly undetectable in peripheral blood lymphocytes, it was upregulated by PHA treatment(63). Non-coding RNAs exert post transcriptional control on gene expression. Small non-coding RNAs (<200 nt) and in particular microRNAs (miRNAs) are well established to have regulatory function. The study of long non-coding RNAs (lncRNA, >200 nt) is an emerging field because of their epigenetic regulatory potential. Both viral and host miRNAs and lncRNAs affect replication of HIV-1(243, 425–427). RNA interference (RNAi) is a post-transcriptional gene silencing mechanism. miRNAs post-transcriptionally suppress or silence gene expression as part of the RNA-induced silencing complex (RISC) forming a protein–RNA complex. Pri-miRNAs are generated by RNAPII and are subsequently processed by microprocessor into pre-miRNAs in the nucleus. Following export to the cytoplasm, they are cleaved by

Dicer and incorporated into RISC. RISC generally binds in the 3'-untranslated region (3'UTR) of a target mRNA. The bound transcript is degraded or transcription is impeded depending on the level of homology, resulting in translational repression. The RNAi affects the infectivity of monocytes and macrophages(428). Comparisons of productively infected, suppressed, and uninfected patients found difference in miRNA profiles, but it is very unlikely that the observed effects are due to viral activity because the number of infected cells is low in elite controllers or under cART(429–431). Knockdown of Dicer or Drosha, a component of microprocessor, stimulates HIV-1 replication, indicating that miRNA generally are responsible for suppression of proviral transcription(425, 426). However, phenotypic effects are hard to interpret due to the pleiotropic side effects of microprocessor depletion. RNAi affects infectivity by targeting transcripts of key host factors and viral proteins involved in HIV-1 repression. In resting T-cells, the polycistronic miRNA cluster miR-17/92 is suppressed by HIV, resulting in PCAF upregulation(425). Additionally, Cyt1 is negatively regulated by miR27b(432). Moreover, during differentiation from monocytes to macrophages, expression of miRNA198 and miR27b decreases relieving suppression of Cyt1(432, 433). In infected cells Tat, and possibly Vpr, inhibit RNAi(434–436). In resting, but not activated, CD4+ T-cells a cluster of five miRNAs (miR-28, miR-125b, miR-150, miR-223, and miR-382) were found to be upregulated. They all target viral mRNAs for degradation; therefore, these miRNAs are contributing to latency maintenance(428). However, further studies are required as results thus far are inconsistent(437–443). Ribozymes might also be involved in the establishment and maintenance of latency as a screen identified 17 hairpin ribozymes targeting different steps of the viral replication cycle, among which integration and transcription(444). lncRNAs can modulate gene expression through different proposed mechanisms: (1) affecting mRNAs through sequence recognition, (2) recruiting proteins to DNA, (3) blocking host factors by assuming a secondary structure, (4) functioning as a scaffold for protein complexes. An anti-sense lncRNA of HIV-1 inhibits viral replication(243). The non-coding repressor of NFAT (NRON) inhibits LTR activity in a NFAT-dependent manner(427). The viral protein Nef is targeted by miR29a which interferes with HIV replication(426, 445). TRIM32 activates HIV-1 expression through the NF-kB pathway and is downregulated by miRNA-155(446). Tat-induced upregulation of miR34a and miR217 inhibits SIRT1 expression, which in turn results in high abundance of NF-kB, enhancing proviral transcription(447, 448). miRNA-182 has a positive effect on LTR activation by Tat(449). miR-1236 restricts viral replication by repressing Vpr (HIV-1)-binding protein expression, VprBP(450). HIV-1-derived miRNAs (vmiRNAs) were predicted *in silico*(451). Applying deep sequencing technologies vmiRNAs were observed in cell line model systems of latency(452, 453). The TAR-derived miRNA-TAR5p and miR-TAR3p are asymmetrically processed and both repress LTR activity(454). The *Nef*-derived miR-N367 inhibits viral

promotor activity(455). Nevertheless, relevance of vmiRNAs is debatable as no vmiRNAs were detected in PBMCs or macrophages(456).

### **Stochastic gene expression**

The initial model of HIV latency proposed that resting memory CD4+ T-cells are deprived of host factors that are necessary for viral expression. An alternative model proposes that expression is highly stochastic. Due to fluctuations in chromatin state and availability of the transcription factors, the latent and productive state co-exist(457, 458). In support, clonal lines (containing the same integration) showed binominal distributions of viral expression(459). Transcriptional bursts of 2–10 mRNA transcripts were estimated to be the source of HIV-1 gene expression(460). In addition to the abundance of host factors, variability in host-cell size contributes to the stochasticity in viral expression, where a larger host-cell size provides a cellular environment conducive for enhanced burst size of viral expression(461). Tat-controlled positive feedback extends the expression reactivation(462). But Tat's noncooperative reversible binding of the LTR generate also contributes to bimodal viral expression(463, 464). The sensitivity to reactivation is also stochastic, as cells derived from patients remained latent during a first round of activation and were reactivatable in the next round of activation(163). Moreover, molecules that increase gene expression fluctuations synergistically enhance HIV-1 reactivation(465).

## **5. HIV cure**

Mechanistic insight into the complex nature of latent HIV-1 infection provides a rationale for eradication strategies. This has led to the proposed strategy of “Shock and Kill””. In which the latent virus is activated – “Shock”. Followed by complete clearance of all HIV infected cells – “Kill”.

### **Activating the latent reservoir – “Shock”**

The identification of molecules that cause latency reversal, without triggering global T-cell activation, is the first objective in eradication strategies. The initial step of LRA discovery is screening drug libraries with cell-line-based models. Positive hits are evaluated further using primary-cell-based models as they better recapitulate the nature of latent reservoirs. If effective, putative LRAs should undergo reactivation studies using primary cells derived from HIV-1-positive individuals that are on cART as well as toxicology studies in animal models, in case of novel molecules. It is advantageous to include molecules that are already approved drugs in such putative LRAs libraries, employing them into clinical practice would be time and resources effective. Moreover, in order to easily diffuse through cell membranes, ideal LRAs are small molecules, with molecular weight below 900 daltons, although clinical practice shows that most effective compounds do not exceed 500 daltons(466, 467). The first attempts to reactivate

proviral DNA failed, due to the use of agents (e.g., IL-2 or a monoclonal antibody against CD3 receptor) which resulted in global T-cell activation. Indeed, viral p24 and plasma HIV-1 RNA levels increased, but the toxicity of such treatment left this approach useless(468–470). Newer LRAs seek to avoid global T-cell activation, instead focus on creating a cellular environment conducive to viral transcription. Grouping them based on their targeted molecular mechanism, they can be grouped into the following:

- chromatin modulators
- activators of transcription
- transcription elongation control

### **Chromatin modulators**

Chromatin modulators are a group of LRAs that aim to change chromatin structure by affecting post translational modifications (PTMs), nucleosome positioning and DNA methylation status. PTMs of nucleosomes can be further subdivided what type of modification of the histone tail they affect.

Several histone methyltransferases (HMTs) such as EZH2, SUV39H1, and G9a interact with 5' LTR contributing to maintenance of latency by deposition of repressive methyl groups on nucleosomal proteins(410–413). Moreover, EZH2 recruits additional repressive proteins such as HDACs and other HMTs(412). Early EZH2 inhibitors, such as DZNep, reactivated latency, but with considerable toxicity(412). Next generation EZH2 inhibitors EPZ-6438 and GSK-343 have improved specificity and reactivated latency in resting memory T cells isolated from HIV-1-infected patients on cART(414). SUV39H1 and G9a can be targeted with Chaetocin and BIX-01294, respectively(410, 471). However, high toxicity, due to pleiotropic effects, makes them unsuitable for clinical practice. A less toxic, BIX-01294 derivative, UNC-0638 reversed latency in CD4+T cells from suppressed patients(414). Another HMT, SMYD2, that mono-methylates lysine 20 at histone H4 (H4K20me1) was found to reactivate latent HIV in an RNAi screen in T cell lines and in primary CD4+ T cells. Interestingly, SMYD2 pharmacological inhibition by AZ391, also caused latency reversal(415).

Histon acetyltransferases (HATs) post translationally modify histone tails by adding an acetyl group, causing chromatin loosening, allowing transcription. The removal of acetyl by histone deacetylases (HDACs) from nucleosomes have a repressive effect. Consequently, the inhibitors of HDAC (HDACi) have latency reversing potential. Interestingly, high levels of the metabolite acetate in the gut and lymph nodes act as HDACi and aid HIV replication(472). Inhibition of HDACs results in an increase of histone acetylation level by HATs. HDACi with a benzamide moiety and pyridyl cap group, exemplified by Chidamide, caused reactivation with the least pronounced toxicity(473). Particularly, HDACs 1 seems to contribute to HIV-1 repression(474, 475). In a very promising study by Archin et al. (2012), a single treatment with Vorinostat resulted in an increase in proviral RNA. Unfortunately,



the follow-up study with additional, multiple-dose rounds of treatment showed that increase on HIV-1 transcription is neither sustained nor elevated(476). It is possible that other mechanisms maintaining latency compensate histone acetylation, in order to restrain proviral transcription. Alternatively such low concentrations of Vorinostat result in activation of pTEF-b instead of HDAC inhibition(477). Fortunately, HDACis are already used in clinical therapies, e.g., VPA is used in epilepsy and bipolar disorders, Vorinostat and Romidepsin are used to treat cutaneous T-cell lymphoma (CTCL) while Panobinostat is used in patients with multiple myeloma. Although initially HDACis, like valporic acid (VPA), Vorinostat (SAHA), Romidepsin, Panobinostat, Givinostat, Droxinostat, or Entinostat, were very promising class of LRAs leading to some (Vorinostat (SAHA), Romidepsin, Panobinostat) undergoing clinical trials(478–480), so far no significant reduction in reservoir has been observed(481, 482). Oral bacteria secrete short-chain fatty acids (SCFAs) including butyric acid, propionic acid, isovaleric acid, and isobutyric acid that are capable of HIV-1 and herpesviruses latency reversal(483, 484). Some of these molecules are known HDACis (e.g. Butyric acid)(485). Moreover, SCFAs not only promotes histone acetylation, but also inhibit repressive histone formation and DNA methylation. Furthermore, they activate P-TEFb resulting in increased elongation of transcription from 5' LTR(476, 483, 486). Therefore, combinatorial treatments are more potent in purging the virus. Since HDACs are involved in general regulation of gene expression; they have pleiotropic effects causing toxicities. Therefore, their use must be strictly controlled and monitored in order to provide maximal safety(487). Nevertheless, HDACis are still under much interest. Especially, finding more specific HDAC inhibitors is very appealing, as current drugs are inhibiting a wide range of different HDACs, contributing to high toxicity(488).

Another complex, belonging to ATP-dependent chromatin remodelers' family, BAF complex (SWI/SNF-A) is a main player in the establishment and maintenance of latency.<sup>480</sup> Several molecules are able to disrupt the BAF complex and thus decrease the frequency of latency establishment and reactivate HIV-1 in cell line and primary cells models of latency(486). Moreover, they synergize with other LRAs – SAHA and prostratin. Furthermore, two most potent inhibitors – caffeic acid phenethyl ester (CAPE) and pyrimethamine (PYR) did not activate T-cells derived from healthy donors. Moreover, PYR is a registered drug used in malaria treatment. Therefore, these inhibitors are promising molecules to include in eradication strategies. However, studies in more relevant primary models of latency are required. More recently a group of inhibitors that target the ARID1A subunit of BAF were identified to reactivate latent HIV in an in vitro T cell line, an ex vivo primary cell model of HIV-1 latency, and in patient CD4+ T cells without toxicity or T cell activation(489).

CpG methylation of the HIV 5'LTR recruits methyl–CpG-binding protein (MBD2) and subsequent recruitment of the repressive NuRD complex(418, 419). Inhibition

of DNA methyltransferases (DNMTs) with 5-aza-2' deoxycytidine (aza-CdR or Decitabine) leads to modest reactivation of latent HIV-1. This activity can be further enhanced with PKC agonists(418). Because methylation of the 5'LTR in the latent reservoir of HIV-infected aviremic individuals on cART is rare, the contribution of LTR methylation to maintaining latency is disputed(419). However, sequential treatment with 5-AzadC and HDACIs was more effective compared to simultaneous treatment both in vitro and ex vivo(410).

### Activators of transcription

The 5'LTR of HIV contains numerous TF binding sites, such as NF- $\kappa$ B, AP-1, STAT5 and NFAT. Stimulating TFs to induce transcription is an obvious route to reactivate latent HIV. Among the TFs targeted for reactivation, NF- $\kappa$ B is arguably the most potent. NF- $\kappa$ B/p65 is a master regulator that takes input from several pathways that can be pharmacologically targeted, such as protein kinase C (PKC), Toll-Like Receptor (TLR) and TNF $\alpha$  signaling. Because NF- $\kappa$ B regulates immune and other functions, there is a risks of serious side effects.

One way to stimulate NF- $\kappa$ B is the use of molecules that are able to selectively activate the protein kinase C (PKC) pathway. PKC pathway agonists trigger nuclear localization of NF- $\kappa$ B, NFAT, and AP-1 transcription factors. Therefore, PKC agonists are one of the most potent activators of HIV-1 transcription. Currently, two PKC agonists are being scrutinized clinically: prostratin and bryostatin, due to their safety and specificity toward HIV-1 reactivation. The latter is a clinically available drug(490). Moreover, these two compounds prevent *de novo* infections, as they downregulate viral receptor and co-receptors CD4, CCR5 and CXCR4 in PBMCs(491). The use of PKC agonists raises concerns about their safety in a clinical setting. The protein kinase enzyme family consists of several isoenzymes that play important roles in signal transduction cascades(492). As activation of latent HIV-1 is mediated via PKC $\alpha$  and PKC $\theta$ , the identification of more specific agonists of PKC $\alpha$  and PKC $\theta$  is needed. Alternatively, lowering the concentration of a-specific agonists might decrease toxicity and contribute to latency reversal(493). The plant derived compound harmine synergizes with PKC agonist to reactive latent HIV(494).

TLRs recently gained more attention, as theirs agonists are strong reactivators of HIV-1(495–497). The main role of these receptors is to activate an immune response against bacterial or viral infections(498). Stimulating TLRs (as adjuvants in immunization) as well as opportunistic bacterial infections elevate plasma HIV-RNA and improve immune function(499–503). Several TLR agonist have LRA functions. For example, TLR 9 agonists were able to reactivate latent HIV and either to activate HIV-specific CD8+ T-cells in peripheral blood or enhance cytotoxic NK cell activation(499, 504). While an agonist of TLR 8 synergized with prostratin in primary cells of HIV-infected patients(505). More recently, in SIV-positive rhesus monkeys undergoing cART treated with GS-9620, a TLR7 agonist,

reversible CD8 cytotoxic T-cells activation alongside with modest CD4 T-cell activation were observed. Moreover, elevated plasma viremia was observed as well as decrease in HIV-1 DNA in blood, colon, and lymph nodes. Interestingly, viral load returned back to undetectable levels when GS-9620 was no longer administered. More strikingly, when cART was stopped, GS-9620-treated monkeys had 0.5 log lower viral set-point than untreated, infected animals. Additionally, in cells isolated from HIV-positive individuals transcription of HIV-1 was observed(506–508). CL413 showed promise as a LRA by targeting TLR 2 and 7 at once and inducing NFkB in CD4+ T cells and TNF $\alpha$  production in monocytes and dendritic cells(509).

Several other compounds target NF- $\kappa$ B for latency reversal. The HIV entry blocker Maraviroc, that can be included in cART, agonizes CCR5 and causes NFkB-mediated latency reactivation(510, 511). In vitro treatment with cocaine leads to increase in HIV- replication in PBMCs as well as increased viral load in mouse models of HIV infection(512–514). Interestingly, in ex vivo infected primary CD4+ cocaine treatment resulted in downregulation of miR125-b expression, which led to enhanced replication of HIV-1(438). In primary human macrophages and myeloid cell systems of latency, cocaine increased replication of HIV-1. Cocaine treatment activates NF- $\kappa$ B and leads to phosphorylation of mitogen- and stress-activated kinase 1 (MSK1). Furthermore, pMSK1 phosphorylates RELA (p65), a subunit of NF- $\kappa$ B promoting the interaction of NF- $\kappa$ B with p300 and recruitment of P-TEFb to the proviral 5'LTR(515). Moreover, treatment with cocaine results in histone H3 phosphorylation, thus increasing accessibility of HIV-1 promoter for transcription factors. Therefore, cocaine not only reverses latency via NF- $\kappa$ B pathway but also causes epigenetic changes on 5' LTR as well as blocks repressive miRNA. A rather controversial molecule that reactivates HIV-1 transcription via NF- $\kappa$ B pathway is arsenic trioxide (As<sub>2</sub>O<sub>3</sub>). In the Jurkat model system of latency, As<sub>2</sub>O<sub>3</sub> activates NF- $\kappa$ B leading to HIV-1 replication. Moreover, it synergizes with prostratin, tumor necrosis factor alpha (TNF $\alpha$ ), and VPA(516). Interestingly, arsenic is already used in clinical practice to treat acute promyelocytic leukemia (APL). Therefore, it would be interesting to test this compound in more relevant models of HIV-1 latency such as primary cells infected *ex vivo* and in cells derived from aviremic patients. A group of compounds called small molecule mimetics of mitochondria-derived activator of caspases (SMAC mimetics) inhibit ubiquitin ligase BIRC2 (cIAP1), a repressor of the noncanonical NF- $\kappa$ B pathway. Treatment of J-Lat cells with SMAC mimetics caused latency reversal without T cell activation(423, 517, 518). SMAC mimetic combined with other LRAs induced apoptosis in reactivated cells and reduced HIV-1 production(519). SMAC induce autophagy and apoptosis to selectively kill latently infected resting memory CD4+ T cells without viral reactivation, while sparing uninfected cells(520).

Studies employing mutagenesis of binding sites for activator protein-1 (AP-1) within the proviral genome showed that the AP-1 transcription factor is a crucial

activator of proviral transcription, as proviruses with altered AP-1 binding sites were less prone to reactivation even if treated with a strong activator such as phorbol 12-myristate 13-acetate – PMA(296). Furthermore, the latent pool of cells infected by virus with deletion in AP-1 sites was bigger, implicating that AP-1 is necessary for provirus transcription(297). Heterodimeric protein AP-1 is formed upon phosphorylation of c-Jun N-terminal kinase (JNK) in JNK/MAPK pathway(298). It is well established that activation of TLR signaling induces nuclear localization of NF- $\kappa$ B and AP-1 mediated via JNK pathway(299, 301, 496, 521). Virtual screening followed by validation of positive hits in cell line model systems for HIV-1 latency discovered 8-methoxy-6-methylquinolin-4-ol (MMQO) as a specific activator of the JNK-AP-1 pathway, which is able to reactivate HIV-1 from its latent state. Interestingly, MMQO inhibits IL-2 and TNF $\alpha$  expression, contributing to maintenance of resting state of CD4+ T-cells(522). The recently synthesized panel of inhibitors of farnesyl transferase (FTase) are able to moderately reactivate HIV-1 transcription via JNK pathway. Interestingly, strong synergy with other LRAs, such as Vorinostat or TNF- $\alpha$ , was observed for these molecules in latency reversal(523).

Several other TFs are targeted to induce latency reversal as well. Treatment with Wnt3A/Rsp (natural stimulators of Wnt pathway) and lithium (inhibitor of Wnt repressor protein GSK3) leads to latency reversal in latent cell lines and enhances the latency reversal potential of HDAC inhibitors in CD4+ T primary cells obtained from patient volunteers when co-treated. This observation shows a functional role for three LEF1 binding sites in the 5'LTR contains, which are downstream targets of the classical Wnt pathway(524). It would be very interesting to find more potent and selective inducers of Wnt pathway, as lithium exhibits many pleiotropic, toxic effects(484, 525). Heat shock factor 1, HSF-1, is activated by resveratrol which also induces histone acetylation, which lead to reactivation(526). By inhibiting SUMOylation STAT5, STAT5 activity and occupancy at the 5'LTR increases, resulting in latency reversal(527).

### **Transcription elongation control**

Elongation is a key step in transcription. Due to the absence of host factors or positive stimuli, promoter-proximal pausing of Pol II at the 5'LTR becomes a major hurdle in overcoming latency(64). Transcription elongation is controlled by P-TEFb. By increasing freely available P-TEFb latency can be reversed. Bromodomain and extra-terminal domain family of proteins (BET), such as BRD4, compete with Tat for recruitment of P-TEFb, their inhibition by BET inhibitors (BETis) reverse latency. Inhibition of BRD4 releases P-TEFb and thus increases its availability to Tat(368, 369). Inhibition of lysine acetyltransferase KAT5 reduces histone H4 acetylation at the 5'LTR, causes dissociation of BRD4 and latency reversal(371). In addition to increasing the availability of P-TEFb, BETis can cause BAF-dependent chromatin remodeling by disrupting BRD4S-BRG1 interaction(374). Several BETis can have

reactivating potential or synergize reactivation in different models of latency(528, 529). The BET inhibitor OTX015 reverses latency through P-TEFb in different cell models and in resting CD4+ T cells from infected patients on cART(530). Treatment with BET protein inhibitor JQ1 reactivates HIV-1 transcription in Tat-independent fashion(370). Furthermore, BET inhibitor activity was positively tested in more relevant primary model system of latency(375). Unfortunately, this molecule is not clinically available, as JQ-1's half-life is short.

Other compounds aim to free up P-TEFb independent of BET proteins. Treatment of cell lines and cells isolated from aviremic patients on cART with hexamethylene bisacetamide (HMBA), an anticancer drug that transiently activates PI3K/Akt pathway, results in phosphorylation of HEXIM1. P-TEFb is subsequently released and interacts with RNAP II, resulting in latency reversal(531–533). Moreover, HMBA provides CDK9 recruitment to the viral promoter by interaction with SP1, which enhances transcription from proviral DNA. Furthermore, Klichko et al. (2006) showed that treatment with HMBA resulted in a decrease of CD4 receptor expression without affecting transcription of CCR5 and CXCR4 co-receptors. Moreover, HMBA does not trigger activation of T-cells. Bortezomib, a clinically approved protease inhibitor, reactivated HIV in latently infected primary CD4+ T cells by increasing levels of CycT1 and activated NF- $\kappa$ B(534). Another protease inhibitor thiostrepton and six of its analogues reactivate latent HIV-1 in cellular models and in primary CD4+ T cells from ART-suppressed individuals *ex vivo*, while it does not induce global T cell activation, severe cytotoxicity, or CD8+ T cell dysfunction(535).

Targeting P-TEFb has shown effective in HIV-1 latency reversal in cell models, however the clinical might be limited as P-TEFb levels are low in resting T-cells, the main reservoir(314, 366, 377, 536).

### **Immune clearance of reactivated cells – “kill”**

The second part of “Shock and Kill” is the clearance of all virus from the body. This is technically challenging because latent cells are rare, while the expression of viral proteins to be recognized by the immune system or therapy might be limited. Approaches to “Kill” either aim to revitalize or repurpose the immune system to clear the infection or in some other way incapacitate the infection. The majority of chronic HIV patients are facing immune exhaustion, characterized by low cytokine secretion, smaller proliferative capacity, and low cytopathic potential of CD8+ T-cells(537–539). Therefore, the first line of action would be reviving normal immune activity.

During chronic HIV-1 infection, immune checkpoint markers, such as PD-1, are upregulated to limit inflammation. PD-1 is associated with latency persistence. Inhibition of PD-1 with pembrolizumab resulted in latency reversal in CD4+ T cells from HIV-positive cART treated individuals(540, 541). Another PD-1 antibody undergoing clinical trials showed promising results as well(542). However, the

latency reversal potential of PD-1 targeting is disputed(543).

In so-called “elite controllers”, CD8+ T-cells effectively restrains infection without intervention of cART, by killing CD4+ T-cells that are actively producing HIV-1 particles(544, 545). The immune system can be boosted by specific amplification of HIV-1-specific CD8+ T-cells. These observations again aroused the idea of developing a vaccine. Indeed, rhesus monkeys vaccinated with CMV vectors resulted in broad cellular immune response to SIV(546–548). However, safety issues related to the use of such vectors remain to be elucidated. Another platform being investigated to increase immune response against HIV-1 are Ad26 vectors, as it was shown that vaccinated rhesus monkeys were protected against infection with SIV as well as viral loads were lowered after vaccination(549, 550). A very interesting group of immunoglobulins to include in eradication strategies are broadly neutralizing monoclonal antibodies (mAbs or bNAbs). New generation of bNAbs exerts higher potency and wider range of activity against many HIV-1 subtypes. bNAbs can combine latency reversal activity with immune boosting, as is the case with Interleukin-15 superagonist N-803 which induced robust and persistent viral reactivation in CD8+ lymphocytes depleted, SIV-positive, cART-treated macaques and had beneficial effect on the immune system(551–553). The combination of other bNAbs is potent enough to transiently suppress viremia in rhesus monkeys as well as to reduce the amount of HIV-1 DNA in the blood, lymph nodes, and gastrointestinal mucosa(549, 550, 554) and in clinical trials(555). However some Nabs have proven unsuccessful in containing escape mutants(556, 557). This could be circumvented by the use of bi- or trispecific dual-affinity re-targeting (DART) molecules that recognize multiple epitopes(558). These antibodies can eliminate reactivated cells from patients on cART(559, 560) and show promise in maintain long-term suppression in the absence of antiretroviral therapy in individuals with antibody-sensitive viral reservoirs(560). To boost the immune-systems capacity for clearance, convertibleCAR-T cells can be employed to effectively kill HIV-infected, but not uninfected, CD4 T cells and extended to include multiple anti-HIV antibodies(561).

## **6. Future perspectives and challenges**

The Berlin and London patients are the only cases where HIV was eradicated from a patient. Although they are a proof of concept that a cure is possible, unfortunately, the treatment is so onerous that to cure every infected patient using a transplant is not safe or possible. Therefore, it is important to come up with a pharmacological strategy towards HIV cure which can be scalable. A reservoir of latent HIV is the main obstacle in finding a pharmacological approach to a functional and/or sterilizing cure. Several challenges need to be addressed in order to overcome this obstacle. Defining the latent reservoir is impeded by the rare occurrence of a latent infection in a high background of defective proviral integration. Although HIV prefers integration in or near transcriptionally active

genes, this leaves ample room for variation in chromatin environment and available host transcription factors. This puts considerable demands on LRAs. LRAs should be effective, yet specific, without being toxic. As LRAs act via pathways involved in distinct cellular processes, pleiotropic effects are to be expected. Currently available LRAs do not reactivate more than 5% of latently infected cells(228) and different LRAs reactivate different CD4+ T cell sub-populations(59). Reactivating effect and toxicity of LRAs differs greatly(562). In cells that are reactivated by LRAs only a small fraction actually produce viral proteins(59, 563). The presence of viral RNA, but lack of viral proteins, points to post-transcriptional blockade that negatively affect stability, splicing, export and translation of viral RNA. This is an avenue of opportunity for therapeutic intervention but needs more research to expand our understanding of the mechanisms involved. One of the concerns arising from “shock and kill” therapy is whether putative LRAs are strong enough to drive virus production to a level at which the immune system will be able to recognize and destroy HIV-1-producing cells. Indeed, trials aiming at testing HDAC inhibitors are inconsistent in showing depletion of latently infected cells while showing increased proviral transcription(564–569). A complementary strategy would be to use multiple LRAs in combination to broadly and potentially synergistically reactivate the diversely integrated latent proviruses. Synergism between LRAs was already identified, e.g., Vorinostat and Prostratin(164). Therefore, the quest for identification and characterization of novel compounds which are able to reactivate HIV-1 transcription as well as identifying combinations of drugs that can synergize to reverse latency is needed. When assessing therapeutic potential of LRAs their reactivating ability is an important factor to consider, however arguably equally important is their toxicity on CD4+ T-cells and CD8+ cells.

Currently, no cell model is able to recapitulate the complexities of latency *in vivo*. A better system that more closely resembles the *in vivo* situation would greatly aid the understanding of molecular mechanisms underlying latency and the screening of new LRA. Moreover, as HIV-1 persists in a silent state, it contributes to a low level of inflammation, which over time leads to immune exhaustion. Furthermore, depletion of cells harboring latent provirus requires antigen-specific CTLs stimulation(545). Most likely successful eradication therapies will be based on the combination of LRAs coupled with boosting HIV-1-specific immune response. A “shock and kill” approach in combination with immune therapies provides hope for reversing HIV-1 infection.

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## Scope of thesis

The latent reservoir that persists in HIV infected patients while under treatment with combination Antiretroviral therapy (cART) is the major hurdle in achieving a definitive cure for HIV-1. A small, elusive, not clearly defined population of cells harbors a transcriptionally inactive, but replication competent HIV-1. Long-lived memory CD4+ T-cells harbor an important part of the latent HIV-1 reservoir and because they are long-lived, cART must be maintained indefinitely in patients. Otherwise, when memory cells become activated, productive HIV replication can re-start leading to de novo infection of target cells. Because it is becoming clear that long-term HIV infection and cART use have negative effects (e.g. neurological and cardiovascular), either a functional cure, in which patients can control the infection, or a sterilizing cure, where one eliminates the latent reservoir, would be sufficient as long as patients can discontinue cART. The strategy “shock and kill” has been proposed as a way to achieve definitive cure. In “shock and kill” the latent virus is “shocked” into active transcription (and virus production) by a cocktail of latency reversal agents (LRAs) and then cleared, “killed” by the immune system or viral cytopathic effects. To accomplish viral re-activation, it is necessary to identify and characterize molecular targets as well as drugs which target them in order for both specific and effective, HIV-1 reactivation. This thesis focuses on elucidating the molecular mechanisms governing HIV latency, identifying and characterizing host molecular targets for drug targeting, identifying new LRAs and devising strategies for optimal activation of the latent reservoir using combinatorial drug targeting.

HIV-1 replication requires viral transcription from the 5'LTR. In the latent state, two nucleosomes, nuc-0 and nuc-1, are positioned on the HIV-1 LTR connected by a region of DNA shown to be hypersensitive to endonucleases, a hallmark of remodeled chromatin(1). The positioned nuc-1, just downstream of the transcription start site (TSS) is transcriptionally repressive(2). The strict nucleosome positioning within the HIV-1 LTR is irrespective of integration site, and actively maintained by the ATP-dependent BAF chromatin remodeling complex(3). Disrupting the BAF complex by drug targeting or by siRNA depletion of the BAF250a subunit leads to reactivation of the HIV-1 LTR transcription(4). The 5'LTR also contains a wide variety of transcription factor binding sites, including NFkB and NFAT(5). The initial burst of transcription leads to the generation of the viral RNA transcript TAR. Although transcription is initiated, NELF (Negative elongation factor) and DISF (DRB sensitivity-inducing factor) recruitment results in pausing of RNA Polymerase II. Once sufficient levels of the viral protein Tat are expressed, TAR recruits Tat to the TSS at the 5'LTR. For transcription elongation to occur, P-TEFb (Positive Elongation Factor b) needs to phosphorylate the carboxy terminal domain (CTD) of RNA Polymerase II (RNAPII)(6). P-TEFb consists of cyclin dependent kinase 9 (CDK9) and cyclin T1. P-TEFb can be sequestered and thus

inhibited by the 7SK snRNP complex(7). The 7SK snRNP complex is a small nuclear ribonucleoprotein (snRNP) and contains among others HEXIM1, HEXIM2, MePCE and LARP7(8). Once HIV Tat recruits P-TEFb, the super elongator complex (SEC) is formed which further enhances RNAPII transcription elongation(9, 10).

Our understanding of the mechanisms governing latency provide a starting point for developing LRAs. LRAs can be divided into three classes according to molecular function in viral transcription: chromatin regulation, transcription initiation and elongation. First, chromatin regulation can be further categorized as affecting post-translational modifications (PTMs) on histone tails, or chromatin remodeling. PTMs such as transcription promoting acetylation can be promoted by histone deacetylase inhibitors (HDACi). Romidepsin, Panobinostat, Vorinostat, and Valproic acid are examples of HDACis that have been studied and have even been in clinical trials in context of HIV-1 cure studies(11, 12). Other modifications potentially interesting for targeting to achieve latency reversal are histone methyltransferase (HMT)(13, 14) and DNA methyltransferases (DNMT)(15). In addition to the contribution on histone modifications, chromatin structure can be actively maintained or altered by ATP dependent remodelers. By disrupting the beforementioned BAF complex, chromatin structure can be changed to one more conducive to transcription. Another example of targeting chromatin remodeling is the inhibition of Bromodomain and extra-terminal domain (BET) proteins BRD2 and BRD4 to promote LTR de-repression(16). Here the BRD4 short form is thought to target the BAF complex to the LTR and is responsible for maintaining a repressive chromatin structure, one which is disrupted in response to BET inhibitors(17).

Additionally, transcription initiation can be facilitated by the binding of sequence specific transcription factors whose consensus sites are present within the LTR. One of these transcription factors, NFκB, whose binding is critical to HIV-1 transcription is capable of transcription initiation and elongation at the LTR through targeting by PKC agonists, TLR agonists and Maraviroc. Interestingly, since canonical activation of NFκB can have serious side effects through general T-cell activation, one class of compounds for HIV-1 latency reversal and cure that has gained much recent attention are those that activate NFκB through the non-canonical pathway(18–20). Finally, promoter-proximal pausing at the 5'LTR TSS can be alleviated and shifted to transcriptional elongation by increasing the amount of freely available P-TEFb. One approach to accomplish this is to target the 7SK snRNP complex, which sequesters and inactivates P-TEFb.

Although several LRAs including those in the chromatin regulator class (SAHA and other HDACis) have been investigated clinically, none have thus far been shown to impact the reservoir. Therefore it is important to identify more potent, more effective drug (combinations) to more robustly reactivate HIV-1 expression in (all) the latently infected cells harboring replication competent virus, as well as to develop strategies to eliminate the re-activated cells. In this thesis we aim to

elucidate the molecular mechanisms that lead to HIV latency using complementary unbiased approaches that focus on molecular mechanisms and small molecules with latency reversal capability. Previous attempts have followed a candidate approach that builds on previous research identifying host factors(3, 21). In this thesis we employ unbiased approaches to gain insight in the molecular mechanisms of latent HIV. To elucidate the link between host cell environment and conduciveness to infection, we use genome wide association data to identify regulatory region in the genome that affect receptiveness to HIV infection. To identify novel host factors we employ an unbiased haploid gene knock-out screen. And finally, to identify novel LRAs we use fungal supernatants as a source of bioactive molecules for latency reversal capacity.

In **Chapter 1** we first provide an in depth overview of current body of knowledge on HIV latency and the molecular mechanisms that contribute to its establishment, maintenance and potential re-activation.

Although some genetic variants have previously been shown to be associated with HIV receptivity, additional variants have thus far proven elusive. Identifying SNPs that affect HIV infectivity using genome wide association studies (GWAS) is difficult, because genetic variants with relatively low minor allele frequencies and low to moderate effects tend to be overlooked, or unattainably large cohort sizes are needed. To uncover novel noncoding HIV-1-associated SNPs with potential regulatory function we integrated data from HIV-1 GWASs with T cell-derived epigenome data. In **Chapter 2** we discover the mechanistic link between an HIV-associated noncoding DNA variant and the expression of IL-32 isoforms that affect receptiveness to HIV infection. Because of its statistically significant association with HIV-1 acquisition and location in a CD4+ T cell-specific deoxyribonuclease I hypersensitive region, the regulatory potential of noncoding intergenic variant rs4349147 is explored. Deletion of a ~400bp region containing rs4349147 in Jurkat cells strongly reduced expression of interleukin-32 (IL-32), located approximately 10-kb upstream. rs4349147 is located in a long-distance enhancer of the IL-32 promoter, as chromosome conformation capture assays revealed a chromatin loop between the two. Generation of single allele clones, rs4349147 G/- or rs4349147 -/A, shows enhancer activity and interaction with the IL-32 promoter are allele dependent. rs4349147 -/A results in reduced IL-32 expression and altered chromatin conformation, compared to rs4349147 G/- cells. Additionally, the ratio of IL-32a to non-a isoforms is lower in rs4349147 G/- cells, while lymphocyte activation factors are increased, leaving cells more conducive to infection with HIV-1. To further explore the functional relationship between IL-32 isoform and HIV infectivity, we exogenously added IL32 isoform a or g via viral transduction or by supplementing recombinant protein. In both cases IL-32g, but not IL-32a, resulted in a proinflammatory T cell cytokine environment concomitant with increased susceptibility to HIV infection. This research shows the impact of a noncoding DNA

variant on the expression of different IL-32 isoforms, which in turn affects HIV susceptibility.

Currently, our understanding of HIV latency is limited to a model of latency centered around the Tat-TAR axis and chromatin modulation and maintenance. However, this model does not fully explain the phenomenon of HIV latency and is based on a rich body of work primarily consisting of building on candidate approaches. In this way, our understanding is limited to the focus of research. To expand on our understanding of HIV latency and to identify novel host factors involved in HIV latency, we performed an unbiased, two color haploid gene trap mutagenesis screen, described in **Chapter 3**. The screen adapts a haploid forward genetic screen approach. This approach unbiasedly screens the whole genome (except chromosome 8) for non-essential genes involved in the mechanisms of interest. Non-essential genes involved in maintenance of HIV latency are valuable therapeutic targets. For this a phenotypical read-out is necessary. In our case, we established a latent infection in haploid KBM7 cells using a minimal HIV with GFP reporter, while maintaining haploidy. To screen for host factors, these cells are subjected to Gene-Trap (GT) mutagenesis. By randomly inserting into the genome, GT truncates genes, effectively knocking them out. If a gene, involved in maintaining latency, is knocked out we hypothesized this would result in reactivation. Indeed, upon GT mutagenesis, some cells become mCherry positive (GT infected) and GFP positive (reactivated HIV). From the reactivated cells we derived two population and determined the enrichment of GT integration sites for each. This resulted in a candidate list of 69 genes. Of 15 candidate genes that were selected for validation, 10 showed reactivation after shRNA knockdown in 2 Jurkat derived cell lines (J-lat), A2 and 11.1. After a literature search three genes, ADK, GRIK5 and NF1, proved druggable by FDA-approved compounds. In A2 and 11.1 cells the inhibitors of GRIK5 and NF1, Topiramate and Trametinib respectively, reactivated latent HIV while not significantly affecting viability. The inhibitor of ADK, 5-Iodotubercidin, was toxic in J-Lat cells and in ex-vivo infected CD4+ T-cells. Trametinib, while reactivating in J-Lat cells, proved ineffective in primary cells. Topiramate reactivated latency in primary cells without inducing substantial cytotoxicity or general T cell activation, and therefore is a promising LRA that should be further explored in a clinical setting. In addition to the druggable targets our screen also identified CHD9. CHD9, a member of the chromodomain helicase DNA-binding protein family is an ATP-dependent chromatin remodeler. Because of the obvious function in chromatin remodeling, we further characterized CHD9's presence on HIV's 5'LTR. ChIP results show association on the LTR in the latent state, that is lost upon PMA treatment. This loss of CHD9 enrichment upon re-activation coincides with a relative increase in H3 acetylation at the HIV-1 LTR. This functional shift in chromatin organization represents a change from repressed to transcriptionally active chromatin.



Currently available LRAs are incapable of fully reactivating the latent reservoir. Additionally the mechanisms in which they reactivate are limited to our current understanding of HIV latency. To expand on the available repertoire of LRAs, in **Chapter 4**, we screened fungal secondary metabolites (extrolites) for bio-active molecules with latency reversal potential. Because fungi excrete a plethora of molecules that have previously been shown to have therapeutic application, we decided to screen supernatants from 115 species of filamentous fungi for reactivating potential using J-Lat cell models of latent HIV. Orthogonal fractionation and mass spectrometry-NMR was then used to fractionate and identify the bio-active molecule responsible for HIV latency reversal. This led to the identification of Gliotoxin (GTX) in active supernatants from the species *Aspergillus Fumigatus*. Gliotoxin's latency reversal potential was confirmed in primary cell models of latency and cells isolated from cART suppressed HIV-1 infected participants, while cytotoxicity was found to be limited. Transcriptome sequencing of GTX treated CD4+ T cells revealed small nuclear RNA 7SK as the most decreased (more than 9-fold) transcript. 7SK RNA is a scaffold of the 7SK snRNP complex that inhibits the activity of positive elongation factor (P-TEFb), by sequestering. We demonstrate that GTX disrupts the binding of LARP7, which destabilizes 7SK snRNP. Consequently, active P-TEFb is released, which in turn phosphorylates RNA Pol II resulting in HIV transcription initiation and elongation. By screening by a library of fungal supernatants, we found a promising LRA which promotes reactivation by increasing the amount of freely available P-TEFb.

The general discussion of this thesis, in **Chapter 5**, explores general themes and future opportunities in HIV latency and cure research.

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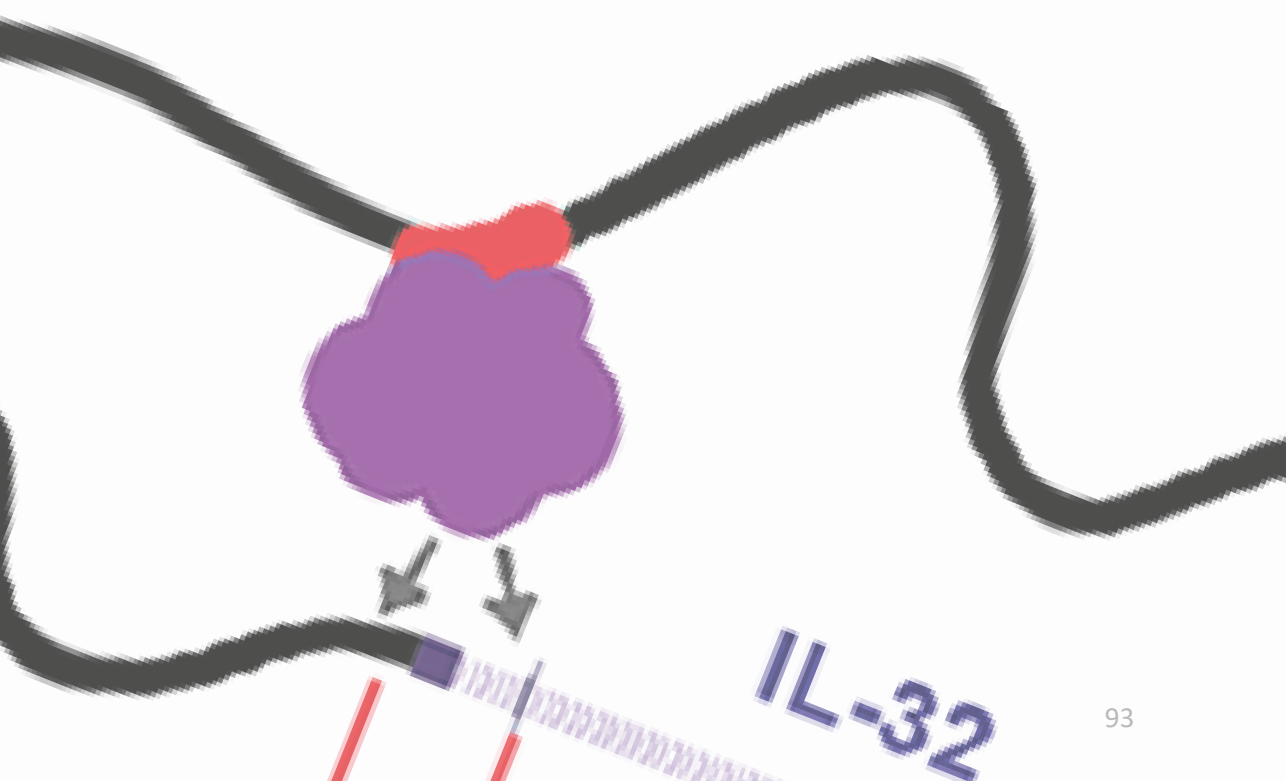
# Chapter 2

ScienceAdvances

**Allele-specific long-distance regulation dictates IL-32 isoform switching and mediates susceptibility to HIV-1**

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## GENETICS

## Allele-specific long-distance regulation dictates IL-32 isoform switching and mediates susceptibility to HIV-1

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We integrated data obtained from HIV-1 genome-wide association studies with T cell-derived epigenome data and found that the noncoding intergenic variant rs4349147, which is statistically associated with HIV-1 acquisition, is located in a CD4<sup>+</sup> T cell-specific deoxyribonuclease I hypersensitive region, suggesting regulatory potential for this variant. Deletion of the rs4349147 element in Jurkat cells strongly reduced expression of interleukin-32 (IL-32), approximately 10-kb upstream, and chromosome conformation capture assays identified a chromatin loop between rs4349147 and the IL-32 promoter validating its function as a long-distance enhancer. We generated single rs4349147-A or rs4349147-G allele clones and demonstrated that IL-32 enhancer activity and interaction with the IL-32 promoter are strongly allele dependent; rs4349147 -/A cells display reduced IL-32 expression and altered chromatin conformation as compared to rs4349147 G/- cells. Moreover, RNA sequencing demonstrated that rs4349147 G/- cells express a lower relative ratio of IL-32 $\alpha$  to non- $\alpha$  isoforms than rs4349147 -/A cells and display increased expression of lymphocyte activation factors rendering them more prone to infection with HIV-1. In agreement, in primary CD4<sup>+</sup> T cells, both treatment with recombinant IL-32 $\gamma$  (rIL-32 $\gamma$ ) but not rIL-32 $\alpha$ , and exogenous lentiviral overexpression of IL-32 $\gamma$  or IL-32 $\beta$  but not IL-32 $\alpha$  resulted in a proinflammatory T cell cytokine environment concomitant with increased susceptibility to HIV infection. Our data demonstrate that rs4349147-G promotes transcription of non-IL-32 $\alpha$  isoforms, generating a proinflammatory environment more conducive to HIV infection. This study provides a mechanistic link between a HIV-associated noncoding DNA variant and the expression of different IL-32 isoforms that display discrete anti-HIV properties.

## INTRODUCTION

Host genetic variation has long been recognized to play a major role in HIV-1 infection susceptibility and disease progression (1). Although it is widely accepted that genetic variants in loci encoding class I human leukocyte antigens (HLAs) and the chemokine receptor CCR5 have a major impact on HIV infection, the contribution of additional genetic variants remains elusive. In the past decade, genome-wide association studies (GWASs) have attempted to identify single-nucleotide polymorphisms (SNPs) that are correlated with various aspects of HIV disease acquisition or progression. In general, a major limitation of GWAS is that they are geared to identify genetic variants with relatively high minor allele frequencies and moderate to high effects, particularly when the sample size is small. This is especially the case for HIV-1 GWAS (1) where collection of large cohorts is challenging. As a consequence, common variants with weak effect size and rare variants fall below the stringent threshold for genome-wide significance ( $P < 5 \times 10^{-8}$ ) and are missed. In addition, the functional significance of identified HIV-associated genetic variants is often unclear because most of the correlated SNPs locate to noncoding regions of the genome with unknown function (1). As a consequence, HIV-centered GWASs fail to deliver distinct candidate loci, which hampers further efforts to characterize the contribution of human genetic variation to HIV susceptibility and disease progression. Integration of additional approaches to prioritize candidate loci is therefore of utmost importance to push the field of HIV-1 host genetics forward.

Recently, it is becoming clear that inclusion of genome-wide functional data into the analysis can circumvent the caveats of GWAS, allowing the identification of candidate noncoding SNPs most likely to be biologically relevant. As we have shown for the pigmentation-associated variant rs12913832 (2), function can be inferred for noncoding SNPs by examining co-occurrence with chromatin marks generally associated with regulatory DNA elements using resources that include the Encyclopedia of DNA Elements (ENCODE) and the Human Epigenome Atlas, among others (3). Thus, epigenomic data are used to identify variants with potential function and strongly reduce the list of noncoding SNPs to analyze. This analysis can also identify the relevant cell types and candidate genes to be investigated. Promising candidate variants are subsequently scrutinized by detailed molecular analysis to elucidate their biological function. Crucially, it was recently demonstrated that such an approach can also prioritize so-called “subthreshold” variants that fail to reach the widely accepted threshold of  $P < 5 \times 10^{-8}$  statistical significance and assign biological function to them (3). A total of 72% of the subthreshold loci that were prioritized in this way contained an allele-specific enhancer, and they were also more likely to replicate in subsequent GWASs than subthreshold loci that do not overlap active enhancers.

Here, we integrated data obtained from HIV-1 GWASs with T cell-derived epigenome data to identify noncoding HIV-1-associated SNPs with potential regulatory function. We performed a detailed molecular analysis of one of the identified variants, rs4349147, which is statistically associated at a subthreshold level with HIV-1 acquisition (4), and demonstrated that this variant is located in a lymphoid-specific enhancer for the interleukin-32 (IL-32) cytokine gene. Haplotypes of rs4349147 not only modulate the transcription level of IL-32 but also alter the ratio of isoforms expressed. This altered expression of IL-32 isoforms modulates the expression of proinflammatory factors that modify susceptibility to HIV-1 infection. Our study demonstrates that the integration of

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GWAS data with epigenome data can assign biological function to sub-threshold HIV-1-associated variants, such as rs4349147, and that allele-dependent differential expression of the noncanonical IL-32 cytokine gene directs HIV-1 susceptibility.

## RESULTS

### Deoxyribonuclease I hypersensitive region encompassing rs4349147 is a long-distance enhancer of IL-32

As a starting point for this study, we collected published HIV-associated variants ( $P < 9 \times 10^{-6}$ ) from the GWAS catalog (www.ebi.ac.uk/gwas/; accessed on May 2014), together with SNPs in strong linkage ( $r^2 > 0.8$ ). Because deoxyribonuclease I (DNase I) “hypersensitivity” is a feature of active cis-regulatory sequences in a given cell type, we compared the HIV-associated variants to DNase I hypersensitivity data obtained from CD4<sup>+</sup> T helper naïve (T<sub>H0</sub>) and T<sub>H1</sub> cells [ENCODE data set; University of California, Santa Cruz (UCSC) genome browser]. This analysis yielded a list of 21 HIV-associated SNPs that are located in potential regulatory regions (table S1). Most of these SNPs are located on chromosome 6 with the majority spread out more than a 1.3-Mb region encompassing the HLA loci, a thoroughly studied region and well known to be associated with HIV infection. Four SNPs are located in a histone cluster, which we reasoned to be of less interest. Two SNPs are located in a 500-kb gene desert on chromosome 1 and two in a 175-kb gene desert on chromosome 8. The remaining three variants are located in a gene-dense region on chromosome 16, and we decided to investigate this region in more detail. The three variants located on chromosome 16 (rs12447486, rs2015620, and rs4349147) are in perfect linkage (HapMap release 22; CEU), and rs4349147 has been reported to be associated at a subthreshold level ( $P = 7.91 \times 10^{-6}$ ) with HIV-1 acquisition in a cohort of African HIV-1 serodiscordant heterosexual couples (4). rs4349147 is located in a region that is strongly DNase I-hypersensitive in the T cell-derived cell lines Jurkat and SupT1 but not in cells not belonging to the immune compartment (Fig. 1A), whereas the other two variants are located in regions with minimal DNase I hypersensitivity. In agreement, formaldehyde-assisted isolation of regulatory elements (FAIRE) assays demonstrated strong enrichment of the rs4349147 DNA region in the T cell lines but not in the unrelated G361 melanoma cells (fig. S1A), highlighting the presence of an active regulatory element and supporting a regulatory role for the rs4349147 region in T cells. To gain more insight into its function, we deleted the DNase I hypersensitive (DHS) region containing rs4349147 using an upstream- and downstream-targeted CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) gene editing approach (Fig. 1B, top). Polymerase chain reaction (PCR) confirmed homozygous deletion of the rs4349147 DHS (Fig. 1B, bottom), and a clonal Jurkat line containing a full knockout (KO) of the rs4349147 DHS was generated. Sequencing confirmed the deletion of the specific region (fig. S1B).

To determine which genes are regulated by this DHS region, we performed high-throughput sequencing of RNA isolated from wild-type (WT) and rs4349147 DHS KO Jurkat cells. We found that of all genes within a 500-kb region centered on rs4349147, specifically, the expression of IL-32 is severely reduced upon KO of the rs4349147 DHS (Fig. 1C and fig. S1C), whereas the expression of surrounding genes remains essentially unchanged (Fig. 1C and fig. S1D). We confirmed this observation by reverse transcription PCR (RT-PCR) (Fig. 1D), Western blotting (Fig. 1E) and intracellular flow cytometry (Fig. 1F) demonstrated that IL-32 expression at the protein level is, as expected, likewise reduced to undetectable levels in the rs4349147 DHS KO cells. In addition, targeting of a dead

Cas9 (dCas9)-Krüppel-associated box (KRAB) fusion protein, which is a strong repressor of enhancer function (5), to the rs4349147 DHS in WT Jurkat cells also resulted in significant reduction of IL-32 RNA expression (fig. S1E), supporting our observations in the rs4349147 KO cells.

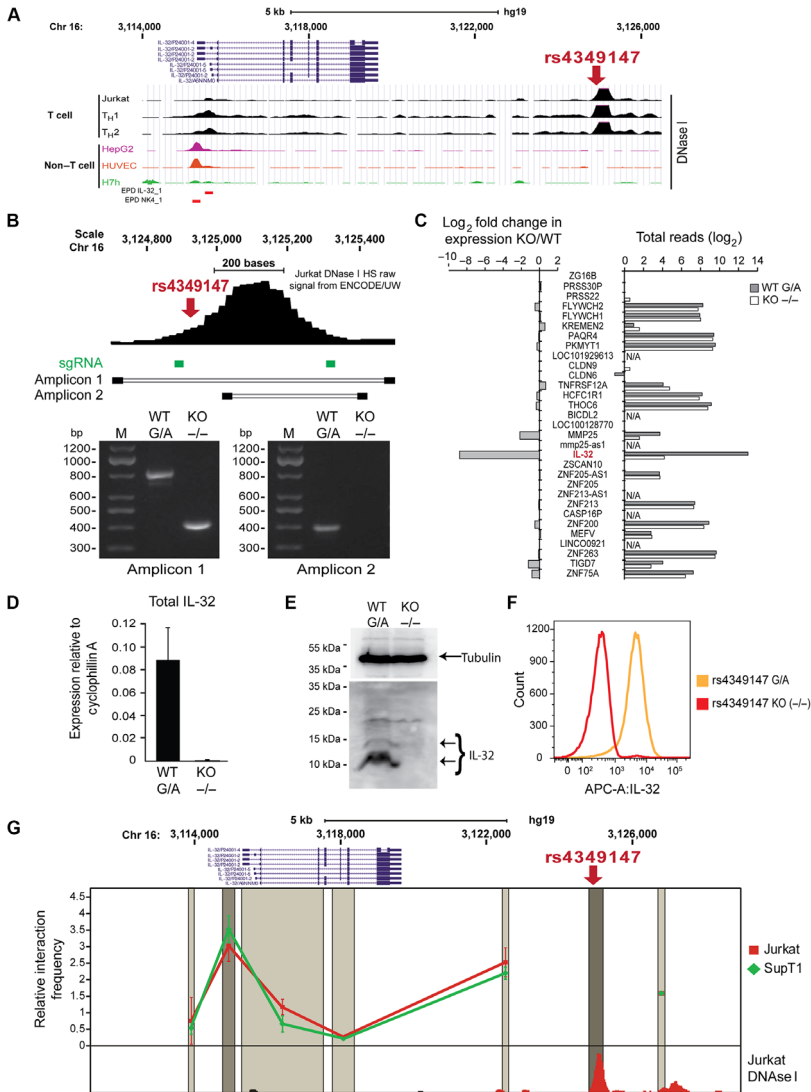
Distal regulatory elements, such as enhancers, are known to communicate with their target genes through chromatin loop formation (6). To determine whether chromatin at the rs4349147 DHS directly contacts the IL-32 promoter, we performed chromosome conformation capture (3C) analysis, which revealed the presence of a chromatin loop between rs4349147 DHS and the IL-32 promoter in WT Jurkat and SupT1 T cells (Fig. 1G). Together, these results demonstrate that the DNA element encompassing rs4349147 is a strong long-distance enhancer essential for the expression of IL-32 in CD4<sup>+</sup> T cells.

### IL-32 enhancer activity is dependent on the rs4349147 haplotype

Jurkat cells are heterozygous rs4349147-G/A that allowed us to generate heterozygous lines harboring a deletion of either the rs4349147-G or rs4349147-A allele enhancer and thus generate single rs4349147-G (G/-) or rs4349147-A (-/A) allele clones. rs4349147-G/A is in perfect linkage disequilibrium (LD) with rs4786376-G/C, which is also situated in the IL-32 enhancer region (Fig. 2A). rs4786376-G/C can be used to genotype clones by restriction fragment length polymorphism because the rs4786376-C allele generates a unique Nla III restriction site (Fig. 2, A to C). RNA and protein expression levels of IL-32 in the single rs4349147 G/- cells were found to be similar to WT levels, whereas rs4349147 -/A cells display significant reduction of IL-32 both at the RNA and protein level, as determined by RT-PCR, Western blotting, and intracellular flow cytometry (Fig. 2, D to F, and fig. S2A). Moreover, 3C analysis revealed that the long-range interaction of the enhancer region with the IL-32 promoter region is significantly altered in rs4349147 -/A cells compared to rs4349147 G/- Jurkat cells (Fig. 2G), suggesting a change in chromatin conformation. To get a better understanding of which variant, rs4349147-G/A or rs4786376-G/C, is responsible for the differential IL-32 expression, we cloned enhancer fragments with different genotypes in an IL-32 promoter-driven luciferase reporter construct and determined their activity. The rs4349147-G allele induces luciferase expression 3.5-fold when compared to the other haplotypes (Fig. 2H), suggesting that this variant is responsible for the main activity of this regulatory element. To further investigate the influence that the rs4349147 and rs4786376 variants have on the activity of the IL-32 regulatory element, we subjected the rs4349147 -/A clone to another round of CRISPR/Cas9 genome editing and obtained a clone designated -/A  $\Delta$ rs4349147 that harbored a 180-base pair (bp) deletion that removed the rs4349147-A variant but left the rs4786376-C variant in place (Fig. 2I and fig. S2B). Real-time PCR analysis of IL-32 expression in this clone revealed a significant 4.5-fold drop in expression as compared to the parental rs4349147 -/A clone (Fig. 2J) and confirmed that rs4349147 is the variant responsible for differential IL-32 expression. We conclude that the activity and physical interaction of the long-distance IL-32 enhancer element are strongly dependent on the allelic status of rs4349147.

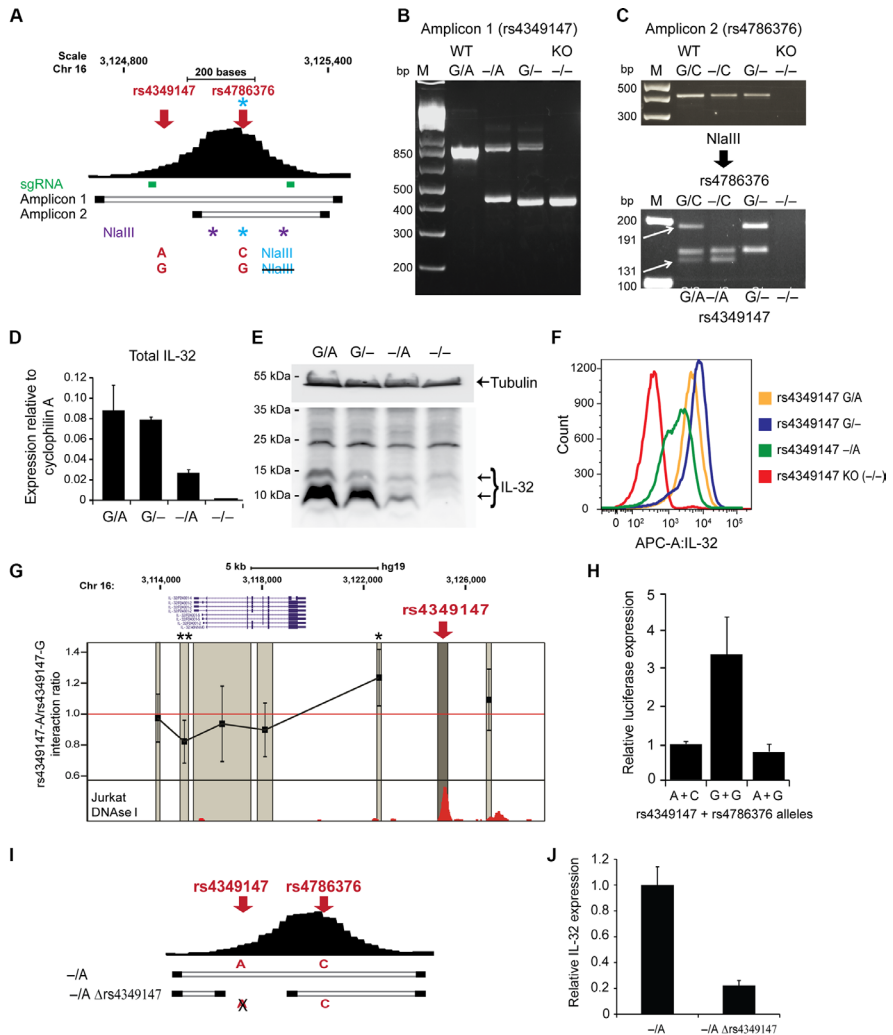
### rs4349147 G/- Jurkat cells are more conductive to HIV infection than rs4349147 -/A cells

When infected with a minimal HIV-derived virus, rs4349147 G/- Jurkat cells are more prone to infection than rs4349147 -/A Jurkat cells (Fig. 3A). The observed differences in infection remained similar over time and with different virus concentrations (fig. S3, A and B). These results support the observations reported in a GWAS, indicating that the rs4349147-G allele confers susceptibility to HIV infection (4).



**Fig. 1. DNA region encompassing rs4349147 is a strong enhancer element for the IL-32 gene in T cells.** (A) UCSC genome browser tracks show that the HIV-associated variant rs4349147 is located in a T cell-specific DHS region. Each track represents the DNase I accessibility profile for a given cell line. Promoters as defined by the Eukaryotic Promoter Database (EPD; <http://epd.vital-it.ch>) are indicated as red bars. HUVEC, human umbilical cord endothelial cell. (B) Deletion of rs4349147 DHS. Location of small-guiding RNAs (sgRNAs) used in the CRISPR-Cas9 KO strategy is indicated. Panels show results from two different PCR assays generating amplicons 1 or 2, as indicated, that confirm homozygous deletion of rs4349147 DHS. (C) RNA expression levels of genes in a 500-kb window surrounding rs4349147 demonstrate a strong reduction in IL-32 expression. For each gene considered in the analysis, right panel shows total number of reads, whereas left panel shows fold change in expression in rs4349147 DHS KO cells compared to WT. N/A, not available. (D to F) Strong reduction of IL-32 expression is confirmed using real-time quantitative PCR (qPCR) (D), Western blotting using an antibody against IL-32 (E), and intracellular staining with biotin-conjugated antibody against IL-32, followed by fluorescence-activated cell sorting (FACS) analysis (F). (G) 3C analysis of the IL-32 locus demonstrates chromatin loop formation between rs4349147 and the IL-32 promoter region in Jurkat and SupT1 cells. The analyzed region of the human IL-32 locus is depicted on the top of each graph. X axis shows the approximate position on chromosome 16 (Chr 16) (UCSC genome browser GRCh37/hg19 assembly). Dark gray shading shows the position and size of the “fixed” Dpn II restriction fragment. Light gray shading indicates position and size of other Dpn II restriction fragments analyzed. The Dpn II restriction fragment containing the IL-32 promoter is indicated in a slightly darker gray color.





**Fig. 2. Activity of the rs4349147 enhancer element is dependent on the haplotype.** (A) Schematic representation of the region encompassing the rs4786376-G/C and rs4349147-G/A SNPs, indicating the size of the PCR products obtained using primers outside (amplicon 1) or inside (amplicon 2) the region targeted for KO. Amplicon 2 contains three Nla III restriction sites (asterisks). The central Nla III site (blue asterisk) includes the rs4786376-G/C SNP, which is in perfect linkage with rs4349147-G/A and can be used to genotype the heterozygous lines. (B) Simultaneous presence of two PCR products of different sizes indicates the occurrence of heterozygous rs4349147 KO lines. (C) Results of restriction reaction of amplicon 2 in WT (heterozygous rs4349147-G/A), rs4349147 -/A, G/-, or rs4349147 enhancer KO Jurkat lines. (D) Expression of IL-32 mRNA in the four lines was measured by real-time PCR. Cells homozygous for rs4349147-A express lower IL-32 RNA levels as compared to those homozygous for rs4349147-G. (E) IL-32 protein levels were determined by Western blotting using an antibody against IL-32. (F) Intracellular staining with biotin-conjugated antibody against IL-32, followed by FACS analysis, shows lower levels of IL-32 in rs4349147 -/A Jurkat cells and rs4349147 KO Jurkat cells as compared to WT and rs4349147 G/- Jurkat cells (Fig. 1F). (G) 3C analysis of the IL-32 locus demonstrates chromatin loop formation between rs4349147-G and the IL-32 promoter region in rs4349147 G/- Jurkat cells and a significant reduction in enhancer-promoter interaction in the rs4349147 -/A Jurkat cells. Ratio of interaction in rs4349147 -/A and G/- Jurkat cells is indicated. For further explanation of the figure, see Fig. 1G. (H) Activity of 476-bp enhancer fragments with different rs4349147 and rs4786376 genotypes in an IL-32 promoter-driven luciferase reporter construct. The rs4349147-G allele results in a 3.5-fold induction of luciferase expression when compared to the other haplotypes. The rs4349147 and rs4786376 alleles present in the reporter construct are indicated below the graph. (I) Schematic depicting the 158-bp deletion in the -/A  $\Delta$ rs4349147 clone. (J) The 158-bp deletion in the -/A  $\Delta$ rs4349147 clone reduces IL-32 expression 4.5-fold as compared to IL-32 expression in the parental rs4349147 -/A Jurkat clone.

Remarkably, deletion of the rs4349147 enhancer region did not result in decreased susceptibility to HIV infection but displayed infection levels similar to the rs4349147 G/A and G/– Jurkat cells (Fig. 3A). This phenotype was not due to a clonal effect in the rs4349147 –/– cells because deletion of a 180-bp fragment that contains rs4349147-A variant from the rs4349147 –/– Jurkat cells (–/– Δrs4349147) reverted the low-rs4349147 –/– HIV susceptibility phenotype to a high-susceptibility phenotype, similar to the rs4349147 G/A, G/–, and –/– Jurkat cells (Fig. 3A).

### Alternative rs4349147 alleles differentially modulate the relative expression of IL-32 isoforms

The observation that the rs4349147 KO cells –/– and –/– Δrs4349147 display similar susceptibility to infection as G/A and G/– cells, despite the fact that IL-32 expression is strongly reduced, compared to the less susceptible rs4349147 –/– cells strongly suggested that the absolute levels of IL-32 expression is not the key factor that determines susceptibility to HIV infection. Multiple protein variants of IL-32 with different but poorly characterized functions have been documented (Fig. 3B) (7, 8). The five major protein isoforms of IL-32 (isoforms A to E) are encoded by multiple RNA splice variants (variants 1 to 9). It is known that these IL-32 isoforms affect the immune system and susceptibility to HIV in different ways (8). In general, IL-32 isoforms are categorized as proinflammatory (represented mainly by IL-32 $\alpha$  and IL-32 $\gamma$ ) or non-proinflammatory (mainly represented by IL-32 $\alpha$ ). Controlling the expression of these distinct isoforms, two different promoters are assigned to the IL-32 gene by the EPD (<http://epd.vital-it.ch>). The upstream promoter is designated EPD NK4\_1, whereas the downstream promoter is designated EPD IL32\_1 (Fig. 1A). Promoter EPD NK4\_1 generates long transcripts such as RNA variant 4 encoding the A isoform of IL-32 (IL-32 $\alpha$ ) and RNA variants 1, 2, and 8 encoding the B isoform of IL-32 (IL-32 $\beta$ ), whereas the downstream promoter EPD IL32\_1 generates shorter transcripts such as RNA variant 3 encoding the main IL-32 $\beta$ , RNA variants 6 and 5 encoding the C isoform of IL-32, RNA variant 7 encoding the D isoform, and RNA variant 9 encoding the isoform E precursor (IL-32 $\gamma$ ) (Figs. 1A and 3B).

The fact that alternative IL-32 isoforms with different effects on the immune system are potentially expressed from two distinct promoters, together with the observation that reduction in absolute IL-32 expression levels is likely not the key determinant of reduced HIV infection susceptibility, we wondered whether the rs4349147 regulatory element could differentially regulate the IL-32 promoters. Therefore, we analyzed the relative expression levels of distinct IL-32 RNA variants in data obtained from high-throughput RNA sequencing (RNA-seq) in rs4349147 G/A, G/–, or –/– and –/– cells (Fig. 3C). This analysis revealed that, concurrent with a decrease in expression of IL-32, a switch occurs in the ratio of isoforms; rs4349147 –/– cells display a shift from “short” IL-32 variants originating from the EPD IL32\_1 promoter to the “longer” RNA variants originating from the EPD NK4\_1 promoter, resulting in a higher relative expression of the IL-32 $\alpha$ -encoding RNA variant 4 (Fig. 3C). The higher relative expression of IL-32 $\alpha$  is confirmed by RT-PCR using primers specific for the IL-32 $\alpha$  and non-IL-32 $\alpha$  isoforms (fig. S3D) and is matched by a relative increase in protein levels of IL-32 $\alpha$  in rs4349147 –/– cells, as demonstrated by Western blotting using an IL-32 $\alpha$ -specific antibody for detection (fig. S3, E and F). The relative ratio of IL-32 $\alpha$  over non-IL-32 $\alpha$  expression does not change after HIV infection of rs4349147 G/– and –/– cells (fig. S3G).

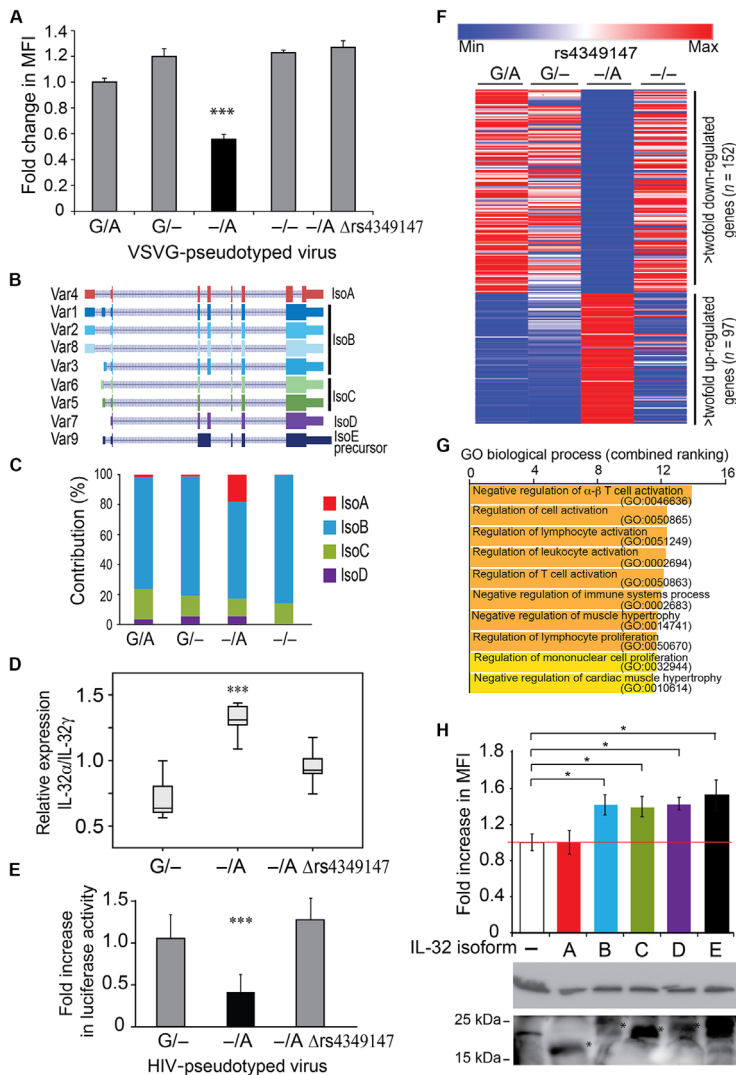
Relative IL-32 isoform expression in rs4349147 KO cells –/– showed a lower relative ratio of IL-32 $\alpha$  expression, consistent with its similar

propensity to HIV infection to G/A and G/– cells but not to –/– cells (Fig. 3, A to C). In agreement, in the –/– Δrs4349147 KO line, both the lower relative IL-32 $\alpha$ -to-IL-32 $\gamma$  ratio and the higher susceptibility to HIV infection are similar to the rs4349147 G/– cells but not to the –/– cells (Fig. 3D and fig. S3, D and E). The observed difference in infection efficiency is independent from the envelope (Env) protein used to generate the pseudoviral particles because rs4349147 –/– cells infected with HIV-1 Env-pseudotyped virus still display a reduced infection rate as compared to rs4349147 G/– or –/– Δrs4349147 Jurkat cells (Fig. 3E). Therefore, the allelic contribution to susceptibility does not involve the interaction between gp120/gp41 and cellular receptor and co-receptors but rather is mediated by a more general mechanism.

### rs4349147 G/– Jurkat cells generate a proinflammatory T cell-activating environment more conducive to HIV infection

To gain further insight into the mechanism by which rs4349147-G promotes HIV infection, we further analyzed the RNA-seq data obtained from unstimulated, uninfected rs4349147 G/A, G/–, –/–, and –/– cells (Fig. 3, F and G). We focused our analysis on genes differentially expressed specifically between rs4349147 –/–, G/–, and G/A Jurkat cells. We found that 97 genes are at least twofold up-regulated, whereas 152 genes are at least twofold down-regulated (Fig. 3F and table S2) in rs4349147 –/– Jurkat cells as compared to rs4349147 G/A and G/– Jurkat cells. Gene ontology (GO) biological process analysis of down-regulated genes revealed overrepresentation of factors involved in the regulation of cell activation (lymphocyte, leukocyte, and T cell), consistent with a relatively lower expression of proinflammatory IL-32 isoforms (Fig. 3G and table S3). RT-PCR confirmed the increased expression of T cell activation genes such as IL-4R (IL-4 receptor), TFFRSF14, STAT5A (signal transducer and activator of transcription 5A), LGALS1, and ANXA1 (fig. S4A) in the WT and rs4349147 G/– cells. Consistent with its reverted higher propensity for infection and increased relative ratio of the proinflammatory IL-32 isoforms, the proinflammatory gene expression profile is restored in the rs4349147 –/– KO cells (Fig. 3F and fig. S4A). An enrichment in GO biological process analysis was observed for the Wnt signaling pathway in up-regulated genes (fig. S4, B and C). Factors previously reported to interact with HIV-1 ([www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/hiv-1/interactions/](http://www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/hiv-1/interactions/)) were also overrepresented (fig. S4D) in the list of down-regulated genes. Cytokine array profiling using supernatants of rs4349147 G/– or –/– Jurkat cultures demonstrates that, in line with the known immune modulatory role of proinflammatory IL-32 isoforms, rs4349147 G/– cells exhibit elevated secretion of several interleukins and other cytokines (fig. S4E and table S4). Together, these experiments demonstrate that Jurkat cells containing the rs4349147 G/– allele display an increased expression of proinflammatory genes and genes involved in T cell activation and proliferation when compared to rs4349147 –/– cells.

We determined the functional consequence of the observed shift in IL-32 isoform expression in context of HIV infection by overexpression of the distinct IL-32 isoforms in rs4349147 enhancer KO Jurkat cells, which express low amounts of endogenous IL-32 (Figs. 1, D to F, and 2, D to F), followed by infection with vesicular stomatitis virus (VSV)-pseudotyped HIV-derived virus (Fig. 3H). Expression of the distinct IL-32 isoforms was confirmed by Western blotting (Fig. 3H, bottom) and RT-PCR (fig. S4F). Exogenous expression of the IL-32 isoforms B to E resulted in an increase in HIV infectability (Fig. 3H). In contrast, when IL-32 $\alpha$  (isoform A) was exogenously expressed, no increase in HIV infection was observed. A similar pattern of susceptibility to infection was observed when IL-32 isoforms were overexpressed in WT



**Fig. 3. The rs4349147-G allele generates a proinflammatory activated T cell environment conducive to HIV infection. (A)** Levels of infection, measured as fold increase in the mean fluorescence intensity (MFI), in rs4349147 G/A, -/A, G/-, -/-, and -/A Δrs4349147 Jurkat lines after challenge with minimal HIV vesicular stomatitis virus glycoprotein (VSVG)-pseudotyped virus. rs4349147 -/A Jurkat cells have reduced susceptibility to infection compared to Jurkat cells with other rs4349147 genotypes. **(B)** Nine RNA variants of IL-32 have been reported that encode for five different IL-32 protein isoforms [isoform A (IsoA), indicated in red; isoform B (IsoB), blue shades; isoform C (IsoC), green shades; isoform D (IsoD), purple; and a precursor isoform E (IsoE precursor), black]. **(C)** IL-32 isoform A expression is relatively elevated in rs4349147 -/A cells as compared to rs4349147 WT (G/A), G/-, or KO(-/-) cells. Bar charts show the contribution of each isoform to the total levels of IL-32 expression. **(D)** RT-PCR demonstrating a reduced ratio of IL-32 $\alpha$  over IL-32 $\gamma$  transcripts upon deletion of the 180 bp rs4349147 containing region from the IL-32 regulatory element. **(E)** Levels of infection in rs4349147 -/A, G/-, and -/A Δrs4349147 Jurkat lines after challenge with HIV Env-pseudotyped virus. Luciferase activity obtained in rs4349147 G/- cells was set to 1. **(F)** RNA-seq heat map representation of differentially expressed genes in rs4349147 G/A, G/-, -/A, and -/- Jurkat cell lines. High-throughput RNA-seq reveals that 97 genes are up-regulated, whereas 152 genes are down-regulated at least twofold in rs4349147 -/A Jurkat cells when compared to rs4349147 G/- and WT (G/A) Jurkat cells. **(G)** GO-based functional classification of genes down-regulated in rs4349147 -/A Jurkat cells compared to rs4349147 G/- Jurkat cells. **(H)** Levels of infection, measured as fold increase in the MFI, following exogenous expression of IL-32 isoforms A to E in rs4349147 enhancer KO cells. Color coding is as for (B). Bottom: Exogenous expression of IL-32 as determined by Western blotting using the pan-IL-32 antibody.

Jurkat cells expressing high levels of IL-32 (fig. S4G), in line with the notion that the relative ratio of IL-32 isoforms, but not their absolute expression levels, determine susceptibility to infection.

### Relative ratio of IL-32 isoforms regulates the proinflammatory cytokine environment and influences susceptibility of primary CD4<sup>+</sup> T cells to HIV infection

Our results thus far demonstrated that the rs4349147 SNP resides within an enhancer region, which, via long-distance interaction, regulates the expression of the IL-32 promoter and controls the expression of distinct isoforms of IL-32 at the RNA level. We also showed that the rs4349147 SNP-driven distinct expression profile of IL-32 RNA isoforms influences HIV infection susceptibility in the Jurkat model system. However, Jurkat cells have been reported to undergo posttranscriptional processing of several IL-32 isoforms at the protein level, resulting in generation of smaller IL-32 peptides (Fig. 1E and fig. S3F) (9), although the expression of IL-32 $\alpha$  is not effected (fig. S3F). Therefore, to validate our results and to examine the functional consequence of the different IL-32 isoforms on HIV infection susceptibility in a more physiologically relevant system, we moved to the *in vivo* relevant primary CD4<sup>+</sup> T cell targets of HIV.

It has previously been reported that stimulation of activated primary CD4<sup>+</sup> T cells with purified IL-32 $\gamma$ , but not with IL-32 $\alpha$ , results in an increased production of proinflammatory cytokines (10). We therefore asked whether supplementing primary CD4<sup>+</sup> T cells with recombinant IL-32 $\gamma$  (rIL-32 $\gamma$ ) or rIL-32 $\alpha$  would have an effect on susceptibility to HIV infection. CD4<sup>+</sup> T cells were isolated from eight healthy donors and stimulated with CD3-CD28-coated beads in the absence or presence of either rIL-32 $\alpha$  or rIL-32 $\gamma$ . After stimulation, luciferase activity was evaluated as a measure of infection efficiency (Fig. 4A). Our experiments in Jurkat cells indicated that rs4349147 has only moderate effects on susceptibility to infection. To maximize the window of opportunity that would allow minor variations in infection susceptibility to be observed while minimizing the influence of factors associated with viral replication, cells were infected with single-round infectious viral particles. Furthermore, to recapitulate, at least in part, the complex mixture of viral variants present at the infection, we used viral particles pseudotyped with an X4 variant derived from the reference strain HXB2 and an R5 Env variant derived from a primary isolate. Similar to what was observed after exogenous expression of IL-32 isoforms in rs4349147 KO Jurkat cells (Fig. 3H), we found that luciferase activity was significantly increased in primary cells stimulated in the presence of rIL-32 $\gamma$ , whereas infection levels similar to untreated control cells were obtained after stimulation with rIL-32 $\alpha$  (Fig. 4B). As expected, treatment of primary CD4<sup>+</sup> T cells with rIL-32 $\gamma$ , but not with rIL-32 $\alpha$ , resulted in significant increase in the transcription of the proinflammatory cytokine IL-8 (Fig. 4C). These data strongly support our observations in the Jurkat T cell line, where the increased relative expression of IL-32 $\alpha$  over non- $\alpha$  isoforms reduced susceptibility to HIV infection. However, the cellular receptor for IL-32 is not yet identified. Therefore, we could not exclude the possibility that the rIL-32 $\alpha$  is unable to enter into the cells. To confirm that the observed HIV-1 infection phenotype is linked to the exogenously provided distinct IL-32 isoforms and to ensure similar exposure to IL-32 isoforms, primary CD4<sup>+</sup> T cells were lentivirally transduced to overexpress IL-32 $\alpha$ , as well as the non- $\alpha$  isoforms  $\beta$  or  $\gamma$ , and subsequently infected with HIV according to the indicated schematic (Fig. 4D and fig. S5B). We examined the proinflammatory environment caused by exogenous overexpression of the specific IL-32 isoforms  $\alpha$  and  $\gamma$  using both intracellular cytokine staining (fig. S5C)

and cytokine arrays of the primary cell supernatants (fig. S5D and table S5). Cytokine arrays demonstrated significantly increased levels of IL-32 (approximately 10- and 6-fold overexpression over control for IL-32 $\alpha$  and IL-32 $\gamma$ , respectively) in the culture supernatant, confirming the IL-32 lentiviral overexpression system (fig. S5D). Although the level of IL-32 $\alpha$  was higher in the culture supernatants only, exogenous expression of IL-32 $\gamma$  resulted in the up-regulation of proinflammatory cytokines, including IL-8, IL-6, IL-10, TNF $\alpha$  (tumor necrosis factor- $\alpha$ ), and IFN- $\gamma$  (interferon- $\gamma$ ) (fig. S5D). Consistent with our data using rIL-32 isoforms, the proinflammatory cytokine environment generated by IL-32 $\gamma$  overexpression resulted in a significant increase in susceptibility to HIV infection (shown for five subjects). Similar to IL-32 $\gamma$ , overexpression of the non-IL-32 $\alpha$  isoform  $\beta$  also resulted in increased susceptibility to HIV infection (fig. S5A).

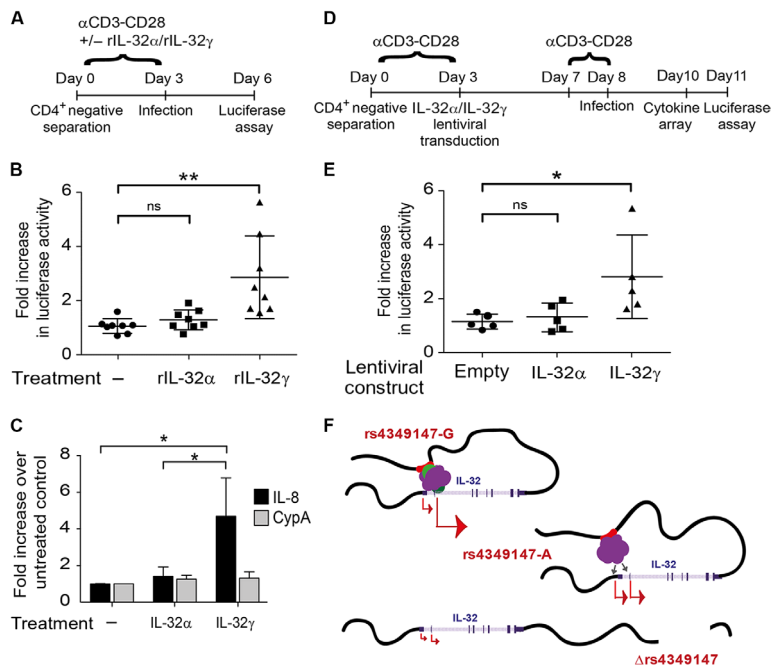
Thus, in remarkable similarity to treatment of primary CD4<sup>+</sup> T cells with rIL-32 $\alpha$  and rIL-32 $\gamma$ , as well as to infections performed after exogenous expression of IL-32 $\alpha$  and non-IL-32 $\alpha$  isoforms in Jurkat cell lines, lentiviral overexpression of non-IL-32 $\alpha$  isoforms  $\gamma$  and  $\beta$ , but not  $\alpha$ , rendered the cells more susceptible to HIV infection (Fig. 4 and fig. S5). These results are consistent with the increased susceptibility to infection observed in rs4349147 G/- cells, which exhibit a lower expression of IL-32 $\alpha$  and higher expression of proinflammatory IL-32 isoforms, and suggest that the rs4349147-A allele generates a subdued proinflammatory environment compared to rs4349147-G, which leads to reduced susceptibility to HIV infection.

## DISCUSSION

Here, we demonstrate that the noncoding variant rs4349147, associated with HIV susceptibility, resides in a DNA region that functions as a long-distance enhancer element for the IL-32 gene, located approximately 10-kb downstream. Deletion of this DNA region results in abrogation of IL-32 gene expression in Jurkat cells, whereas 3C experiments demonstrated direct long-distance interaction between this enhancer region and the IL-32 promoter. Because Jurkat cells heterozygously harbor both rs4349147-A and rs4349147-G alleles, we were able to examine the effect of each allele on IL-32 expression and HIV infection efficiency by generating single-allele KO clones using the CRISPR-Cas9 technology. The presence of the rs4349147-A allele alone was associated with an increased relative expression of nonproinflammatory  $\alpha$  IL-32 isoform, whereas the rs4349147-G allele cells displayed a switch in the relative expression of IL-32 isoforms to the proinflammatory non- $\alpha$  isoforms. We found that the functional consequence of this switch in IL-32 isoforms is enhanced lymphocyte activation, which directs increased susceptibility to HIV infection.

### IL-32 isoforms and HIV infection

IL-32 has previously been reported to play a role in HIV infection and disease progression, although its precise role has remained controversial (7, 8). Comparison of IL-32 expression levels in HIV-infected patients and healthy individuals indicated a rise in IL-32 levels in peripheral blood mononuclear cells (PBMCs), gut, and lymph nodes in different stages of HIV infection (11–13). IL-32 was suggested to mediate immune suppression by promoting the expression of immunosuppressive molecules, leading to decreased immune activation and, as a consequence, increased HIV replication (13). However, several pieces of evidence also pointed to an inhibitory role for IL-32 in HIV replication; small interfering RNA (siRNA) depletion of IL-32 in Jurkat, human embryonic kidney (HEK) 293T, and latently infected U1 macrophage cell lines and infected



**Fig. 4. Relative ratio of IL-32 isoforms influences susceptibility of primary CD4<sup>+</sup> T cells to HIV infection and alters the proinflammatory cytokine environment.** (A) CD4<sup>+</sup> T cells isolated from eight healthy donors were stimulated with CD3-CD28-coated beads in the presence or absence of rIL-32 $\alpha$  or rIL-32 $\gamma$  and infected with an HIV virus harboring a luciferase reporter, as shown in the schematic. Efficiency of infection was determined by measuring luciferase activity 72 hours after infection. (B) CD4<sup>+</sup> T cells exogenously incubated with rIL-32 $\gamma$  are more susceptible to HIV infection than mock-treated cells and cells treated with rIL-32 $\alpha$ . ns, not significant. (C) Real-time PCR analysis demonstrates that CD4<sup>+</sup> T cells incubated with rIL-32 $\gamma$ , but not those treated with rIL-32 $\alpha$ , show increased IL-8 expression. CypA, cyclophilin A. (D) CD4<sup>+</sup> T cells isolated from five healthy donors (rs4349147-G/A) were stimulated with CD3-CD28-coated beads and infected with a control lentiviral vector or a lentiviral vector expressing IL-32 $\alpha$  or IL-32 $\gamma$ . After 4 days, the cells were restimulated for 24 hours and infected with an HIV virus harboring a luciferase reporter, as shown in the schematic. Efficiency of infection was determined by measuring luciferase activity 72 hours after infection. (E) Exogenous lentiviral overexpression of IL-32 $\gamma$  results in an increase in HIV infection as compared to CD4<sup>+</sup> T cells overexpressing IL-32 $\alpha$  or control cells infected with an empty lentiviral construct. (F) Model depicting rs4349147 regulation of IL-32 expression. See Discussion for explanation.

PBMCs led to an increase in HIV replication (11, 14). IL-32 depletion also caused a reduction in levels of T<sub>H1</sub> cells and proinflammatory cytokines including IL-6 and TNF $\alpha$  (14). Resolving these seemingly contradictory observations on the function of IL-32 in the context of HIV replication, IL-32 was proposed to play a dual immune regulatory role (13), inducing an antiviral immune response primarily via type I and type III IFNs (12, 15, 16) but also moderating immune activation during HIV infection (13). The siRNA depletion studies relied on depletion of all isoforms of IL-32 (11, 14), and many studies have used RT-PCR primers and antibodies that recognize common IL-32 sequences or epitopes and do not distinguish between different IL-32 isoforms and their potentially distinct functional roles in immune regulation.

Key to deciphering the role of IL-32 in immune function and regulation, IL-32 exists in multiple isoforms, which, it is becoming increasingly clear, are functionally distinct (17). IL-32 $\alpha$ , which we found to be predominantly expressed in rs4349147-A allele Jurkat cells, has been in particular and in agreement with our findings, shown to be less potent than either IL-32 $\beta$  and IL-32 $\gamma$  isoforms in inducing a proinflammatory state in PBMCs (18, 19). A recent study has shown that plasma levels of

the proinflammatory non-IL-32 $\alpha$  isoforms are correlated with higher viral loads and significantly increased in HIV-infected slow progressors that experience virological failure (10). In addition, consistent with our data, IL-32 $\gamma$  was shown to be a much more potent inducer of IL-6 and IL-17F in CD4<sup>+</sup> T cells than IL-32 $\alpha$  (10). T<sub>H17</sub> cells are known to be a preferential target susceptible to HIV infection (20), although it is long known that IL-6, alone or in combination with other cytokines, promotes HIV replication in macrophages and latently infected CD4<sup>+</sup> T cells (21–23). Thus, as suggested by our cytokine array data comparing the expression of proinflammatory cytokines induced by exogenous expression of IL-32 $\alpha$  and IL-32 $\gamma$ , one can envision that in vivo, the rs4349147-G allele by its induction of IL-32 $\gamma$  could augment the production of IL-6 and other proinflammatory cytokines, producing a sustained inflammatory environment ideal for HIV replication. rs4349147 is not readily picked up with high significance in HIV acquisition GWAS, which indicates that the effect of rs4349147 is small and it is not the main driver of HIV resistance. Likewise in our study, looking only at single rs4349147 alleles, we observe modest effects on IL-32 isoform ratio and infection susceptibility, although the fact that these effects

may be enhanced in individuals that carry homozygous G/G or A/A alleles of rs4349147 cannot be excluded. We therefore envision that rs4349147 nuances the combinatorial effect that includes the contribution of additional host factors toward resistance to HIV acquisition.

### Transcriptional regulation of IL-32 isoform expression

How the different alleles of rs4349147 (or the associated variant rs4786376) modulate the activity of the IL-32 enhancer remains to be understood. Bioinformatic transcription factor consensus site analysis of the region using PROMO (<http://algenen.lsi.upc.es/>) indicated that the rs4349147-G allele is part of a potential binding site for YY1 (Yin Yang 1), STAT, and ETS (E26 transformation-specific) family transcription factors, which are compromised when the rs4349147-A allele is present. Because similar analysis of rs4786376 did not yield a potential transcription factor binding site that is altered, rs4349147, and not the linked rs4786376, is likely to be the functionally relevant allele within the IL-32 enhancer. This is consistent with our data obtained in the luciferase reporter assays where only the rs4349147-G variant is increasing the expression from the reporter construct. Data from genome-wide binding studies performed in T cells indicate that STAT5, ETS, and other factors of the TAL1 (T cell acute lymphocytic leukemia 1) complex (HEB, E2A, LMO1, RUNX1, and GATA3) bind to the rs4349147 DHS (24–27). YY1 has been implicated in long-range chromatin interaction within the  $T_H2$  cytokine locus and V(D)J recombination in immature B cells (28, 29). Which of the above transcription factors are involved in the long-range regulation of IL-32 expression is the subject of an ongoing investigation.

The observed rs4349147 allele-specific switch in ratio of IL-32 isoform expression is in agreement with data from the International Human Epigenome Consortium that identified the IL-32 gene (among others) as having a significant genetic and epigenetic contribution to its expression levels and to its isoform expression in CD4<sup>+</sup> T cells (30). Our data provide mechanistic insight into this phenomenon: In cells carrying only rs4349147-G, expression of “short” IL-32 variants presumably originating from the downstream EPD IL32\_1 promoter is favored (most notably, variants that encode the proinflammatory IL-32 isoforms). Conversely, expression of “long” IL-32 variants originating from the upstream EPD NK4\_1 promoter is favored in cells that contain the rs4349147-A allele [variants 2 and 8 encoding the B isoform of IL-32 (IL-32β) and variant 4 encoding the A isoform of IL-32 (IL-32α)]. This notion that these “long” and “short” IL-32 RNA transcripts are transcribed using different promoters is supported by the presence of a dual DNase I hypersensitivity peak at the IL-32 proximal upstream region in T cells (fig. S6). The alternative use of promoters has long been described to be a mechanism to generate diversity in transcriptional output, as well as complexity and nuance in the regulation of gene expression (31). We envision three models that can explain the observed IL-32 isoform switching. First, depending on its allelic nature, the enhancer (rs4349147-G) contacts either the EPD IL32\_1 “short” isoform promoter or the EPD NK4\_1 promoter of the long IL-32 RNA isoforms (rs4349147-A). Second, the rs4349147-G enhancer interacts with the EPD IL32\_1 promoter of the short IL-32 RNA isoforms, and no interaction with either promoter is established when the rs4349147-A allele is present. Third, the rs4349147-A enhancer interacts with the EPD NK4\_1 promoter of the long isoforms, and this interaction is lost with rs4349147-G. We deem the latter scenario unlikely because a strong reduction in IL-32 RNA expression is observed in rs4349147 –/A Jurkat cells, suggesting that the enhancer element is no longer fully functional. Unfortunately, 3C experiments lack the resolution to distinction between the two remaining models. However, 3C experiments indicate

a significant reduction in the interaction between the enhancer and the general IL-32 promoter region, indicating a major change in the chromatin conformation in rs4349147 –/A Jurkat cells. In cell types that are not DNase I-hypersensitive at the rs4349147 region but still express low levels of IL-32, a major DHS peak is observed at the EPD NK4\_1 promoter, which suggests that this is a “default” promoter for IL-32 expression (fig. S6). However, in cell lines lacking the rs4349147 regulatory element, IL-32 expression is strongly reduced compared to cells containing the rs4349147-A allele, which indicates that in these cells, the rs4349147 regulatory element still contains significant enhancer activity. The switch in IL-32 promoter usage in rs4349147 –/A cells suggests that this residual enhancer activity is now less focused, potentially by loss of binding of a tethering transcription factor, on the EPD IL32\_1 short isoform promoter, which allows the EPD NK4\_1 long isoform promoter to profit from the now more promiscuous regulatory element. Therefore, our results are consistent with the model depicted in Fig. 4F, where the rs4349147-G enhancer interacts with and specifically activates the EPD IL32\_1 promoter while this interaction is altered in rs4349147 –/A Jurkat cells, resulting in relatively increased IL-32 expression from the EPD NK4\_1 long isoform promoter and a relative increase in the expression of IL-32α. In the absence of the rs4349147 regulatory element, IL-32 expression from both promoters is strongly reduced (Fig. 4F).

### Implications for HIV GWAS

We took a three-step approach to demonstrate that HIV-1-associated variants that fall below the accepted statistical threshold for genome-wide significance can still have biological relevance. We collected HIV-1-associated variants and compared them to epigenomic features to extract the variants with potential biological function. This integration of GWAS data with epigenomic data allowed us to discriminate true biological signals from noise. Promising candidate variants are subsequently scrutinized by detailed molecular analysis to elucidate their biological function. GWASs are geared to identify genetic variants with relatively high minor allele frequencies and moderate to high effects, especially when the sample size is small. As generally seen in HIV-1 GWASs (1), which are underpowered because of the difficulty in collection of large cohorts, rare variants and common variants with weak effect size are missed because they fall below the stringent threshold for genome-wide significance ( $P < 5 \times 10^{-8}$ ). The study of Lingappa *et al.* (4), which initially pointed out the association between the rs4349147 and HIV-1 susceptibility in discordant couples, was likely affected by the low number of individuals included in the study. Furthermore, our data underline a modest effect of rs4349147 on HIV-1 susceptibility, which has likely further hampered the achievement of level of significance. The general approach to identify rarer variants with a low effect size is to enlarge the sample size (for example, by replication studies and/or meta-analysis), which is not always practical and still can only provide a statistical association and not actual biological relevance. Our current study, as well as the work of others (3), provides an additional approach to identify and characterize biologically important sub-threshold variants. Using our approach, functional data can be extracted from previous GWASs, which is of utmost importance to push the field of HIV-1 host genetics forward.

In summary, we identify here, for the first time, the molecular mechanism by which a GWAS-identified HIV susceptibility allele functions. Our findings underscore the power of using a combined approach in which GWAS data and genome-wide functional data, together with functional molecular and phenotypic analysis, are leveraged to understand and unravel the underlying biological basis of disease. Our data provide

the key mechanistic insight that allelic differences in a distal enhancer can direct a switch in promoter usage and, as a consequence, modulate differential isoform expression. This can subsequently lead to complex phenotypic changes, such as, as we show for IL-32, differential susceptibility to HIV infection but also potentially for other IL-32-related pathologies such as viral and bacterial infections, cancer, rheumatoid arthritis, and atherosclerosis (7). Our methodological approach can be expanded to identify additional loci associated with HIV susceptibility, pathogenesis, and latency, allowing the development of therapies and interventions tailored to specific patient populations.

## MATERIALS AND METHODS

### Cell culture and plasmids

Jurkat cells (a human T cell–derived line) were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. rIL-32 $\alpha$  and rIL-32 $\gamma$  were purchased from R&D Systems and resuspended according to the manufacturer's instructions. For the IL-32 overexpression constructs, gBlocks gene fragments (Integrated DNA Technologies) corresponding to the complementary DNA (cDNA) sequence of the corresponding IL-32 isoforms were cloned into IRES-puro-GFP (a gift from B. Vogelstein; plasmid #16616, Addgene). Plasmids were sequence-verified. pHR-SFFV-KRAB-dCas9-P2A-mCherry was a gift from J. Weissman (plasmid #60954, Addgene).

### Analysis of GWAS SNPs

HIV/AIDS implicated DNA variants were retrieved from the GWAS catalog ([www.ebi.ac.uk/gwas/](http://www.ebi.ac.uk/gwas/); June 2014). A total of 117 SNPs from 16 studies were recovered, 59 of which were classified as intergenic/non-coding. SNPs in strong LD ( $r^2 > 0.8$ ) were obtained from the CEU population panel of the 1000 Genomes Pilot 1 and HapMap3 (release 2) data sets using SNAP (SNP Annotation and Proxy Search) (<http://archive.broadinstitute.org/mpg/snap/>; distance limit, 500 kb). Human hg18 genome coordinates were lifted to hg19 using the lift-over function in the standard Galaxy tools of the Hypergenome browser (<http://hyperbrowser.uio.no/3d>). CD4<sup>+</sup> T<sub>H</sub>0 and T<sub>H</sub>1 DNase I hypersensitivity peaks (<http://genome.ucsc.edu/ENCODE/>; wgEncodeAwgDnaseDukeTh0UniPk and wgEncodeAwgDnaseDukeTh1UniPk) containing HIV-associated SNPs were identified using the intersect function under Operate on Genomic intervals in the standard Galaxy tool, returning the overlapping intervals. Fifteen and 16 DHS regions were recovered for T<sub>H</sub>0 and T<sub>H</sub>1 cells, respectively. Subsequently, the SNPs overlapping these DHS regions were retrieved using the join function under Operate on Genomic intervals in the standard Galaxy tool, resulting in 17 SNPs for T<sub>H</sub>0 cells and 19 SNPs for T<sub>H</sub>1 cells, totaling 21 unique SNPs for both cell types (table S1).

### Formaldehyde-assisted identification of regulatory elements

FAIRE was performed as described previously (32), except that selected genomic sites were analyzed by quantitative real-time PCR using GoTaq qPCR Master Mix (Promega) under the following cycling conditions: 95°C for 5 min, 45 cycles of 10 s at 95°C, and 30 s at 60°C, followed by a melting curve analysis. Enrichment was calculated relative to Axin2, and values were normalized to input measurements. PCR primers are available on request.

### Amata nucleofection

Nucleofection of Jurkat cell clones was conducted as described previously (33). Cells were split to  $3 \times 10^5$  cells/ml 24 hours before Amata

nucleofection. Five to 8 million cells were centrifuged at 1000 rpm for 10 min at room temperature, resuspended in 100  $\mu$ l of solution R, and nucleofected with 2  $\mu$ g of expression plasmid using program O28. Nucleofected cells were resuspended in 500  $\mu$ l of prewarmed, serum-free RPMI 1640 lacking antibiotics and allowed to recover at 37°C in a 5% CO<sub>2</sub> incubator for 15 min. Prewarmed complete RPMI 1640 (4 ml) was then added to the cells.

### Generation of rs4349147 KO cells using CRISPR-Cas9

The sgRNA cassette, the puro selection marker containing Cla I/Kpn I fragment from pLKO.1-puro U6 sgRNA BfuAI large stuffer, was cloned into pBluescript to yield pBS-puro U6 sgRNA BfuAI large stuffer. Oligos for guide RNAs targeting the rs4349147 containing DHS region were designed using ZiFiT (Zinc Finger Targeter) (<http://zifit.partners.org/ZiFiT/CSquare9Nuclease.aspx>) (34) and cloned into the BfuAI sites of pBS-puro U6 sgRNA BfuAI large stuffer. Sequences for the oligos used are available in table S6. sgRNA plasmids were nucleofected with pSpCas9(BB)-2A-Puro (PX459) into Jurkat cells. Nucleofected cells were treated with puromycin (1  $\mu$ g/ml) for 24 hours. After recovery, the polyclonal population was single cell–sorted using a BD FACSAria III cell sorter. Clones were screened for deletion of the rs4349147 DHS using PCR, and breakpoints were sequenced. Selected clones displayed similar growth characteristics.

pLKO.1-puro U6 sgRNA BfuAI large stuffer was a gift from S. Wolfe (plasmid #52628, Addgene). pSpCas9(BB)-2A-puro (PX459) was a gift from F. Zhang (plasmid #48139, Addgene) (35).

### Primer sequences

Primer sequences used in this study are available in table S6.

### RNA sequencing

RNA was isolated using TRI Reagent (Sigma-Aldrich). RNA-seq was performed according to the manufacturer's instructions (Illumina) using the TruSeq Stranded mRNA Library Prep kit. The resulting DNA libraries were sequenced according to the Illumina TruSeq v3 protocol on an Illumina HiSeq 2500 sequencer. Reads of 50 bp in length were generated. Reads were mapped against the UCSC genome browser hg19 reference genome with TopHat (version 2.0.10 or 2.0.13). Gene expression was quantified using both Cufflinks (version 2.1.1) and HTSeq-count (version 0.6.1). For the WT Jurkat and rs4349147 DHS KO samples, 13 to 18 million reads were generated, whereas approximately 20 million reads were generated for the Jurkat rs4349147  $-/A$  and  $G/-$  lines. In all cases, more than 97% of these reads aligned to the hg19 genome. Differential expression analysis of the RNA-seq data was performed using edgeR package run under Galaxy (<https://bioinf-galaxian.erasmusmc.nl/galaxy/>). False discovery rate cutoff was set to 0.05.

Heat maps were generated using MORPHEUS (<https://software.broadinstitute.org/morpheus/index.html>). Enrichment of GO terms for the differentially expressed genes was assessed using Enrichr (<http://amp.pharm.mssm.edu/Enrichr/>) (36).

### Western blotting

Whole-cell extracts were fractionated by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane according to the manufacturer's guidelines (Bio-Rad). Membranes were blocked in 5% nonfat milk in PBST [phosphate-buffered saline (PBS), 0.1% Tween 20] for 30 min and then washed three times in PBST. Membranes were incubated with antibodies against IL-32 (513501, BioLegend; or MAB30401, R&D Systems), IL-32 $\alpha$  (AF3040, R&D Systems), and tubulin (1:1000; TS168, Sigma-Aldrich) overnight

at 4°C. After washing the membranes three times for 10 min, they were incubated with horseradish peroxidase–conjugated anti-mouse (A9044, Sigma-Aldrich), anti-rat (A 9037, Sigma-Aldrich), or anti-goat (V8051, Promega) antibodies for at least 1 hour. Membranes were washed three times with TBS-T (tris-buffered saline, 0.1% Tween 20) and imaged using ImageQuant LAS 4000 Mini (GE Healthcare) with SuperSignal West Pico Chemiluminescent Substrate or SuperSignal Weste Femto Maximum Sensitivity Substrate (34080 and 34095, respectively, Thermo Fisher Scientific).

### cDNA analysis

Total cellular RNA was isolated from the different cell lines with TriPure Isolation Reagent according to the manufacturer's instructions (Roche Diagnostics). Subsequent DNase I digestion was performed with amplification grade DNase I (Invitrogen). The reverse-transcriptase reaction was performed using SuperScript II RNase H reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR reactions for gene expression analysis were performed on a CFX96™ Real-Time System (Bio-Rad) with the GoTaq qPCR Master Mix (Promega) using the following parameters: denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, and 60°C for 30 s, followed by a melting curve analysis. The reference gene  $\beta_2$ -macroglobulin or cyclophilin A was used to normalize the amplification signal between the samples of different cell lines, differences in treatment, and amount of input cDNA.

### Intracellular stain

WT, rs4349147  $-/A$ , rs4349147  $G/-$ , and rs4349147 DHS KO Jurkat clones were harvested, counted, and directly stained for intracellular IL-32 expression. Briefly,  $1 \times 10^6$  cells were first washed with Hanks' balanced salt solution (HBSS)/3% FBS/0.015 M  $\text{NaN}_3$  and then fixed with IC Fixation Buffer (eBioscience). After washing with HBSS/3% FBS/0.015 M  $\text{NaN}_3$ , cells were incubated with biotinylated anti-human IL-32 antibody (clone Ku32-52, BioLegend) for 60 min at 4°C, followed by two washes with permeabilization buffer (eBioscience) and incubation with allophycocyanin (APC)–conjugated streptavidin (Thermo Fisher Scientific) for 60 min at 4°C. Cells were also incubated with only APC-conjugated streptavidin as control. Cells were washed twice with permeabilization buffer and resuspended in 1% paraformaldehyde. A total of 100,000 cells were collected per sample on an LSRFortessa flow cytometry instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star).

### 3C analysis

3C analysis was performed as described previously (37, 38) using Dpn II as the restriction enzyme. Quantitative real-time PCR (CFX96™ Real-Time System, Bio-Rad) was performed using GoTaq qPCR Master Mix (Promega) under the following cycling conditions: 50°C for 2 min, 95°C for 10 min, 45 cycles of 15 s at 95°C, and 1 min at 60°C, followed by a melting curve analysis. A random template was generated as described previously (39) using gBlocks gene fragments (Integrated DNA Technologies). PCR primers are available in table S6.

### Luciferase reporter assays

Custom 500-bp gBlocks gene fragments (Integrated DNA Technologies) containing different haplotypes of the enhancer region were cloned into a modified pGL3-promoter vector [in which the SV40 promoter and the SV40 3' untranslated region (3'UTR) were replaced by the

IL-32 promoter and an HSP 3'UTR] (Promega). Jurkat cells are heterozygous for two additional common variants within the hypersensitive region: rs12598118 and rs12917910 (see fig. S1B). These variants are in high linkage to each other but are linked to rs4349147 at a much lower level, and there is no association with HIV acquisition reported for these SNPs. The genotypes for rs12598118 and rs12917910 were fixed to their ancestral C alleles while we varied the alleles for rs4349147 and rs4786376. Inserts in each construct were verified by sequencing (BaseClear). Constructs were nucleofected into Jurkat cells using the Cell Line Nucleofector Kit R (Lonza) and an Amaxa Nucleofector device (Lonza). Luciferase expression was measured after 24 and 48 hours in a GloMax 96 microplate luminometer (Promega) and normalized to *Renilla* luciferase expression. Data represent at least three independent experiments. Student's two-tailed *t* test was used to determine statistical significance.

### Virus production

HIV Env-pseudotyped particles were obtained by cotransfecting Env together with the HIV-1 backbone plasmid (pNL4.3.Luc.R-E-) into HEK 293T cells using FuGENE transfection reagent (Promega). To produce X4 tropic–pseudotyped viruses, pNL4.3.Luc.R-E- was cotransfected with HBXB2-Env expression construct, whereas cotransfection with pTRO11 Env expression vector resulted in production R5 tropic pseudoviruses. Twenty-four, 36, and 60 hours after transfection, the pseudovirus-containing supernatant was collected, filtered through a 0.45- $\mu\text{m}$  filter, aliquoted, and stored at  $-80^\circ\text{C}$ . HIV-1 molecular clone pNL4.3.Luc.R-E- and HIV-1 HXB2-Env expression vector were provided by the Centre for AIDS Reagents, National Institute for Biological Standards and Control. HIV-1 molecular clone pNL4.3.Luc.R-E-, HIV-1 HXB2-Env, and pTRO11 Env expression vector were donated by N. Landau; K. Page and D. Littman; and D. Montefiori, F. Gao, and M. Li, respectively.

HIV-derived virus particles were generated as described previously (40). Briefly, HEK293T cells were transfected with VSVG, the NL4.3 packaging vector R8.9, and the minimal HIV retroviral vector LTR-Tat-IRES-EGFP (pEV731). Virus was harvested every 12 hours, starting at 24 hours after transfection, filtered through a 0.45- $\mu\text{m}$  filter, aliquoted, and stored at  $-80^\circ\text{C}$ . Plasmids used to generate HIV-derived virus particles [VSV Env (VSVG), the NL4-3 packaging vector (R8.9), and the retroviral vector LTR-Tat-IRES-EGFP (pEV731)] were described previously (40).

### HIV infection of Jurkat rs4349147 clones

Jurkat rs4349147 clones were infected with the minimal HIV VSVG-pseudotyped virus at low multiplicity of infection such that less than 10% of cells were infected. Samples were analyzed on a Fortessa flow cytometer with FACSDiva software (Becton Dickinson). The live population was defined by forward versus side scatter profiles. Cells were further gated by using forward scatter versus green fluorescent protein (GFP) to differentiate between GFP-positive and GFP-negative cells. GFP expression in the Jurkat rs4349147 clones was analyzed by FACS 24 and 48 hours after infection.

For HXB2 HIV Env–pseudotyped virus infection, 500,000 cells were infected by spinoculation (1200g, 90 min,  $37^\circ\text{C}$ ), incubated in a humidified  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  incubator for 2 hours, washed, and cultured for 48 hours in RPMI 1640/10% fetal calf serum (FCS). To evaluate infection levels, luciferase activity was measured using Luciferase Assay System (Promega). Relative light units were normalized to protein content determined by Bradford assay (Bio-Rad).



### Primary CD4<sup>+</sup> T cell isolation and infection

Primary CD4<sup>+</sup> T cells were isolated from buffy coats from healthy donors by Ficoll gradient, followed by magnetic separation with Rosette Sep Human CD4<sup>+</sup> T cell Enrichment Cocktail (STEMCELL Technologies) according to the manufacturer's instructions. Cells were kept in culture in RPMI 1640/10% FCS for 1 week and subsequently stimulated for 3 days with anti-CD3-CD28-coated beads and IL-2 in the absence or presence of 500 ng/ml (10) of either rIL-32 $\alpha$  or rIL-32 $\gamma$  (R&D Systems). After stimulation, cells were infected with a mixture of R5 and X4 Env-pseudotyped virus by spinoculation (1200g, 90 min, 37°C), incubated in a humidified 37°C, 5% CO<sub>2</sub> incubator for 2 hours, washed, and then cultured in RPMI 1640/10% FCS supplemented with IL-2. Seventy-two hours after infection, cells were harvested and washed once with PBS, and luciferase activity was measured using Luciferase Assay System (Promega). Relative light units were normalized to protein content determined by Bradford assay (Bio-Rad).

### Lentiviral overexpression of IL-32 isoforms

cDNAs encoding different isoforms of IL-32 were PCR-cloned into Mlu I/Spe I restriction sites of the lentiviral vector pLenti6.3/V5-DEST (Invitrogen). Virus particles were produced using the ViraPower Lentiviral Packaging Mix (Invitrogen) according to the supplier's recommendations. Primary CD4<sup>+</sup> T cells were isolated from buffy coats from healthy donors by Ficoll gradient, followed by magnetic separation with Rosette Sep Human CD4<sup>+</sup> T cell Enrichment Cocktail (STEMCELL Technologies) according to the manufacturer's instructions. Cells were subsequently stimulated for 3 days with anti-CD3-CD28-coated beads and IL-2. After stimulation, cells were infected by spinoculation with IL-32 $\alpha$ -, IL-32 $\beta$ -, or IL-32 $\gamma$ -expressing lentivirus. After 4 days, cells were restimulated for 24 hours and infected with an HIV virus harboring a luciferase reporter, as described above. Seventy-two hours after infection, cells were harvested and washed once with PBS, and luciferase activity was measured using Luciferase Assay System (Promega). Relative light units were normalized to protein content determined by Bradford assay (Bio-Rad).

### Intracellular cytokine detection

Purified CD4<sup>+</sup> T cells were treated as described for the lentiviral IL-32 isoform overexpression. During the last 5 hours of the final bead activation step, a protein transport inhibitor (GolgiPlug, BD Biosciences) was added to prevent the release of cytokines. Cells were harvested and incubated with annexin V-phycoerythrin (PE) in FACS wash (HBSS, 3% FBS, 0.02% NaN<sub>3</sub>, and 2.5 nM CaCl<sub>2</sub>) for 20 min at 4°C in the dark. For intracellular staining for cytokines, cells were then fixed (IC Fixation Buffer, eBioscience), washed (permeabilization buffer, eBioscience), incubated with the antibodies (TNF $\alpha$ -APC, clone MAb11; IFN- $\gamma$ -PE-Cy7, clone 4S.B3, eBioscience) for 45 min, washed two times with permeabilization buffer, and fixed with 1% paraformaldehyde. At least 100,000 events were collected per sample within 24 hours after staining on a LSRFortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star).

### Proteome profiler Human XL Cytokine Array

Jurkat rs4349147 -/A and rs4349147 G/- clones or purified CD4<sup>+</sup> cells were grown to equal density (8 × 10<sup>5</sup> cells/ml for Jurkat cells and 2 × 10<sup>6</sup> cells/ml for CD4<sup>+</sup> cells). Cells were spun down, and 500  $\mu$ l of supernatant was incubated with the Human XL Cytokine Array membrane (R&D systems). Membranes were processed according to the manufacturer's instructions and imaged on a ImageQuant LAS 4000 (GE Healthcare). Signal was quantified using ImageQuant TL software (GE Healthcare).

### Statistical analysis

All data are means  $\pm$  SD of three or more independent biological replicates. For 3C and cDNA qPCR experiments, comparison of fold change data was performed by a Student's two-tailed *t* test or by one-way analysis of variance (ANOVA).

For infection experiments, comparison of fold change data was performed by one-way ANOVA (Kruskal-Wallis test) using logarithmically transformed values. Statistical significance was reported as ns, *P* > 0.05, \**P*  $\leq$  0.05, \*\**P*  $\leq$  0.01, and \*\*\**P*  $\leq$  0.001.

### SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/4/2/e1701729/DC1>

- fig. S1. Characterization of the rs4349147 DHS and the rs4349147 KO clone.  
fig. S2. Characterization of the rs4349147 G/-, rs4349147 -/A, and -/A  $\Delta$ rs4349147 clones.  
fig. S3. HIV infectability and differential expression of IL-32 isoforms between WT, rs4349147 G/-, and rs4349147 -/A clonal Jurkat cell lines.  
fig. S4. Analysis of genes that are at least twofold differentially expressed in rs4349147 -/A compared to rs4349147 G/- and WT clonal Jurkat cell lines.  
fig. S5. Endogenous lentiviral overexpression of IL-32 isoforms in CD4<sup>+</sup> cells results in altered HIV susceptibility and proinflammatory cytokine expression.  
fig. S6. UCSC genome browser graphic of DNase I hypersensitivity (upper track, black, blue, purple) and mRNA sequencing (lower track, green) data obtained from the Human Epigenome Atlas.  
table S1. Overview of SNPs.  
table S2. List of differentially regulated genes.  
table S3. GO term analysis of -/A versus WT and G/- down-regulated genes.  
table S4. Cytokine secretion in Jurkat cells as measured by Proteome Profiler Human XL Cytokine Array kit.  
table S5. Cytokine secretion in CD4<sup>+</sup> T cells after stimulation and infection with HIV as measured by Proteome Profiler Human XL Cytokine Array kit.  
table S6. Primer sequences used.

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**Supplementary Materials**

Supplementary Materials available at::

<https://advances.sciencemag.org/content/suppl/2018/02/16/4.2.e1701729.DC1>

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HapLa

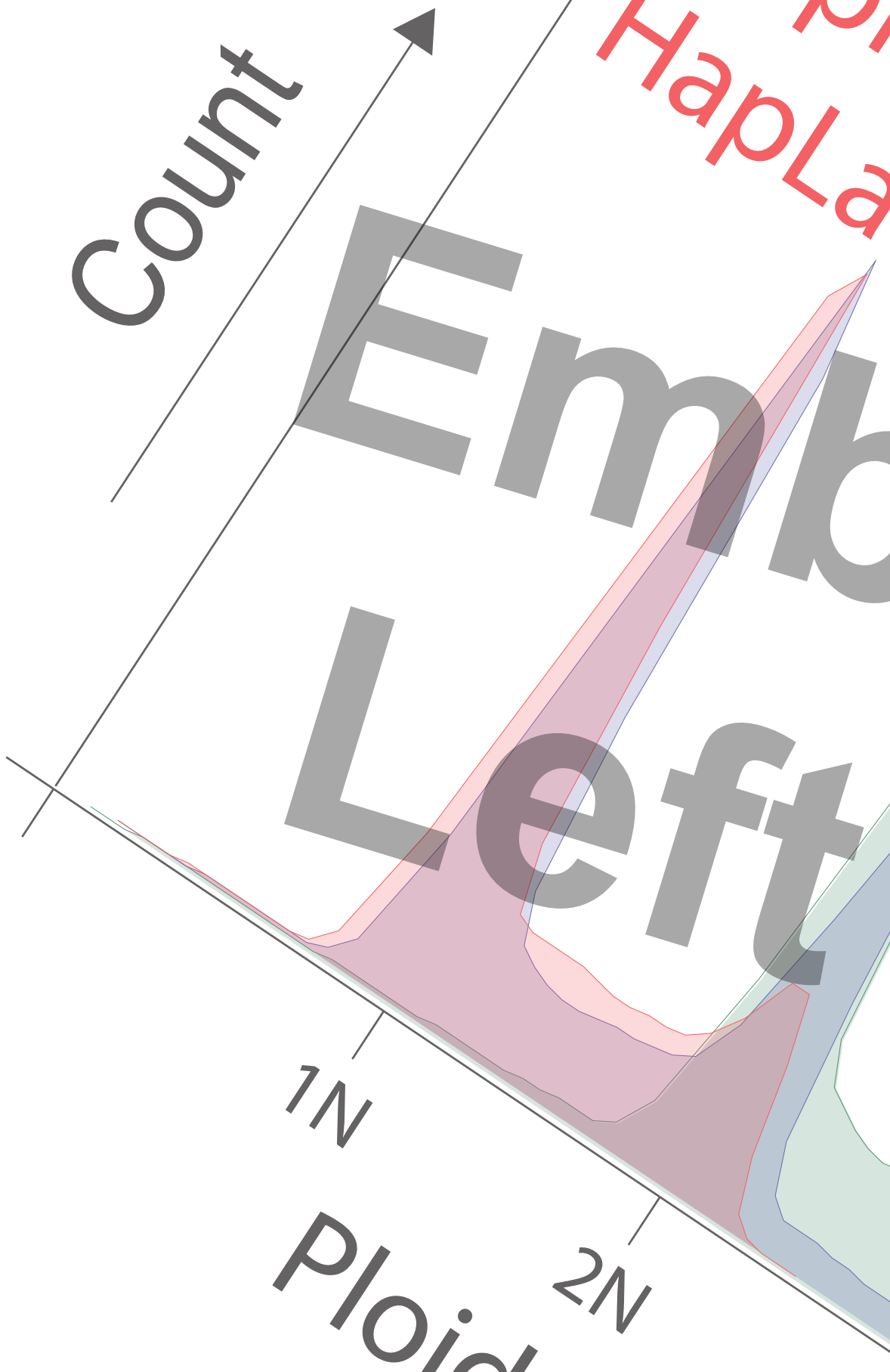
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1N

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Ploid



# Chapter 3

## A two-color haploid genetic screen identifies novel host factors involved in HIV latency

Running Title: Identification of novel HIV latency host factors.

Authors: Michael D Röling<sup>1</sup>, Mahsa Mollapour Sisakht<sup>1#</sup>, Enrico Ne<sup>1#</sup>, Panagiotis Moulos<sup>2#</sup>, Mateusz Stoszko<sup>1</sup>, Elisa De Crignis<sup>1</sup>, Helen Bodmer<sup>1</sup>, Tsung Wai Kan<sup>1</sup>, Maryam Akbarzadeh<sup>1</sup>, Vaggelis Harokopos<sup>2</sup>, Thijn Brummelkamp<sup>3</sup>, Pantelis Hatzis<sup>2</sup>, Robert-Jan Palstra<sup>1</sup>, Tokameh Mahmoudi<sup>1\*</sup>

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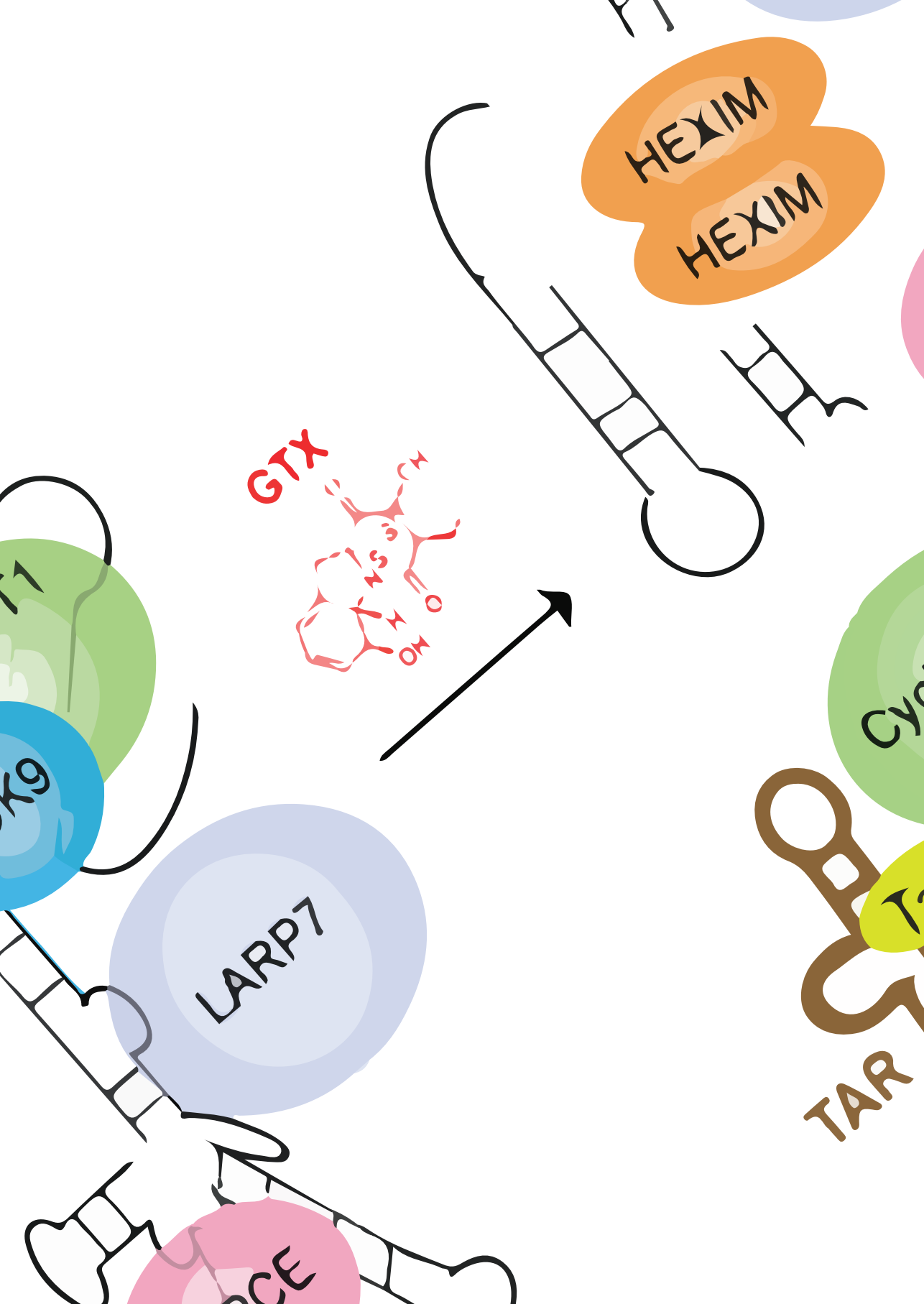
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*Submitted*

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Jurkat



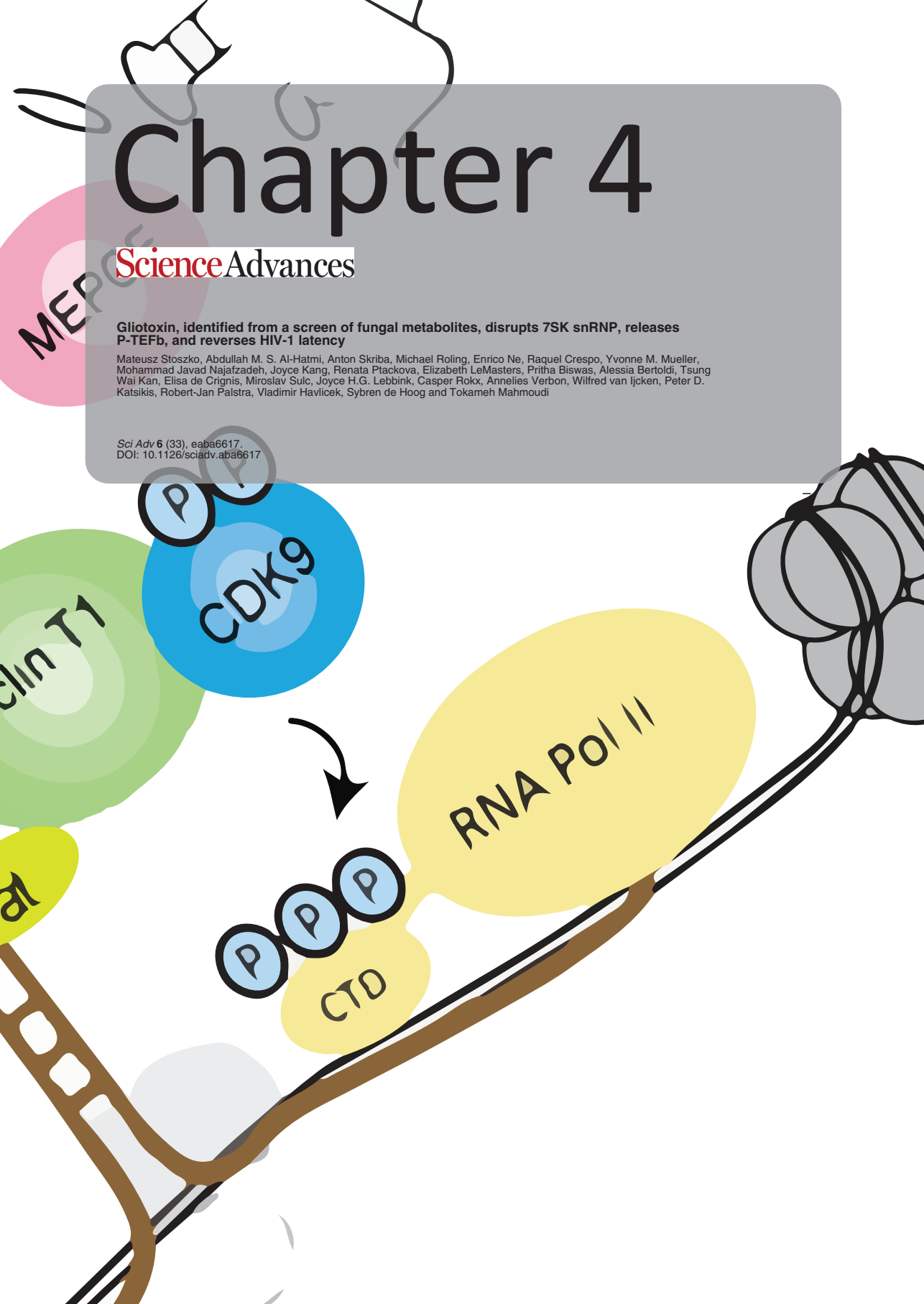
# Chapter 4

Science Advances

**Gliotoxin, identified from a screen of fungal metabolites, disrupts 7SK snRNP, releases P-TEFb, and reverses HIV-1 latency**

Mateusz Stoszko, Abdullah M. S. Al-Hatmi, Anton Skriba, Michael Roling, Enrico Ne, Raquel Crespo, Yvonne M. Mueller, Mohammad Javad Najafzadeh, Joyce Kang, Renata Ptackova, Elizabeth LeMasters, Pritha Biswas, Alessia Bertoldi, Tsung Wai Kan, Elisa de Crignis, Miroslav Sulc, Joyce H.G. Lebbink, Casper Rokx, Annelies Verbon, Wilfred van Ijcken, Peter D. Katsikis, Robert-Jan Palstra, Vladimir Havlicek, Sybren de Hoog and Tokameh Mahmoudi

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## MOLECULAR BIOLOGY

# Gliotoxin, identified from a screen of fungal metabolites, disrupts 7SK snRNP, releases P-TEFb, and reverses HIV-1 latency

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A leading pharmacological strategy toward HIV cure requires “shock” or activation of HIV gene expression in latently infected cells with latency reversal agents (LRAs) followed by their subsequent clearance. In a screen for novel LRAs, we used fungal secondary metabolites as a source of bioactive molecules. Using orthogonal mass spectrometry (MS) coupled to latency reversal bioassays, we identified gliotoxin (GTX) as a novel LRA. GTX significantly induced HIV-1 gene expression in latent ex vivo infected primary cells and in CD4<sup>+</sup> T cells from all aviremic HIV-1<sup>+</sup> participants. RNA sequencing identified 7SK RNA, the scaffold of the positive transcription elongation factor b (P-TEFb) inhibitory 7SK small nuclear ribonucleoprotein (snRNP) complex, to be significantly reduced upon GTX treatment of CD4<sup>+</sup> T cells. GTX directly disrupted 7SK snRNP by targeting La-related protein 7 (LARP7), releasing active P-TEFb, which phosphorylated RNA polymerase II (Pol II) C-terminal domain (CTD), inducing HIV transcription.

## INTRODUCTION

Combination antiretroviral therapy (cART) causes a drastic and immediate viral decrease by targeting distinct steps in the HIV-1 life cycle, effectively blocking replication and halting disease progression (1–3). However, cART does not target or eliminate HIV that persists in a latent state in cellular reservoirs. Because some of the proviruses are replication competent, latent HIV-infected cells inevitably rebound once cART is interrupted, leading to necessity for lifelong therapy (4). Particularly in resource-limited countries, which are also disproportionately affected, this is translated into an insurmountable medical, social, and financial burden. To achieve a scalable cure for HIV infection, it will be necessary to reduce or eliminate the latent HIV-infected reservoir of cells and/or equip the immune system with the

robustness and effectiveness necessary to prevent viral rebound such that cART can be safely discontinued.

An important breakthrough in HIV-1 cure was the unequivocal proof that it is possible to mobilize the latent patient HIV reservoir by treatment with agents that activate HIV gene expression [latency reversal agents (LRAs)] (5). However, clinical studies thus far have shown little to no reduction in the latent reservoir in patients (6, 7). This is consistent with limited potency and specificity of currently tested drugs, which appear to be unable to reach a significant proportion of latently infected cells or to induce HIV-1 expression in latent reservoir at sufficient levels to produce viral proteins for recognition by the immune system (8). Furthermore, transcriptional stochasticity and heterogeneity of latent HIV integrations (9) may pose additional barriers to reactivation of the latent reservoir as a whole; sequential rounds of stimulation yield new infectious particles (10), while certain LRA combinations result in more efficient latency reversal when administered in intervals rather than at once (11). In addition, pleiotropic functions and toxic effects of LRAs may compromise the ability of CD8<sup>+</sup> T cells to eliminate HIV protein-expressing cells (12, 13). Therefore, it is critical to identify and develop novel therapeutics, which strongly induce HIV-1 gene expression to effectively disrupt HIV latency without dampening the immune response.

The pharmaceutical industry is highly equipped for high-throughput screens using defined synthetic libraries. While this is an effective approach, it is important to record that approximately half of the novel small molecules introduced to the market between 1981 and 2014 are natural or nature-derived (14). Biological systems represent an invaluable source of functional molecules with high chemical diversity and biochemical specificity, evolved during millions of years of adaptation. In particular, fungi represent a largely unexplored source of compounds with potential therapeutic use. Fungi secrete a gamut

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of extracellular compounds and other small molecular extrolites (15). While some of these compounds have been shown to have antibiotic (ex. penicillin) or carcinogenic (ex. aflatoxin) properties, little is known in general about their biological activities and possible molecular targets. In addition, a single fungal strain often produces a wide array of secondary metabolites that are not essential for its growth but are exuded as a consequence of specific environment, such as nutrient-rich versus minimal growth conditions (16, 17). Fungal extrolites might target various signaling pathways in mammalian cells, such as those influencing HIV-1 gene expression. Fungal supernatants are an ideal source for an expert academic setting, where low- and medium-throughput biological screening systems, academic knowledge of evolutionary mycology, and state-of-the-art fractionation and purification techniques are routinely combined. Studies of regulation of HIV-1 gene expression have identified distinct molecular mechanisms and cellular pathways at play, which can be targeted pharmacologically to activate expression of latent HIV (18, 19). The rich diversity of fungal extrolites therefore may prove an untapped source of new compounds that target HIV for reactivation. In search of novel LRAs, here, we have performed an unbiased medium-throughput screen of fungal extrolites, coupled to HIV latency reversal bioassays and orthogonal fractionation and mass spectrometry (MS)/nuclear magnetic resonance (NMR), and identified gliotoxin (GTX). GTX potently reversed HIV-1 latency in multiple *in vitro* latency models as well as *ex vivo* in cells obtained from all aviremic HIV-1-infected patients examined without associated cytotoxicity. We demonstrate that GTX disrupts the positive transcription elongation factor (P-TEFb)—sequestering 7SK small nuclear ribonucleoprotein (snRNP) complex by targeting the La-related protein 7 (LARP7) component, leading to degradation of its scaffold 7SK RNA and release of active P-TEFb, which is then recruited by Tat to phosphorylate the Ser<sup>2</sup> residue of the RNA polymerase II (Pol II) C-terminal domain (CTD) and activate HIV transcription elongation. Our data highlight the power of combining a medium-throughput bioassay, mycology, and orthogonal MS to identify novel potentially therapeutic compounds.

## RESULTS

### Growth supernatant of *Aspergillus fumigatus* identified in a medium-throughput screen of fungal secondary metabolite has HIV-1 latency reversal activity

We screened 115 species of filamentous fungi for their ability to induce HIV-1 proviral expression; of the species that appeared promising, 2 to 4 additional strains were tested (table S1). The species belonged to 28 orders (43 families) of the fungal kingdom (Fig. 1A) and were chosen on the basis of their evolutionary position, ecological trends, and known active production of extracellular compounds. The majority of fungi were of ascomycetous affinity, four species were of basidiomycetous affinity, and two belonged to the lower fungi. Selected fungi were grown in both complete yeast media and minimal media (RPMI 1640), as they are known to produce distinct extrolites depending on their growth conditions (fig. S1). Culture supernatants were then screened for latency reversal activity using Jurkat-derived 11.1 and A2 cell line models of HIV-1 latency (J-Lat) in a low-medium throughput assay setup, in which expression of green fluorescent protein (GFP) is controlled by the HIV-1 promoter and indicates latency reversal. We identified the supernatant of *Aspergillus fumigatus* CBS 542.75 to strongly activate the latent HIV-1 5 long

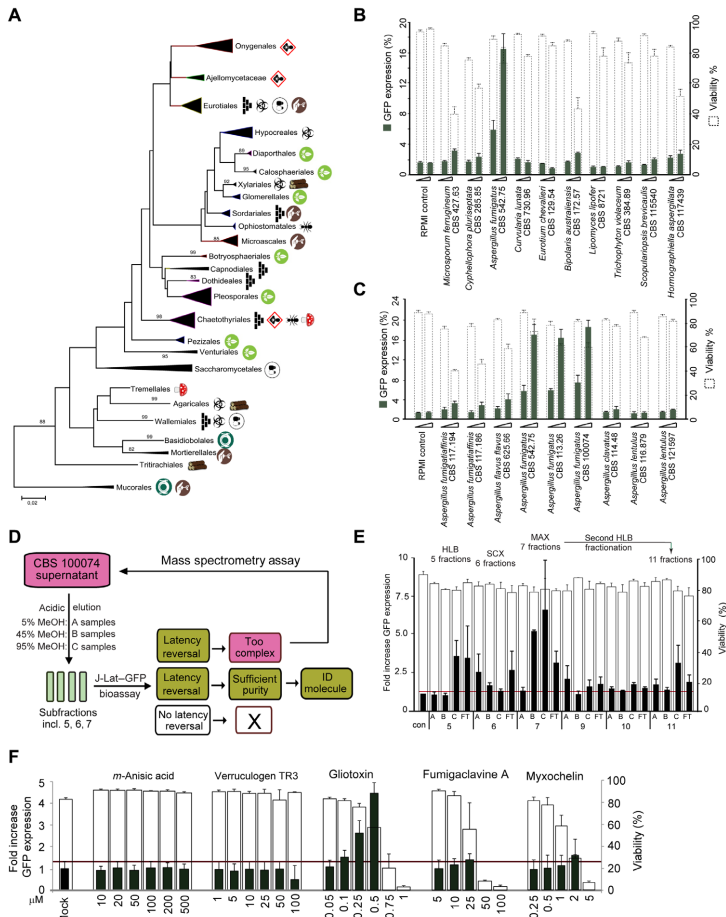
terminal repeat (LTR) (Fig. 1B). We also compared other *Aspergillus* species growth supernatants indicated for their potential to induce HIV-1 expression (Fig. 1C) and observed that only strains of *A. fumigatus* (CBS 542.75, CBS 113.26, and CBS 100074) had latency reversal activity (Fig. 1C).

### Orthogonal liquid chromatography–MS/NMR strategy coupled to latency reversal bioassays identifies GTX from growth supernatant of *A. fumigatus* as a putative LRA

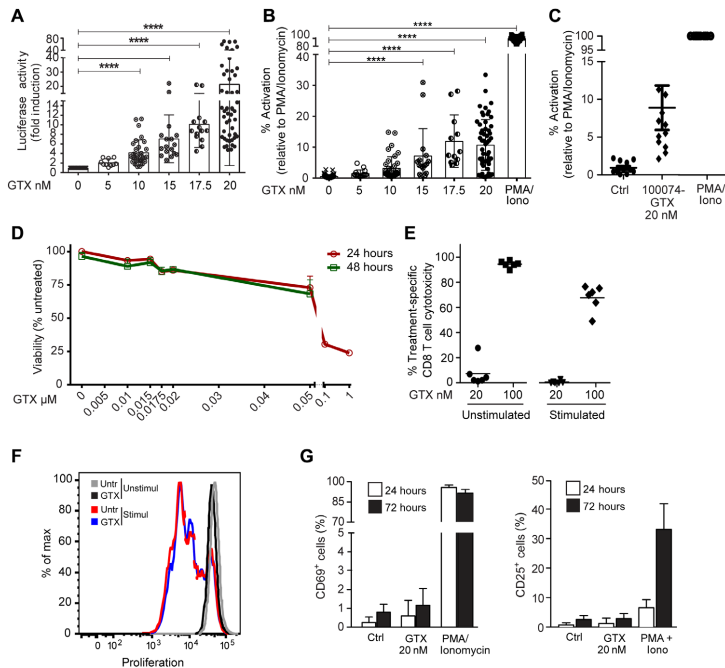
Because of the chemical complexity of the positive fungal supernatants, direct MS analysis of their constituents proved to be impossible. Therefore, *A. fumigatus* CBS 100074 growth supernatant was fractionated several times by means of orthogonal MS (Fig. 1D). We selected this particular supernatant as it showed the highest potency to reverse latency in the J-Lat models. After each round of fractionation, all samples/fractions were again tested in latency reversal bioassays, followed by quantitation of the GFP expression and identification of fractions retaining latency reversal activity. As expected, originally less active fractions became more active during the fractionation/enrichment process (Fig. 1E). The most active 7B/7C fractions were further fractionated on a hydrophilic-lipophilic balance (HLB) cartridge (11 samples) and components of 7B/7C and 11C fractions dereplicated by CycloBranch software (Fig. 1E and fig. S2, A and B) (20). Compound matching against the annotated database of *Aspergillus* secondary metabolites revealed a set of candidate compounds further selected for latency reversal testing (fig. S2C and table S2). Among the candidate molecules identified, GTX, obtained from a commercially available synthetic source, was able to induce expression of the latent provirus in a concentration-dependent manner (Fig. 1F). Notably, GTX was found in all positive fractions (table S2). While GTX isolated from supernatant of CBS 100074 showed strong induction of HIV-1 transcription, supernatant of *Aspergillus flavus* (CBS 625.66), a close relative of *A. fumigatus* that was inactive in latency reversal (Fig. 1C), did not contain GTX (fig. S2, D and E), providing further support that GTX is the main mediator of LRA activity in *A. fumigatus* supernatants.

### GTX reverses latency in *ex vivo* infected primary CD4<sup>+</sup> T cells without associated cytotoxicity

To examine the latency reversal potential of GTX in a more clinically relevant system, we used a modified primary *ex vivo* infected latency model, in which primary CD4<sup>+</sup> T cells are infected with a full-length replication-incompetent HIV-1 virus driving expression of the luciferase reporter (fig. S3A) (21). Treatment of latently infected CD4<sup>+</sup> T cells with GTX resulted in significant, concentration-dependent latency reversal at lower concentrations than necessary to achieve reactivation in latently infected cell lines (Fig. 2, A to C). GTX (20 nM) treatment resulted in more than 20-fold induction of HIV-1 expression (Fig. 2A), which translated into more than 10% latency reversal of that observed upon maximal stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin (Fig. 2B). Similar to the latency reversal observed using commercially available synthetic GTX (Fig. 2, A and B), treatment of HIV-1-infected latent primary CD4<sup>+</sup> T cells with GTX isolated from growth supernatant of *A. fumigatus* CBS 100074, estimated at 20 nM by NMR in comparison to commercially available GTX, resulted in significant latency reversal, approximately 10% of maximal PMA/ionomycin stimulation (Fig. 2C). At high concentrations, GTX is known to be toxic to immune cells (22–24), ascribed to its unusual disulfide



**Fig. 1. Medium-throughput screen of fungal secondary metabolites combined with orthogonal fractionation and MS strategy coupled to latency reversal bioassays identifies GTX from growth supernatant of *A. fumigatus* to reverse HIV-1 latency.** (A) Phylogenetic tree representing the main orders of the fungal kingdom with strains used in the current study, collapsed per order. Orders selected from the tree published (57), with some of the lower orders included for structural reasons. Approximate ecological trends in the orders are summarized by symbols, as follows: vertebrate pathogenicity prevalent, climatic extremotolerance prevalent, frequent production of extracellular metabolites or mycotoxins, frequent osmotolerance or growth in sugary fluids, numerous members with soilborne lifestyle, numerous members inhabiting decaying wood rich in hydrocarbons, frequent insect association, frequent mushroom decomposition or hyperparasitism on fungi or lichens, and frequent inhabitants of foodstuffs or vertebrate intestinal tracts. (B) Latency reversal bioassay performed by treatment of J-Lat A2 cells with increasing volumes of growth [normalized by O.D. (optical density)] supernatants obtained from selected fungal strains. (C) Latency reversal bioassay in J-Lat A2 cells with growth supernatants obtained from members of the *Aspergillus* genus. Cells were treated as in (B). (D) Schematic representation of the orthogonal MS strategy coupled to latency reversal bioassays used to identify putative LRA. See main text for full description. (E) Three preconcentration cartridges (HLC, SCX, and MAX) were combined with variable content of extracting solvent (A: 5% MeOH, B: 45% MeOH, and C: 95% MeOH; FT, flowthrough). Latency reversal potential of fractionated secondary fungal metabolites was tested via treatment of J-Lat A2 cells. Latency reversal (fold increase percentage of GFP, left axis, black bars) and cell viability (percentage of viability, right axis, empty bars) were assessed by flow cytometry analysis. (F) Commercially obtained versions of five common molecules identified in active fractions were tested for LRA activity in J-Lat A2 cells. Data are presented as fold increase percentage of GFP expression and percentage of viability as indicated,  $\pm$ SD from at least three independent experiments.

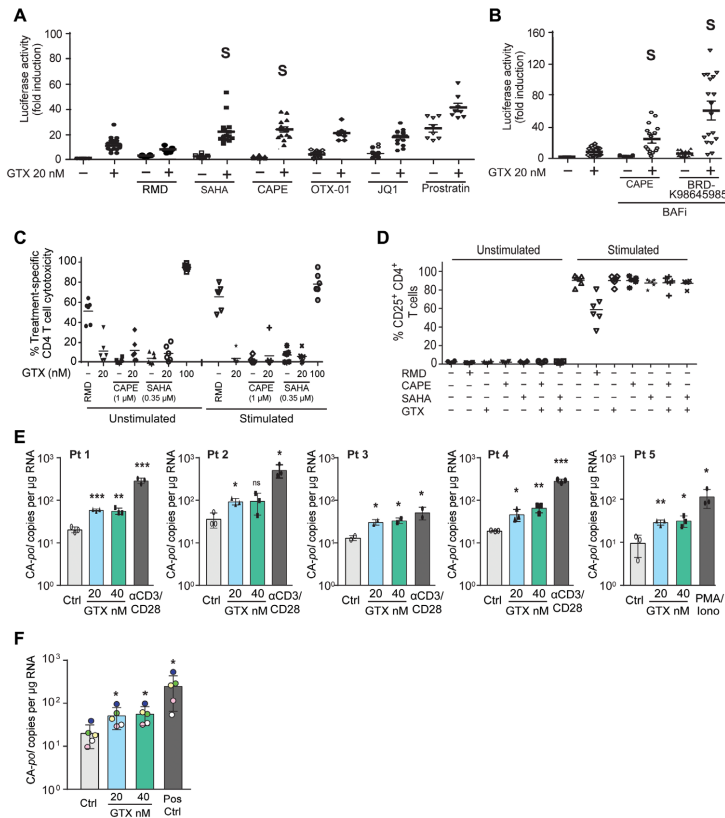


**Fig. 2. GTX (20 nM) reverses HIV-1 latency in ex vivo infected primary CD4<sup>+</sup> T cells without associated cytotoxicity, T cell activation, or inhibition of proliferative capacity.** (A) Latency reversal after treatment of latently infected primary CD4<sup>+</sup> T cells with increasing concentrations of GTX for 24 hours as indicated, shown as fold induction over untreated control luciferase activity. Statistical analysis was calculated using Kruskal-Wallis one-way analysis of variance (ANOVA) (\*\*\*\* $P < 0.0001$ ). (B) Data presented in (A) normalized and shown as percent latency reversal activity relative to treatment with PMA/ionomycin. (C) Latency reversal after treatment of HIV-1-infected latent primary CD4<sup>+</sup> T cells with 20 nM GTX isolated from growth supernatant of *A. fumigatus* CBS 100074, measured as increased luciferase activity normalized to and shown as percentage of activation of PMA/ionomycin ( $n = 6$ ). Wide horizontal lines represent average, and shorter horizontal lines represent SD. (D) Viability of the primary CD4<sup>+</sup> T cells treated for 24 (red line) and 48 (green line) hours with indicated increasing concentrations of GTX, assessed by flow. (E) Unstimulated or  $\alpha$ CD3/ $\alpha$ CD28-stimulated PBMCs were treated with 20 and 100 nM GTX for 72 hours, stained with AnnexinV, and cell death (AnnexinV<sup>+</sup> CD3<sup>+</sup> CD8<sup>+</sup>) was determined by flow cytometry. Treatment-specific cell cytotoxicity was calculated as indicated in the Materials and Methods section. Each symbol represents one healthy donor ( $n = 6$ ), and horizontal lines depict means. (F) Representative fluorescence-activated cell sorting (FACS) plot overlay showing the proliferation of unstimulated or  $\alpha$ CD3/ $\alpha$ CD28-stimulated CD8<sup>+</sup> T cells in the presence or absence of GTX. Cells were stained with a proliferation dye and analyzed 72 hours later by flow cytometry to define nonproliferating cells (proliferation dye undiluted, bright stain intensity) and dividing cells (proliferation dye diluted, reduced stain intensity). (G) Activation status of primary CD4<sup>+</sup> T cells upon GTX treatment as indicated ( $n = 6$ ).

bridge, and responsible for pleiotropic effects on cellular and viral systems (25).

Consistent with the literature, GTX concentrations upward of 100 nM induced significant toxicity as indicated by annexin V staining (Fig. 2D and fig. S3B). However, primary CD4<sup>+</sup> T cell viability was not significantly affected after GTX treatment at concentrations up to 20 nM, with moderate toxicity observed at 50 nM (Fig. 2D and fig. S3B). Thus, at lower concentrations in which GTX did not show toxicity on CD4<sup>+</sup> T cells, strong latency reversal was induced (Fig. 2, A to D, and fig. S3B). CD8<sup>+</sup> T cells play a central role in eliminating HIV-infected cells (26). Therefore, it is of utmost importance to evaluate the potential toxicity of newly developed LRAs on CD8<sup>+</sup> T cells (13). GTX at a low concentration of 20 nM did not reduce the viability or proliferation capacity of CD8<sup>+</sup> T cells whether unstimulated or  $\alpha$ CD3/ $\alpha$ CD28-stimulated peripheral blood mononuclear cells

(PBMCs) were examined (Fig. 2, E and F, and fig. S4, A and B). Consistent with the literature (22–25), treatment with higher concentrations of GTX at 100 nM and 1  $\mu$ M caused apoptosis and death of primary CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as B cells, natural killer (NK) cells, and monocytes, as shown by annexin V staining followed by flow cytometry (Figs. 2, D and E, and 3C and figs. S3B and S4, A to C). Potential for clinical applicability of a candidate LRA also requires that it does not induce global T cell activation nor should it interfere with CD8<sup>+</sup> T cell activation. Treatment of unstimulated primary CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells with GTX (20 nM), which significantly reversed latency, did not induce expression of the T cell activation markers CD69 and CD25 (Figs. 2G and 3D and fig. S5, A and B), nor did it induce proliferation of resting CD4<sup>+</sup> and CD8<sup>+</sup> T cells (fig. S5, C and D), while, as expected, PMA/ionomycin treatment activated T cells (Fig. 2G). Conversely, GTX treatment of activated PBMCs also did



**Fig. 3. GTX strongly synergizes with HDAC and BAF inhibitors and reverses latency in primary CD4<sup>+</sup> T cells obtained from all tested aviremic participants.** (A and B) HIV-1 latency reversal in latent ex vivo HIV-1-infected primary CD4<sup>+</sup> T cells in response to 24-hour cotreatment with 20 nM GTX and distinct LRA class compounds [2 nM RMD, 350 nM SAHA, 1 µM CAPE, 2 µM OTX-01, 0.5 µM JQ-1, and 0.2 µM prostratin (A) and 1 µM CAPE and 5 µM BRD-K98645985 (B)] and shown as fold increase in luciferase activity. S indicates compound synergism in latency reversal according to the Bliss independence score. (C) Cytotoxicity of GTX alone and combined with indicated LRAs in CD4<sup>+</sup> T cells. Unstimulated or αCD3/αCD28-stimulated PBMCs were cotreated as indicated for 72 hours followed by annexin V staining, and cell death (AnnexinV<sup>+</sup> CD3<sup>+</sup>CD4<sup>+</sup>) was determined by flow cytometry. (D) GTX (20 nM) does not alter activation of CD4<sup>+</sup> T cells. PBMCs from healthy donors were incubated with the indicated LRAs for 72 hours, either unstimulated or stimulated with αCD3/αCD28 antibodies. Panels depict pooled data showing the frequency of CD25<sup>+</sup> cells within CD4<sup>+</sup> T cells. (E) Absolute, cell-associated (CA) pol copy number in CD4<sup>+</sup> T cells isolated from five aviremic participants that were treated in vitro with vehicle control (Untr), GTX (20 and 40 nM), or positive control for 24 hours as indicated. Statistical significance was calculated using t test (\**P* < 0.05; \*\**P* < 0.005; \*\*\**P* < 0.0005). ns, not significant. (F) Data presented in (D) had been averaged and plotted together. Each symbol represents aviremic participant: green, participant 1; blue, participant 2; white, participant 3; yellow, participant 4; and pink, participant 5. Statistical significance was calculated using unpaired Mann-Whitney test (\**P* < 0.05).

not inhibit CD25 expression (Fig. 3D and fig. S5, A and B) or proliferation of activated CD4<sup>+</sup> or CD8<sup>+</sup> T cells (fig. S5, C and D).

**GTX enhances activity of other LRA class molecules and synergizes strongly with histone deacetylase and BAF inhibitors**

To investigate possible synergies, we tested the latency reversal potential of GTX (20 nM) in combination with a panel of known LRAs in the J-Lat A2 and 11.1 models of latency (fig. S6) as well as in ex vivo infected primary CD4<sup>+</sup> T cells (Fig. 3, A and B). GTX cotreatment

enhanced the latency reversal activity observed after single treatments with all compounds (Fig. 3, A and B). When latent HIV-1-infected primary CD4<sup>+</sup> T cells were cotreated with GTX (20 nM) and either the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) or BAF (BRG1- or BRM-associated factors) inhibitors caffeic acid phenethyl ester (CAPE) [at 1 µM concentration in which it does not inhibit nuclear factor κB (NF-κB)] (27) or BRD-K98645985 (28), synergistic reversal of HIV-1 latency was observed (Fig. 3, A and B). Cotreatments with BET (bromodomain and extra-terminal proteins) inhibitors JQ-1 and OTX-015, as well

as prostratin, resulted in an additive effect on HIV-1 provirus expression. In the primary CD4<sup>+</sup> T cell model of HIV-1 latency, GTX treatment alone showed more potent latency reversal activity than SAHA, CAPE, OTX-015, JQ-1, or romidepsin (RMD) alone at tested nontoxic concentrations (Fig. 3, A and B). RMD treatment showed modest latency reversal activity (Fig. 3A) and, consistent with the literature (13), significant CD4<sup>+</sup> and CD8<sup>+</sup> T cell cytotoxicity (Fig. 3C and fig. S4, A and B). With the exception of RMD, we did not observe any negative impact of these cotreatments on viability and proliferation of CD4<sup>+</sup> T cells (Fig. 3C and fig. S5C), CD8<sup>+</sup> T cells (figs. S4, A and B, and S5D), and other immune subpopulations, including CD19<sup>+</sup> B cells, CD56<sup>+</sup> NK cells, and CD14<sup>+</sup> monocytes (fig. S4C). Moreover, none of the cotreatments altered the activation status of either resting or activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 3D and fig. S5, A and B). Our observed synergistic effects upon GTX cotreatment with BAF inhibitors and the HDAC inhibitor SAHA suggested that GTX may target a different pathway for HIV-1 latency reversal, as targeting the same pathway with different molecules would more likely result in additive effects. Unlike SAHA, GTX treatment of CD4<sup>+</sup> T cells did not result in increased histone acetylation (fig. S7A). Similarly, we excluded that GTX behaves as a protein kinase C (PKC) agonist, as treatment with GTX did not induce T cell activation or the expression of PKC pathway target genes (Fig. 2G and fig. S7B).

### GTX reverses HIV-1 latency after ex vivo treatment of CD4<sup>+</sup> T cells from all aviremic HIV-1-infected study participants

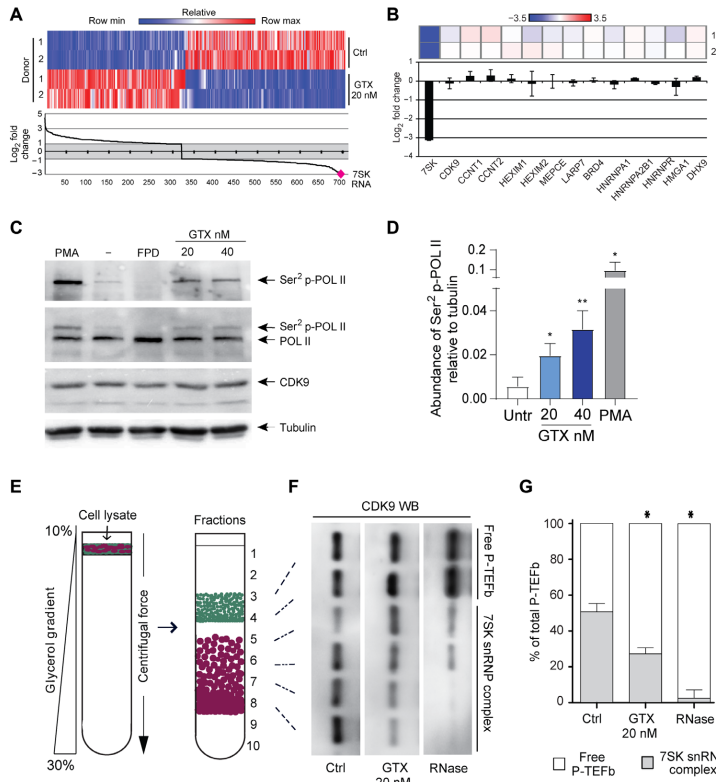
We next examined the potential of GTX to reverse latency after ex vivo treatment of CD4<sup>+</sup> T cells obtained from people living with HIV-1. All five participants enrolled were treated with cART and maintained HIV-1 viremia below 50 copies/ml for at least 2 years. Despite differences in the size of the latent pool, assessed by maximal stimulation of the cells with  $\alpha$ -CD3/CD28 beads or PMA/ionomycin, GTX treatment at nontoxic 20 and 40 nM concentrations significantly increased the levels of cell-associated HIV-1 *pol* RNA copies (CA-*pol* copies) in CD4<sup>+</sup> T cells obtained from aviremic HIV-1<sup>+</sup> participants (Fig. 3, E and F). Notably, the observed GTX effect is systematic, as latency reversal was uniformly observed in cells of all tested participants after 24 hours of stimulation (Fig. 3, E and F). This observed latency reversal at the RNA level is the first step consistent with induction of protein expression, although this is not formally examined here. In addition, we observed no increase in expression of genes related to T cell-specific responses, reactive oxygen species, or apoptosis, previously reported pleiotropic effects of GTX observed at significantly higher concentrations (fig. S7C). These results therefore indicate that GTX treatment at nontoxic concentrations reverses latency without inducing immune cell toxicity or activation and without affecting T cell proliferation, rendering GTX a promising potential candidate for further clinical investigation in context of HIV-1 latency reversal and inclusion in “shock and kill” strategies.

### GTX reverses HIV-1 latency via disruption of 7SK snRNP, leading to release of active P-TEFb

To gain insight into the molecular mechanism by which GTX reverses latency, we performed RNA sequencing of primary resting CD4<sup>+</sup> T cells isolated from healthy blood donors that were treated for 4 hours with 20 nM GTX. This short incubation time was chosen to focus on primary effects of GTX on the global transcriptome,

decreasing the presence and likelihood of secondary transcriptional effects. We observed a very good correlation between treatments of two independent healthy blood donors (Fig. 4A, top). Moreover, less than 700 genes showed an altered differential expression pattern (Fig. 4A, bottom). Small nuclear RNA 7SK was the most decreased (more than ninefold) transcript after GTX treatment in CD4<sup>+</sup> T cells from the independent donors (Fig. 4, A and B). 7SK RNA serves as a scaffold for the 7SK snRNP complex that sequesters the P-TEFb and inhibits its activity (29–33). Among all components of the 7SK snRNP complex and its close interactors, only the 7SK transcript was affected by treatment with GTX (Fig. 4B and fig. S9A). Consistent with our observations after treatment of CD4<sup>+</sup> T cells obtained from aviremic participants (fig. S7C), we did not detect significant change in expression of NF- $\kappa$ B, oxidative stress, apoptosis, and T cell effector function-related genes after GTX treatment (figs. S7B and S8), indicating that GTX (20 nM) treatment does not influence these pathways after 4 or 24 hours of stimulation. As P-TEFb is an essential cofactor for Tat-mediated HIV-1 transcription elongation (34, 35), we examined whether GTX treatment affects P-TEFb activity. Phosphorylation of Ser<sup>2</sup> residues within the CTD of RNA Pol II is a prerequisite for activation of transcription elongation and is mediated by the kinase activity of cyclin-dependent kinase 9 (CDK9), a component of P-TEFb (36). To examine whether GTX treatment resulted in Ser<sup>2</sup> RNA Pol II phosphorylation, we treated resting CD4<sup>+</sup> T cells with GTX, the CDK9 inhibitor flavopiridol (FPD), and PMA or  $\alpha$ CD3/ $\alpha$ CD28 as positive control (Fig. 4C and fig. S9B). As expected, FPD treatment abrogated RNA Pol II phosphorylation, while PMA stimulation led to strong Ser<sup>2</sup> RNA Pol II phosphorylation. Treatment with GTX caused an increase in phosphorylation of the CDK9 target RNA Pol II Ser<sup>2</sup> in three independent donors tested (Fig. 4, C and D, and fig. S9B). We also examined potential Tat-independent effects of GTX on basal HIV-1 transcription by comparing GTX-induced reactivation in J-Lat A72 cells, which lack Tat, with that in J-Lat A2 cells (fig. S9C). While GTX robustly reactivated transcription in Tat-containing A2 cells, in comparison, A72 cells were not significantly reactivated. These results support a Tat-dependent mechanism of GTX function consistent with a role in enhancing Tat–P-TEFb-mediated HIV-1 transcription.

The fact that short-term GTX treatment enhanced P-TEFb activity in resting CD4<sup>+</sup> T cells without cellular activation (Figs. 2G, 3D, and 4C) led us to hypothesize that GTX treatment may cause destabilization of the 7SK snRNP complex, leading to the release of free (active) P-TEFb. Active P-TEFb would then become available for transcription elongation at the latent HIV-1 LTR, a critical step required for HIV-1 latency reversal (37–39). To test this, we performed glycerol gradient sedimentation experiments after treatment of resting CD4<sup>+</sup> T cells with GTX (Fig. 4, E to G, and fig. S9D). GTX treatment (20 nM) of CD4<sup>+</sup> T cells for 4 hours resulted in release of free P-TEFb from its inhibitory higher-molecular weight 7SK snRNP complex, as shown by Western blot analysis depicting distribution of the P-TEFb component CDK9 over high- and low-molecular weight fractions (Fig. 4F and fig. S9D). As expected, control treatment of CD4<sup>+</sup> T cell lysates with ribonuclease A (RNase A), which digests the 7SK RNA scaffold, resulted in release of free P-TEFb, which eluted at lower-molecular weight fractions (Fig. 4F and fig. S9D). Quantification of free versus 7SK snRNP-sequestered P-TEFb demonstrates a significant GTX-induced release of free P-TEFb from the 7SK snRNP complex in CD4<sup>+</sup> T cells from three independent donors (Fig. 4G). CDK9 T-loop phosphorylation at Thr<sup>186</sup> is critical for the kinase

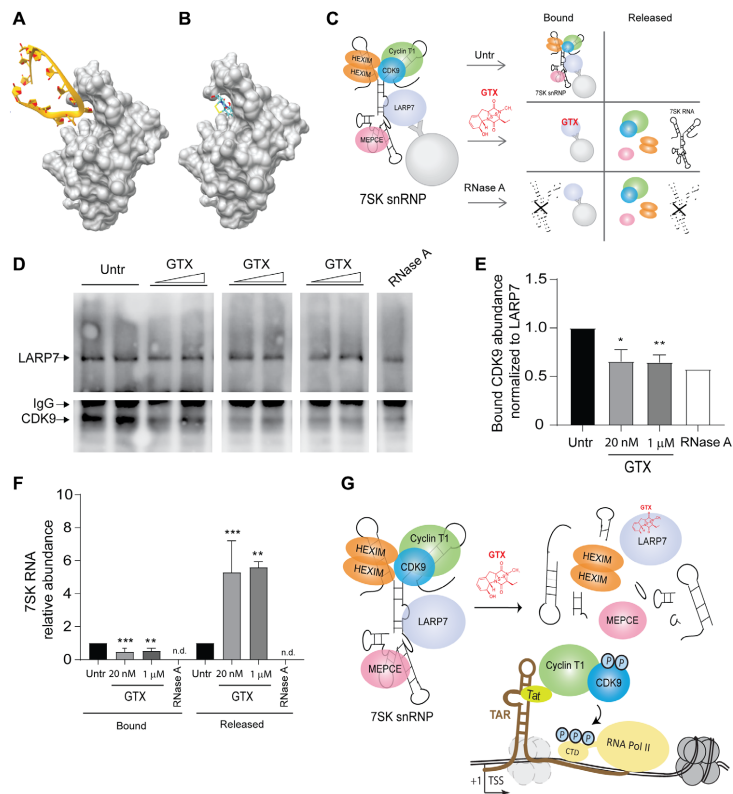


**Fig. 4. GTX treatment of resting CD4<sup>+</sup> T cells causes decrease in 7SK RNA and activation of P-TEFb activity.** (A) Heatmap of differentially expressed genes obtained from RNA sequencing analysis of primary CD4<sup>+</sup> T cells treated as indicated for 4 hours (top). RNA sequencing indicates 7SK RNA to be the most differentially decreased gene in response to GTX treatment of CD4<sup>+</sup> T cells in two independent donors (bottom). (B) GTX treatment of primary CD4<sup>+</sup> T cells for 4 hours leads to specific depletion of 7SK RNA levels and not mRNA levels of other components of the 7SK snRNP complex. (C) Representative Western blot (WB) analysis of CTD Ser<sup>2</sup> phosphorylation of RNA Pol II mediated by CDK9 in primary CD4<sup>+</sup> T cells treated for 6 hours with GTX as indicated. PMA was used as a positive control, and FPD was used as a negative control. (D) Quantification of bands representing phosphorylated RNA Pol II normalized to tubulin and relative to untreated control from three independent experiments (C; fig. S9C). Statistical significance was calculated using unpaired *t* test (\**P* < 0.05; \*\**P* < 0.01). (E and F) GTX treatment causes release of P-TEFb from sequestration within the 7SK snRNP complex. (E) Schematic of glycerol gradient experiments, in which cell lysates (from GTX-treated or untreated cells) are loaded on top of generated glycerol gradients (10 to 30%) and ultracentrifuged, followed by collection of fractions, trichloroacetic acid protein precipitation, and subsequent analysis by SDS-polyacrylamide gel electrophoresis Western blotting. (F) Representative Western blot analysis of glycerol gradient sedimentation of lysates from primary CD4<sup>+</sup> T cell, which were treated (6 hours) with GTX (20 nM) or vehicle control as indicated using antibodies specific for the P-TEFb component CDK9. As control, untreated lysates were treated with RNase for 1 hour to digest 7SK RNA and release P-TEFb. (G) Quantification of free versus total CDK9 in primary CD4<sup>+</sup> T cells treated as indicated, as shown in (F) and fig. S9E, from three independent donors. Statistical significance was calculated using unpaired *t* test (\**P* < 0.05).

activation and its dissociation from the 7SK snRNP (40). In resting CD4<sup>+</sup> T cells, cyclin T1 levels are low, as are the levels of CDK9 T-loop phosphorylation (39). To check whether GTX-induced release of P-TEFb involves increased CDK9 T-loop phosphorylation or increase in cyclin T1 levels, we treated primary CD4<sup>+</sup> T cells with increasing concentrations of GTX for 6 hours and used PMA as positive control. At 6 hours after treatment, at a time point in which we observe phosphorylation of RNA Pol II CTD (Fig. 4, C and D), GTX treatment does not cause phosphorylation of CDK9 at Thr<sup>186</sup>, nor does

it lead to increased cyclin T1 levels (fig. S9, E and F), demonstrating that these mechanisms are not involved in GTX-induced latency reversal at this early time point.

To understand better which component of the 7SK snRNP complex may be targeted by GTX, we performed *in silico* docking experiments using two independent software packages, Chimera and Achilles. We modeled GTX against all essential components of the complex separately, as complete crystal structure of 7SK snRNP is not yet available. Notably, we observed preferential binding of GTX



**Fig. 5. GTX disrupts 7SK snRNP, causing release of P-TEFb and enhanced HIV-1 transcription.** (A) Crystal structure of human LARP7 CTD in complex with 7SK RNA SL4 (Protein Data Bank ID code 6D12). LARP7 is shown in space-filling representation (gray) with bound RNA as yellow cartoon representation with bases indicated as flat rings (oxygen in red). (B) Proposed binding mode of GTX into the deep pocket on the surface of LARP7 as predicted by Chimera's AutoDock Vina function and the Achilles Blind Docking server. Color code for GTX: carbon (turquoise), oxygen (red), sulfur (yellow), hydrogen (white), and nitrogen (blue). (C) Schematic representation of the 7SK snRNP coimmunoprecipitation CDK9 release experiment. (D) 7SK snRNP complex is immunoprecipitated using beads coated with anti-LARP7. Beads are divided and left either untreated or treated with GTX 20 nM or 1 μM or RNase A as indicated. Bead-bound fractions are separated and subjected to Western blot analysis to detect presence of LARP7 and CDK9. IgG, immunoglobulin G. (E) Quantification of CDK9 abundance normalized to immunoprecipitated LARP7 and relative to untreated control ( $n = 3$ ). Statistical significance was calculated using unpaired *t* test (\* $P < 0.05$ ; \*\* $P < 0.01$ ). (F) 7SK RNA release assay from immunoprecipitated 7SK snRNP complex, as represented in (C). Bound and released fractions are subjected to reverse transcription quantitative polymerase chain reaction to quantitate the levels of bead-bound and released 7SK RNA in reaction supernatant. Input 7SK RNA (pre-reaction beads) was used for normalization. Statistical significance was calculated with unpaired *t* test ( $n = 3$ , \*\*\* $P < 0.01$ ; \*\* $P < 0.001$ ; n.d., not detected). (G) Proposed model for GTX-mediated transcription activation of the latent HIV-1 LTR via degradation of 7SK RNA and release of CDK9 from the 7SK snRNP complex. Free P-TEFb is then recruited to the HIV-1 Tat-TAR axis, leading to phosphorylation of RNA Pol II at Ser<sup>2</sup> and subsequent stimulation of transcription elongation.

into the hydrophobic pocket of LARP7, which, in physiological conditions, is responsible for binding stem loop 4 (SL4) of the 7SK RNA (Fig. 5, A and B, and fig. S10). LARP7 is a critical component, responsible for stabilization of the 7SK snRNP complex, as its depletion has been shown to lead to decreased levels of 7SK RNA with concomitant increase in free P-TEFb levels (41). To test whether GTX interferes with LARP7-7SK RNA binding, we performed a 7SK RNA release assay (Fig. 5, C to F). 7SK snRNP complex was immunoprecipitated and immobilized on protein G beads

using a LARP7 antibody. 7SK snRNP-bound beads were then either left untreated or treated with GTX or RNase A as indicated, and the presence of LARP7-bound CDK9 was examined by Western blot analysis of the bead-bound fraction (Fig. 5, D and E). As expected, RNase A treatment resulted in direct disruption of the 7SK snRNP complex as measured by release of the CDK9 component into the supernatant and its separation from the bead-bound LARP7 component (Fig. 5, D and E). GTX treatment also resulted in release of CDK9 as observed in relative decrease in LARP7-bound

CDK9 as determined by Western blot analysis (Fig. 5, D and E). In agreement, treatment with GTX resulted in a concomitant significant decrease in abundance of scaffold 7SK RNA from the bead-bound 7SK snRNP fraction as well as an approximately sixfold increase in levels of 7SK RNA in the released fraction (Fig. 5F).

Our experimental data, together with this modeling exercise and previously published data, are consistent with a model in which GTX interferes with the binding of SLA of 7SK RNA into the hydrophobic pocket of LARP7, which results in destabilization of the complex and subsequent release of P-TEFb and 7SK RNA (Fig. 5, D to G). In resting CD4<sup>+</sup> T cells, 7SK RNA is then degraded and free P-TEFb made available for recruitment to the RNA Pol II at the latent HIV LTR by the Tat-TAR (trans-activation response element) axis. CDK9 then phosphorylates CTD of RNA Pol II, leading to activation of proviral transcription elongation (Fig. 5G).

## DISCUSSION

Distinct classes of LRAs have been shown to target different subpopulations of proviruses (9, 19, 42, 43). Thus far, none of the clinically tested LRAs have been able to induce strong viral expression or to significantly deplete the latent reservoir in patients, pointing to potential limitations of single treatments (6, 7). The heterogeneous nature of latent HIV integrations and the complex molecular mechanisms that contribute to maintaining HIV latency, together with individual genetic variability, dictate that a cocktail of stimulatory compounds targeting distinct cellular and HIV gene regulatory pathways would be most effective to activate the latent reservoir in HIV-1-infected patients (19). Preclinical studies demonstrate that combination treatments can result in synergism and lead to stronger HIV-1 latency reversal (11, 19, 27, 28, 44–48). Here, we found that GTX, a molecule that targets the HIV-1 transcription elongation step, strongly synergized with LRAs that derepress chromatin structure, namely, BAF and HDAC inhibitors, which respectively target complexes that position the latent LTR chromatin in a repressive configuration (49) or deacetylate histones compacting LTR chromatin. Cotreatment of primary latently infected CD4<sup>+</sup> T cells with GTX and BET inhibitors resulted in additive and not synergistic increase in HIV transcription. This observation is consistent with the fact that these compounds target the same mechanistic step: transcription elongation at the HIV-1 LTR. Our data highlight the attractiveness of simultaneous pharmacological targeting of mechanistically distinct steps in HIV-1 transcription regulation, namely, at the level of chromatin structure, transcription factor-induced activation and transcription elongation. We postulate that more robust latency reversal will be observed when compounds targeting these three mechanistic steps are combined, and further optimized by interventions that address blocks occurring posttranscriptionally, at the polyadenylation and splicing stages, which may hinder translation of HIV-1 genes (38). In addition, consistent with the importance of targeting transcription elongation, shown recently to be a major rate-limiting step in HIV-1 latency reversal in patient CD4<sup>+</sup> T cells (38), ex vivo GTX treatment of CD4<sup>+</sup> T cells obtained from cART-suppressed aviremic HIV-1-infected patients demonstrated significant increase in levels of cell-associated HIV-1 RNA in all patients examined.

GTX causes disruption of the 7SK snRNP complex with subsequent release of active P-TEFb by directly targeting LARP7. Other molecules including HMBA (hexamethylene bisacetamide) (50) and DRB (5,6-Dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole) and acti-

nomycin D (30, 32) have also previously been shown to cause 7SK snRNP disruption although through indirect mechanisms. Given the significance of P-TEFb in HIV transcription elongation and the current lack of available molecules able to mediate its release from the inhibitory 7SK snRNP complex, GTX may be a promising candidate not only in the context of HIV-1 latency reversal but also in other diseases in which P-TEFb plays a prominent regulatory role (51). Our modeling exercises provide insight toward a mechanism where GTX competes with 7SK RNA for the hydrophobic pocket of LARP7, causing destabilization and disassembly of the 7SK snRNP complex, leading to the release of P-TEFb and subsequent degradation of the 7SK RNA scaffold as observed in our experiments (Figs. 4 and 5 and figs. S9 and S10). LARP7 has been shown to be a critical scaffold required for the stability of the 7SK snRNP complex; consistent with our observations, RNA interference-mediated depletion of LARP7 resulted in elevated levels of free P-TEFb and increased Tat-mediated HIV-1 expression, concomitant with degradation of 7SK RNA (41).

Thus far, GTX has been regarded as a toxin and a virulence factor of *Aspergillus* spp. fungi, shown to function as an immunosuppressant that inhibits phagocytosis, blocks NF- $\kappa$ B signaling and cytokine production, and induces reactive oxygen species formation (22, 24, 25, 52–55). These properties are ascribed to the molecule's unusual disulfide bridge, which may mediate the activity of GTX by cycling between a reduced and oxidized state (25). However, it is important to note that all of the above-mentioned effects of GTX were observed at high concentrations in the 100 nM to 5  $\mu$ M range, concentrations at which we also observed significant toxicity and cell death (Fig. 2 and figs. S3, S4, and S6). Serum concentrations of GTX reported in patients with aspergillosis are found to be between 200 nM and 2.4  $\mu$ M (56), more than 10 to 100 orders of magnitude higher than concentrations that we have shown to effectively reverse HIV-1 latency in primary T cells and cells of cART-suppressed HIV-1-infected participants (Figs. 3 and 4). Together, these observations suggest that lower GTX concentrations in the 20 nM range, at which effective HIV-1 latency reversal is observed in primary CD4<sup>+</sup> T cells without associated toxicity, global T cell activation, or interference with capacity for CD8<sup>+</sup> T cells activation, will be physiologically achievable in a therapeutic context. Our data therefore support the potential of GTX for inclusion in combination latency reversal therapeutic approaches in a safe concentration range. Last, our data underlie the power of coupling a screen of fungal extrolites, which comprise a largely unexplored plethora of bioactive chemical entities, with bioassays and state-of-the-art fractionation and MS/NMR as a strategy to identify and characterize novel compounds with therapeutic potential.

## MATERIALS AND METHODS

### Cells and culture conditions

Jurkat latent (J-Lat) cell lines A2 and 11.1 were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100  $\mu$ g/ml) at 37°C in a humidified 95% air–5% CO<sub>2</sub> incubator. Primary CD4<sup>+</sup> T cells were cultured in RPMI 1640 media supplemented with 7% FBS and penicillin-streptomycin (100  $\mu$ g/ml) at 37°C in a humidified 95% air–5% CO<sub>2</sub> incubator.

### Preparation of fungal supernatants

We screened 115 species of filamentous fungi (table S1 and fig. S1A). The species belonged to 28 orders (43 families) of the fungal kingdom.



The majority were of ascomycetous affinity including ascomycetous yeasts (Saccharomycetales: 4 species), 12 were of basidiomycetous affinity including 3 basidiomycetous yeasts (Trichosporonales and Tremellales) and 3 species of lower fungi (Mucorales) (57). Rationale for choice was the expected production of a wide array of metabolites, which are known to be more pronounced in fungi living in habitats with environmental stress or microbial competition. Particularly versatile nutrient scavengers in Eurotiales and Hypocreales are established metabolite and toxin producers. Onygenales are cellulose and keratin degraders and contain a large cluster of mammal pathogens with alternating environmental life cycles. Members of Capnodiales are saprobes in environments with stressful microclimates such as rock, glass, and indoor. Identity of all strains was verified by recombinant DNA internal transcribed spacer and partial nuLSU (D1/D2 of large subunit) sequencing. Strains were derived from lyophilized storage in the reference collection of the Centraalbureau voor Schimmelcultures (housed at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands). After opening, contents of vials were taken up in 1 ml of malt peptone and distributed on Malt Extract Agar (MEA; Oxoid) culture plates. Strains were maintained on MEA slants and subcultured regularly until use. Mycelial fragments of 1-week-old colonies grown on MEA at 25°C were used as inocula for 500-ml flasks containing 150 ml of RPMI 1640 (with glutamine), shaken at 180 rpm and incubated at 25°C for 7 days, with one negative control. Flasks were checked daily until biomass reached one-third to one-fourth of the volume and then harvested by centrifugation at 14,000g and filtered using 0.2- $\mu$ m metal filters. Supernatants were transferred to Falcon tubes and used for latency screens. One strain per genus was used in a first round; additional isolates, some of which were close relatives and others located at larger phylogenetic distance, were tested in case of positive response. Positive tests of strain *A. fumigatus* CBS 113.26 was thus followed by *A. fumigatus* CBS 154.89, CBS 117884, and CBS 100074, *Aspergillus lentulus* CBS 117884, and *A. flavus* CBS 625.66.

#### Fractionation and MS characterization of active components

Complex fungal supernatants (3.3 ml) were twice extracted with ethylacetate (3 ml) in glass tubes (5-min vortex) and centrifuged at 3000 rpm. Water phase was discarded and organic extract dried in a vacuum concentrator. Lipids were removed by consequent hexane/water extraction [50/50 (v/v), 4-ml total volume], and aqueous phase was collected and dried. The dried material was reconstituted in 50% methanol (MeOH) and such a solution loaded onto HLB, MCX (Mixed-mode, strong Cation-eXchange), and MAX (Mixed-mode, strong Anion-eXchange) cartridges (Fig. 2, A and B) obtained from Waters Corporation (Prague, Czech Republic). The adsorbed compounds were desalted and stepwise eluted with increasing (5, 45, and 95%) organic solvent (MeOH) gradient (1% HCOOH), providing A, B, and C sample variants, respectively (Fig. 2B). HLB was based on *N*-vinylpyrrolidone–divinylbenzene copolymer and provided 5 fractions. MCX was a cation-exchange sorbent that represented the HLB material modified with SO<sub>3</sub>H<sup>-</sup> groups (6 fractions). The best performance was provided by anion-exchange MAX cartridge, providing 7 fractions. After each round of fractionation, all samples were tested in J-Lat/S-Lat latency models, followed by quantitation GFP/luciferase expression and identification of fraction-retaining activating component. As expected, originally less active fractions have become positive, as the active components became populated during the enrichment process.

The same phenomenon was observed when working with less or more active *Aspergillus* strains (fig. S2B). The most active 7B/7C fractions were further fractionated on HLB cartridge (11 samples) and components dereplicated by CycloBranch software (20). Compound matching against the annotated database of *Aspergillus* secondary metabolites has revealed a set of candidate compounds further selected for latency reversal testing. GTX was present in active fractions (Fig. 2B and fig. S2B). Detailed examination of commercial (GTX, Cayman and ApexBio) and natural GTXs was performed by high-performance liquid chromatography and Fourier transform ion cyclotron resonance (FTICR) MS. A solarix XR 12T FTICR instrument (Bruker Daltonics, Billerica, USA) was operated in a positive ion mode with electrospray ionization. Separation of GTX components was performed on an Acquity UPLC HSS T3 analytical column (Waters, Prague, Czech Republic) with 1.0 mm by 150 mm dimensions and 1.8- $\mu$ m particle size. The analysis was carried out at 30°C and a 30  $\mu$ l/min flow rate with the following A/B gradient: 0 min, 30% B; 30 min, 95% B; 40 min, 95% B; 50 min, 30% B; 60 min, 30% B. The gradient components A and B were represented by 0.1% formic acid in water or acetonitrile (ACN), respectively. One-minute time windows around S2 and S3/S4 GTXs were used for fraction isolation. The preparative chromatography was performed both with commercial GTX and with *A. fumigatus* 100074 strain.

#### Flow cytometry and annexin V staining

GFP expression of cell lines J-Lat A2 and 11.1 and phenotype of spin-infected primary CD4<sup>+</sup> T cells at the moment of reactivation were analyzed by fluorescence-activated cell sorting (FACS) as described previously (27). For annexin V staining, 10<sup>6</sup> cells were washed with phosphate-buffered saline (PBS) supplemented with 3% FBS and 2.5 mM CaCl<sub>2</sub> and stained with annexin V-PE (phycoerythrin) (Becton and Dickinson) for 20 min at 4°C in the dark in the presence of 2.5 mM CaCl<sub>2</sub>. Stained cells were washed with PBS/FBS/CaCl<sub>2</sub>, fixed with 1% formaldehyde, and analyzed by FACS.

#### Primary CD4<sup>+</sup> T cell isolation and infection

Primary CD4<sup>+</sup> T cells were isolated from buffy coats from healthy donors by Ficoll gradient (GE Healthcare) followed by negative selection with RosetteSep Human CD4<sup>+</sup> T Cell Enrichment Cocktail (STEMCELL Technologies). Isolated cells were left overnight for recovery and used for spin infection according to Lassen and Greene method as described previously (27, 58). Briefly, CD4<sup>+</sup> T cells were infected with the pNL4.3.Luc.R-E- virus by spinoculation (120 min at 1200g) and cultured for 3 days in RPMI 1640, 10% FBS, and penicillin-streptomycin (100  $\mu$ g/ml) in the presence of saquinavir mesylate (5  $\mu$ M). Three days after infection, cells were treated as indicated in the presence of raltegravir (30  $\mu$ M). Cells were harvested 24 hours after treatment, and luciferase activity was measured using the Luciferase Assay System (Promega, Leiden, The Netherlands). Infections were performed using pseudotyped particles obtained by cotransfecting HXB2-Env with pNL4.3.Luc.R-E- plasmid into human embryonic kidney 293T cells using polyethylenimine 48 and 72 hours after transfection, and supernatants containing pseudotyped virus were collected, filtered with a 0.45- $\mu$ m filter, and stored at -80°C. Molecular clones pNL4.3.Luc.R-E- and HIV-1 HXB2-Env and antiretroviral drugs saquinavir mesylate and raltegravir were provided by the Centre for AIDS Reagents, National Institute for Biological Standards and Control. HIV-1 molecular

clone pNL4.3.Luc.R-E- and HIV-1 HXB2-Env expression vectors were donated by N. Landau and by K. Page and D. Littman, respectively.

#### HIV-1 latency reversal in primary CD4<sup>+</sup> T cells isolated from aviremic patients

Primary CD4<sup>+</sup> T cells were isolated as described previously (27). Three million CD4<sup>+</sup> T cells were plated in triplicate at a cell density of 10<sup>6</sup>/ml and treated as indicated. After 24 hours, cells were lysed, and total RNA was isolated as described below. Written informed consent was obtained from all patients involved in the study. The study was conducted in accordance with ethical principles of the Declaration of Helsinki. The study protocol and any amendment were approved by The Netherlands Medical Ethics Committee (MEC-2012-583).

#### Total RNA isolation and quantitative reverse transcription polymerase chain reaction

Cells were lysed in TRI reagent, and RNA was isolated with the Total RNA Zol-Out kit (A&A Biotechnology); complementary DNA (cDNA) synthesis and quantitative polymerase chain reaction (qPCR) was performed as described previously (27). Gene expression was calculated using the 2<sup>-ΔΔC<sub>T</sub></sup> method (59), and expression of glyceraldehyde phosphate dehydrogenase (GAPDH) was used for normalization. Absolute quantification of cell-associated *pol* RNA was performed as described previously (27). Briefly, qPCR was performed in a final volume of 25 μl using 4 μl of cDNA, 2.5 μl of 10× PCR buffer (Life Technologies), 1.75 μl of 50 mM MgCl<sub>2</sub> (Life Technologies), 1 μl of 10 mM deoxynucleotide triphosphates (Life Technologies), 0.125 μl of 100 μM Pol forward primer (HXB2 genome 4901 → 4924), 0.125 μl of 100 μM Pol reverse primer (HXB2 genome 5060 → 5040), 0.075 μl of 50 μM of Pol probe, and 0.2 μl of Platinum Taq polymerase (Life Technologies). The lower limit of detection of this method was of 20 copies of HIV-1 RNA in 1 μg of total RNA. The absolute number of *pol* copies in PCR was calculated using standard curves ranging from 7 to 480 copies of a plasmid containing the full-length HIV-1 genome (pNL4.3.Luc.R-E-). The amount of HIV-1 cell-associated RNA was expressed as the number of copies per microgram of input RNA in reverse transcription. Preparations of cell-associated RNA were tested for potential contamination with HIV-1 DNA and/or host DNA by performing the PCR amplification in the presence and absence of reverse transcriptase.

Primers used for reverse transcription (RT)-qPCR were as follows: *pol* forward, 5 GGTTTATTACAGGGACAGCAGAGA3 and *pol* reverse, 5 ACCTGCCATCTGTTTTCCATA3; *pol* probe, [6FAM] AAA ATT CGG TTA AGG CCA GGG GGA AAG AA[BHQ1]; tumor necrosis factor-α (TNFα) forward, 5 TAGGCTGTCCCATGTAGCC3 and TNFα reverse, 5 CAGAGCTCAGCAATGAGTG3; interleukin-2 (IL-2) forward, 5 ACCTCAACTCTGCCACAAT3 and IL-2 reverse, 5 GCACTTCCTCCAGAGGTTTG3; IFNγ forward, 5 ATAATGCAGACGCCAAATTGTCTCC3 and IFNγ reverse, 5 ATGCTTCCTTGATGGTCTCCAC3; CD25 forward, 5 ATCAGTGGTCCAGGGATAC3 and CD25 reverse, 5 GACGAGGAGGAAGTCTCAC3; BAK1 (Brassinosteroid Insensitive 1-associated receptor kinase 1) forward, 5 GGTTCCTCCGACGTACGTTTTT3 and BAK1 reverse, 5 GCAGAGGTAAGGTGACCATCTC3; BAX (BCL-2-associated X protein) forward, 5 CCCGAGAGGTCTTTTCCGAG3 and BAX reverse, 5 CCAGCCCATGATGTTTCTGAT3; BCL2 (B-Cell lymphoma 2 protein) forward, 5 GGTGGGTCATGTGTGTGG3 and BCL2 reverse,

5 CGGTTCAGGTAAGTACTCAGTCAATCC3; CASP-3 (Caspase-3 protein) forward, 5 CATGGAAGCGAATCAATGGACT3 and CASP-3 reverse, 5 CTGTACCAGACCGAGATGTCA3; AKT1 (RAC-alpha serine/threonine-protein kinase) forward, 5 AGC-GACGTGGCTATTGTGAAG3 and AKT1 reverse, 5 GCCAT-CATTCTTGAGGAGGAAGT3; NRF2 (NFE2-related factor 2 protein) forward, 5 TTCCCGTTCACATCCGAGAG3 and NRF2 reverse, 5 TCCTGTTGCATACCGTCTAAATC3; FOXO3a forward, 5 CGGACAAACGGCTCACTCT3 and FOXO3a reverse, 5 GGACCCGCATGAATCGACTAT3; KEAP1 (Kelch-like ECH-associated protein 1) forward, 5 GCAATGAACACCATCCGAAGC3 and KEAP1 reverse, 5 ACCATCATAGCCTCCAAGGAC3; CYC1 (Cyclin T 1 protein) forward, 5 GGCCTGGACCCAGATAAAG3 and CYC1 reverse, 5 CTGTGTGAAGGACTGAATCAT3; CDK9 forward, 5 ATGGCAAAGCAGTACGACTCG3 and CDK9 reverse, 5 GCAAGGCTGTAATGGGGAAC3; GAPDH forward, 5 CAAGAAGGTGGTGAAGCAG3 and GAPDH reverse, 5 GC-CAAATTCGTTGTCAATACC3; and B2M forward, 5 ATGAG-TATGCCTGCCGTGTG3 and B2M reverse, 5 CCAATGCGG-CATCTTCAAC3.

#### Glycerol gradient sedimentation

Glycerol gradients were prepared as described previously (50). Briefly, around 40 × 10<sup>6</sup> primary CD4<sup>+</sup> T cells isolated from healthy donors were either untreated or treated with GTX (20 nM) for 4 hours for RNase A treatment. Cells were lysed for 30 min in buffer A supplemented either with RNasin (100 U/ml; Promega) for untreated and GTX conditions or with RNase A (100 μg/ml). RNase-supplemented lysates were incubated for 10 min at room temperature to ensure efficient degradation of RNA. Lysates were fractionated by centrifugation in a SW41 Ti rotor at 38,000 rpm for 20 hours. Fractions (1 ml) were collected in 2-ml tubes and subjected to trichloroacetic acid (TCA) precipitation of proteins as described previously (60). Briefly, to each 1 ml of fraction, 110 μl of ice-cold 100% TCA was added and incubated on ice for 10 min. Then, 500 μl of ice-cold 10% TCA was added to each sample and incubated on ice for 20 min, followed by centrifugation at 20,000g for 30 min. Supernatants were carefully removed, and precipitates were gently washed with 500 μl of ice-cold acetone followed by centrifugation at 20,000g for 10 min. Supernatants were gently removed and dried at room temperature for about 10 min. Protein precipitates were then resuspended in 50 μl of Laemmli loading buffer and subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separation and detection of CDK9 (C-20, sc-484, Santa Cruz Biotechnology).

#### RNA Pol II and CDK9 T-loop phosphorylation

For RNA Pol II phosphorylation, 10 million primary CD4<sup>+</sup> T cells were either untreated or treated with GTX, OTX-015 (1 μM), FPD (500 nM), PMA (10 nM), or αCD3/CD28 beads (cell:bead ratio of 1:1) for 6 hours. For CDK9 phosphorylation, 5 million primary CD4<sup>+</sup> T cells were either untreated or treated with GTX (20 and 40 nM) or PMA (10 nM) for 6 hours. Four million cells were lysed for 30 min on ice with immunoprecipitation buffer [25 mM Hepes (pH 7.9), 150 mM KCl, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 5% glycerol, 1% NP-40, 0.5 mM dithiothreitol (DTT), PhosSTOP phosphatase inhibitor (Sigma-Aldrich), and a Complete protease inhibitor cocktail (Sigma-Aldrich)] and subjected to Western blot analysis using the following antibodies: total RNA Pol II (N-20, sc-899, Santa Cruz Biotechnology), phospho-Ser<sup>2</sup> RNA Pol II (H5, ab-24758, Abcam), CDK9 (C-20, sc-484,

Santa Cruz Biotechnology) (Fig. 4C and fig. S9B), total CDK9 (D7, sc-13130, Santa Cruz Biotechnology) (fig. S9E), cyclinT1 (H-245, sc-10750, Santa Cruz Biotechnology),  $\alpha$ -tubulin (ab6160, Abcam), and phospho-CDK9 Thr<sup>186</sup> (#2549, Cell Signaling Technology).

### 7SK snRNP complex immunoprecipitation and 7SK RNA / CDK9 release assay

Fifty million Jurkat cells were lysed in 5 ml of immunoprecipitation buffer [1% NP-40, 25 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 5% glycerol, and EDTA-free protease inhibitor cocktail (1 U/ml; Roche)] for 30 min on ice and centrifuged for 10 min at 14,000 rpm. Protein G Sepharose beads (200  $\mu$ l) were washed two times with lysis buffer. Lysates were incubated with beads and 2  $\mu$ g of antibody against human LARP7 (A303-723A, Bethyl Laboratories) at 4°C overnight. Next day, the beads were washed two times with lysis buffer, resuspended in 400  $\mu$ l of lysis buffer containing 1 mM DTT, and divided in four reactions of 100  $\mu$ l each. The presence of DTT is critical to the activity of GTX in agreement with its lability and known unusual disulfide bridge. Aliquots were left untreated or treated with GTX for 1 hour or with RNase A for 15 min at room temperature. Next, supernatant was discarded, and beads were washed twice with lysis buffer. Beads were resuspended in 100  $\mu$ l of Laemmli loading buffer and subjected to 12% SDS-PAGE separation and detection of CDK9 (sc-484, Santa Cruz Biotechnology) and LARP7 by Western blot. Ten microliters of beads suspension before and after treatment and 10  $\mu$ l of supernatant after treatment were taken and resuspended in TRI reagent (Sigma) for subsequent RNA isolation (Total RNA Zol-Out kit, A&A Biotechnology). cDNA was synthesized using 10  $\mu$ l of RNA solution as described above and diluted 560 times for quantitative RT-PCR. 7SK RNA was amplified using specific primers described elsewhere (61). qPCR was performed as described above. Values were normalized to the input RNA (post-treatment bead suspension).

### Histone acetylation

Ten million primary CD4<sup>+</sup> T cells were treated with GTX concentration gradient, SAHA, or left untreated for 4 hours and then washed twice in PBS. Cells were lysed for 10 min at 4°C in TBE (Tris-borate-EDTA) buffer [PBS, 0.5% Triton X-100 (v/v), 2 mM phenylmethylsulfonyl fluoride, and 0.02% (w/v) Na<sub>3</sub>N] at a density of 10<sup>7</sup> cells per 1 ml of the buffer. Samples were centrifuged at 425g for 10 min at 4°C. Supernatants were discarded, and cell pellets were washed in half the volume of TBE buffer used for lysis and centrifuged as before. Supernatants were discarded, and pellets were resuspended in 0.2 N HCl at a density of 4  $\times$  10<sup>7</sup> cells/ml. Histones were extracted overnight at 4°C and then centrifuged at 425g for 10 min at 4°C. Supernatants were collected, protein concentration was determined by Bradford assay, and samples were subjected to SDS-PAGE Western blot. The following antibodies were used in Western blot analysis: anti-acetyl-histone H4 (06-598, Millipore) and anti-histone H2B (ab52484, Abcam).

### RNA sequencing and data analysis

Ten million primary CD4<sup>+</sup> T cells were stimulated with 20 nM GTX or left unstimulated for 4 hours. Experiment was performed in duplicate on cells isolated from two buffy coats from healthy donors as described above. RNA was isolated as described above. cDNA libraries were generated using the Illumina TruSeq Stranded mRNA Library Prep kit (Illumina). The resulting DNA libraries were se-

quenced according to the Illumina TruSeq Rapid v2 protocol on an Illumina HiSeq 2500 sequencer. Reads were generated of 50 base pairs in length. Reads were mapped against the GRCh38 human reference using HiSat2 (version 2.0.4). We calculated gene expression values using htseq-count (version 0.6.1) using Ensembl transcript annotation. Heatmaps were generated using MORPHEUS (<https://software.broadinstitute.org/morpheus/index.html>).

### Toxicity assay

PBMCs were isolated by density gradient centrifugation (Ficoll-Hypaque, GE Healthcare Life Sciences) from buffy coats from healthy donors (Sanquin, Amsterdam) and either used immediately or frozen in freezing media [90% FBS/10% dimethyl sulfoxide (DMSO)] and stored short term at -80°C. For cytotoxicity testing, cells were cultivated in culture media RPMI 1640 (Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin-sulfate (100 g/ml) at a concentration of 1  $\times$  10<sup>6</sup> cells/ml in 24-well plates (Thermo Scientific) that were either uncoated (unstimulated cells) or coated with anti-human CD3 (1  $\mu$ g/ml; clone UCHT1, no azide/low endotoxin, BD Biosciences) and anti-CD28 (10  $\mu$ g/ml; clone CD28.2, no azide/low endotoxin, BD Biosciences) monoclonal antibodies (stimulated cells). PMA/ionomycin was added at 50 ng/ml and 1  $\mu$ g/ml, respectively, and cells were exposed for 16 hours. The following LRAs were added at the concentrations indicated in figures: GTX (ApexBio), SAHA-Vorinostat (Selleck Chemicals), CAPE (MP Biomedicals), and RMD (Sigma-Aldrich). LRAs at indicated concentrations were added to the cultures, and cells were continuously exposed for 72 hours. Since GTX was reconstituted in ACN and all other LRAs in DMSO, both solvents were added to the DMSO/ACN control culture (ACN, 1:10000; DMSO, 1:2500) to control for the effect that these solvents may have on cell viability.

### Flow cytometry for cytotoxicity assay

To examine the effects the LRAs have on immune cell subpopulations, cell viability, activation, and proliferation were analyzed by flow cytometry. To determine the cytotoxic effect of the LRAs on cells, AnnexinV staining was used to define apoptotic and dead cells. Surface antigens were detected by incubating 0.8 to 1.0  $\times$  10<sup>6</sup> cells with predetermined optimal concentrations of monoclonal antibody mixes in FACS wash [FW; Hank's Balanced Salt Solution (Life Technologies), 3% FBS (Life Technologies), 0.02% Na<sub>3</sub>N, and 2.5 mM CaCl<sub>2</sub>] for 20 min at 4°C, washed one time with FW, and fixed with 1% paraformaldehyde. To determine proliferation, cells were stained with 0.07  $\mu$ M CellTrace Far Red Cell Proliferation dye according to the manufacturer's protocol (Thermo Fisher Scientific) before cultivating for 72 hours with either unstimulated or stimulated conditions in the presence of LRA. Upon cell division, the proliferation dye intensity decreases in daughter cells, and this was measured by flow cytometry. The following directly conjugated monoclonal anti-human antibodies were used to analyze CD8<sup>+</sup> T cells (CD3<sup>+</sup>CD8<sup>+</sup>), CD4<sup>+</sup> T cells (CD3<sup>+</sup>CD4<sup>+</sup>), B cells (CD3<sup>+</sup>CD19<sup>+</sup>), monocytes (CD14<sup>+</sup>), and NK cells (CD3<sup>+</sup>CD56<sup>+</sup>): CD3-BV421 (clone UCHT1), CD4-BV650 (SK3), CD8-BV786 (RPA-T8), CD14-PE-Cy7 (61D3, eBioscience), CD19-PerCP-Cy5.5 (H1B19, eBioscience), CD56-PE-Cy5.5 (CMSSB, eBioscience), annexin V-PE, and CD25<sup>+</sup> Super Bright 600 (BC96, eBioscience). All antibodies were purchased from BD Biosciences unless otherwise indicated. Between 2  $\times$  10<sup>5</sup> and 4  $\times$  10<sup>5</sup> events were collected per sample within 24 hours after staining on a LSRFortessa (BD Biosciences, 4 lasers, 18 parameters) and analyzed using FlowJo

software (version 9.7.4, Tree Star). Data are represented as frequency within a defined population. Treatment-specific cell cytotoxicity was calculated using the following formula:  $[(\% \text{ drug-induced cell death} - \% \text{ cell death in DMSO/ACN only}) / (100 - \% \text{ cell death in DMSO/ACN only})] * 100$ .

### Molecular docking

Molecular docking was used to predict the most likely binding mode of GTX to LARP7 CTD. The crystal structure of human LARP7 CTD in complex with 7SK RNA SL4 [Protein Data Bank (PDB) ID code 6D12] was optimized using PDB-REDO (62) and used as a template to define the receptor for the docking simulation. The resulting pdb file was manually adapted for input into the docking procedure by elimination of protein chain B and RNA domain C and replacement of selenium atoms (present as selenomethionin, incorporated for phasing purposes) (63) by sulfur. GTX ligand structure was built and energy minimized using the program Chimera (64). Molecular docking of GTX to LARP7 CTD was performed using the Achilles Blind Docking server (<https://bio-hpc.ucam.edu/achilles/>) and Chimera's AutoDock Vina function (65). The resulting solutions were ranked on the basis of the highest binding affinity (or lowest binding energy). Figures were created using PyMol software (66).

### Quantification and statistical analysis

#### Western blot quantification

Differential band density was quantified by ImageQuant TL software using area and profile-based toolbox. For glycerol gradient Western blot quantification, an area frame was defined for all bands (total CDK9 protein content in all fractions), and complex-bound CDK9 bands (heavy fractions) or free CDK9 bands (light fractions) were defined. The three area frames were measured for total density after background subtraction (local average). Relative complex-bound CDK9 or free CDK9 percentage was calculated regarding the untreated control (total CDK9 abundance). For RNAPII Ser<sup>2</sup> phosphorylation, CDK9 T-loop phosphorylation, or immunoprecipitation Western blot quantification, an area for each respective band and loading controls were defined, and total density was measured after background subtraction (local average). LARP7 abundance was first normalized to total CDK9 abundance for each lane, and relative abundance was calculated regarding untreated control. RNAPII Ser<sup>2</sup> abundance was first normalized to tubulin abundance for each lane, and relative abundance was calculated with respect to untreated control. Thr<sup>186</sup> P-CDK9 and cyclin T1 band density was normalized to total CDK9 abundance, and relative abundance was calculated with respect to untreated control.

#### Statistical significance

Statistical significance was calculated as indicated in the figure legends. Analyses were performed using Prism version 7.03 (GraphPad software).

#### Data and code availability

Sequencing data that support the findings of this study were deposited in Gene Expression Omnibus and are available under the accession code GSE135184.

### SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/33/eaba6617/DC1>

[View/request a protocol for this paper from Bio-protocol.](#)

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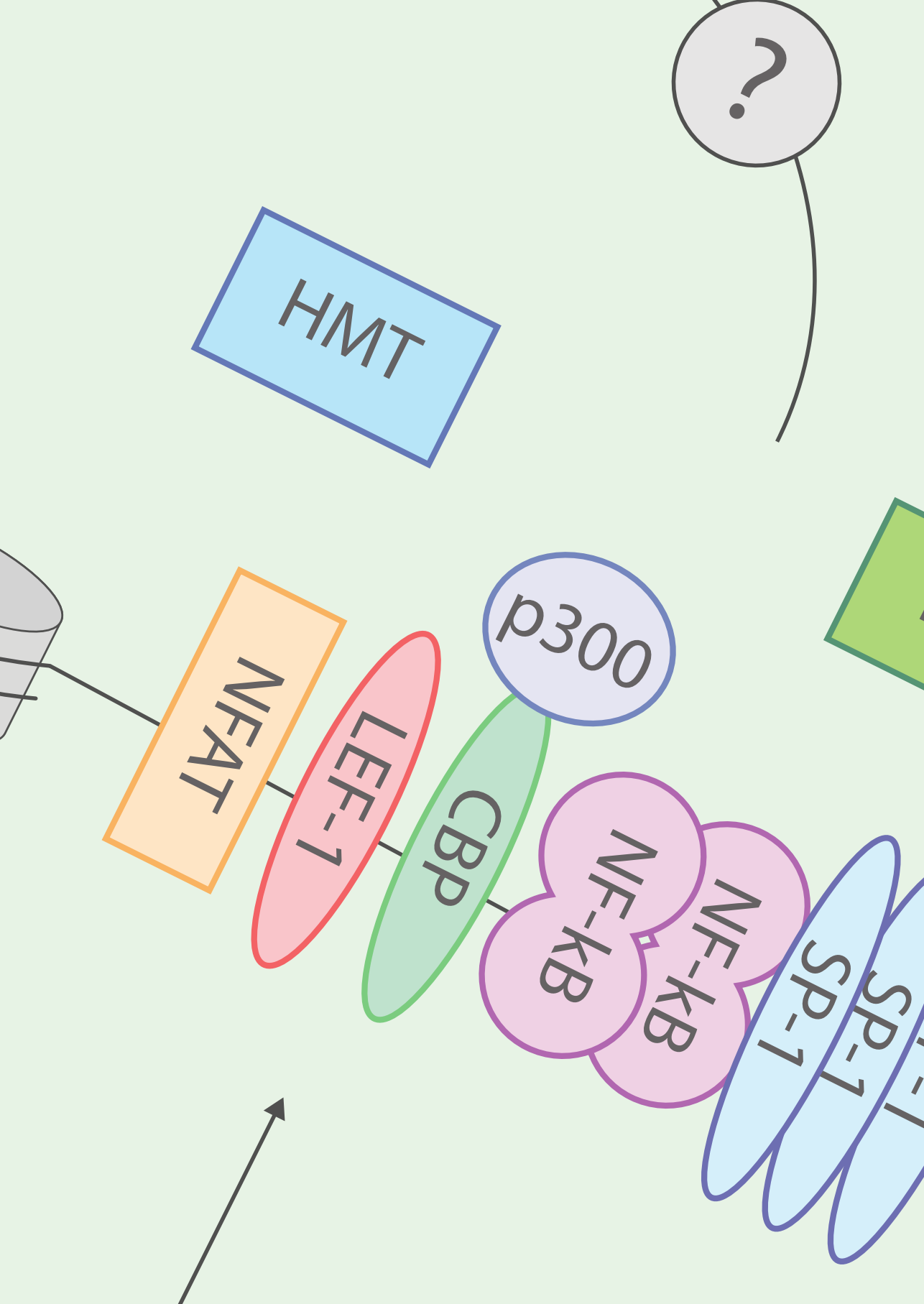
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**Supplementary Materials**

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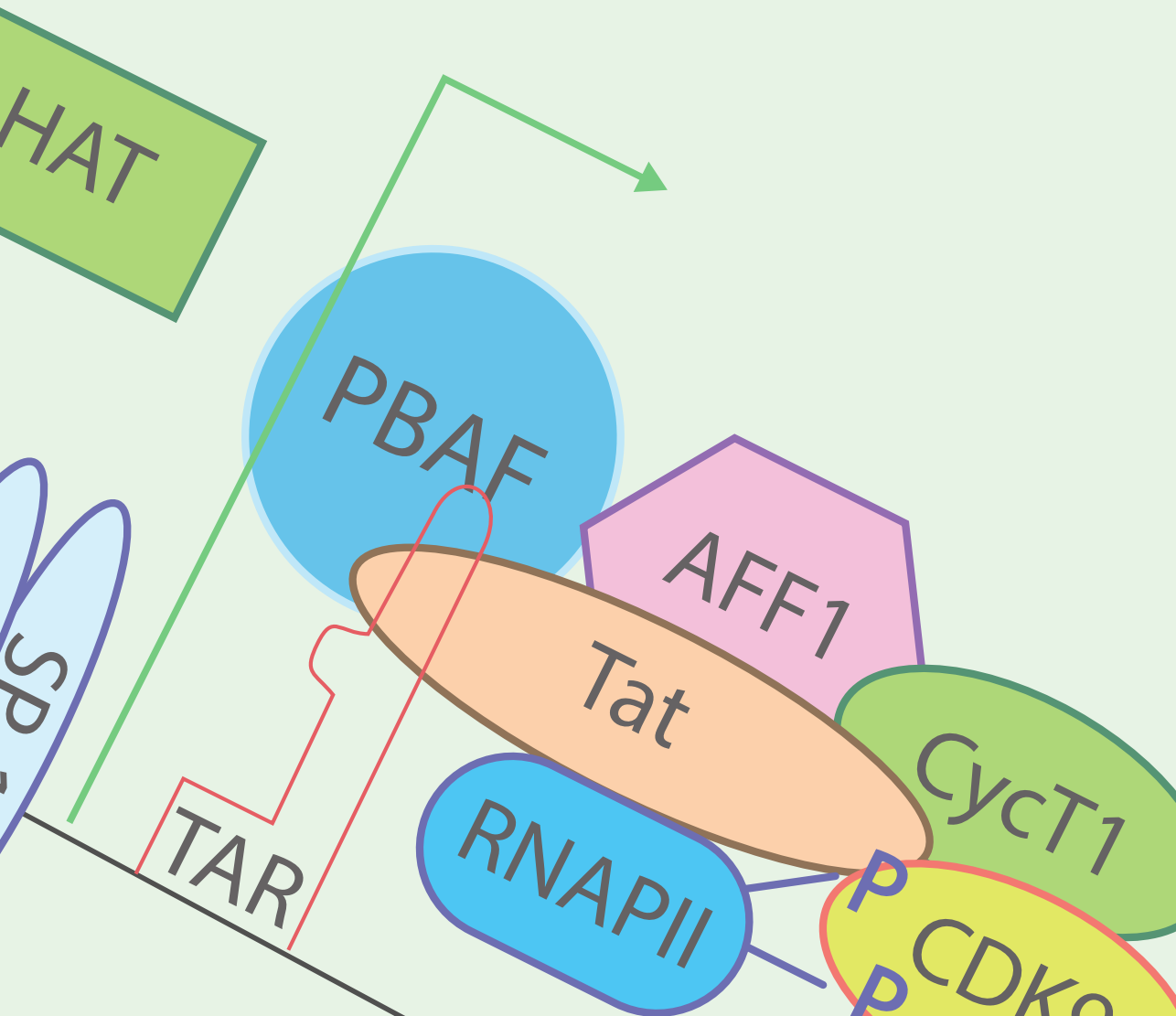
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# Summary & General Discussion



## Summary and general discussion

The earliest well-described case of an HIV infected individual dates back to 1959(1). The HIV/AIDS pandemic became public in 1981. Since the start of the pandemic 75 million people have been infected and about 32 million people have died(2). In 1987 the first anti-retroviral drug to treat HIV infection, azidothymidine (AZT), was introduced. In the early nineties combinatorial antiretroviral therapy (cART) was developed. Since then HIV infection has become a chronic disease for those who have access to treatment and can afford it. While cART does block viral replication, it does not lead to definitive cure. Once treatment is stopped, viral replication can resume, even after a long time of treatment. In addition to the danger of recurring infection, it is becoming apparent that long-term cART treatment has negative neurological and cardiovascular effects(3). The source of the persistent infection is a latent reservoir of infected cells. This reservoir consists of long-lived memory CD4+ T-cells that contain transcriptionally inactive, but replication competent HIV-1. The need for either a functional cure (in which patients control the infection) or a sterilizing cure (where the virus is eliminated entirely from the body) is clear. "Shock and kill" is a proposed strategy towards a definitive cure. It proposes the latent virus is "shocked" into active transcription (and virus production) and then "killed" by the immune system or viral cytopathic effects. The first step, of reactivating latent virus requires a specific and complete reactivation of the latent reservoir. Currently no such treatment exists. The focus of this thesis is to gain insight into the molecular mechanisms of latent HIV, to identify druggable targets using unbiased approaches and identify compounds that reactivate HIV-1 gene expression.

**Chapter 1** summarizes the considerable body of knowledge about the structure and function of both latent and active HIV. Our understanding of the molecular regulation of HIV starts with the HIV-1 replication cycle. After HIV RNA has entered the cell, it is reverse transcribed and the pre-integration complex (PIC), consisting of a double stranded DNA copy of HIV bound by host factors is assembled. The PIC enters the nucleus, where HIV stably integrates into the host genome(4). Transcription of HIV starts at its 5'LTR. It contains numerous transcription factor binding sites. Transcription is initiated which gives rise to the viral RNA transcript TAR and viral protein Tat. TAR binds to the Transcription Start Site (TSS) and recruits Tat. However, NELF (Negative elongation factor) and DISF (DRB sensitivity-inducing factor) cause pausing of RNA Polymerase II(5). To overcome this blockade the carboxy terminal domain (CTD) of RNA Polymerase II (RNAPII) needs to be phosphorylated by P-TEFb (Positive Elongation Factor b)(6). P-TEFb consists of cyclin dependent kinase 9 (CDK9) and cyclin T1. P-TEFb can be inhibited by sequestration in the 7SK snRNP complex. When P-TEFb is recruited to the HIV TSS by Tat, subsequently the super elongation complex (SEC) can be

formed further enhancing RNAPII transcription elongation(7, 8). In the latent state, two nucleosomes are positioned on the LTR, this positioning is irrespective of integration site and actively maintained by the BAF-complex(9). Disrupting the BAF-complex leads to reactivation.

Our knowledge of the molecular mechanism of HIV latency has helped in the development of latency reversal agents (LRAs), compounds with potential to induce HIV-1 gene expression from a latent state. By inhibiting repressive host factors, inducing factors that promote transcription or creating a chromatin organization conducive to transcription, LRAs reactivate latent HIV. According to their effect on viral transcription, LRAs can currently be subdivided into chromatin regulators, transcription initiators and -elongators. Chromatin regulators affect post-translational modifications (PTMs) on histone tails or chromatin remodeling. Acetylation is a transcription promoting PTM and can be promoted by Histone Deacetylase inhibitors (HDACi). Examples of HDACis are Romidepsin, Panobinostat, Vorinostat, and Valproic acid. Other PMTs to target for latency reversal are histone methyltransferase (HMT) and DNA methyltransferases (DNMT). Chromatin is actively remodeled by ATP dependent remodelers. For example, the BAF complex actively maintains a repressive chromatin structure. Disrupting the BAF-complex leads to reactivation of latent HIV. Similarly, inhibition of Bromodomain and extra-terminal domain (BET) proteins BRD2 and BRD4 results in de-repression. The second category are drugs that recruit transcription factors to the LTR and promote transcription initiation. For example, NFκB can reactivate latent HIV independent of Tat. PKC agonists, TLR agonists and Maraviroc can activate NFκB but also cause T-cell activation, which is a serious side effect. And more recently, the use of SMAC mimetics that trigger the non-canonical NFκB pathway are very promising due to their dual effect of reactivation and clearance of latent cells. Because transcription initiation does not necessarily lead to transcription elongation, promoter-proximal pausing at the 5'LTR TSS needs to be overcome. By increasing freely available P-TEFb the CTD of RNA Polymerase II can become phosphorylated triggering transcription elongation. Several molecules like hexamethylene bisacetamide (HMBA), 5,6-Dichloro-1-β-d ribofuranosylbenzimidazole (DRB) and actinomycin D have previously been shown to increase freely available P-TEFb(10–12). Our discovery of Gliotoxin as an LRA is the first of its kind that in mechanistically targeting LARP7, disrupting the P-TEFb sequestering 7SK snRNP complex resulting in its release and activation of HIV-1 transcription elongation. Combinations of LRAs have been shown to synergize to reactivate latent HIV in a research setting. It is conceivable that a combination of LRAs will be administered as treatment in the future to eradicate the latent reservoir. Some LRAs have made it to clinical studies, but unfortunately none so far have shown a meaningful impact on the reservoir. Therefore we need to find more potent and effective LRAs. To improve the chance of success, this search should also include the identification of novel druggable targets.

To increase the number of druggable targets and increase our understanding of the molecular mechanisms of HIV latency we employed complementary, unbiased approaches to identify novel host factors and drugs involved in HIV latency. Previous research into HIV has followed candidate approaches, which use previous findings to build on and expand our knowledge of viral replication and interaction with the host environment. A candidate approach takes a known element, say the immune master regulator NF $\kappa$ B or HIV's promoter, the 5' LTR, and tries to expand on existing knowledge by, for example, identifying novel binding partners or key mutations that affect function. This at once provides an avenue of opportunity to explore but also limits the possible outcomes, because factors outside of the known mechanisms might not come to light. In contrast to candidate approaches, unbiased approaches make no assumptions about host mechanisms involved beforehand to start tweezing apart the mechanism of interest. Unbiased approaches do not look at a specific gene or compound but rather look for the underlying mechanism of a specific phenotype. So unbiased approaches don't exclude any candidate genes or compounds available to us beforehand, casting the net as wide as possible. As such, unbiased screens are hypothesis-generating and exploratory whereas candidate approaches are hypothesis-driven and investigative. In this thesis we employ independent but complementary unbiased approaches to elucidate the molecular mechanism of latent HIV infection by looking at 1) what makes the cellular environment permissive to HIV infection, 2) what host factors are involved in latency and 3) what compounds are potential novel LRAs. To understand what makes a cell permissive to HIV infection, we used previously published genome wide association study (GWAS) data to identify regulatory genomic regions for genes that affect conduciveness to infection. To identify novel host factors, we employed an unbiased two-color haploid screen. And to find new LRAs, we screened a compound library of bioactive molecules from diverse species in the fungal Kingdom. Each of our approaches starts out with a collection, whether it's SNPs, genes or compounds. Each item in the collection is screened to assess its effect on latency reversal in a biological assay that recapitulates the molecular mechanism governing latency. Thus each of our approaches are unbiased, hypothesis-generating and exploratory, leading to some surprising finds.

**Chapter 2** applies the power of genome-wide association studies (GWAS) to statistically link genetic variants in different individuals to a phenotype. However, because the collection of large cohorts can be challenging, common variants with weak effect size and rare variants do not meet the widely accepted stringent threshold for genome-wide significance ( $P < 5 \times 10^{-8}$ ). Additionally, the majority of the identified SNPs lack a clear function because they are often located in noncoding regions of the genome. Regulatory DNA elements are generally associated with chromatin marks. By combining this functional data of the

genome from Encyclopedia of DNA Elements (ENCODE) and the Human Epigenome Atlas with GWAS, the potential regulatory function of noncoding SNPs can be predicted, which reduces the list of candidate SNPs to study. By combining data from HIV-1 GWASs and T cell-derived epigenome data, we identify and functionally characterize the regulatory potential of noncoding HIV-1-associated intergenic variant rs4349147 on HIV infectivity in Chapter 2. Deletion of a region containing rs4349147 strongly reduced expression of IL-32, but also revealed a shift in which IL-32 isoforms were expressed. IL-32 has five major isoforms, A to E, that are encoded by multiple RNA splice variants (variants 1 to 9). Broadly speaking, IL-32b and IL-32g are pro-inflammatory whereas IL-32a is non-pro-inflammatory. Chromosome conformation capture reveals a chromatin loop between rs4349147 and IL-32 promoter. This interaction is allele dependent: rs4349147 -/A clones have reduced IL-32 expression, a lower IL-32a to non-a isoforms ratio and altered chromatin conformation compared to rs4349147 G/- cells. Exogenous addition of IL-32g, but not IL-32a, by recombinant protein supplementation or viral transduction improved HIV infectivity of Jurkat cells by inducing a proinflammatory T cell cytokine environment. We functionally show how HIV infectivity is dependent on the enhancer function of a non-coding DNA variant by mediating expression of IL-32 isoforms.

rs4349147 does not reach the gold standard significance of  $P < 5 \times 10^{-8}$  in HIV acquisition GWASs. HIV-1 GWASs are underpowered because the gathering of samples from large cohorts is difficult. As a consequence, rare variants and common variants with weak effect size fall below the threshold for genome-wide significance ( $P < 5 \times 10^{-8}$ ) and are missed. Using the approach shown in chapter 2, combining GWAS data with epigenomic data, it becomes possible to distinguish true biological signals from noise. This allows inclusion of weak SNPs and narrows down the list of candidate SNPs to screen.

Previous findings report contradictory or dual roles of IL-32 in inflammation. IL-32 was observed to be higher in peripheral blood mononuclear cells (PBMCs), gut, and lymph nodes at different stages of infection of HIV positive individuals, compared to healthy controls(13–15). IL-32 was thought to promote expression of immunosuppressive molecules, resulting in decreased immune activation and increased HIV replication(15, 16). Other findings point to an inhibitory role for IL-32 in HIV replication. Knockdown of IL-32 by small interfering RNA (siRNA) caused increased HIV replication in Jurkat, human embryonic kidney (HEK) 293T, latently infected U1 (macrophage cell line) and infected PBMCs(13). After knockdown of IL-32 the number of T<sub>H</sub>1 cells were decreased as well as levels of proinflammatory cytokines including IL-6 and TNF $\alpha$ (17). However these studies do not distinguish between IL-32 isoforms in their knockdown or detection by antibodies and PCR primers. The functionally distinct roles of the IL-32 isoforms are becoming clear(18). In comparison to other isoforms such as IL-32b or IL-32g, IL-32a is not as strong an inducer of a proinflammatory state in PBMCs (19, 20). Proinflammatory

isoforms of IL-32 (primarily b and g) correlated with decreased CD4 T-cell counts, increased viral load, lower CD4/CD8 ratio and levels of inflammatory markers (sCD14 and IL-6) in HIV infected slow progressors experiencing virological failure(16). The HIV replication promoting potential of IL-6 in macrophages and latently infected CD4+ T cells is well documented(21–23). Results from our cytokine array revealed increased levels of proinflammatory cytokines IL-8, IL-6, IL-10, TNF $\alpha$  (tumor necrosis factor- $\alpha$ ), and IFN-g (interferon-g) after overexpression of IL-32g, but not IL-32a. It is conceivable that rs4349147-G allele cells, the induction of IL-32g could boost levels of IL-6 and other proinflammatory cytokines, resulting in a proinflammatory environment, conducive to HIV replication. But the effects of this shift in isoform production is modest. Our results provide a unifying explanation of previous seemingly contradictory functions of IL-32 and its isoforms in infection susceptibility. Future studies will determine whether the proinflammatory effect of non-IL-32a isoforms also contributes to latency reversal.

**Chapter 3** shows the results of a gene knock out screen in a haploid myeloid human cell line in order to identify novel host factors in the maintenance of HIV latency. Because the screen identifies non-essential genes, their corresponding proteins are promising drug targets. To perform this screen, we adapted the haploid KBM7 cells by introducing a latent HIV infection using a minimal HIV with GFP reporter, all the while maintaining haploidy. Next, the cells were subjected to Gene-Trap (GT) mutagenesis. GT is a murine stem-cell virus, which contains a splice acceptor site, mCherry and a polyA terminator. When GT infects a cell, it preferably inserts in the sense orientation of expressed genes in the host genome(24–26). The splice acceptor site truncates the native transcript of the mutagenized gene, effectively knocking out the gene while producing a mCherry fluorescent protein. When a repressive, latency maintaining host factor is knocked-out, HIV would be reactivated. After GT mutagenesis a small percentage of cells become mCherry positive (GT infected) and GFP positive (reactivated HIV). We sorted double positive cells and used high throughput sequencing to identify the GT integration sites in the GT-re-activated population. This led to the identification of 69 candidate genes as potentially involved in maintenance of HIV-1 latency. We choose 15 candidates whose potential roles in HIV-1 latency we validated by shRNA knockdown in two Jurkat derived latently HIV-1 infected cell lines (J-lat), J-LatA2 and J-Lat11.1. Ten genes showed reactivation of HIV-1 in latently infected T cell lines after knockdown. We also performed a literature search for compounds to target our candidate genes. For three genes, ADK, GRIK5 and NF1, FDA-approved compounds were available. In both J-Lat cell lines and in ex-vivo infected CD4+ T-cells the ADK inhibitor 5-Iodotubercidin was toxic. The inhibitors of GRIK5 and NF1, Topiramate and Trametinib respectively, reactivated latent HIV while not negatively affecting viability in both A2 and 11.1 J-Lat cells.

Trametinib, although active in latently infected cell lines, was ineffective in primary cells, highlighting the importance of validating the function of putative compounds in more relevant primary CD4+ T cell models of HIV-1 latency. Finally, Topiramate showed significant latency reversal in primary cells without inducing significantly cytotoxicity, nor did it cause general T cell activation, a dangerous potential side effect candidate latency reversal agents should be checked for. The LRA potential of this compound should be further investigated in a clinical setting. Another candidate we found, CHD9, is a member of the chromodomain helicase DNA-binding protein family. Because of the ATP-dependent chromatin remodeler function of the candidate we decided to validate the candidate. Upon validation, we further characterize CHD9's presence on HIV's 5'LTR. ChIP results show association on the LTR, that is lost upon PMA treatment. In all, these results show that employing the haploid screen format based on myeloid leukemic KBM7 cells to identify novel host factors is a viable strategy.

Historically, RNAi has been the go-to method for loss-of-function screens. However, due to RNAi's high off-target effects, short-term and incomplete gene suppression, screens suffer from high false-positive rates(27). While we were performing our haploid screen, the CRISPR gene editing revolution was unleashed. CRISPR gene editing is the adapted guided nuclease complex from antiviral defense mechanism from bacteria, CRISPR-Cas9. It consists of a sgRNA that contains a fixed sequence to bind to the Cas9 protein, a PAM sequence and the host sequence that is meant to be targeted. The PAM sequence is a fixed nucleotide sequence needed for the CRISPR complex to recognize the targeted sequence. The nuclease activity of the complex causes double-strand breaks (DSBs) or single-strand nicks. The repair of DSBs and nicks by non-homologous end joining (NHEJ) and homology directed repair (HDR) results in mutations that cause altered function or knockout of the targeted gene. Because CRISPR-Cas9 is so specific, it's potential is tremendous. However, limitations of screening using CRISPR-Cas9 are emerging. Technical difficulties associated with CRISPR-Cas9 screens are sgRNA coverage of the genome and cell population. To screen the entire genome sgRNA need to be spread evenly over the genome. However due to the necessity of a PAM sequence for targeting, genes or regions depleted in PAM sequences are underrepresented in CRISPR screens. Additionally to turn up in a screen a gene needs to be hit in multiple cells, resulting in the need for large scale experiments with great numbers of cells<sup>28</sup>. To tweeze apart the molecular mechanisms involved in a phenomenon of interest, knockdown or knock-out screens are very effective to identify novel factors.

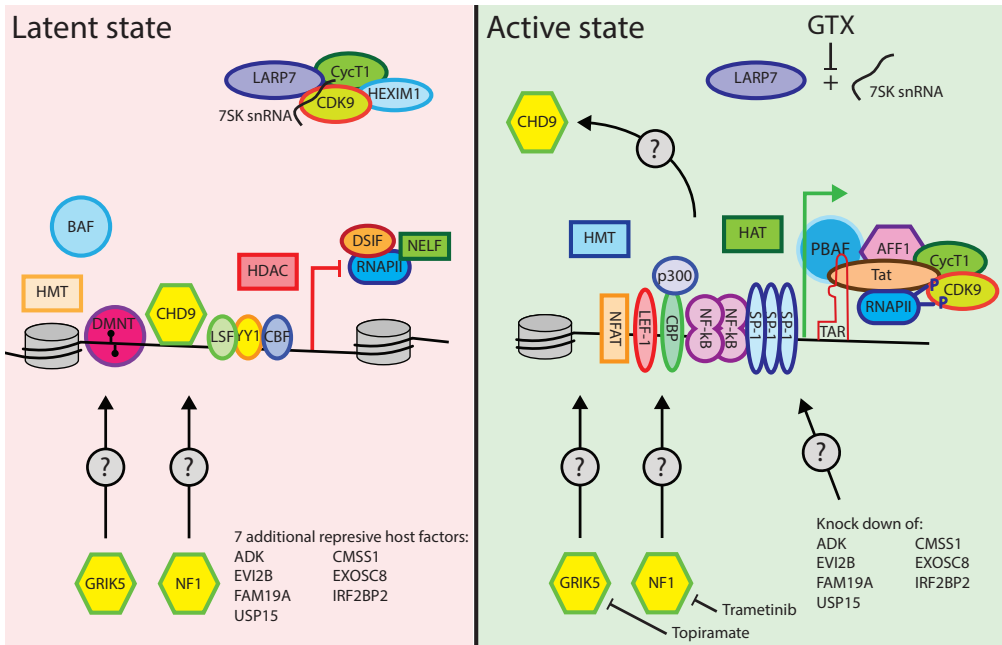
The myeloid leukemic origin of KBM7 cells are a cell type distinct from HIV's preferred T-cells, although they do share lineage with other cells that are conducive to HIV infection such as monocytes and macrophages. From the 15 candidate genes we selected for further characterization, 10 proved to be involved in maintaining latency in Jurkat derived cell lines of latency (J-Lat). Our

finding that two thirds of the tested candidates are validated suggests that the remaining 54 candidates contain more host factors. Of the candidates that appear to be “false positive” after testing in J-lat cells, it would be of interest to see how these host factors behave in latent models of monocytic or macrophagic origin. This would distinguish between true false positives in our screen and differences in cell lineage environment (myeloid vs lymphocytic). The forward genetic screen platform based on the haploid KBM7 cells is a straightforward system that allows relative easy adaptation to studying the phenomenon of interest, whether it’s viral entry (28, 29), toxicity(24, 30) or cell signaling(31, 32). Because KBM7 cells are near haploid (diploid for chromosome 8 and a small part of chromosome 10), the screen, using the GT-virus to randomly knock-out genes, surveys the whole genome except for the diploid chromosomes, although recently complete haploid lines have been developed(33). Our endeavor was hampered by the small percentage of cells that reverted after mutagenesis by the GT virus. We tried to overcome this by massive amounts of cell sorting to collect as many cells as possible. However, this means our screen is by no means exhaustive. Because we used an unbiased approach the host factors we identify the be involved in HIV latency sometimes do not immediately fit into our “canonical” understanding of the molecular mechanism of HIV latency. Some such as CHD9, an ATP-dependent chromatin remodeler, or IRF2BP, a transcription factor, intuitively makes sense as a novel host factor in latent HIV. In particular IRF2BP2 (Interferon regulatory factor 2 binding protein 2) has been studied in relation to lymphocytes as it was previously identified as a repressor of NFAT1(34). In mice, IRF2BP2 overexpression in CD4 T lymphocytes repressed the expression of IL-2 high affinity receptor  $\alpha$ -chain , decreased STAT5 phosphorylation and down-regulated CD69 expression(35), suggesting a role for IRF2BP2 in CD4 T-cell activation.

Plenty of our validated candidate do not immediately fit into the currently known molecular mechanism of latency. Some, such as GRIK5, a G-coupled ion channel, or ADK, an Adenosine Kinase, have such pleiotropic functions it is imaginable they are in one way or another involved in maintaining latency. Others such as EXOSC8, an 3'-5' exoribonuclease in the exosome complex, or CMSS1, Cms1 Ribosomal Small Subunit Homolog, offer no immediate path towards connecting to the known mechanism of HIV latency. How these hits mechanistically link to the “canonical” pathway/chromatin structure of latency is an outstanding and challenging research question.

**Chapter 4** describes the untapped potential of screening the vast number of metabolites produced by members of the fungal Kingdom reveal latency reversal agents. Depending on growth conditions fungi excrete small compounds many of which have therapeutic potential. In Chapter 4 we identified GTX from a screen of bio-active fungal secondary metabolites (extrolites) from diverse species in de





Visual summary of the findings from this thesis in regards to novel host factors and LRAs. Although the exact mechanism by which the validated candidate genes from the haploid screen act to maintain latency is an outstanding question, the knock-out by shRNA and inhibition by FDA-approved compounds in relevant cell models do confirm the candidates as biologically relevant and therapeutically promising in latency maintenance and reversal. The identification of Gliotoxin as disruptor of 7SK snRNA, which causes latency reversal, is a promising therapeutic potential.

fungal Kingdom, as an LRA with a novel method of action. In a primary cell model of latency and cells isolated from cART suppressed HIV-1 infected participants GTX caused reactivation of latent HIV without considerable toxicity. Release of active P-TEFb after GTX treatment was confirmed by glycerol gradient. According to our *in silico* modelling experiments, GTX presumably functions by destabilizing the 7SK snRNP complex by disrupting the binding of LARP7 to 7SK RNA. Indeed, GTX disrupted LARP7-7SK RNA binding in a release assay with immunoprecipitated 7SK snRNP complex using anti-LARP7 antibody. These results show GTX reactivates latent HIV by increasing freely available P-TEFb through disruption of the 7SK RNA complex. Additionally, we show how combining bioassays and state-of-the-art fractionation and MS/NMR to screen fungal extrolites which contain an abundance of bioactive compounds is promising strategy to uncover novel compounds with therapeutic potential.

GTX is to the best of our knowledge the first compound to directly free P-TEFb by disrupting binding to the 7SK complex. Other compounds such as HMBA, DRB and actinomycin D promote P-TEFb availability too, but by indirect mechanism. Whereas GTX synergizes with LRAs in other classes such as BAF inhibitors (altering repressive chromatin configuration) and HDAC inhibitors (repression of deacetylation of histones), co-treatment with BET inhibitors (Inhibition of BRD4 releases P-TEFb) only has additive effects on latency reversal. The additive effect points to similar mechanism of action and highlights the importance of targeting distinct steps in the molecular mechanism of HIV latency when developing a reversal therapy. Previously, GTX was identified as an immunosuppressant inhibiting phagocytosis, NFkB signaling and cytokine production(36–39). However, these effects were observed at much higher concentrations (100 nM to 5 µM range) than the therapeutic concentrations (20 nM range) in our experiments. Reported serum concentrations in patients with aspergillosis are in the range of 200 nM to 2.4 µM GTX(40). So the intended therapeutic concentrations are well below levels associated with toxicity, global T cell activation, or interference with capacity for CD8+ T cells activation while reactivating latent HIV, making GTX a promising LRA. As P-TEFb plays a role in diverse and various cell function such as diverse cellular responses (stress, cytokines, cell communication), disease and development(41), GTX's application can stretch beyond latency reversal. Gliotoxin's capability to specifically activate P-TEFb and positively affect transcription gives it great therapeutic potential for a wide range of diseases that stem from inhibited transcription such as infectious diseases, inflammation/autoimmune diseases, cardiac hypertrophy, and cancer(42, 43).

**Chapter 5** concludes this thesis. Reactivating and clearing the latent reservoir are outstanding therapeutic challenges. In order to overcome these challenges we need to increase our knowledge of the exact cells that maintain the reservoir, the molecular mechanisms governing latency and finally what method to apply to

achieve eradication of HIV from the patient and hopefully one day the world. To define the latent reservoir is in itself a challenge due to the heterogenous nature of cells involved. A matter that is further complicated by the rarity of latently infected cells that contain replication competent virus. The latent reservoir is established early in the acute phase of HIV infection in various cell types in different anatomical sites. HIV-infected cells have been found in the brain (and cerebrospinal fluid), lungs, kidneys, liver, adipose tissue, gastrointestinal tract, the male and female genitourinary systems and bone marrow. However, during active infection viral replication primarily takes place in the lymphoid tissues (spleen, thymus, lymph nodes, GALT), and HIV DNA remains detectable after many years of cART. Although the reservoir resides in many different cell types, central memory CD4+ T cells (TCM) are considered the primary reservoir(44, 45). More recently, research has focused on cell markers to define the latent reservoir. Although these markers only enrich for latent infection, they are a step in the right direction to further define and target the reservoir. A futuristic and for now conceptual approach that would definitively answer where the reservoir exactly resides and what cell types it is composed of is whole body in-situ sequencing of patients on cART. First developed for single cells(46), the technique has been extended for tissues(47, 48). However physical, practical and in silico challenges need to be resolved to meet this challenge.

Central memory CD4+ T cells are the primary cells harboring the latent reservoir. Their longevity and quiescent state, that is transcriptionally poised to proliferate upon antigen encounter, makes latent HIV all the more difficult to resolve. T-cell activation is a serious complication that has to be avoided when applying a latency reversal treatment. Proper latency reversal therapy will prime and activate the transcriptional machinery in central memory CD4+ T cells, without triggering T-cell activation. However, transcriptional machinery, such as P-TEFb and its components CycT1 and CDK9, is mostly absent or inactive in central memory CD4+ T-cells(49–53). Compound that would boost levels of CDK9 and CycT1, promote P-TEFb formation and activation are promising avenues of therapeutic opportunity. In parallel alternative active transcription pathways need to be explored. One promising pathway is to activate HIV without negative side effects such as T-cell activation is the non-canonical NFkB pathway. Currently the development of SMAC mimetics is very promising, because of the dual function of reactivation and clearance of latently infected cells. In addition to transcriptional activation and elongation, post-translational blocks involved in latency need to be better understood. The observation that only a fraction of reactivated cells produce viral protein indicates that there are post transcriptional blocks despite the presence of viral RNA(54, 55). These blocks can occur at each stage of the viral protein production process, such as stability, splicing, export and translation of viral RNA and possibly extend to viral protein processing as well.

Currently available LRAs individually activated no more than 5% of latent cell in an *in vitro* primary model(56). Not surprisingly, in a clinical setting there is no single LRA that clears the latent reservoir. Multiple LRAs are known to synergize to reactivate latency. A future latency reversal therapy will be composed of a combination of LRAs that is able to clear the reservoir. Modeled after cART, which consists of a combination of compounds, each targeting a distinct part of the replication cycle, and thus creating an insurmountable block to viral replication, combinatorial latency reversal therapy will comprise of a number of compounds targeting different elements of the HIV latency molecular mechanism. This cocktail will be tailored to mold the cellular environment of the cell types harboring the latent reservoir (primarily central memory CD4+ T cells) to compel latency reversal. While each compound of the latency reversal cocktail targets a specific aspect of latency, that alone might not affect latency much, together, working in concert, they create a cellular environment that reactivates HIV. Because LRAs have the ability to synergize, the perfect mix of compounds could be administered at such low concentrations in such a composition that the cellular environment of latently infected cells is switched to a state where HIV is reactivated with little to no side effects (i.e. Immune activation). To achieve this therapy our knowledge of the molecular mechanisms governing latency and activation, the precise composition and nature of the reservoir and available compounds and their function needs to be extended.

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## Samenvatting en algemene discussie

Het vroegste goed gedocumenteerde geval van een HIV-geïnfecteerd persoon dateert van 1951. De HIV/AIDS-pandemie werd openbaar in 1981. Sinds het begin van de pandemie zijn 75 miljoen mensen besmet en ongeveer 32 miljoen mensen overleden. In 1987 werd het eerste antiretrovirale geneesmiddel voor de behandeling van HIV-infectie, azidothymidine (AZT), geïntroduceerd.

Combinatorische antiretrovirale therapie (CART) werd ontwikkeld in de vroege jaren negentig. Sindsdien is HIV-infectie een chronische ziekte geworden voor wie toegang heeft tot behandeling en deze kan betalen. Hoewel cART virale replicatie blokkeert, leidt dit niet tot definitieve genezing. Zodra de behandeling is gestopt, kan de virale replicatie worden hervat, zelfs na lange behandeling. Naast het gevaar van terugkerende infectie, wordt duidelijk dat langdurige cART-behandeling van HIV infectie negatieve neurologische en cardiovasculaire effecten heeft. De bron van de aanhoudende infectie is een latent reservoir van geïnfecteerde cellen. Dit reservoir bestaat uit langlevende CD4 + T- geheugen-cellen die transcriptioneel inactief, maar replicatiecompetent HIV-1 bevatten. De noodzaak van een functionele genezing (waarbij patiënten de infectie onder controle houden) of een steriliserende genezing (waarbij het virus volledig uit het lichaam wordt verwijderd) is duidelijk. "Shock and kill" is een voorgestelde strategie voor een definitieve genezing. Het stelt voor dat het latente virus wordt wakker geschokt, "Shock", tot actieve transcriptie (en virusproductie) en vervolgens wordt gedood, "Kill" door het immuunsysteem of virale cytopathische effecten. De eerste stap, het reactiveren van latent virus, vereist een specifieke en volledige reactivering van het latente reservoir. Momenteel bestaat een dergelijke behandeling niet. De focus van dit proefschrift is om met behulp van onbevooroordeelde onderzoeksbenaderingen inzicht te krijgen in de moleculaire mechanismen van latent HIV te identificeren en om nieuwe geneesmiddelen en hun methode van werking te vinden.

**Hoofdstuk 1** vat onze huidige kennis samen over latente als actieve HIV-1 infecties. Ons begrip van de moleculaire regulatie van HIV begint met de HIV-1-replicatiecyclus. Nadat HIV-RNA de cel is binnengekomen, wordt het ge-reverse-transcribeerd en wordt het pre-integratiecomplex (PIC), bestaande uit een dubbelstrengs DNA-kopie van HIV gebonden door gastheerfactoren, geassembleerd. De PIC komt de kern binnen, waar HIV stabiel integreert in het gastheergenoom. De 5'LTR van HIV is de promotor voor virale gen-expressie. De 5'LTR bevat talrijke bindingsplaatsen voor transcriptiefactoren. Transcriptie wordt geïnitieerd die aanleiding geeft tot het virale RNA-transcript TAR en het virale eiwit Tat. TAR bindt zich aan de Transcription Start Site (TSS) en werft Tat. NELF (negatieve verlengingsfactor) en DISF (DRB-gevoeligheidsinducerende factor) veroorzaken echter een onderbreking van RNA-polymerase II. Om deze blokkade



te overwinnen, moet het carboxy-terminale domein (CTD) van RNA Polymerase II (RNAPII) worden gefosforyleerd door P-TEFb (positieve verlengingsfactor b). P-TEFb bestaat uit cycline-afhankelijk kinase 9 (CDK9) en cycline T1. P-TEFb wordt geremd wanneer het gebonden wordt in het 7SK snRNP-complex. Wanneer P-TEFb door Tat wordt gerekruteerd op de HIV TSS, kan vervolgens het super elongatie complex (SEC) worden gevormd, waardoor de transcriptie-verlenging van RNAPII verder wordt versterkt. In de latente toestand worden twee nucleosomen op de LTR gepositioneerd, deze positionering is onafhankelijk van de integratieplaats en wordt actief onderhouden door het BAF-complex. Het verstoren van het BAF-complex leidt tot reactivering.

Onze kennis van het moleculaire mechanisme van latent HIV heeft geholpen bij de ontwikkeling van latentie-reversal agents (LRA's), werkzame moleculen die de potentie hebben om HIV-1-genexpressie vanuit een latente toestand te induceren. Door repressieve gastheerfactoren te remmen, factoren te induceren die transcriptie bevorderen of een chromatineorganisatie te creëren die bevorderlijk is voor transcriptie, reactiveren LRA's latente HIV. Volgens hun effect op virale transcriptie kunnen LRA's momenteel worden onderverdeeld in chromatine-regulatoren, transcriptie-initiatoren en -verlengers. Chromatine-regulatoren beïnvloeden post-translationele modificaties (PTM's) op histonstaarten of chromatine-hermodellering. Acetylering is een transcriptie-bevorderende PTM en kan worden bevorderd door Histone Deacetylase-remmers (HDACi). Voorbeelden van HDACis zijn Romidepsin, Panobinostat, Vorinostat en Valproïnezuur. Andere PMT's die als doelwit dienen voor latentieomkering zijn histon-methyltransferase (HMT) en DNA-methyltransferasen (DNMT). Chromatine wordt actief opnieuw gemodelleerd door ATP-afhankelijke remodelers. Het BAF-complex houdt bijvoorbeeld actief een repressieve chromatinestructuur in stand. Verstoring van het BAF-complex leidt tot reactivering van latent HIV. Evenzo resulteert remming van bromodomein en extra-terminale domein (BET) -eiwitten BRD2 en BRD4 tot de-repressie. De tweede categorie zijn geneesmiddelen die transcriptiefactoren voor de LTR rekruteren en transcriptie-initiatie bevorderen. NFκB kan bijvoorbeeld latent HIV onafhankelijk van Tat reactiveren. PKC-agonisten, TLR-agonisten en Maraviroc kunnen NFκB activeren, maar ook T-celactivering veroorzaken, wat een ernstige bijwerking is. Meer recentelijk is het gebruik van SMAC-mimetica die de niet-conventionele NFκB-route activeren, veelbelovend vanwege hun dubbele effect op reactivering en klaring van latente cellen. Omdat transcriptie-initiatie niet noodzakelijk leidt tot transcriptie-verlenging, moet promotor-proximale pauze bij de 5'LTR TSS worden overwonnen. Door het vrij beschikbare P-TEFb te vergroten, kan de CTD van RNA Polymerase II gefosforyleerd worden, wat transcriptieverlenging teweegbrengt. Van verschillende moleculen zoals hexamethyleenbisacetamide (HMBA), 5,6-dichloor-1-β-d ribofuranosylbenzimidazol (DRB) en actinomycine actinomycine D is eerder aangetoond dat ze het vrij beschikbare P-TEFb verhogen. Onze ontdek-

king van Gliotoxine als een LRA is de eerste in zijn soort die mechanisch richt op het verstoren van LARP7 aan 7SK RNA. Door de verstoring kan gebonden P-TEFb vrijkomen, wat resulteert in activering van HIV-1-transcriptie-elongatie. In het laboratorium is aangetoond dat combinaties van LRA's synergetisch werken om latente HIV in een te reactiveren. Het is denkbaar dat in de toekomst een combinatie van LRA's zal worden toegediend als behandeling om het latente reservoir uit te roeien. Sommige LRA's hebben klinische studies gehaald, maar helaas hebben deze tot dusver geen betekenisvolle impact op het reservoir getoond. Daarom moeten we krachtigere en effectievere LRA's vinden. Om de kans op succes te vergroten, moet deze zoektocht ook de identificatie doelen waar medicijnen op aangrijpen omvatten.

Om het aantal doeleiwitten voor medicijnen te vergroten en ons begrip van de moleculaire mechanismen van latent HIV te vergroten, hebben we complementaire, onbevooroordeelde onderzoeksbenaderingen gebruikt om nieuwe gastheerfactoren en geneesmiddelen die betrokken zijn bij HIV-latentie te identificeren. Eerder onderzoek naar HIV heeft kandidaat-gedreven onderzoeksbenaderingen gevolgd, die eerdere bevindingen gebruiken om voort te bouwen op onze kennis van virale replicatie en interactie met de gastomgeving en deze uit te breiden. Een kandidaat-benadering neemt een bekend element, bijvoorbeeld de immuunmeesterregulator NFkB of de promotor van HIV, de 5'LTR, en probeert bestaande kennis uit te breiden door bijvoorbeeld nieuwe bindingspartners of mutaties te identificeren die de functie beïnvloeden. Dit biedt een mogelijkheid om te verkennen, maar beperkt ook de mogelijke uitkomsten, omdat factoren buiten de bekende mechanismen mogelijk niet aan het licht komen. In tegenstelling tot kandidaat-benaderingen, maken onbevooroordeelde onderzoeksbenaderingen vooraf geen aannames over gastheermechanismen om het mechanisme van interesse te onderzoeken. Onbevooroordeelde benaderingen kijken niet naar een specifiek gen of een specifieke verbinding, maar zoeken eerder naar het onderliggende mechanisme van een specifiek fenotype. Dus onbevooroordeelde benaderingen sluiten geen kandidaat-genen of verbindingen uit en werpen het net zo breed mogelijk. Als zodanig zijn onbevooroordeelde onderzoeksbenaderingen hypothese-genererend en verkennend, terwijl kandidaat-benaderingen hypothese-gestuurd en onderzoekend zijn. In dit proefschrift gebruiken we onafhankelijke maar complementaire onbevooroordeelde benaderingen om het moleculaire mechanisme van latente HIV-infectie op te helderen door te kijken naar 1) wat de cellulaire omgeving HIV-infectie gedijt, 2) welke gastheerfactoren betrokken zijn bij latentie en 3) welke werkzame stoffen latent HIV kunnen reactiveren. Om te begrijpen waarom een cel HIV-infectie toelaat, hebben we eerder gepubliceerde gegevens van genomwijde associatiestudies (GWAS) gebruikt om genomische regulator regio's te identificeren voor genen die de ontvankelijkheid voor infectie beïnvloeden. Om nieuwe gastheerfactoren te identificeren, hebben we een onbevooroordeeld

tweekleurig haploïde onderzoeksbenadering gebruikt. En om nieuwe LRA's te vinden, hebben we een verzameling van bioactieve moleculen van verschillende soorten in het schimmelkoninkrijk gescreend. Elk van onze benaderingen begint met een verzameling, of het nu gaat om SNP's, genen of werkzame stoffen. Elk item in de collectie wordt gescreend om het effect ervan op de reactivatie van HIV-latentie te beoordelen. Al onze benaderingen zijn dus onbevooroordeeld, hypothese-genererend en verkennend, wat tot verrassende vondsten heeft geleid.

**Hoofdstuk 2** past de kracht van GWAS toe om genetische varianten in verschillende individuen statistisch te koppelen aan een fenotype. Omdat het verzamelen van grote cohorten echter uitdagend kan zijn, voldoen veel voorkomende varianten met een zwakke effectgrootte en zeldzame varianten niet aan de algemeen aanvaarde stringente drempel voor genoombrede significantie ( $P < 5 \times 10^{-8}$ ). Bovendien ontbreekt bij de meerderheid van de geïdentificeerde SNP's een duidelijke functie omdat ze zich vaak in niet-coderende regio's van het genoom bevinden. Regulerende DNA-elementen worden gekenmerkt door chromatine-markeringen. Door deze functionele gegevens van het genoom uit Encyclopedia of DNA Elements (ENCODE) en de Human Epigenome Atlas te combineren met GWAS, kan de potentiële regulerende functie van niet-coderende SNP's worden voorspeld, waardoor de lijst van kandidaat-SNP's om te bestuderen wordt verminderd. Door gegevens van HIV-1 GWASs en T-cel-afgeleide epigenoomgegevens te combineren, identificeren we het regulerende potentieel van niet-coderende HIV-1-geassocieerde intergene variant rs4349147 op HIV-infectiviteit in Hoofdstuk 2. Deletie van de regio waarin rs4349147 zich bevindt, verminderde de expressie van IL-32 sterk, maar onthulde ook een verschuiving in weke IL-32-isovormen tot expressie werden gebracht. IL-32 heeft vijf isovormen, A tot E, die worden gecodeerd door meerdere RNA-splitsingsvarianten (varianten 1 tot 9). In het algemeen zijn IL-32b en IL-32g pro-inflammatoir, terwijl IL-32a niet-pro-inflammatoir is. Biochemische bepaling van de chromosoomconformatie toont een chromatinelus tussen rs4349147 en IL-32-promotor. Deze interactie is allel-afhankelijk: rs4349147 - / A-klonen hebben verminderde IL-32-expressie, een lagere IL-32a tot niet-a-isovorm verhouding en veranderde chromatine-conformatie in vergelijking met rs4349147 G / - -cellen. Exogene toevoeging van IL-32g, maar niet IL-32a, doormiddel van recombinant eiwitsuppletie of virale transductie verbeterde de HIV-infectiviteit van Jurkat-cellen door een pro-inflammatoire T-celcytokine-omgeving te induceren. We laten zien hoe HIV-infectiviteit beïnvloed wordt doordat de expressie van IL-32-

isovormen die op hun beurt gereguleerd worden door niet-coderende genomische elementen.

rs4349147 bereikt niet de gouden standaard van significantie van  $P < 5 \times 10^{-8}$  in GWAS's voor HIV-acquisitie. HIV-1 GWAS's hebben te weinig kracht omdat het moeilijk is om genoeg monsters uit grote cohorten te verzamelen. Als gevolg hiervan vallen zeldzame varianten en veel voorkomende varianten met een zwakke effectgrootte onder de drempel voor genomische significantie ( $P < 5 \times 10^{-8}$ ) en worden ze gemist. Door gebruik te maken van de in hoofdstuk 2 getoonde benadering, waarbij GWAS-gegevens worden gecombineerd met epigenomische gegevens, wordt het mogelijk om echte biologische signalen te onderscheiden van achtergrondruis. Hierdoor kunnen zwakke SNP's worden opgevoerd en wordt de lijst met te screenen kandidaat-SNP's beperkt. Eerdere bevindingen melden tegenstrijdige of dubbele rollen van IL-32 bij ontstekingen. IL-32 bleek hoger te zijn in mononucleaire cellen in perifeer bloed (PBMC's), darmen en lymfeklieren in verschillende stadia van infectie van HIV-positieve individuen, vergeleken met gezonde controles. Van IL-32 werd gedacht dat het de expressie van immunosuppressieve moleculen bevordert, wat resulteert in verminderde immuunactivering en verhoogde HIV-replicatie. Andere bevindingen wijzen op een remmende rol voor IL-32 bij HIV-replicatie. Knockdown van IL-32 door interfererend RNA (RNAi) veroorzaakte verhoogde HIV-replicatie in Jurkat, menselijke embryonale nier (HEK) 293T cellen, latent geïnfecteerde U1 (macrofaagcellijn) en geïnfecteerde PBMC's. Na het uitschakelen van IL-32 was het aantal TH1-cellen afgenomen, evenals de niveaus van pro-inflammatoire cytokines, waaronder IL-6 en TNF $\alpha$ . Deze onderzoeken maken echter geen onderscheid tussen IL-32-isovormen wat betreft hun knockdown of detectie door antilichamen en PCR-primers.

In vergelijking met andere isovormen zoals IL-32b of IL-32g, is IL-32a niet zo sterk als inductor van een pro-inflammatoire toestand in PBMC's. Pro-inflammatoire isovormen van IL-32 (voornamelijk b en g) correleren met verlaagde CD4 T-celtellingen, verhoogde virale belasting, lagere CD4 / CD8-ratio en niveaus van inflammatoire markers (sCD14 en IL-6) in HIV-geïnfecteerde langzame progressoren die virologisch falen ervaren. Het potentieel van IL-6 om HIV-replicatie te bevorderen in macrofagen en latent geïnfecteerde CD4 + T-cellen is goed gedocumenteerd. Resultaten van onze cytokine-array lieten verhoogde niveaus van pro-inflammatoire cytokines IL-8, IL-6, IL-10, TNF $\alpha$  (tumornecrosefactor- $\alpha$ ) en IFN-g (interferon-g) zien na overexpressie van IL-32g, maar niet IL-32a. Het is denkbaar dat in rs4349147-G-allelcellen, de inductie van IL-32g zorgt voor hogere niveaus van IL-6 en andere pro-inflammatoire cytokines, wat resulteert in een pro-inflammatoire omgeving die bevorderlijk is voor HIV-

replicatie. Maar de effecten van deze verschuiving in de productie van isovormen zijn bescheiden. Onze resultaten bieden een uniforme verklaring van eerdere schijnbaar tegenstrijdige functies van IL-32 en zijn isovormen in infectiegevoeligheid. Toekomstige studies zullen bepalen of het pro-inflammatoire effect van niet-IL-32a isovormen ook bijdraagt aan het omkeren van de latentie.

**Hoofdstuk 3** laat de resultaten zien van een gen-knock-out-screen in een haploïde myeloïde menselijke cellijn om nieuwe gastheerfactoren te identificeren bij het in stand houden van HIV-latentie. Omdat de screen niet-essentiële genen identificeert, zijn de gevonden eiwitten veelbelovende doelwitten voor geneesmiddelen. Om deze screening uit te voeren, hebben we de haploïde KBM7-cellen aangepast door een latente HIV-infectie te introduceren met behulp van een minimale HIV met GFP-reporter, terwijl haploïditeit werd gehandhaafd. Vervolgens werden de cellen onderworpen aan Gene-Trap (GT)-mutagenese. GT is een muizenstamcelvirus dat een splicing-acceptorsequentie, mCherry en een polyA-terminator bevat. Wanneer GT een cel infecteert, wordt het bij voorkeur geïntegreerd in de sense-oriëntatie van tot expressie gebrachte genen in het gastheergenom. De splicing-acceptorsequentie kapt het transcript van het gemutageniseerde gen af, waardoor het gen effectief wordt uitgeschakeld terwijl een mCherry-fluorescerend eiwit wordt geproduceerd. Wanneer een repressieve gastheerfactor wordt uitgeschakeld, wordt HIV geactiveerd. Na GT-mutagenese wordt een klein percentage van de cellen mCherry-positief (GT-geïnfecteerd) en GFP-positief (gereactiveerd HIV). We sorteerden dubbele positieve cellen en gebruikten high throughput sequencing om de GT-integratieplaatsen in de GT-geactiveerde populatie te identificeren. Dit leidde tot de identificatie van 69 kandidaat-genen die mogelijk betrokken zijn bij het in stand houden van de latentie van HIV-1. We hebben 15 kandidaten gekozen om hun rol in latentie van HIV-1 te valideren doormiddel van shRNA knockdown in twee van Jurkat afgeleide latent HIV-1 geïnfecteerde cellijnen (J-lat), J-LatA2 en J-Lat11.1. Tien genen vertoonden reactivering van HIV-1 in latent geïnfecteerde T-cellijnen na knockdown. We hebben ook in de literatuur gezocht naar werkzame stoffen die gericht zijn op onze kandidaatgenen. Voor drie genen, ADK, GRIK5 en NF1, waren door de FDA goedgekeurde geneesmiddelen beschikbaar. In zowel J-Lat-cellijnen als in ex-vivo geïnfecteerde CD4 + T-cellen was de ADK-remmer 5-Iodotubercidin toxisch. De remmers van GRIK5 en NF1, respectievelijk Topiramaat en Trametinib, reactiveerden latent HIV zonder de levensvatbaarheid in zowel A2- als 11.1 J-Lat-cellen negatief te beïnvloeden. Trametinib, hoewel actief in latent geïnfecteerde cellijnen, was niet effectief in primaire cellen, wat het belang

benadrukt van het valideren van de functie van vermeende werkzame stoffen in meer relevante primaire CD4 + T-celmodellen van HIV-1-latentie. Ten slotte vertoonde Topiramaat een significante omkering van de latentie in primaire cellen zonder significante cytotoxiciteit te induceren, noch veroorzaakte het algemene T-celactivering, een gevaarlijke potentiële bijwerking van kandidaat-LRAs. Het LRA-potentieel van deze verbinding moet verder worden onderzocht in een klinische setting. Een andere kandidaat die we hebben gevonden, CHD9, is een lid van de chromodomeinhelicase-DNA-bindende eiwitfamilie. Vanwege de ATP-afhankelijke chromatine remodeler-functie van de kandidaat hebben we besloten om de kandidaat verder te karakteriseren. We bekeken de aanwezigheid van CHD9 op de 5'LTR van HIV. ChIP-resultaten tonen associatie op de LTR, die verloren gaat bij PMA-behandeling. Al met al laten deze resultaten zien dat het gebruik van de haploïde screen een haalbare strategie is om nieuwe gastheerfactoren betrokken bij HIV-latentie te identificeren.

Historisch gezien was RNAi de go-to-methode voor verlies-van-functie screens. Vanwege de hoge off-target-effecten van RNAi, korte en onvolledige genonderdrukking, hebben screens echter te kampen met hoge foutpositieve percentages. Terwijl we ons haploïde screen uitvoerden, voltrok zich de CRISPR-revolutie voor het bewerken van genen. CRISPR-genbewerking is het aangepaste geleide nucleasecomplex van het antivirale verdedigingsmechanisme van bacteriën, CRISPR-Cas9. Het bestaat uit een sgRNA dat een vaste sequentie bevat om te binden aan het Cas9-eiwit, een PAM-sequentie en de gastheersequentie die gebruikt wordt om te richten. De PAM-sequentie is een vaste nucleotidesequentie die nodig is voor het CRISPR-complex om de beoogde sequentie te herkennen. De nuclease-activiteit van het complex veroorzaakt dubbelstrengs breuken (DSB's) of enkelstrengs inkepingen. De reparatie van DSB's en inkepingen door niet-homologe end-verbinding (NHEJ) en homologie gericht herstel (HDR) resulteert in mutaties die een veranderde functie of knock-out van het beoogde gen veroorzaken. Omdat CRISPR-Cas9 zo specifiek is, is het potentieel enorm. Er zijn echter beperkingen aan het screenen met CRISPR-Cas9. Technische problemen in verband met CRISPR-Cas9-screens zijn sgRNA-dekking van het genoom en de celpopulatie. Om het volledige genoom te screenen, moet sgRNA gelijkmatig over het genoom worden verspreid. Vanwege de noodzaak van een PAM-sequentie voor targeting, zijn genen of regio's die uitgeput zijn in PAM-sequenties ondervertegenwoordigd in CRISPR-schermen. Om in een scherm te verschijnen, moet een gen in meerdere cellen worden geraakt, waardoor grootschalige experimenten met een groot aantal cellen nodig zijn. Om de moleculaire mechanismen die betrokken zijn bij een interessant fenomeen uit elkaar te halen,

zijn knockdown- of knock-out-schermen zeer effectief om nieuwe factoren te identificeren.

De myeloïde leukemische oorsprong van KBM7-cellen is een celtype dat verschilt van de T-cellen die de voorkeur hebben van HIV, hoewel ze een afstamming delen met andere cellen die ontvankelijk zijn voor HIV-infectie, zoals monocyten en macrofagen. Van de 15 kandidaatgenen die we selecteerden voor verdere karakterisering, bleken er 10 betrokken te zijn bij het handhaven van latentie in Jurkat-afgeleide cellijnen met latentie (J-Lat). Onze bevinding dat tweederde van de geteste kandidaten gevalideerd is, suggereert dat de overige 54 kandidaten meer gastfactoren bevatten. Van de kandidaten die "vals-positief" lijken te zijn na testen in J-lat-cellen, zou het interessant zijn om te zien hoe deze gastheerfactoren zich gedragen in latente modellen van monocytische of macrofagische oorsprong. Dit zou een onderscheid maken tussen echte valse positieven in onze screen en verschillen in de celomgeving (myeloïde versus lymfocytisch). Het voorwaartse genetische screeningsplatform op basis van de haploïde KBM7-cellen is een eenvoudig systeem dat relatief gemakkelijk aan te passen is om een fenomeen van interesse te bestuderen, zoals virale toegang, toxiciteit of celsignalering. Omdat KBM7-cellen bijna haploïde zijn (diploïde voor chromosoom 8 en een klein deel van chromosoom 10), onderzoekt het screen, dat het GT-virus gebruikt om willekeurig genen uit te schakelen, het hele genoom behalve de diploïde chromosomen, hoewel recentelijk complete haploïde lijnen zijn ontwikkeld. Ons onderzoekstreven werd belemmerd door het kleine percentage cellen dat werd gereactiveerd na mutagenese door het GT-virus. We hebben geprobeerd dit te verhelpen veel te celsorteren om zoveel mogelijk cellen te verzamelen. Dit betekent echter dat ons screen zeker niet uitputtend is. Omdat we een onbevooroordeelde onderzoeksbenadering gebruikten, passen de gastheerfactoren die we identificeren die betrokken zijn bij HIV-latentie soms niet onmiddellijk in ons "conventionele" beeld van het moleculaire mechanisme van HIV-latentie. Sommige, zoals CHD9, een ATP-afhankelijke chromatine-remodeler, of IRF2BP, een transcriptiefactor, zijn intuïtief logisch als een nieuwe gastheerfactor bij latent HIV. In het bijzonder is IRF2BP2 (Interferon regulerende factor 2 bindend proteïne 2) bestudeerd in relatie tot lymfocyten aangezien het eerder werd geïdentificeerd als een repressor van NFAT133. Bij muizen onderdrukte IRF2BP2-overexpressie in CD4 T-lymfocyten de expressie van de hoge affiniteit  $\alpha$ -keten van IL-2 receptor, verminderde het STAT5-fosforylering en nam CD69-expressie af, wat een rol suggereert voor IRF2BP2 bij CD4 T-celactivering. Veel van onze gevalideerde kandidaten passen niet onmiddellijk in het momenteel bekende moleculaire mechanisme van latentie. Sommige, zoals GRIK5, een G-gekoppeld ionenkanaal, of ADK, een adenosinekinase, hebben zulke

wijdverspreide effecten, dat het goed denkbaar is dat ze op de een of andere manier betrokken zijn bij dehandhaving van latentie. Anderen, zoals EXOSC8, een 3'-5'-exoribonuclease in het exosoomcomplex, of CMSS1, Cms1 Ribosomal Small Subunit Homolog, bieden geen directe weg om verbinding te maken met het bekende mechanisme van HIV-latentie. Hoe deze hits mechanistisch verband houden met de "conventionele" route / chromatinestructuur van latentie is een uitstaande en uitdagende onderzoeksvraag.

**Hoofdstuk 4** beschrijft het potentieel van het screenen van het grote aantal metabolieten dat wordt geproduceerd door leden van het schimmelkoninkrijk om te zoeken naar LRAs. Afhankelijk van de groeiomstandigheden scheiden schimmels stoffen uit, waarvan er vele therapeutisch potentieel hebben. In Hoofdstuk 4 hebben we Gliotoxin (GTX) geïdentificeerd op basis van een screening van bioactieve secundaire metabolieten (extrolieten) van verschillende soorten in het schimmelrijk, als een LRA met een nieuwe werkmethode. In een primair celmodel van latentie en cellen geïsoleerd uit cART-onderdrukte HIV-1-geïnfekteerde vrijwilligers veroorzaakte GTX reactivatie van latent HIV zonder aanzienlijke toxiciteit. Vrijkomen van actief P-TEFb na GTX-behandeling werd bevestigd door glycerolgradiënt. Volgens onze in silico-modelleringsexperimenten functioneert GTX vermoedelijk door het 7SK snRNP-complex te destabiliseren door de binding van LARP7 aan 7SK-RNA te verstoren. GTX verstoort inderdaad de LARP7-7SK RNA-binding in een afgiftetest met immunoprecipitatie 7SK snRNP-complex met behulp van anti-LARP7-antilichaam. Deze resultaten tonen aan dat GTX latent HIV activeert door het vrij beschikbare P-TEFb te verhogen door verstoring van het 7SK RNA-complex. Bovendien laten we zien hoe het combineren van bioassays en state-of-the-art fractionering en MS/NMR om schimmel-extrolieten te screenen een veelbelovende strategie is om nieuwe verbindingen met therapeutisch potentieel te ontdekken.

GTX is naar ons beste weten de eerste werkzame stof die P-TEFb direct vrijmaakt door de binding aan het 7SK-complex te verstoren. Andere verbindingen zoals HMBA, DRB en actinomycine D bevorderen ook de beschikbaarheid van P-TEFb, maar via een indirect mechanisme. Terwijl GTX synergiseert met LRA's in andere klassen, zoals BAF-remmers (wijziging van repressieve chromatine-configuratie) en HDAC-remmers (onderdrukking van deacetylering van histonen), heeft gelijktijdige behandeling met BET-remmers (remming van BRD4 zorgt voor vrijkomen van P-TEFb) alleen additieve effecten op latentie omkering. Het additieve effect wijst vermoedelijk op een vergelijkbaar onderliggend werkingsmechanisme en benadrukt het belang van het includeren van werkzame stoffen die zich richten op verschillende stappen in het moleculaire mechanisme van HIV-latentie bij het ontwikkelen van een therapie. Eerder werd GTX geïdentificeerd als een immunosuppressivum dat fagocytose, NFκB-signalering en



cytokineproductie remt. Deze effecten werden echter waargenomen bij veel hogere concentraties (100 nM tot 5  $\mu$ M) dan de therapeutische concentraties (tot 20 nM) in onze experimenten. De gerapporteerde serumconcentraties bij patiënten met aspergillose liggen in het bereik van 200 nM tot 2,4  $\mu$ M GTX. De beoogde therapeutische concentraties liggen dus ruim onder de niveaus die zijn geassocieerd met toxiciteit, globale T-celactivering of interferentie met het vermogen om CD8+ T-cellen te activeren, maar activeert alsnog latent HIV, waardoor GTX een veelbelovende LRA is. Omdat P-TEFb een rol speelt bij verschillende celfuncties, zoals diverse cellulaire reacties (stress, cytokines, celcommunicatie), ziekte en ontwikkeling, kan GTX toegepast worden om veel meer ziektes te behandelen dan alleen HIV latentie. Het vermogen van GTX om specifiek P-TEFb te activeren en de transcriptie positief te beïnvloeden, geeft het een groot therapeutisch potentieel voor een breed scala aan ziekten die voortkomen uit geremde transcriptie, zoals infectieziekten, ontstekingen / auto-immuunziekten, cardiale hypertrofie en kanker.

**Hoofdstuk 5** besluit dit proefschrift. Het reactiveren en leegmaken van het latente reservoir zijn uitstaande therapeutische uitdagingen. Om deze uitdagingen het hoofd te bieden, moeten we onze kennis vergroten van de exacte cellen die het reservoir bevatten, de moleculaire mechanismen die de latentie beheersen en tot slot welke methode we kunnen toepassen om de uitroeiing van HIV bij patiënt en hopelijk ooit de wereld te bewerkstelligen.

Het definiëren van het latente reservoir is op zichzelf een uitdaging vanwege de heterogene aard van de betrokken cellen. Een kwestie die verder wordt gecompliceerd door de zeldzaamheid van latent geïnfecteerde cellen die replicatiecompetent virus bevatten. Het latente reservoir wordt vroeg in de acute fase van HIV-infectie gevormd in verschillende celtypen op verschillende anatomische plaatsen. Met HIV geïnfecteerde cellen zijn aangetroffen in de hersenen (en hersenvocht), longen, nieren, lever, vetweefsel, maagdarmkanaal, het mannelijke en vrouwelijke urogenitale systeem en beenmerg. Tijdens actieve infectie vindt virale replicatie echter voornamelijk plaats in de lymfoïde weefsels (milt, thymus, lymfeklieren, GALT) en HIV-DNA blijft detecteerbaar na vele jaren cART. Hoewel het reservoir zich in veel verschillende celtypen bevindt, worden CD4+ centraal geheugen T-cellen beschouwd als het primaire reservoir. Meer recentelijk richt onderzoek zich op celoppervlakte markers om het latente reservoir te definiëren. Hoewel deze markers alleen verrijken voor latente infectie, zijn ze een stap in de goede richting om het reservoir verder te definiëren. Een futuristische en voorlopig conceptuele benadering die definitief zou antwoorden waar het reservoir zich precies bevindt en uit welke celtypen het bestaat, is in-situ sequencing van het hele lichaam van patiënten op cART. Voorlopers op deze techniek zijn eerst ontwikkeld voor individuele cellen, maar

zijn al uitgebreid naar weefsels. Fysieke, praktische en in silico-uitdagingen moeten echter worden opgelost om deze uitdaging aan te gaan.

Centraal geheugen CD4 + T-cellen zijn de primaire cellen die het latente reservoir herbergen. Hun lange levensduur en metabole rusttoestand maakt latente HIV des te moeilijker op te lossen. T-celactivering is een ernstige complicatie die moet worden vermeden bij het toepassen van een LRA-behandeling. Correcte LRA-therapie zal de transcriptiemachinerie van CD4+ centrale geheugen T-cellen stimuleren en activeren, zonder T-celactivering op gang te brengen.

Transcriptiecomponenten, zoals P-TEFb en zijn subcomponenten CycT1 en CDK9, zijn echter meestal afwezig of inactief in het CD4+ centrale geheugen T-cellen. Werkzame stoffen die de niveaus van CDK9 en CycT1 verhogen en/of P-TEFb-vorming en activering bevorderen zijn veelbelovende therapeutische mogelijkheden. Tegelijkertijd moeten alternatieve actieve transcriptieroutes worden onderzocht. Een veelbelovende route om HIV te activeren zonder negatieve bijwerkingen, zoals T-celactivering, is de niet-conventionele NFkB-route. Momenteel is de ontwikkeling van SMAC-mimetica veelbelovend, vanwege de dubbele functie van reactivering en klaring van latent geïnfecteerde cellen. Naast transcriptionele activatie en verlenging moeten post-translationele blokkades die betrokken zijn bij latentie beter worden begrepen. De waarneming dat slechts een fractie van gereactiveerde cellen viraal eiwit produceert, geeft aan dat er post-transcriptionele blokkades zijn ondanks de aanwezigheid van viraal RNA. Deze blokkades kunnen voorkomen in elke fase van het productieproces van virale eiwitten, zoals stabiliteit, splitsing, export en translatie van viraal RNA, en kunnen zich mogelijk ook uitstrekken tot virale eiwitproductie.

Momenteel beschikbare LRA's activeerden afzonderlijk niet meer dan 5% van de latente cellen in een in vitro primair celmodel. Het is niet verrassend dat er in klinische settings geen enkele LRA is die het latente reservoir volledig activeert. Het is bekend dat meerdere LRA's synergistisch werken om latentie te reactiveren. Een toekomstige LRA-therapie zal bestaan uit een combinatie van LRA's die het gehele reservoir kunnen reactiveren. Gemodelleerd naar cART, dat bestaat uit een combinatie van geneesmiddelen, elk gericht op een bepaald deel van de virale replicatiecyclus, zal combinatorische latency reversal-therapie bestaan uit een aantal geneesmiddelen die zich richten op verschillende elementen in het moleculair mechanisme van HIV-latentie. Deze cocktail zal worden samengesteld om de cellulaire omgeving van de celtypen die het latente reservoir herbergen (voornamelijk centraal geheugen CD4 + T-cellen) om te vormen om latentieomkering af te dwingen. Elke verbinding van de LRA-cocktail richt zich op een specifiek aspect van latentie, samen creëren ze een cellulaire omgeving die HIV reactiveert. Omdat LRA's synergetisch werken, zou de perfecte mix van verbindingen in zulke lage concentraties in een zodanige samenstelling kunnen worden toegediend dat de cellulaire omgeving van latent geïnfecteerde cellen wordt omgeschakeld naar een toestand waarin HIV wordt gereactiveerd

met weinig tot geen bijwerkingen (bijv. T cell activering). Om deze therapie te bereiken, moet onze kennis van de moleculaire mechanismen die latentie en activering bepalen, de precieze samenstelling en aard van het reservoir en de beschikbare geneesmiddelen en hun functie worden uitgebreid.

## List of Abbreviations

ADK	Adenosine kinase
AIDS	acquired immuno-deficiency syndrome
cART	combinatorial antiretroviral therapy
CHD9	Chromodomain-helicase-DNA-binding protein 9
DSIF	DRB sensitivity-inducing factor
GALT	gut-associated lymphoid tissues
GRIK5	Glutamate receptor, ionotropic kainate 5
GT	Gene-Trap
GTX	Gliotoxin
HAT	Histone Acetyl transferase
HDAC	histone deacetylase
HIV-1	Human immunodeficiency virus-1
HMT	histone methyltransferase
LRA	latency reversal agent
NELF	Negative elongation factor
NF1	Neurofibromin 1
PBMCs	peripheral blood mononuclear cells
P-TEFb	positive transcription elongation factor b
snRNP	small nuclear ribonucleoprotein
TCM	central memory T-cell
TSCM	T memory stem cells
TSS	Transcription start site

## Curriculum Vitae

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# PhD Portfolio

## Summary of PhD training an teaching activities

Name: M Röling	PhD period: June 2013-Jan 2021
Department: Biochemistry	Promotor: Peter Verrijzer
Graduate school: Biomedical sciences	Supervisor: Tokameh Mahmoudi

### Training

Biochemistry and Biophysics	2013
Genetics	2014
Cell and Developmental Biology	2014

### Seminars, workshops and conferences

Dutch chromatin meeting	2014, 2016
HIV meeting	2014
MGC symposium	2013-2017
MGC workshop	2014, 2016

### Presentations

Mahmoudi Lab work discussions	2013-2017
Inderdepartmental Monday morning meetings	2013-2017
MGC Workshop	2016

### Teaching

Supervision of Master and Bachelor internships	2014-2016
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