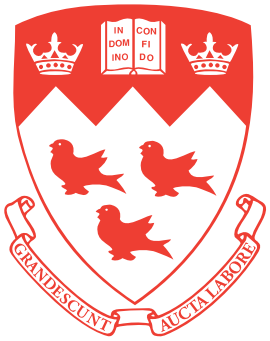
Experimental and Computational Investigations into Interactions between HIV-1 and the RNA Interference Pathway



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December 2021

A thesis submitted to McGill University in partial fulfillment
of the requirements of the degree of Master of Science

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Abstract

As an obligate intracellular pathogen, the human immunodeficiency virus type 1 (HIV-1) targets and co-opts a diverse set of host processes to overcome cellular barriers to infection, including pathways involved in the regulation of gene expression. It remains controversial whether HIV-1 affects the RNA interference (RNAi) pathway, a key post-transcriptional regulatory mechanism that is being used to develop a new class of gene therapies. To better understand the replication cycle of the virus and inform the development of future antiviral therapies, we investigated the hypothesis that HIV-1 changes the substrates and functionality of the RNAi system in specific contexts. To investigate our hypothesis, we first aimed to identify regulatory networks associated with blocks in virus replication during latency. We characterized a novel cellular model for HIV-1 infection that was designed to study events in HIV-1 latency reversion that follow transcription initiation. We then reactivated this model using differently acting latency reversing agents, to then sequence long and short RNA transcriptomes associated with latency maintenance and reversion. These data contributed new genes and regulatory RNA networks to our understanding of latency, and possibly new targets for RNA therapies. We next analyzed an interaction between the HIV-1 protein Gag and the RNAi enzyme Dicer, which leads to the specific enrichment of three microRNAs (miRNAs) on Dicer. A combination of bioinformatic analyses were used to identify the targets of these miRNAs and to define a target-specific hypothesis for the function of this interaction. Using gene reporter assays, Western blots and reverse transcription quantitative polymerase chain reactions, we explored one miRNA-target interaction in depth, showing that this miRNA inhibits HIV-1 expression and that Gag promotes viral expression by increasing expression of the target, possibly by inhibiting the antiviral miRNA. Finally, we sought to develop a testing platform to distinguish between RNAi substrates that could be used in combination therapies against HIV-1. We designed a novel protocol that can score RNAi substrates with four primary measures: cellular toxicity, inhibition of HIV expression, inhibition of HIV replication, and ability to lock proviruses in a latent state. Due to delays related to cloning, this protocol could not be fully employed in this manuscript. However, we individually tested the first three endpoints and used a surrogate fourth endpoint to make preliminary assessments of the therapeutic potential of several molecules. Here, we describe two workflows used to gain better insight into interactions between HIV-1 and the RNAi pathway, and further designed a protocol for testing small RNAs for their potential to lock the HIV-1 in a latent state.

Résumé

Plusieurs virus qui infectent les humains interfèrent avec la voie d'interférence par ARN (iARN). Bien que l'iARN reste fonctionnelle dans des cellules infectées par le virus de l'immunodéficience humaine (VIH-1), des protéines virales pourraient en modifier le processus et l'efficacité. Ici, nous explorons l'hypothèse que le VIH-1 modifie la fonctionnalité du système iARN dans des contextes spécifiques pendant l'infection. Nous avons d'abord cherché à identifier les réseaux de régulation associés aux blocages de la réplication virale pendant la latence. Notre groupe a récemment caractérisé un nouveau modèle de VIH-1 qui a été modifié pour éliminer des boucles de rétroaction qui amplifient l'expression virale et ainsi étudier les évènements de réactivation de latence après l'initiation de la transcription. Nous avons réactivé ce modèle dans des lymphocytes latents en utilisant différents agents de réversion de la latence. Ensuite, nous avons isolé en parallèle les transcriptomes entiers déplétés de l'ARN ribosomal et les transcriptomes de petits ARNs pour chaque condition. Ces données ont contribué à la compréhension de nouveaux réseaux régulatoires qui affectent la latence virale. Nous avons ensuite analysé une interaction que notre laboratoire a découverte entre la protéine Gag du VIH-1 et la protéine cellulaire Dicer, un membre de la voie de l'iARN. Nous avons effectué une immunoprécipitation avec un anticorps anti-Dicer, suivi de l'isolement de l'ARN (RIP) et du séquençage à grande échelle (RNA-Seq) pour identifier les microARN liés préférentiellement à Dicer dans les cellules exprimant Gag comparés à ceux trouvés dans les cellules sans Gag. Pour comprendre le rôle de ces microARNs enrichis avec Gag, nous avons identifié leurs cibles potentielles à l'aide de plusieurs analyses bioinformatiques. Nous avons ensuite confirmé l'activité des microARNs et de leurs cibles à l'aide de mimiques et d'inhibiteurs d'un microARN en combinaison avec des essais RT-qPCR, des gènes rapporteurs avec des fragments d'un ARNm cible prédit, et de *Western Blot* (WB). Nous avons testé l'effet d'un microARN sur l'expression du VIH-1 avec ou sans Gag et les effets indirects de Gag sur l'ARNm ciblé et sur l'expression du VIH-1 par RT-qPCR et WB. Nous montrons qu'en interagissant avec Dicer, la protéine Gag séquestre un microARN spécifique, ce qui diminue son efficacité sur sa cible et en conséquence augmente l'expression du VIH-1. Ces résultats suggèrent que ce microARN pourrait être utilisé en thérapie pour bloquer la transcription du VIH. Enfin, nous avons cherché à développer une plate-forme pour distinguer entre les microARNs et d'autres petits ARNs qui pourraient être utilisés dans des thérapies contre le VIH-1. Ici, nous avons conçu un nouveau protocole dans lequel

chaque petit ARN pourrait être testé pour sa toxicité cellulaire, son efficacité d'inhibition de l'expression du VIH-1, son efficacité d'inhibition de la réplication du VIH-1 et sa capacité à maintenir la latence. En raison de problèmes liés au clonage, nous n'avons pas pu utiliser ce protocole dans ce manuscrit. Cependant, nous avons pu tester les critères de ce protocole individuellement pour faire des évaluations préliminaires du potentiel thérapeutique de quelques molécules. En conclusion, nos travaux ont contribué à deux études qui permettent de mieux comprendre les interactions entre la voie de l'iARN et le VIH-1. En plus, nous avons commencé le développement d'un protocole pour tester les petits ARNs pour leur potentiel à empêcher la réactivation du VIH-1 latent.

Acknowledgments

I would firstly and most enthusiastically like to thank my supervisor, Dr. Anne Gatignol, for her availability, support and direction over the last three years of my scientific endeavors in undergraduate and graduate studies. Among my past and present professors and supervisors, Anne has had an outsized influence on my appreciation of the merits and demands of becoming a rigorous scientist. Anne encouraged my academic attraction to retrovirology and emerging infectious diseases, allowed me to see the light in the coming age of oligonucleotide therapies, and supported my interest and development in both bioinformatics and traditional wet lab techniques, all the while nurturing my love of science; a collective feat for which I am profoundly grateful.

I would also like to thank everyone else in the Gatignol lab for contributing to a friendly and supportive work environment, most notably including Aïcha Daher and Dr. Robert Scarborough, who contributed to important discussions on the direction of my research and helped me overcome challenges faced during the course of my experiments. I am likewise indebted to my encouraging academic advisor, Dr. Sonia del Rincon, my thesis committee members, Drs. Claudia Kleinman and Daniel Kaufmann, and numerous lab workers and administrative staff at the Lady Davis Institute and the Division of Experimental Medicine who helped me graduate from this degree unscathed. The same goes for my funders at the Canadian Institutes of Health Research, the Fonds de la recherche en santé du Québec, the division of Experimental Medicine and the Post Graduate Students' Society of McGill who helped pay for my studies and my attendance at conferences so that money worries did not plague my time in the lab.

Last but not least, I would also like to thank my family and every one of my close friends who helped me overcome the heightened challenges of being a graduate student during a drawn-out *annus horribilis* of pandemic lockdowns.

Preface and Author Contributions

This thesis was written in English according to the traditional format at McGill, beginning with a literature review on HIV-1 and the RNA interference pathway, followed by a study rationale and hypothesis broken into three specific aims. The materials & methods, conclusion and bibliography sections are not separated into aims, while the results and discussion sections are, except for a global overview that brings together the divergent aims. This thesis was written and edited under the guidance of Dr. Anne Gatignol.

The project for Aim 1 was conceived by Dr. Elodie Rance and Dr. Gatignol before the start of my graduate studies. Figure 7C was generated by Dr. Rance. The raw RNA-seq data used in my preliminary bioinformatic analyses were collected by Dr. Rance before I joined the lab, although I collected samples for this project to complete a biological replicate for our findings. RNA sequencing was performed at Génome Québec (McGill University). Bioinformatic analysis for this aim was performed by myself in collaboration with Dr. Bernard Mari and his student Marin Truchi at l'Institut de pharmacologie moléculaire et cellulaire (CNRS, Valbonne, France). Figure 9B & C were generated by Mr. Truchi, although I performed similar analyses separately. Figure 9D-F were generated by Dr. Mari using Mr. Truchi's data from Figures 9B & C, so Mr. Truchi's data were shown for consistency. All other figures are my own. Figures 5 & 6 contributed to a paper authored principally by Dr. Rance and Dr. Gatignol (under review), while Figures 7-9 will contribute to a second paper to be authored primarily by myself and Mr. Truchi that is still in data analysis steps.

The project for Aim 2 was conceived by Dr. Gatignol, Dr. Sergio P. Alpuche-Lazcano and myself, based on an interaction Dr. Alpuche-Lazcano had found between HIV-1 $p55^{Gag}$ and Dicer. Data for Figures 10A-E, 12C, 13A and certain replicates of 14D were provided by Dr. Alpuche-Lazcano, among which I plotted Figures 10B, 12C, 13A and 14D. All other figures, data and interpretations referred to in Aim 2 are my own. Findings from Aim 2 contributed to a paper authored by Dr. Alpuche-Lazcano and myself, which includes his discovery of the Gag-Dicer interaction and the RIP-seq/IP-RT-qPCR experiments from which we derived our hypothesis, as well as Figures 11-15 & 18, with certain modifications for publication (to be submitted shortly).

I conceived of the project for Aim 3 with the advice of Dr. Gatignol, Aïcha Daher and Dr. Robert J. Scarborough, based on a similar protocol designed by Dr. Ian Tietjen at the Wistar Institute (Philadelphia, USA) for testing small molecules against latency. All data and figures from Aim 3 are my own.

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List of Abbreviations

AAV: adeno-associated virus
AF9: ALL1-fused gene from chromosome 9
AFF1/4: AF4/FMR2 Family Members 1/4
Ago1-4: Argonaute proteins 1-4
AIDS: Acquired Immunodeficiency Syndrome
ANOVA: analysis of variance
ATP: adenosine triphosphate
AZT: Zidovudine, also known as azidothymidine
B&L: Block & Lock
BAF: BRG/BRM-associated factor
bp: base pairs
BP: Biological Process gene ontology
BSA: Bovine Serum Albumin
cART: combination Antiretroviral Therapy
Cas: CRISPR-associated
CCR5: C-C chemokine receptor type 5
CD4: cluster of differentiation 4
CDC: Center for Disease Control (USA)
CDK9: cyclin-dependent kinase 9
cDNA: complementary DNA
COVID-19: Coronavirus disease 2019
CRISPR: clustered regularly interspaced short palindromic repeats
CXCR4: C-X-C chemokine receptor type 4
CycT1: cyclin T1
dCA: didehydro-cortistatin A
DDX6: DEAD-box helicase 6
Dm: Dox GFP-
DMEM: Dulbecco's Modified Eagle Medium
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
Dox: Doxycycline
DPm: Dox prostratin GFP-
DPp: Dox prostratin GFP+
DPSm: Dox prostratin SAHA GFP-
DPSp: Dox prostratin SAHA GFP+
DSIF: DRB sensitivity inducing factor
DSm: Dox SAHA GFP-
DSp: Dox SAHA GFP+
dsRNA: double stranded RNA
dTTP: deoxythymidine triphosphate
EDTA: Ethylenediaminetetraacetic acid
ELL2: eleven-nineteen Lys-rich leukaemia protein 2
ENL: eleven-nineteen leukemia protein
Env: HIV envelope protein
EU: European Union
FACS: fluorescence-activated cell sorting
FBS: Fetal Bovine Serum
Gag: HIV uncleaved $p55^{Gag}$ polyprotein
GagPol: HIV uncleaved $p160^{GagPol}$ polyprotein
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GFP: Green Fluorescent Protein
GO: Gene Ontology
GTP: guanosine triphosphate
GW182: Glycine-Tryptophan protein of 182
HAT: histone acetyl transferase
HDAC: Histone Deacetylase
HDACi: HDAC inhibitor
HDF: host dependency factor
HEXIM1: hexamethylene bis-acetamide–inducible protein 1
HIV(-1 (M)): Human Immunodeficiency Virus (species 1, major group)
HMBA: Hexamethylene bisacetamide
HMT: histone methyltransferase
HMTi: HMT inhibitor
HSCT: haematopoietic stem-cell transplantations
HTLV: Human T-cell Leukemia Virus
ICAM-1: intercellular adhesion molecule 1
IN: HIV Integrase
IP: immunoprecipitation
IPA: Ingenuity Pathway Analysis (Qiagen)
IRES: internal ribosome entry site
kb: kilobase pairs
kDa: kilodalton
LPA: latency promoting agent
LRA: latency reversing agent
LTR: HIV long terminal repeat
MDM: Monocyte-derived macrophages
miRISC: miRNA-bound RISC
miRNA: microRNA

mRNA: messenger RNA
mTOR: mechanistic target of rapamycin
Nef: HIV negative factor
NELF: negative elongation factor
NeoR: neomycin/kanamycin/G418 resistance
NFAT: nuclear factor for activated T-cells
NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells
nt: nucleotides
p24: HIV capsid protein
PABP: poly(A)-binding protein
PANTHER: Protein Analysis Through Evolutionary Relationships
PBS: Phosphate-buffered saline
PEP: post-exposure prophylaxis
PIC: pre-initiation complex
piRNA: PIWI-interacting RNA
PMA: Phorbol 12-myristate 13-acetate
pre-miRNA: precursor miRNA
PrEP: pre-exposure prophylaxis
pri-miRNA: primary miRNA
P-TEFb: positive transcription elongation factor b
PTGS: post-transcriptional gene silencing
R: repeat element of the HIV LTR
R5: CCR5-tropic HIV-1
R5X4: CCR5 & CXCR4 dual-tropic HIV-1
Rev: HIV regulator of expression of viral proteins
RIN: RNA integrity number
RIP-seq: RNA immunoprecipitation followed by sequencing
RISC: RNA induced silencing complex
RLC: RISC-loading complex
RNA: Ribonucleic acid
RNAi: RNA interference
RNA Pol II/III: RNA Polymerase II/III
RNA-seq: RNA sequencing
RNP: ribonucleoprotein
RPMI: Roswell Park Memorial Institute medium
RRE: HIV Rev response element
rRNA: ribosomal RNA
RT: HIV reverse transcriptase
RT-ddPCR: reverse-transcription droplet digital PCR
RT-qPCR: reverse transcription - quantitative polymerase chain reaction
rtTA: reverse tetracycline-controlled transactivator
SAHA: suberanilohydroxamic acid, also known as vorinostat
SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEC: Super Elongation Complex
shRNA: short hairpin RNA
shRNAmiR: shRNA built onto an miRNA backbone
siRNA: small interfering RNA
SIV(cpz): Simian Immunodeficiency Virus (lineage found in chipanzees)
sncRNA: short non-coding RNA
snRNP: small nuclear ribonucleoprotein
Sp1: specificity protein 1
ssRNA: single-stranded RNA
TAR: HIV trans-activation response element
TasP: Treatment as Prevention
Tat: HIV trans-activator of transcription
TBST: tris-buffered saline with 1% Tween 20
TetO: tetracycline operator
TGS: transcriptional gene silencing
TRBP: TAR RNA binding protein
TSS: transcription start site
U3: unique region 3 of the HIV LTR
U5: unique region 5 of the HIV LTR
UNAIDS: United Nations programme on HIV/AIDS
USA: United States of America
UTR: untranslated region
Vif: HIV viral infectivity factor
Vpr: HIV viral protein regulatory
Vpu: HIV-1 viral protein unique
vRNA: viral RNA
VSV-G: Vesicular Stomatitis Virus Glycoprotein
WST-1: Water-soluble tetrazolium salt 1
X4: CXCR4-tropic HIV-1

Chapter 1: Introduction and Literature Review

1 The Human Immunodeficiency Virus (HIV)

1.1 The HIV/AIDS Pandemic: A Historical Perspective

1.1a Discovery

At the turn of the 1980's, physicians in New York City and California noticed an alarming outbreak of immune suppression in previously healthy young adults. These first clusters of patients presented with darkened skin lesions associated with a rare opportunistic cancer and/or pneumonia caused by an opportunistic fungus, although later cases would expand clinical presentations into a long list of opportunistic diseases (1-3). An important underlying marker that connected these cases was a severe and specific loss of T helper lymphocytes (Figure 1A) (4). Early reports remarked that afflicted patients were almost uniquely men who have sex with men, people who used injection drugs, haemophiliacs who required regular pooled blood transfusions or Haitian immigrants; the former three indicators suggesting that this new outbreak of immune deficiency could somehow be acquired sexually and percutaneously (2, 5, 6).

By January 1983, a group of virologists at the Pasteur Institute in Paris isolated and grew a virus from the enlarged lymph node of a patient at risk for the immunosuppressive disease (7, 8). This virus had an abnormal conical core and could reverse transcribe RNA into DNA, suggesting to this team that it was a new retrovirus. At the same time, a lab at the National Institutes of Health (Maryland) considered that the isolated agent was the Human T-cell Leukemia Virus (HTLV), a virus with a polyhedral core that enhances T-cell replication (9). HTLV, the first pathogenic human retrovirus, had recently been identified in 1980 based on key discoveries made in the previous decade of the reverse transcriptase enzyme and a growth factor that could maintain T-cells in culture (10-14). These discoveries gave precedence to identify the novel immunosuppressive agent as a human retrovirus and to study it in cells. Over several publications, the retrovirus described in Paris was finally confirmed in late 1984 to be a new virus and the cause of the epidemic of immune suppression (15-17). The novel retrovirus in question would be called Human Immunodeficiency Virus (HIV) and the disease was Acquired Immune Deficiency Syndrome (AIDS). Despite the characterization and culture of HIV in the early 1980's, an accessible cure remains elusive on the outbreak's 40th anniversary. HIV/AIDS has spread worldwide, affected sexual and social practices at large, become a major obstacle for the economic and social development of many African countries, and killed approximately 33 million people (18, 19).

1.1b Evolution & Classification

AIDS is caused by two retroviruses of the *Lentivirus* genus: *HIV-1* and *HIV-2*. HIV-1 is further subdivided into four groups: M, N, O and P. The 'major' (M) group is the only pandemic group of HIV and is the cause for the vast majority of current AIDS diagnoses, while groups N, O and P are still largely confined to the Congo Basin, where they were discovered (20, 21). Group M is further subdivided into subgroups with different pathogenicities and geographical prevalences (Figure 1B) (22). The four HIV-1 groups likely emerged in the human population as a result of distinct blood-borne cross-species transmissions of Simian Immunodeficiency Virus (SIV) from chimpanzees (M & N) or gorillas (O & P) to humans (20, 23, 24). Based on sequence identity, all known lineages of the less prevalent and locally constrained HIV-2 are thought to have originated from sooty mangabey monkeys, which harbour a species-specific lineage of SIV (SIVsmm) that is distinct from those most common in chimpanzees (SIVcpz) and gorillas (SIVgor) (20).

SIV rarely establishes transmissible infection in humans despite regular spillovers of SIV into bushmeat hunters in Central Africa (25). On rare instances when such a zoonotic infection takes hold in a human host, SIV can progressively adapt to the environment due to high mutation and replication rates common to lentiviruses (20, 26-28). Sampling of modern SIVcpz from chimpanzee species around Central Africa suggested that the SIV ancestor of HIV-1 (M) first entered humans from *troglodytes* chimpanzees in southeastern Cameroon (23). The first confirmed case of HIV-1 infection worldwide was a patient in 1959 in Léopoldville, what is now Kinshasa, the capital of the Democratic Republic of the Congo, whose preserved lymph tissue was retrieved in the 1990s for retrospective diagnosis (29). This preserved HIV-1 genome and a divergent genome sample from 1960 were used to constrain molecular clock models to estimate that HIV-1 (M) began circulating in the population near Léopoldville around 1920 (20, 21, 30). Between the 1920's and 1980's, HIV-1 (M) would spread and silently evolve into a devastatingly efficient group of human pathogens that would erupt into the pandemic we know today. Central Africa continues to retain the highest genomic diversity of HIV-1 worldwide (Figure 1B) (22, 30, 31). It is estimated that HIV-1 (M) circulated from Central Africa to the Caribbean in the mid-to-late 1960's and from there to New York in the late 1960's to early 1970's, explaining the higher incidence of AIDS among Haitian immigrants in the early American epidemic (27, 32, 33).

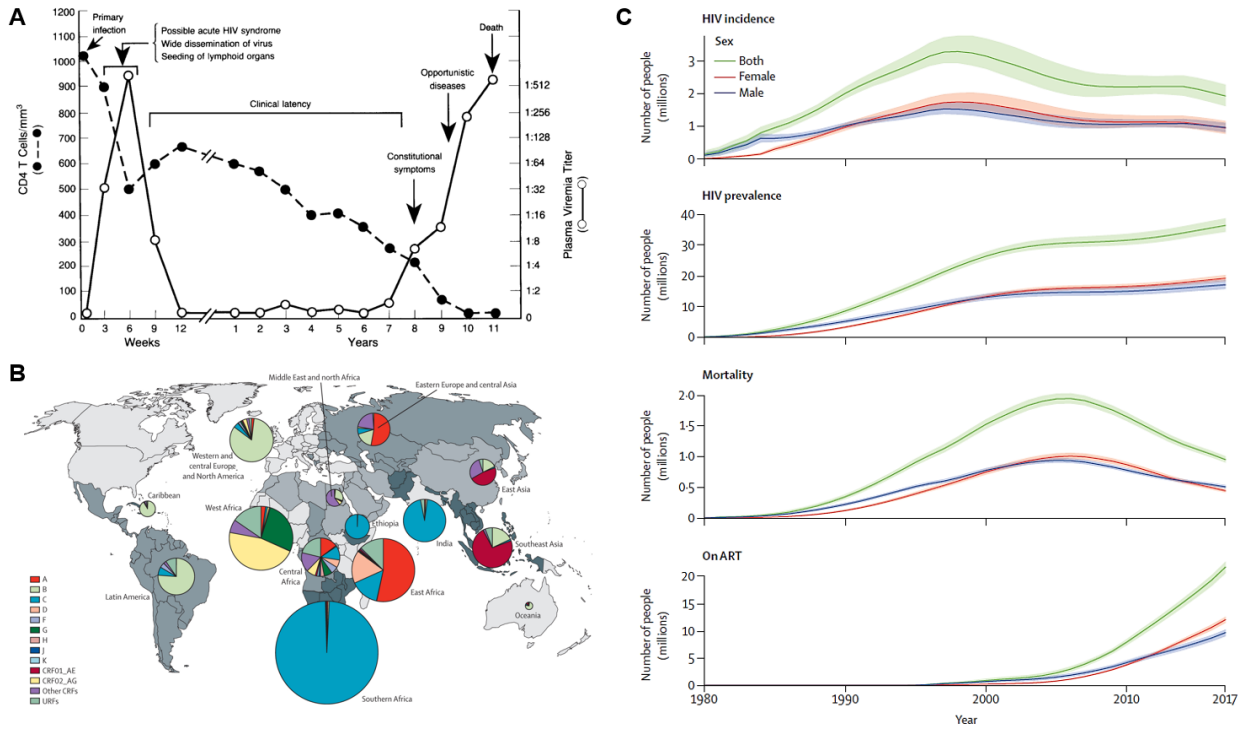


Figure 1: HIV/AIDS epidemics. A) Chart showing a typical course of HIV infection and development of AIDS following an acute phase of HIV replication and a long phase of clinical latency with a continually depreciating count of T helper cells ($CD4^+$ T-cells). Figure from Pantaleo, Graziosi & Fauci *N Eng J Med* 1993 (4). **B)** Regional abundances and distributions of HIV-1 (M) subtypes (A-K), circulating recombinant forms (CRFs) or unique recombinant forms (URFs) from data collected between 2010 and 2015. Figure from Hemelaar et al. *Lancet Infect Dis* 2019 (22). **C)** Global HIV incidence, prevalence, mortality, and people on ART, by sex, for all ages, 1980–2017. Shaded areas are 95% confidence intervals. Figure and legend from GBD 2017 HIV collaborators *Lancet HIV* 2019 (34).

HIV-1 rapidly mutates and can become a diverse population or "quasispecies" of variably mutated virus lineages within a single infected person before effective viral suppression is achieved with combination antiretroviral therapies (cART) (28). Rapid mutation is an important consideration for the development of antiretroviral drugs because in combination with pre-existing genetic diversity, this phenomenon is contributing to a rising prevalence of drug resistance in developing countries and has implications on vaccine development, diagnostic assays and responses to antiretroviral treatments (22, 35-37).

1.1c The Ongoing Pandemic

The rollout of effective cART beginning in the mid-1990's alongside a wide range of science-based societal, public health and medical interventions, have collectively brought down

the global rate of new HIV cases and AIDS-related deaths from their respective maxima in 1998 and 2004-2006 (Figure 1C) (34, 38). However, not everyone living with HIV knows their status, not everyone who knows their status is accessing sustained cART, and not everyone accessing sustained cART is able to achieve successful viral suppression (18, 39). HIV continues to infect more than a million people every year and remains most prevalent in Sub-Saharan Africa (18). The virus has recently been increasing in incidence in Eastern Europe, Central Asia, Latin America and several high-income countries including Canada (34, 38).

In 2014, the Joint United Nations Programme on HIV/AIDS (UNAIDS) proposed to the United Nations General Assembly a set of targets for ending AIDS as a public health threat by 2030, in which they included interim goals for 2020 (40). None of these interim goals were met in 2020 due to a combination of stagnating funding for continued interventions against the virus and an unequal landscape of support from governments that had agreed to these targets (34, 38). The COVID-19 pandemic dealt an additional blow to the UNAIDS' 2030 target in the form of an acute volatility of healthcare spending and support for people living with HIV (38). As the largest single cause of immune deficiency in humans, HIV adds a considerable burden to healthcare systems and can contribute to the emergence, re-emergence and evolution of other infectious diseases (41-45). HIV/AIDS should thus remain of concern to all those infected or not by the virus. If we hope to one day end AIDS as a public health threat, we must reverse the current trend of letting HIV/AIDS slip down the international agenda, we must continue to support evidence-based interventions in the prevention of HIV spread, and we must continue to research new ways of ending the pandemic.

1.2 The HIV-1 Virion and its Replication Cycle

1.2a The Viral Genome and its Products

Like other members of the *Lentivirus* genus, HIV-1 is an enveloped virus whose duplicated positive single-stranded (ss)RNA genome is enclosed by a conical core of capsid proteins in mature virions (Figure 2A). As part of the virus' replication cycle, the HIV-1 RNA genome is copied into DNA that can be integrated into the human genome to become a provirus. This proviral genome encodes nine principal genes (*gag*, *pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, *env* and *nef*) and is flanked by a pair of identical long terminal repeats (LTRs) that can be structurally subdivided into a region uniquely found at the 3' end of the ssRNA genome (U3), a repeat region found at both ends of the ssRNA genome (R), and a region unique to the 5' end of the ssRNA genome (U5) (Figure 2B).

The U3 region encodes a virus-specific promoter, enhancer and modulatory region; the R region spans the virus' +1 transcriptional start site (TSS) and an important RNA stem loop called the transactivation response (TAR) element; and the U5 region encodes several downstream transcriptional modulators.

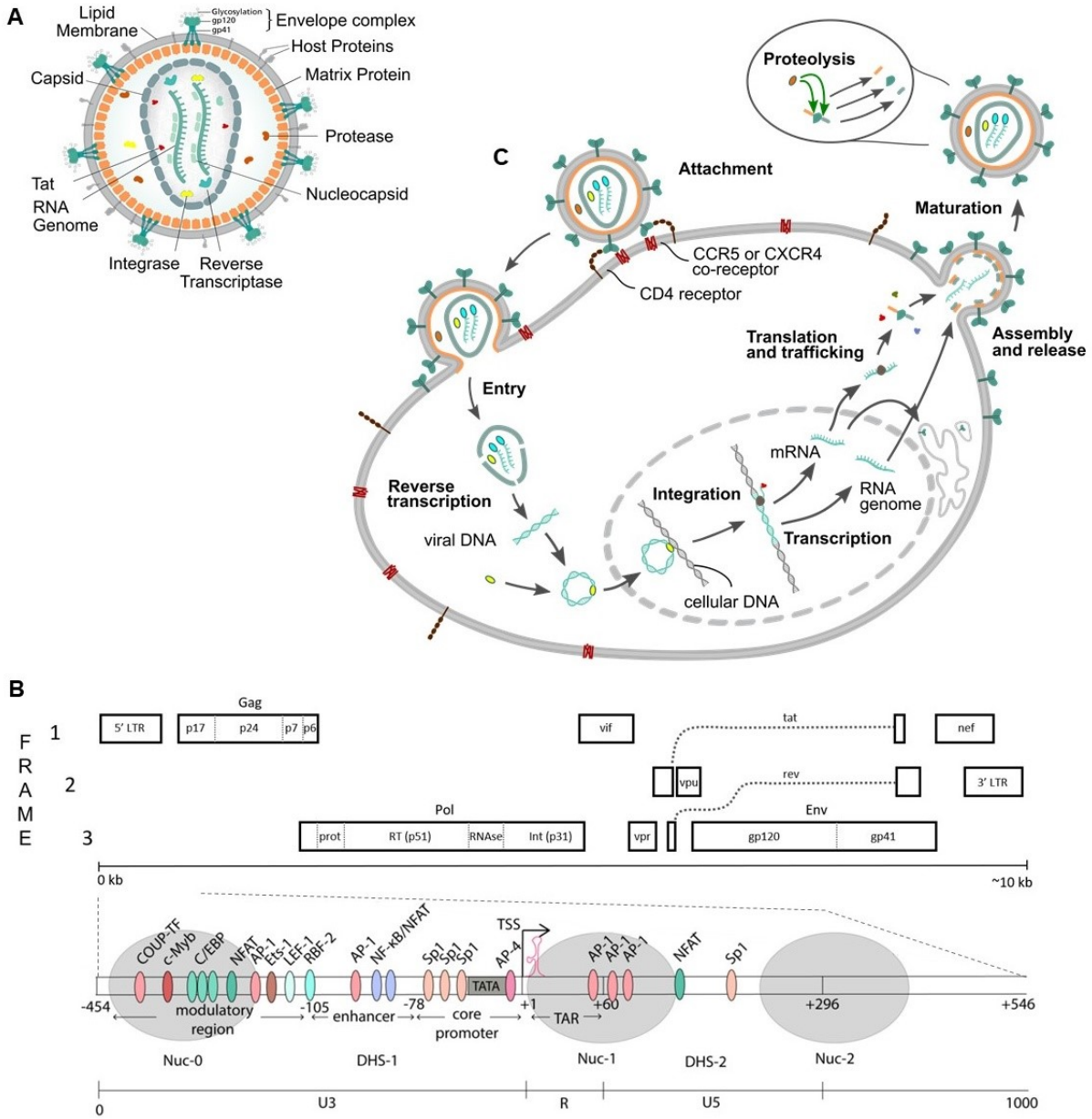


Figure 2: The HIV-1 virion, elements of its genome and its replication cycle. A) Components of the HIV-1 particle. Figure adapted from HI-virion-structure_en.svg (by Thomas Splettstoesser, CC BY-SA 4.0). **B)** Map of the HIV-1 genome adapted from reference locations on the HIV sequence database (hiv.lanl.gov). Below are annotated structural and functional regions of the LTR, adapted from Mori & Valente *Viruses* 2020 (46). **C)** Important events in the HIV replication cycle. Figure adapted from HIV-replication-cycle-en.svg (by Jmarchn, CC BY-SA 3.0).

The viral gene *gag* encodes a 55kDa polyprotein $p55^{Gag}$ (Gag), which is cleaved by the virus' protease into a nucleocapsid, a capsid (p24), a matrix protein and several smaller peptides. *pol* is translated as a frameshift readthrough product of the *gag* gene and so results in a less abundant larger $p160^{GagPol}$ (GagPol) polyprotein, which itself can be cleaved into the above Gag products in addition to an integrase enzyme important for integrating the reverse-transcribed genome into the host genome, the reverse transcriptase (RT) enzyme, and the protease. The *env* gene encodes a precursor gp160 protein, which is cleaved by cellular furin and furin-like proteases into two products (gp120 and gp41) that assemble into trimers of heterodimers on the viral envelope to bind and infect new host cells (47, 48). Accessory proteins of the virus perform the following functions: the viral infectivity factor (Vif) stimulates reverse transcription (49); the viral protein regulatory (Vpr) facilitates nuclear import of the pre-integration complex and induces apoptosis (50, 51); the trans-activator of transcription (Tat) is primarily a regulator of gene transactivation guided by the TAR RNA (section 1.2c) (52); the regulator of expression of viral proteins (Rev) facilitates the export of viral (v)RNAs from the nucleus into the cytoplasm by associating with an RNA secondary structure encoded in *env* called the Rev response element (RRE) (53); the viral protein unique (Vpu) inhibits cellular regulatory factors (54, 55); and the negative factor (Nef) enhances viral infectivity and modulates the expression of host proteins by engaging host trafficking pathways (56, 57).

1.2b The Replication Cycle

When HIV-1 enters a new host through blood or other body fluids, the first step in the viral replication cycle is for a virion to meet a target immune cell that carries cluster of differentiation 4 (CD4) receptors on its surface, including T helper cells (also known as $CD4^+$ T lymphocytes), monocytes, macrophages and dendritic cells (Figure 2C) (58-61). Here, gp120 subunits of the envelope complex on the viral surface interface with CD4, which causes a rearrangement of variable loops on the gp120 surface such that the complex can bind to one of two chemokine co-receptors on the cell surface: CCR5 or CXCR4 (58, 62). Common mutations in gp120 favour binding of HIV-1 envelope complexes to either CCR5, CXCR4 or both, corresponding respectively to the *R5 HIV*, *X4 HIV* and *R5X4 HIV* nomenclature of viral tropism (63). However, likely due to several host restrictions on X4 viruses, only R5 and R5X4 viruses are frequently sexually transmitted (64). Thus, R5 viruses predominate in early infection while X4 & R5X4

viruses only begin to predominate in later stages of infection (58, 65). Once both the host receptor and the co-receptor are bound, the viral envelope is either endocytosed or fuses with the host cell membrane and the core is released into the cytoplasm (66-68).

Within the cytoplasm, the viral core migrates towards the nucleus of the cell either using microtubules or by some other means (69, 70). While this is happening, the core transitions into a reverse transcription complex in which the viral RNA genome is copied into double-stranded DNA. Reverse transcription is effectuated by the error-prone viral reverse transcriptase enzyme in a complex set of transfer RNA and end-to-end priming steps that contribute to HIV population heterogeneity through mutation and recombination (71-74). Although the details of uncoating are not well understood, the reverse transcription complex gradually loses components during or after reverse transcription to become a pre-integration complex that associates with nuclear pore complexes (75, 76). Capsid proteins, other components of the pre-integration complex, the viral integrase and the DNA genome then translocate through nuclear pore complexes into the nucleus where integrase inserts the viral genome into accessible host DNA (77, 78).

In a favourable environment (section 1.2c), the proviral genome is transcribed into vRNAs including unspliced RNAs that are incorporated into virions and encode Gag and GagPol; incompletely spliced sub-genomic mRNAs encoding Env, Vif, Vpr and Vpu; and multiply spliced RNAs that encode Tat, Rev and Nef (79). Multiply spliced vRNAs are first shuttled out of the nucleus like cellular mRNAs until enough Rev accumulates to interact with RREs in unspliced and partially spliced vRNAs to overcome cellular restrictions on intron-containing RNAs exiting the nucleus (79). Sub-genomic vRNAs are transcribed into viral proteins either in the cytoplasm or across the endoplasmic reticulum in the case of gp160 (80).

The assembly and release of virus particles are both primarily coordinated by the uncleaved Gag or the larger unprocessed frameshift product GagPol. At the membrane, the Gag polyprotein can form spherical virus-like particles on its own, facilitate concentration of Env complexes on the envelope, recruit duplexed RNA genomes to budding virions, and signal to host endosomal sorting complexes required for transport (ESCRT) to release the virion from the plasma membrane (81). Envelope complexes are shuttled from the Golgi apparatus to the outer leaflet of the plasma membrane and are concentrated on virions by trans-membrane interactions with the matrix domain of Gag (81). Two full-length non-translating copies of the capped and polyadenylated RNA genome are incorporated into each virion as dimers by means of interactions between RNA

secondary structure packaging signals and Gag's nucleocapsid domain at the cell membrane (82-84). Virions carry cellular proteins on their envelopes alongside other host factors in their cores. The contributions of these host factors to the virus replication cycle are not clearly defined, with notable exceptions of host transfer RNAs used in reverse transcription and host intercellular adhesion molecule 1 (ICAM-1) that increases HIV-1 infectivity (71, 85).

Further steps of maturation are required after the virus buds from the cell before it can go on to infect a new cell (81, 86). The viral protease cleaves Gag into its separate protein fragments for the virus core to develop its signature conical shape given by the assembly of hexamers and pentamers of capsid proteins (81). During transcription, RNA processing and virion production/maturation, genomic RNAs begin with R-U5 regions and end with U3-R regions. The duplicate LTRs at either end of the viral DNA genome are completed as part of the reverse transcription process at the start of the next cellular infection so that the genome can be integrated into cellular DNA (71).

1.2c Regulation of Viral Transcription

HIV-1 has evolved many strategies to fine-tune virus gene expression through host regulatory processes, starting before the virus is integrated into the human genome. After HIV-1 enters the nucleus and before it is integrated into the human genome, recent data suggests that host nucleosomes are recruited to specified loci on the unintegrated virus genome that prevent transcription until the virus is integrated (87, 88).

The genomic environment surrounding a virus integration site can have a profound effect on its expression (89, 90). Although integration is not specific to individual sites in the human genome, the virus favours integration into genomic environments that are generally favourable to virus expression. The complete proviral genome is most often found in RNA Polymerase (Pol) II-dependent transcriptional units, in regions with high GC content, in regions with active histone marks, and in regions at the outer shell of the active T-cell nucleus; all of which are associated with transcription (78).

Once integrated, several factors determine whether the provirus will remain transcriptionally silent or will express viral genes (summarized in Figure 3A). The fate of transcription-competent viruses are regulated basally by epigenetic factors, such as the occupancy of nucleosomes at specific sites on the HIV-1 promoter, the recruitment of histone-binding

proteins, and DNA methylation (46, 78, 87, 90, 91). In patients receiving cART, intact proviruses are most often found in resting $CD4^+$ T-cells, in which the virus promoter is often associated with repressive histone deacetylases (HDACs) and histone methyltransferases (HMTs) (92-94). Following integration and in unfavourable environments for virus transcription, the ATP-dependent chromatin remodeler BRG/BRM-associated factor (BAF) positions a nucleosome at an energetically unfavourable site adjacent to the transcription start site (TSS), which contributes to a block in transcription elongation (46, 88).

Transcription initiation at the TSS is further subject to regulation by binding of numerous necessary, accessory and modulatory transcription factors at the 5'LTR (46, 95-97). Upon T-cell activation, transcription factors such as nuclear factor for activated T-cells (NFAT), specificity protein 1 (Sp1), and heterodimers of nuclear factor (NF)-κB bind to the promoter. With the help of Tat, these transcription factors recruit elements of the pre-initiation complex (PIC) and histone acetyl transferases (HATs) to remodel the nucleosome at the TSS and ultimately form an active promoter (46, 88, 93, 97-100). However, even in favourable conditions for transcription initiation, transcription of full HIV-1 transcripts is especially weak in the absence of Tat (101-103). Tat has been shown have a stronger effect on proviral expression than does cell state in computational, synthetic and patient cell models of infection (103).

The Tat protein has evolved several multitasking functions within the cell, including recruitment of HATs and the PIC to the HIV-1 promoter for initiation, recruitment of a BAF-related virus promoting remodeling complex to the TSS-adjacent nucleosome, and activation or repression of cellular genes (46, 88, 96, 97, 100, 104, 105). However, the most recognized function performed by Tat is the formation of an effective processive RNA Pol II complex for transcriptional elongation of paused ~60nt vRNAs by recruitment of the positive transcription elongation factor (P-TEFb) to TAR stem loops (52, 101, 106-110). When the promoter is open and active, RNA Pol II initiates transcription, but stalls after 50-60 nucleotides to form short transcripts containing TAR (111). These transcripts are often aborted and make up the majority of HIV-1 transcripts in cells isolated from patients on suppressive cART (112). This pausing is thought to be effectuated primarily by the inhibitory actions of DRB Sensitivity Inducing Factor (DSIF) and Negative Elongation Factor (NELF), two negative elongation factors bound to RNA Pol II that pause transcription at human genes following initiation (91, 109, 113-115).

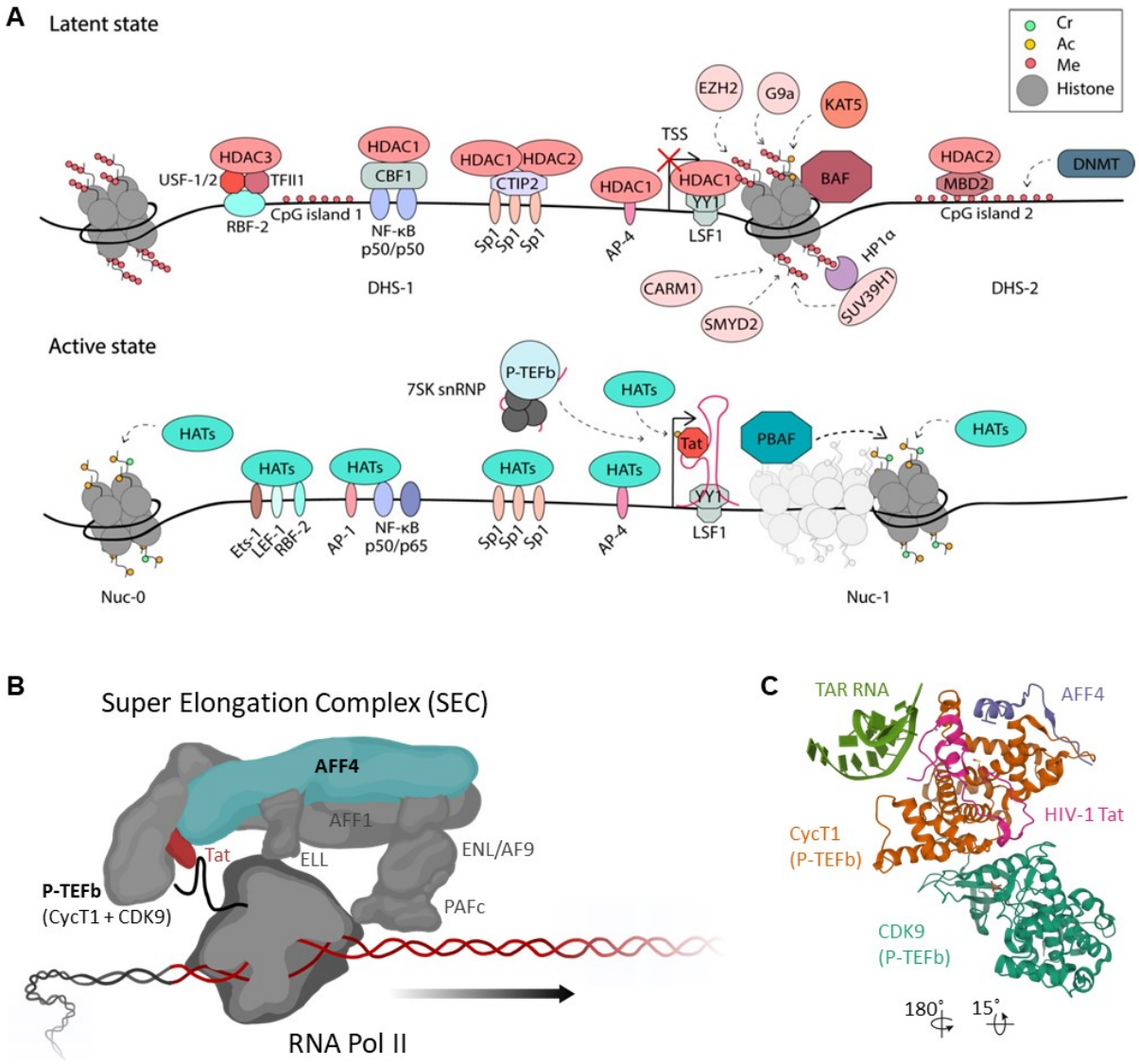


Figure 3: Regulation of HIV-1 expression. A) Simplified overview of some of the more important factors involved in the epigenetic regulation of HIV-1 transcription. Figure from Mori & Valente *Viruses* 2020 (46). **B)** Proposed model of transactivation by Tat/TAR through recruitment and stabilization of the SEC to paused TAR-containing RNAs. Drawing informed by He et al. *PNAS* 2011 (116), Chou et al. *PNAS* 2013 (117), Qi et al. *Nat Commun* 2016 (118) and Schulze-Gahmen & Hurley *PNAS* 2018 (119). **C)** Annotated ribbon diagram of TAR and Tat in complex with P-TEFb and a disordered region at the N-terminus of AFF4 that becomes ordered in this complex. Structure determined by X-ray crystallography to a resolution of 3.5 Å (PDB ID 6CYT) (119). Approximate rotation angles shown with respect to Figure 3B.

P-TEFb is a complex composed of cyclin-dependent kinase 9 (CDK9) and cyclin T1 (CycT1), which regulates the transition from transcription initiation to elongation by phosphorylating and thereby relieving the inhibitory effects of NELF and DSIF on RNA Pol II, as well as phosphorylating Ser2 residues of the C-terminal domain of the largest subunit of RNA Pol

II (96, 113, 120). P-TEFb has also been implicated in assembling complexes important for transcription initiation at the HIV-1 promoter (97). Transcription regulation, RNA interference, inhibitory kinases and the proteasome maintain low levels of available CycT1 and CDK9 in resting $CD4^+$ T-cells, while reactivation of T-cells increases these levels (121-124). In an active T-cell, P-TEFb can assemble, but its ability to promote transcription initiation and elongation is further fine-tuned by hexamethylene bis-acetamide-inducible protein 1 (HEXIM1), which recruits most free P-TEFb complexes onto the 7SK small nuclear ribonucleoprotein (snRNP) at inactive promoters for functional sequestration (96, 125). Signaling cascades and cell states including stress (apoptosis, ultraviolet light, transcriptional blockers), drug treatments and changes to cellular chromatin can release P-TEFb from the 7SK snRNP to return cells to homeostasis or shift the state of cell growth and differentiation (126, 127). However, regulation by HEXIM1 and 7SK is in equilibrium in cells and the release of P-TEFb leads to increased expression of HEXIM1 until P-TEFb is returned to the 7SK snRNP (126, 128). In the context of HIV-1, Tat can directly compete with HEXIM1 for P-TEFb and can further induce the non-degradative monoubiquitination of HEXIM1, liberating free P-TEFb from HEXIM1 to stimulate HIV-1 transcription (125, 129).

Tat forms two stable complexes with P-TEFb that can separately act to elongate HIV-1 transcripts. The first complex is composed of P-TEFb, Tat and the 7SK snRNP lacking HEXIM1 (109). The second complex is the host Super Elongation Complex (SEC), which is an important regulator of transcription elongation for host cell genes in the absence of Tat (91, 130). The SEC is composed of Tat, two different transcription elongation factors P-TEFb and eleven-nineteen Lys-rich leukaemia protein 2 (ELL2), and members of the eleven-nineteen leukemia/ALL1-fused gene from chromosome 9 (ENL/AF9) family, built onto a flexible scaffold of AF4/FMR2 Family Members AFF1 and/or AFF4 (Figure 3B) (91, 116, 118, 131-133). Without Tat, an intrinsically disordered N-terminal region of AFF4 comes together into a semi-stable complex with CycT1 to form a transient SEC (134). Tat associates with CycT1 and AFF4 in this binding groove and pulls TAR into close association with CycT1 to form an active SEC (Figure 3C) (119, 135). AFF4 binding stabilizes CycT1-Tat-TAR so that SEC-bound Tat-P-TEFb complexes have 50-fold higher affinity for TAR than do Tat-P-TEFb alone (135, 136). Furthermore, AFF4 inefficiently recruits ELL2 into fully functional SECs in the absence of Tat, while Tat increases the expression of ELL2 and recruitment of ELL2 to P-TEFb through AFF4, stimulating the assembly of competent SECs (118). AFF1/4-built SECs are thus important co-regulators of HIV-1 gene expression with Tat.

1.2d Reservoirs & Latency

In the early stages of infection in a new human host, HIV-1 virions associate with dendritic cells, which efficiently carry the virus to $CD4^+$ T-cells in lymph nodes as part of the canonical antiviral immune response (61, 137, 138). Virions then hijack immune synapses formed between mature dendritic cells and T-cells to infect this latter preferred target cell type (61, 137, 138). Most T-cells activated during infection die off quickly following acute stages of infection (Figure 1A), but sub-populations of infected cells survive and maintain diverse reservoirs of intact proviral genomes (139, 140). Resting memory $CD4^+$ T-cells remain the most well-defined reservoir cell type for HIV-1 proviruses, although naïve T-cells can also contribute to the reservoir population and there is some controversy as to whether macrophages can also contribute to persistence (61, 94). Resting T-cells are able to persist for years and may further maintain proviral reservoirs through low levels of proliferation and clonal expansion in organs with low cART penetration, although the contribution of compartmentalized replication is disputed (139, 141-143). Of importance to cure strategies, defective proviruses harbouring hypermutated genomes or large internal deletions rapidly make up the majority of integrated genomes in people living with HIV-1 (74). These replication incompetent proviruses contribute to a reservoir that can express viral epitopes and stimulate immune activation despite sustained cART, which may support proliferation of intact reservoir cells and may have long-term immunomodulatory consequences for people living with the virus (144). Although the intact reservoir declines over time, the average reservoir half-life in people on sustained cART is 3.6 years, and given the average estimated reservoir size at the start of cART, should be understood as being a life-long reservoir (145-147).

As cells that do not express viral proteins can avoid virus-mediated cytopathic effects and immune clearance, the persistence of the intact reservoir over years of sustained cART relies on low or no expression of vRNAs or virus antigens from intact genomes, a state broadly termed latency. Latency is an important subject of study for HIV-1 cure research but suffers from unclear and sometimes varied definitions. Since virus reservoirs exist as quasispecies in diverse cell subsets, diverse microenvironments and diverse chromosome locations; the latent reservoir should be understood as a variable population of infected cells in an array of different HIV-1 expression states ranging from “deep latency” with no vRNA expression, to low protein expression and defective virion production. To this point, there is no consensus about which state of expression is most common amongst latent populations. It was initially believed that transcriptionally silent

HIV-1 genomes make up the bulk of latent viruses, but a recent study by Yukl et al. using droplet digital PCR (RT-ddPCR) put this into question. By assessing the abundance of initiated, elongated, completed, polyadenylated and spliced vRNAs in treated primary T-cells from HIV-1-infected individuals, the authors reported that blocks to transcription initiation contributed less to reversible latency than did a series of blocks to transcription elongation, distal transcription, polyadenylation and splicing (112). Using assays targeting elongated vRNAs, a different group similarly found that significant fractions of infected cells in peripheral blood and lymph nodes express vRNAs during cART, indicating that regulatory events after transcription initiation contribute under-appreciated effects to latency (148, 149).

Latency is rarely sustained in individuals who do not maintain regular treatment. Sustained cART is effective at inhibiting reservoir proviruses from replicating and spreading, but it has been well described that most people who pause or terminate antiretroviral therapies eventually have a rebound in virus expression from seemingly stochastic events (150, 151). Proviral expression in reservoir cells can be triggered by drugs, or stochastically by a multitude of possible changes to the cellular microenvironment that may lead to changes in the regulation of transcription (section 1.2c) or translation (46, 94, 152).

1.3 Taming HIV

1.3a Current Therapies and Drug Regimens

Zidovudine (AZT) was the first antiretroviral drug approved for use in the United States against HIV-1 in 1987. AZT can safely prolong the lives of people living with HIV-1, but its use is associated with notable toxicities and it was soon discovered that drug resistance would rapidly develop against monotherapies using the drug (153). Progression to AIDS remained inevitable for most HIV-infected individuals until it was demonstrated in 1996/1997 that triple therapies including the newly approved nucleoside reverse-transcriptase inhibitor lamivudine could restore $CD4^+$ T-cells and suppress HIV-1 expression for up to a year without reversion (154). There are now approximately 30 unique antiretroviral drugs that have been approved for use in the USA, EU and Canada (155). These drugs broadly comprise seven classes that target different steps in the viral replication cycle (155-157). Modern therapies are largely effective at keeping HIV-1 expression at undetectable levels for most newly infected individuals and offer numerous combinations in the event that a first-line combination results in toxicities, resistance, or dosing

regimen issues (155, 156). Cabenuva, the first relatively safe and effective nanoformulated long-acting cART regimen, was recently approved for use in Canada and the USA in 2020 and 2021, respectively, providing an alternative once-a-month solution for individuals who have trouble adhering to daily regimens (158, 159). Year-long extended-release regimens are also being developed in animals to improve upon this most recent advancement (160). Importantly, the continued administration of cART in people living with HIV is an effective measure for inhibiting transmission between sexual partners, known as Treatment as Prevention (TasP) (157).

1.3b Prevention Measures

Effective drug combinations are not the only factors affecting a limited spread of HIV-1 (M) in regions where the spread of infection has been dwindling. In the absence of effective vaccines, many evidence-based measures have helped prevent new infections, including measures to promote increased screening and counselling for at-risk populations, condom use, male circumcision, needle exchanges for injection drug users, and prevention of spread by blood transfusion or from mother to child through screening and treatment (34, 157, 161). Pre-Exposure Prophylaxis (PrEP) and Post-Exposure Prophylaxis (PEP) composed of cART, can be used in individuals who are exposed to HIV and can inhibit early replication of the virus in a new host to prevent the establishment of a reservoir (157).

1.3c The Need for a Cure

Despite evidence that the above measures that can be pivotal to the strength of a public health campaign aimed at limiting the spread of new infections, there are still 38 million people living with HIV, of which only 68% are receiving ART of some form (18). With recent advancements in cART, most individuals living with HIV can look forward to a life expectancy approaching that of non-infected people (162). However, for a person to avoid progressing to AIDS and prevent transmitting the virus to sexual partners, they must follow a strict schedule of cART administration indefinitely. Furthermore, people living with HIV in areas with little access to reliable healthcare follow-up often have difficulties adhering to treatment regimens, while others fail to follow their treatment for other reasons such as overly complex dosing schedules, treatment related side-effects, stigma and depression (163, 164). Given the complexities, medical expenses and stigma associated with lifelong cART, people infected with HIV need and deserve a cure.

1.3d Strategies Towards a Cure

In order to eliminate the need for life-long drug administration, three major HIV cure strategies have emerged: to pharmaceutically reactivate virus expression in all reservoir cells for a controlled clearance of these cells by immune and/or cytotoxic effects (termed the "Shock & Kill" strategy); to excise the viral genome from latently infected cells using gene editing; and to functionally inhibit the reservoir in the absence of cART by preventing of virus expression ("Block & Lock" or "B&L") and/or more broadly by preventing the spread of infection (a functional cure).

Shock & Kill strategies have garnered much attention for their theoretical potential to eliminate reservoirs from infected individuals (termed a sterilizing cure). Various attempts at Shock and later Shock & Kill strategies have been made in clinical trials from the late 1990's onwards, using latency reversing agents (LRAs) that are known to reactivate latent HIV-1 proviruses to varied extents. Trials included LRAs such as chromatin modifying HDAC inhibitors (HDACis) and histone methyltransferase inhibitors (HMTis), P-TEFb agonists and transcriptional activators that stimulate activation of the HIV-1 promoter and T-cell proliferation signals through NF-κB (165-169). All human Shock & Kill trials to date have had limited success at reducing the size of the latent reservoir and no success at complete elimination (170, 171). A successful sterilizing cure would require highly effective reactivation of the host reservoir in order to ensure that no deeply latent proviruses remain, a challenge that has yet to be achieved. Highly targeted LRAs have recently shown promise in animal models of infection by stimulating reactivation in all reservoir tissues with limited toxicity, but these advanced LRAs have not yet been tested in humans (172). If these interventions prove effective at reactivating the reservoir, resulting high levels of virus expression might counterbalance the clearance of infected cells by re-seeding a larger reservoir if not properly managed with effective cART in difficult-to-penetrate reservoir tissues. A successful Shock & Kill intervention would thus also hinge on effective clearance of activated cells and virus particles, which has not proven to be a simple requirement in patients at risk for immune suppression and AIDS (173).

The second strategy to reduce the size of the reservoir consists of direct *in vivo* gene editing of reservoir proviruses. At present, the most commonly used technology for gene editing in experimental settings is CRISPR-Cas, an easily programmable RNA-guided system that cleaves sequences in the genome that are homologous to a ~20 nt sequence in guide RNAs carried by the CRISPR-associated (Cas) effector enzyme (174). CRISPR-Cas was developed as a gene editing

tool in 2012 and has since inspired the development of numerous modified Cas proteins that have different targeting-dependent functions or that have more precise genome editing capabilities than their predecessor, which are promising developments for an eventual HIV-1 sterilizing cure (175-177). A recent publication reported an unprecedented successful reduction of viral reservoirs in humanized HIV-1 infected mice treated sequentially with lipophilic reservoir penetrating long-acting slow effective release ART and an adeno-associated virus (AAV) vector carrying a *Streptococcus pyogenes* Cas in combination with multiplexed guide RNAs targeting conserved regions of the HIV-1 genome (178). This proof-of-concept study demonstrated that a reduction in reservoir sizes may be possible using gene editing technologies, however this combination is in early stages of research and optimisations will be needed to improve the delivery of this combination therapy in humans without sacrificing safety. Although *in vivo* gene editing technologies show promise for reducing the HIV-1 reservoir, these technologies remain in their infancy in the clinic. Over the coming years, gene editing approaches will have to overcome concerns over low delivery efficiency to a heterogeneous reservoir, construct immunogenicity, off-target effects and viral escape due to the quasispecies nature of HIV-1 populations (179).

Functional cure strategies have emerged as tractable alternatives to the above reservoir reduction/eradication approaches. Although interventions that merely repress viral rebound in patients may appear less attractive than those that have a potential to eradicate latent reservoirs, approaches taken in the near-term to negate the need for regular cART may reduce costs and associated side-effects for patients while the safety and efficacy of sterilizing cure technologies are improved for later combined efforts.

Functional cure strategies are inspired by a small fraction of HIV-infected individuals who naturally control virus replication in the absence of cART (elite controllers). Elite control is stratified into subgroups of individuals with more or less virus suppression, several of whom are known to have particularly small reservoirs and normal immune systems, and can thus be used as examples for how a functional cure to HIV infection may be achieved through a variety of interventions (180, 181). Similarly, a small fraction of people who previously had high levels of viremia, termed “post-treatment controllers”, can interrupt cART after many years of suppression and not suffer from viral rebound despite remaining silent reservoirs (182-184). It is not well understood how elite controllers and post-treatment controllers are differently able to suppress viral rebound without cART, however, it is believed that a combination of a strong host immune

response, virus integration sites unfavourable to transcription in remaining reservoir cells, and low starting reservoir size may contribute to these states (185-187). Recent data additionally suggests that intact HIV-1 genomes that have become transcriptionally silent and show multiple features suggestive of deep latency are selected for in patients receiving sustained cART and gradually become the majority of intact integrated genomes, which may help explain post-treatment control and may inform a B&L cure (188). Functional cure strategies base on heightened immune regulation of low-level reservoir proliferation are reviewed in (39, 94, 189).

Other biological precedence to consider in functional cure research are two HIV-infected individuals, Adam Castillejo and the late Timothy Ray Brown, who received allogeneic haematopoietic stem-cell transplantations (HSCT) from donors harboring a homozygous mutation in their CCR5 co-receptors (CCR5Δ32/Δ32) and subsequently remained in remission from reservoir reactivation for years following cART interruption (190-192). CCR5Δ32/Δ32 mutations are rare but not pathogenic in the human population and confer resistance to R5 HIV infection, suggesting that these two HSCT recipients maintained a stable HIV-1 remission by limiting the number of cells the virus can re-seed during low-level reservoir proliferation (190, 193). Unfortunately, HSCT can not currently be used for large-scale cure efforts because it is limited by the requirement for highly specialized medical personnel, by the high cost and high risk of the procedure and by the need for CCR5Δ32/Δ32 stem cells derived from rare immune-compatible donors or from expensive *ex vivo* gene therapy procedures (194). Furthermore, R5X4 and X4 strains of HIV made up no more than 2.9% of Brown's virus population before he received two rounds of full body irradiation and HSCT, while no X4 HIV proviruses were detectable among Castillejo's virus population before his irradiation-free HSCT (191, 192). No mutations in CXCR4 that confer resistance to X4 HIV have been reported to circulate non-deleteriously in the human population and targeting CXCR4 prevents proper maturation and differentiation of hematopoietic stem cells, so a similar treatment may not be possible for people living with large populations of R5X4 and X4 viruses (194, 195). Functional cure strategies that include the removal of susceptible HIV target cells are reviewed in (39, 189, 195, 196).

In addition to therapeutics that can augment the antiviral immune response and limit the number of HIV-susceptible cells, an effective combination functional cure may require elements that extendedly prevent virus expression from reservoirs at the level of transcription or translation. Several potential latency promoting agents (LPAs) have been proposed to deepen latency for a

Block & Lock cure approach, notably including didehydro-cortistatin A (dCA), a potent synthetic inhibitor of Tat-TAR interactions that has been shown to reduce vRNA expression from reservoirs and delay viral rebound in primary cells and in humanised mice; rapamycin and other non-eponymous inhibitors of the mechanistic target of rapamycin (mTOR), which reduce CCR5 & CXCR4 surface expression and inhibit CDK9 phosphorylation; several promising signaling pathway kinase inhibitors recently identified in high-throughput screens; and small RNA drugs that will be explored in the following sections (46, 195-202). Research into LPAs as possible curative therapies has only recently been given renewed interest after years of funding stagnation in favour of research into sterilizing cure approaches. Due to continued failures of Shock & Kill interventions and known limitations of current gene editing technologies, increased attention needs to be attributed to the identification of new LPAs and to the development of therapies that combine LPAs with advanced antiviral immunotherapies and/or cART with the aim of eventually eliminating the need for continued cART after a treatment schedule is complete.

2 RNA Interference (RNAi)

2.1 Complexity, Regulation and the Discovery of RNAi

Over long evolutionary time, organisms have diverged to effectuate countless biological roles and survive in varied environments. To adapt to fluctuations in surrounding conditions, organisms have evolved multilayered regulatory mechanisms that can define extremely complex regulatory programs and resulting cellular phenotypes, while making use of a relatively small number of genes and simple regulatory mechanisms. For example, the human genome encodes approximately 20 000 protein coding genes, while the human body is composed of tens of trillions of cells that exhibit innumerable distinct phenotypes and functions which can each change in response to external cues from the cellular microenvironment. Here, we explore RNAi, a critically important regulatory pathway in sculpting the transcriptome for development or homeostasis which appears to have been present in the last common ancestor of all eukaryotes and has left a distinct imprint on most of the genes in our genomes (203-206).

RNAi was first identified in 1998 by Andrew Fire and Craig Mello as a curious phenomenon by which long exogenous double-stranded (ds)RNAs showed potent above-stoichiometric post-transcriptional gene silencing (PTGS) of complementary genes in *Caenorhabditis elegans*, an occurrence which could not be solely explained by antisense RNA

hybridization (207). It was soon discovered that RNA silencing by dsRNAs was more effective than antisense inhibition by ssRNAs in invertebrates and in vertebrates, suggesting that RNAi was a convergent or conserved enzymatic system across eukaryotes (208). Despite this discovery, long (>30 bp) dsRNA stretches were not found to stimulate sequence-specific PTGS in mammalian cells because they triggered antiviral interferon signaling pathways and non-specific shutdowns of global protein synthesis (209-213). This non-specific restriction was overcome when a minimal length (~21 nt) dsRNA called a short interfering RNA (siRNA) was shown to effectively stimulate PTGS in mammals while remaining a far less important stimulator of cellular antiviral pathways than longer dsRNAs (214). That same year, microRNAs (miRNAs) were discovered as an evolutionarily conserved class of short regulatory RNAs that are transcribed from the genome and are processed enzymatically into short (20-22 bp) dsRNA constructs for PTGS (215-219). Over the last two decades since these discoveries, the RNAi pathway has been shown to readily incorporate both exogenous and endogenous RNAs to effectuate numerous regulatory functions in many eukaryotes, including fine-tuning gene expression, determining cell developmental cascades and defending against viruses and transposons (205).

2.2 Mechanism of RNAi

Three general classes of RNAi substrates exist in human cells. The largest and most diverse class of RNAi substrate short non-coding (snc)RNAs expressed in animal cells is a group of genome-encoded ssRNAs called PIWI-interacting (pi)RNAs. piRNAs feed into silencing effector proteins related to those used by both miRNAs and siRNAs, which are expressed abundantly in testes and primarily function to silence transposable elements and maintain fertility in mammals (220). It is disputed whether the piRNA class and their processing enzymes are expressed at sufficient levels in human somatic cells to be important in determining cell phenotypes, so these will not be further discussed in this thesis (220-222). Stretches of dsRNAs that are derived from genomic repeats, transcription of overlapping or otherwise complementary sequences, virus genomes or experimental constructs, can be processed into siRNAs that are loaded into RNAi machinery and can effectuate slicing of fully complementary RNAs in human cells (223, 224). Therapeutic siRNAs will be discussed further in later sections. miRNAs are the best-defined group of RNAi substrates, of which an estimated 2300 take part in regulating the human transcriptome and 519 meet stringent criteria to be called canonical miRNAs (219, 225, 226).

Canonical miRNA biogenesis in humans begins with the transcription by RNA Pol II of primary (pri-)miRNAs; long non-coding RNA transcripts composed of single or multiple inverted repeats that fold over each other to form bulged stem loops (Figure 4A). While most pri-miRNAs are non-coding RNAs whose only known function is to be processed into miRNAs, more than a quarter of well conserved miRNAs are derived from the introns of protein-coding genes (227). pri-miRNA stem loops that will eventually go on to form miRNAs are most often 35 ± 1 bp, contain up to four conserved recognition motifs, and are flanked by ssRNA segments (205, 218). These stem loops are recognized by the Microprocessor, a heterotrimeric nuclear complex that contains the endoribonuclease Drosha and two molecules of its essential binding partner DGCR8 which ensures the fidelity of stem loop processing (218, 228). Drosha has two RNase III domains that each cut a flanking strand from either end of the pri-miRNA-encoded stem loop to liberate ~60 nt imperfectly paired hairpin RNAs called precursor (pre-)miRNAs (218, 228). pre-miRNAs are then shuttled out of the nucleus by Exportin 5 and RAN-GTP in a GTP-dependent manner (218).

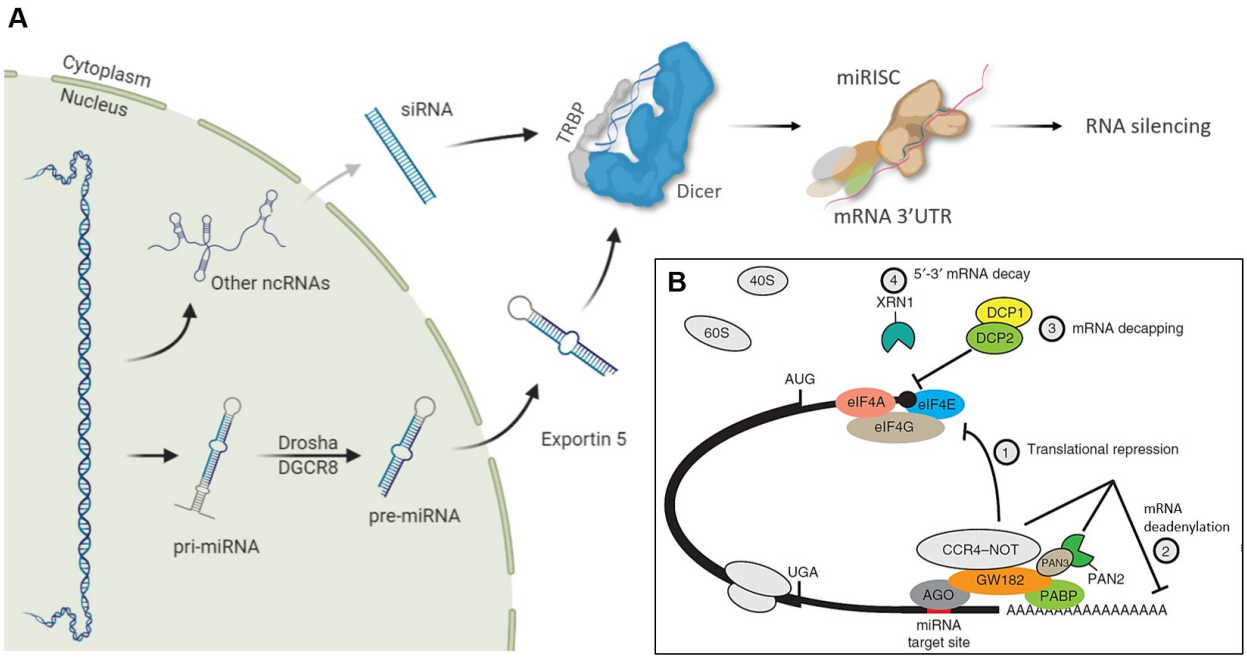


Figure 4: Fundamental mechanisms of RNA interference. A) Simplified schematic of the miRNA biogenesis pathway including processing of pri-miRNAs into miRNAs, as well as processing of siRNAs from the genome or external sources. dsRNAs are loaded into Dicer by TRBP and are cleaved by Dicer before being loaded in a strand-dependent fashion onto Argonaute for RNA silencing. **B)** Dominant steps in miRNA-RISC repression of mRNAs, including 1) direct repression of mRNA cap-dependent translation by CCR4–NOT with the help of DDX6, 2) mRNA deadenylation primarily by CCR4–NOT with additional contributions from PAN2-PAN3, 3) mRNA decapping, and 4) exonuclease-mediated mRNA decay. Figure 4B from Duchaine & Fabian. *Cold Spring Harb Perspect Biol* 2019 (219).

In the cytoplasm, pre-miRNAs are recognized by the ~1900 amino acid endoribonuclease Dicer and a binding partner involved in substrate selectivity, cleavage site determination and strand selection; the HIV-1 TAR RNA-binding protein (TRBP) or its homologue PACT (229-232). This complex removes the terminal loop of pre-miRNAs to form short duplexes consisting of an miRNA and its complementary passenger strand. Dicer can then interact with one of four Argonaute proteins (Ago1-4) to form an RNA-Induced Silencing Complex (RISC)-Loading Complex (RLC) consisting of Dicer, Ago, the duplexed RNA, TRBP/PACT and other possible cofactors, although it is debated whether the RLC is essential to load canonical dsRNAs into Ago in humans or whether certain miRNAs are ejected from Dicer and loaded into Ago separately (218, 233, 234). miRNAs are loaded into Ago with the help of chaperone proteins (HSC70/HSP90), which use ATP to temporarily open a binding pocket on Ago that is relaxed after loading to kick out or slice the passenger strand (218). While either strand of an miRNA duplex can be loaded into Ago for effective RNAi, the Ago binding pocket preferentially associates with whichever miRNA is less thermodynamically stable at its 5' terminus, among other less important factors (218). As Ago can accept either strand, miRNA naming conventions include the end of the pre-miRNA stem loop from which the miRNA is derived. For example, "mmu-miR-155-5p" is the 5' miRNA from mouse pre-miRNA 155 and "hsa-miR-766-3p" is the 3' miRNA from human pre-miRNA 766.

The 'minimally necessary' RISC components in mammals are Ago2 and a loaded ssRNA, as Ago2 has retained its ancestral ability to 'slice' target strands that have extensive complementarity to the loaded ssRNA (205, 219, 235, 236). Slicing is the primary silencing mechanism used by siRNAs, but not by miRNAs (205, 219, 235). Most miRNA-bound RISC (miRISC) targets in the genome are the 3' untranslated regions (3'UTRs) of genes. These 3'UTRs often retain conserved sequences that hybridize with 'seed' regions ranging miRNA nucleotides 2-8 and occasionally also retain pairing sites for supplementary 3' miRNA nucleotides, but mRNA target sites rarely ever retain complete complementarity to expressed miRNAs (204, 205, 219). It has been estimated that more than 60% of mammalian protein coding genes are under selective pressure to maintain pairing to one or many miRNAs in these regions, supporting the importance of miRNA regulation through seed-pairing in mammalian gene regulation (204). As seed pairing is not sufficient to catalyze slicing by Ago2 or the other Ago species, additional RISC binding partners are needed to effectuate miRNA silencing. The Glycine-Tryptophan protein of 182 kDa (GW182) coordinates this task by directly binding to Ago1-4 and acting as a scaffold protein for

several silencing effectors (219, 237). GW182 binding partners include the poly(A)-binding protein (PABP), several decapping and translation repressing enzymes such as DEAD-box helicase 6 (DDX6), and two deadenylation complexes: poly(A) nuclease (PAN)2-PAN3 and CCR4–NOT (205, 219, 237). There is still much to be explained about how individual GW182-binding effectors affect the kinetics of silencing, although it has been shown that miRISC silencing begins in cells with translational inhibition, effectuated by several activities of DDX6 (recruited indirectly to GW182 by CCR4-NOT); by a dissociation between PABP and translation initiation factors; and by other less well explained mechanisms (205, 219). Inhibition of translation is soon followed by mRNA decay through further deadenylation, decapping and exonuclease activity, making up the more dominant mode of miRISC-mediated repression than translation inhibition in post-embryonic cells (Figure 4B) (205, 219, 238). In mammals, this silencing is largely effectuated in glycine- and tryptophan-rich liquid-liquid phase-separated cytoplasmic foci alternatively called GW bodies or mammalian processing (P) bodies (239).

A less studied and less understood sncRNA-mediated silencing mechanism in mammals is transcriptional gene silencing (TGS), which in contrast to canonical RNAi, can induce stable and long-term epigenetic locks on gene expression that can be passed on to daughter cells during cell division (240). TGS can be induced by dsRNA species including miRNAs and siRNAs, as well as antisense ssRNAs (240). Although the mechanisms involved in TGS are yet to be described in detail, both miRNAs and siRNAs that are complementary to promoter sequences effectuate TGS by associating with Ago1/2 and recruiting HMTs, HDACs and DNA methyltransferases to targeted promoters in the nucleus (240-245)(Reviewed in Goguen RP et al. Submitted). Data from human cells suggest that TGS complexes initiates the silencing of promoter regions by hybridizing with low-abundance promoter-associated RNAs (produced largely by the initiation of RNA Pol II in the opposite orientation from that of normal transcription at active promoters), rather than invading dsDNA (246-249).

2.3 RNAi as Treatment

2.3a Manipulating RNAi

RNAi can easily be employed in experimental or therapeutic settings to knock down genes with high specificity by the introduction of a short double-stranded RNA substrate into cells. In an experimental setting, RNAi is thus a useful and simple alternative to mutagenesis or directed gene

editing that allows for the study of transient or permanent loss-of-function phenotypes for chosen genes (208). In the clinic, this ease of design translates to a highly flexible drug platform that can reduce the expression of deleterious protein coding genes and ncRNA targets that might otherwise not have the appropriate binding pockets to be 'druggable' by small molecule drugs (250, 251).

For experimental purposes, one of the simplest ways to test an siRNA of choice against its target is to express the siRNA from a plasmid or viral vector directly from an RNA Pol III promoter as a short hairpin (sh)RNA (224, 252). shRNAs can also be expressed by RNA Pol II on miRNA backbones (shRNAmiRs), which are processed by the Microprocessor as pri-miRNAs and have been shown to more effectively knock down target genes with fewer cytotoxic effects than RNA Pol III-transcribed shRNAs (253, 254). Exogenous chemically modified siRNAs can also be synthesized *ex vivo* as overlapping oligonucleotides and introduced into cells or animals if the use of vectors is not possible. Overexpression of endogenous miRNAs can be studied or used therapeutically in a similar way by the introduction of oligonucleotide miRNA mimics or RNA Pol II expression vectors. Antisense oligonucleotide seed targets, sometimes called antagomiRs or antimiRs, can alternatively inhibit miRNA/siRNA activity by crowding out the effects of specific RISCs, allowing for a temporary disinhibition of the sncRNA's target genes (224, 255, 256).

For clinical purposes, the first RNAi therapy to have been approved for systemic delivery in humans was patisiran (Onpattro; Alnylam Pharmaceuticals), a chemically modified siRNA delivered intravenously in a lipid nanoparticle for the treatment of hereditary transthyretin amyloidosis with polyneuropathy. This drug was approved by USA and Canadian regulatory agencies in 2018 and 2019 respectively and was soon followed by several other oligonucleotide drugs with similar nucleic acid modifications and particle formulations (224, 257). Recent advancements in biochemistry have led to new understandings of RNA modifications and nanoparticle formulations that can improve tissue-specific siRNA delivery and knockdown efficiency, while limiting construct immunogenicity and toxicity, paving the way for further developments in the field (224, 257). No viral or nanoparticle-packaged plasmid vectors have yet been approved for systemic RNAi delivery in clinical settings. However, these therapies may soon become available, as recombinant tissue-specific AAVs and other similar delivery vectors that can stably express gene therapies in post-mitotic cells without replicating or integrating into the host genome, have repeatedly been shown to have limited immunogenic effects and effective tissue-specific delivery of genes (258-260). Delivery of RNAi substrates to lymphocytes may prove to

be more difficult because of their diffuse dissemination in the body, but advancements in systemic viral and non-viral delivery techniques are promising (260-262). Modified stem cells expressing RNAi substrates from immobile vectors have also been explored for use in HSCTs, often in therapies targeting HIV infection (224, 263-265), but these *ex vivo* edited cells have yet to reach large cohort clinical trials for antiviral treatments. As research into the mechanisms of RNAi began just over two decades ago, RNAi treatments are only now starting to reach the clinic. Although underdeveloped at present, these sequence-specific therapies hold great potential as part of a new era of oligonucleotide drugs.

2.3b Applications of RNAi in HIV-1 Treatment

Within a year of the development of RNAi in mammals, early studies showed that siRNAs could be designed to target HIV to directly inhibit viral expression and replication, stimulating a wave of optimism about future antiviral RNAi therapeutics (266-270). Unfortunately, it was soon discovered that the HIV-1 genome could rapidly overcome PTGS restriction by maintaining a high mutation diversity in quasispecies populations and by adopting a complex secondary structure that is refractory to RISC binding (196, 270-276). Different therapeutic design strategies emerged to increase the threshold for viral resistance, including combining multiple directly antiviral RNAi substrates into a single multivalent treatment (277-281); targeting evolutionary restrained and accessible regions of the viral genome (196, 282, 283); and targeting host dependency factors (HDFs) that promote viral replication rather than directly targeting the virus (196, 264, 265, 284-288). Several therapies intended to support a functional cure that include RNAi components are now making their way through clinical trials, all of which are in the form of HSCTs of cells transduced with lentiviral vectors. These include a tat/rev-specific shRNA, a CCR5-specific ribozyme, and a TAR decoy (NCT01961063, NCT02337985, NCT00569985); a CCR5-specific shRNA, a TAR decoy and a chimeric mimic of the antiviral host TRIM5α protein that binds to the viral capsid and inhibits ingress/integration (NCT02797470) (289); a CCR5-specific shRNA and multiplexed shRNAs targeting the HIV-1 genome (NCT03517631); or a CCR5-specific shRNA alongside a fusion-inhibiting peptide (C46) (NCT03593187, NCT02390297, NCT01734850).

Another RNAi strategy tested for a functional cure to HIV-1 is to target the HIV-1 promoter via TGS to inhibit the early epigenetic and transcriptional steps involved virus reactivation (241, 242, 244, 290-292). As a proof of concept for this mechanism, HIV-1 TAR RNA can be fed into

RNAi machinery and has been implicated in negative feedback TGS of the HIV-1 promoter, indicating a potential virus mechanism for favouring latency establishment (293, 294). However, conflicting reports have contested the importance of such virus-derived RISCs due to their low abundance (276). The most developed investigation into the induction of deep HIV-1 latency using TGS was a series of publications that used two non-overlapping si/shRNAs, PromA and 143 that targeted regions of the HIV-1 5'LTR upstream of the TSS (241, 295). Independently, PromA can lead to long-term HIV-1 promoter-specific epigenetic regulation *in vitro* and can reduce HIV-1 reactivation *in vivo*, leading to the preservation of several subsets of hematopoietic cells from cytopathic effects, including bone marrow stem/progenitor cells and $CD4^+$ T cells (296-299). In combination *in vitro*, siPromA and si143 were able to resist LRA-mediated viral reactivation by maintaining epigenetic repressive marks at the virus promoter (292). Although TGS using combined promoter-targeting sncRNAs shows promise in experimental models, further *in vivo* research is needed before TGS-guiding therapeutic constructs can be attempted in humans.

2.3c Treatments Informed by RNAi-Virus Interactions

Another way of identifying HDFs and effector molecules that might be useful in antiviral RNAi therapeutics is to explore interactions between RNAi pathway members and HIV-1 during infection. By identifying miRNA regulatory networks that are correlated with reactivation or maintenance of latency, a systems biology approach can be used to identify host pathways, network functions and environmental determinants of virus fate that can later be targeted or appropriated in directed treatments or in miRNA treatments. Numerous studies have explored the dysregulation of miRNAs in different stages of HIV-1 infection and latency establishment or reactivation *in vivo* and *in vitro* (122, 276, 300-307). However, these explorations have largely identified miRNAs that directly target HIV-1 transcripts. Among these, miR-29a has received outsized attention for its ability to target a conserved region of the HIV-1 3'UTR (308). miR-29a has repeatedly been shown to inhibit HIV-1 expression and replication *in vitro* and has been shown to be controlled by an antiviral miR-29a-specific immune signaling cascade *in vivo* (308). Numerous directly antiviral miRNAs have been identified since, the most promising candidates of which are listed in (309, 310). Although directly antiviral miRNAs have received much attention, it is important to caution that the therapeutic relevance of these miRNAs have been put into question for similar reasons to previously mentioned siRNA therapeutics that directly target

vRNAs: HIV-1 genomes are mutationally diverse and retain complex secondary structures that are refractory to RISC targeting (275, 276, 306, 311). In fact in experiments that sequenced RNAs bound to the RISC in HIV-1 infected cells, vRNAs were heavily underrepresented (50-160 fold) with respect to cellular RNA reads, likely due to virus evolution and/or RNA secondary structure (276). The few reads that mapped to the HIV-1 genome in these experiments were found to be clustered into four specific binding sites, which correspond to direct binding sites for miR-29a, miR-155, miR-301a and miR-423 (276).

More promisingly, multiple publications have shown that the inhibition of Dicer and DGCR8 in infected and/or latent T-cells augments HIV-1 expression, suggesting that the RNAi pathway might at least indirectly maintain HIV-1 latency by regulating HDFs at non-cytotoxic levels (301, 312). One such study demonstrated that a pri-miRNA that encodes several miRNAs including miR-17 and miR-20a was substantially downregulated upon HIV-1 infection (301). miR-17 and miR-20a were then shown to regulate among other host factors PCAF, a histone acetylase and Tat cofactor known to enhance HIV-1 gene expression (301). Another study identified eight miRNAs that were affected by latency reversal, among which miR-155 showed the most potent anti-reversal effect in their cellular model of latency. Interestingly, miR-155 was principally active in reactivated cells, suggesting that the miRNA promotes a 'return-to-latency' effect on replicating genomes rather than a latency maintenance effect for non-reactivated genomes (312). miR-155 has been suggested to promote HIV-1 latency through an NF-κB-stimulating agent (TRIM32), alongside four HDFs involved in trafficking and/or nuclear import of the HIV-1 pre-integration complex (ADAM10, TNPO3, Nup153, and LEDGF/p75) in addition to directly regulating an accessible binding site on the HIV-1 genome (276, 312, 313). A different group was able to identify a latency promoting miRNA by directly exploring regulators of previously identified HIV regulatory mechanisms. The activation of memory $CD4^+$ T-cells upregulates CycT1 protein expression independently of an increase in its mRNA levels, which suggests some form of post-transcriptional regulation of the gene in resting T-cells independently of mRNA levels (314, 315). Chiang et al. quantified miRNAs differentially expressed in resting or activated T-cells and filtered these for their predicted regulation of CycT1 (122). By these means, miR-27b was identified as a direct modulator of CycT1 and a potent inhibitor of HIV-1 expression and replication (122). Treatment of primary resting T-cells with miR-27b antimiRs led to an increased reactivation of HIV-1 expression, suggesting that its targets including CycT1 may

be useful markers to study. Several other latency promoting miRNAs and targets have been identified in similar studies, which are reviewed in (308-310).

RNAi can interfere with numerous host regulators of HIV-1 infection, integration, latency disruption and replication, both via artificial inhibitors and via inhibitors already present within the cell. More research is needed to understand the sequences and targets by which siRNAs, shRNAs, miRNAs and antagomiRs can maintain or reverse HIV-1 latency before effective combination antiviral therapeutics can be developed.

3 Hypothesis and Objectives

3.1 Rationale

Achieving a cure of HIV infection will require a deep understanding of fundamental mechanisms that lead to either latency or productive infection. As an effective sequence-specific regulatory pathway present in our cells, the RNAi pathway may become a critical component in a curative solution for people living with HIV. At present, interactions between HIV-1 and the RNAi pathway remain poorly defined and the processes that determine whether an RNAi therapy will be effective against HIV-1 are not well understood. To better understand host-virus interactions used during the virus replication cycle and to better inform the development of future RNA therapies, we investigated the hypothesis that HIV-1 changes and/or is changed by the substrates and functionality of the RNAi system in specific contexts during infection.

3.2 Aim 1: Identifying Networks Associated with Blocks in Virus Expression during Latency

The characterization of RNA transcriptomes associated with latency in distinct models of infection can help identify common factors and pathways that are important in the maintenance or reversion of latency. Several studies have explored host mRNA profiles associated with HIV-1 latency and reversion in primary cells and cell-line models of infection (316-320), though few have been able to distinguish between steps involved in latency reversion. Therefore, RNAi regulatory networks involved in latency remain poorly defined. Here, we describe and characterize a novel cellular model of HIV-1 latency that uses a Tat/TAR-deficient reporter lentivirus which permits the study of post-initiation events in HIV-1 transcription. Once characterized, we reactivated this model using LRAs targeting different pathways in latency reversion and sequenced rRNA depleted

transcriptomes in parallel with short RNA transcriptomes associated with reactivation by each treatment. We hypothesized that host genes and short RNAs associated with post-initiation events in HIV-1 latency reactivation studied here would show unique signatures distinct from those previously identified in studies exploring Tat/TAR intact models of latency.

3.3 Aim 2: Determining the RNA-Mediated Downstream Effects of an Interaction between HIV-1 Gag and the RNAi Protein Dicer

Our lab recently discovered an interaction between Gag and Dicer that leads to the specific enrichment of three miRNAs on Dicer in Gag-transfected cells. The purpose of this Gag-Dicer interaction was not clear, but the resulting enrichment of specific RNAs on Dicer implied that a Gag-mediated post-transcriptional regulation of specific miRNAs in the RNAi pathway may influence the HIV-1 replication cycle. We hypothesized that Gag evolved to interact with Dicer, which may consequently affect regulatory events in HIV-1 expression or processing. To answer this hypothesis, we investigated enriched functions among the gene targets of these miRNAs and further explored one miRNA-target pair in detail.

3.4 Aim 3: Developing an HIV-1 Locking Strategy Using ncRNAs

Studies to date have largely ascribed latency maintenance functions to specific RNAs by reporting the inhibition of LRA-mediated reactivation from latency during the transient expression of these therapeutic RNAs. Such experiments can show the potential of specific RNAi substrates for preventing drug-mediated latency reactivation, but it is unclear whether these assays correlate well with the therapeutic potential of these sequences for the long-term suppression of stochastic proviral reactivation events. Research into Block & Lock interventions will need to re-define the experiments used to identify potential LPAs. We therefore designed a novel protocol that can score shRNAs and miRNAs together in a single experiment with four primary scoring endpoints: inhibition of HIV-1 expression, inhibition of HIV-1 replication, the ability to lock HIV-1 proviruses in a latent state, and cellular toxicity in multiple cell models.

Chapter 2: Materials and Methods

Cell Culture

HEK 293T cells (ATCC CRL-11268) were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Hyclone) supplemented with 10% heat-treated fetal bovine serum (FBS) (Hyclone) and 100 U/mL Penicillin & Streptomycin (Gibco). Jurkat (ATCC TIB-152), J-Lat 10.6 (321), CEM T4 GagzipGFP and THP-1 GagzipGFP cell lines were maintained in RPMI-1640 (Hyclone) with identical supplementation (complete RPMI).

To differentiate monocytes, THP-1 GagzipGFP cells were plated in 6-well plates at 5×10^5 cells/well in complete RPMI supplemented with 200 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich). After 24 h, cells became adherent and the medium was replaced by complete RPMI without PMA. Culture medium was replaced again on day 4 following differentiation. These monocyte-derived macrophages would then be used seven days after differentiation.

Latency Reversing Agents, Drug Exposures and Transfection

Cell lines were treated with 2 μg/mL of doxycycline (Dox) (MultiCell). PMA was used at 32.4 nM (20 ng/mL) for 24 h (322). LRA treatments were used either alone or in a combination with Dox. These included either 500 nM (+) JQ1 (CaymanChemical) (323), 5 mM HMBA (Sigma-Aldrich) (324, 325), 4 μM SAHA (Sigma-Aldrich) (324), 90 nM chaetocin (CaymanChemical) (326), 500 nM disulfiram (Sigma-Aldrich) (327), or 1 μM prostratin (Sigma-Aldrich) (324). PMA, JQ1, SAHA, chaetocin, disulfiram and prostratin were kept in dimethyl sulfoxide (DMSO) (Sigma Aldrich), while Dox and HMBA were diluted in phosphate buffered saline (PBS) (MultiCell).

For Western blots and reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR), CEM T4 GagzipGFP, THP-1 GagzipGFP and J-Lat 10.6 were plated at 5×10^5 to 1×10^6 cells/well in 6-well plates, while 12-well plates were seeded with 2.5×10^5 cells/well. Cells were immediately treated with the above drugs and incubated for 3 or 3.5 days. The final concentration of DMSO was 0.04% for Figure 6B and 0.0005% for Figure 6C, D.

For fluorescence-activated cell sorting (FACS) in preparation for RNA sequencing, 4.5×10^7 CEM T4 GagzipGFP cells were incubated in T182.5 cell culture flasks with 90 mL RPMI for 3.5 days before cell sorting and RNA isolation. Cells were immediately given no treatment (Mock) or Dox in combination with SAHA, prostratin, or both SAHA and prostratin. The final concentration of DMSO was adjusted to 0.062% for all treatments.

Cells were transfected with plasmids using 0.25-1 µg of DNA for every mL of media in 6-well plates (2 mL media) with 0.5 or 1 X 10^6 cells/well using polyethyleneimine MAX 40K (PEI MAX) (Polysciences) at a ratio of 3 µL PEI to 1 µg total DNA following manufacturer's protocol. Supernatants and lysates were then collected 24 h or 48 h after transfection as indicated in figures.

Cells were transfected with mirVana® miRNA mimics (ThermoFisher #4464066) and Anti-miR™ miRNA inhibitors (ThermoFisher #AM17000) using Lipofectamine RNAiMAX (Thermo Fisher) according to the manufacturer's protocols 48 h prior to further manipulations.

Immunoblotting

Cells were washed with PBS and lysed in cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8.0, 10% Glycerol, 1% IGEPAL® CA-630 (Sigma)) with phosphatase and protease inhibitor cocktails (Roche), or lysed in cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% IGEPAL® CA-630, 0.1% sodium deoxycholate, 0.5% SDS) with phosphatase and protease inhibitor cocktails. Lysates in either buffer were then freeze-thawed three times between liquid nitrogen and ice. Prior to centrifugation, RIPA lysates were incubated with 1 µL Benzonase and 1 µL $MgCl_2$ for 30 min at room temperature. All samples were then centrifuged on a tabletop centrifuge for 30 min at 13000 rpm to remove debris. Proteins in preserved supernatants were quantified by Bradford assay using a BioMate 3 Spectrophotometer (Thermo). 15-125 µg of protein mixed with Laemmli sample buffer were incubated for 5 min at 100 °C. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fast-transferred onto 0.2 µm nitrocellulose membranes (Bio-Rad) using pre-programmed Turbo protocols on a Trans-Blot Turbo transfer system (Bio-Rad). Membranes were washed with water, dyed with Ponceau S to confirm effective transfer and blocked for 1h with 5% milk in Tris-buffered saline with 1% tween 20 (TBST) followed by two washes with TBST (5min each). Membranes were then incubated overnight at 4 °C or for one hour at RT with primary antibodies listed in Table 1. After five additional washes with TBST, membranes were incubated for one hour with horseradish peroxidase-conjugated secondary antibodies (Table 1). After five further washes with TBST, the bands were visualized with Western Lightning Plus-ECL reagent (Perkin-Elmer) or Pierce™ ECL Western Blotting substrates (Thermo Fisher). Western Blots shown in manuscript are representative of at least three independent replicates.

Table 1: Antibody information

Protein target	Antibody name	Dilution	Species	Manufacturer	Cat #
GFP	GFP (B-2)	1:1000	mouse	Santa Cruz Biotechnology	sc-9996
p24 (Capsid)	183-H12-5C	1:1000	mouse	NIH AIDS reagent program	3537
GAPDH	GAPDH (6C5)	1:1000	mouse	Santa Cruz Biotech.	sc-32233
AFF4	Anti-AFF4 (7D)	1:500	rabbit	Dr. M. Estable lab (Ryerson U)	none
HA	Anti-HA tag	1:1000	rabbit	Abcam	ab137838
RT	Anti HIV-1 RT	1:500	rabbit	Dr. L. Kleiman lab (LDI)	none
Mouse IgG	Mouse IgG (H+L)	1:3000	goat	KP Laboratory	KP-474-1806
Rabbit light chain	Rabbit Light Chain HRP	1:5000	mouse	Millipore (Sigma Aldrich)	MAB201P
Rabbit IgG	Rabbit IgG (H+L)	1:5000	goat	KP Laboratory	KP-474-1506

Reverse Transcription - Quantitative Polymerase Chain Reaction

Cells were lysed using 750 µL of TRIzol reagent per well (Life Technologies). RNA extraction was performed using the miRNeasy® mini kit (QIAGEN) according to the manufacturer's protocol, including a digestion of DNA using an RNase-free DNase set (QIAGEN). RNA was diluted from spin columns in ultrapure distilled water. These samples were used to compare relative rates of pre- and post-pause transcription between two latent cell models and not to quantify their absolute RNA quantities, so a polyadenylation step was not required. Reverse transcription reactions of 1 µg of total RNA were performed as per the SuperScript™ II Reverse Transcriptase kit protocol (ThermoFisher), using 1 µL each of Oligo(dT)12-18 and random hexamers (ThermoFisher). Quantitative PCR reactions contained 5 µL of 2X BrightGreen qPCR MasterMix (ABM), 50 ng of cDNA and 125 nM of each primer (Table 2) and were filled to 10 µL with ultrapure distilled water. Samples were loaded in triplicate technical replicates. qPCR conditions were as follows: 95 °C for 5 min followed by 49 cycles of 95 °C for 10 s, 60 °C for 15 s, 72 °C for 5 s. A melting curve cycle followed with 5s increments of 0.5 degrees from 65 °C to 95 °C. Data acquisition and analysis were performed using Bio-Rad CFX96 and CFX maestro software respectively. β-actin was used as a housekeeping gene control.

Table 2: List of primers used in qPCR analyses

Primer	Target transcripts	Sequence
TAR-F7	HIV HXB2 456 → 474	GTCTCTCTGGTTAGACCAG
TAR-R6	HIV HXB2 496 ← 513	TGGGTTCCCTAGTTAGCC
LTR3-F	HIV HXB2 738 → 757	CGACTGGTGAGTACGCCAAA
LTR3-R	HIV HXB2 801 ← 820	CCCCGCTTAATACTGACGCT
AFF4-F	AFF4 cds	GCAGCAAAGCACATCTCACC
AFF4-R	AFF4 cds	AGGCCATGAATGCGTCATCT
β-Actin-F	Housekeeping control	GAGCGGTTCCGCTGCCCTGAGGCACTC
β-Actin-R	Housekeeping control	GGGGCAGTGATCTCCTTCTGCATCCTG

Cell Sorting and RNA Isolation

For RNA sequencing, CEM T4 GagzipGFP cells treated as above were washed twice with PBS (5 min, 1200 rpm, at RT), then once with PBS + 2% FBS (5 min, 1200 rpm, at RT) and resuspended in 2 mL PBS + 2% FBS. The resuspended cells were filtered through a 35 μM cell strainer. Cells were then sorted by FACSAria Fusion (BD Biosciences) into GFP^+ and GFP^- populations as depicted in Figure 7B by Christian Young (Lady Davis Institute Flow Cytometry facility manager).

Sorted cells were washed once with PBS (5 min, 5000 rpm, at RT) and re-suspended in 750 μL of TRIzol reagent (Life Technologies). RNA extraction was performed using the miRNeasy® mini kit (QIAGEN) according to the manufacturer's protocol, including digestion of contaminating DNA using an RNase-free DNase set (QIAGEN). RNA was diluted from the columns in ultrapure distilled water. Samples were kept at -80 ˚C and submitted to Génome Québec for quality assessment, short RNA sequencing (RNA-seq) and ribosomal (r)RNA-depleted RNA-sequencing.

RNA-seq and Short RNA-seq Data Processing

RNA integrity numbers (RINs) were assessed for each sample by Bioanalyzer 2100 (Agilent). All samples met a threshold of RIN > 7 and were deemed of sufficient quality to be sequenced. Short RNAs were prepared for sequencing using a NEB miRNA library preparation kit with size selection for Illumina sequencing. Short RNA libraries were sequenced on an Illumina HiSeq single read sequencing lane for 50 cycles. Total RNAs were prepared by KAPA HMR stranded library preparation for Illumina sequencing to deplete rRNAs. The remaining ribo-depleted RNAs were sequenced by Illumina HiSeq v4 paired-end 125 bp sequencing.

RNA-seq processing is depicted in Figure 7D, E. Ribo-depleted RNA-seq reads were checked for quality using FastQC (328) and MultiQC (329) before and after trimming by FastP (330), and then mapped to the human genome (build hg38) using STAR version 2.7.8a (331). Mapped reads were counted using FeatureCounts (332). Differential expression analysis was performed using DESeq2 (333) in the R 4.0.4 computing environment (334). Short RNA-seq reads were similarly checked for quality using FastQC (328) and MultiQC (329) before and after trimming by FastP (330), then mapped the human genome (build hg38) using Bowtie2 (335).

Reads were quantified using FeatureCounts (332) to miRBase v22.1 (336) before normalization and differential expression using DESeq2 (333).

RNA-seq Network Generation, Pathway and Ontology Analysis

Differentially expressed long RNAs and miRNAs between controls and SAHA-treated groups were quantified and compared using R with the following packages installed: tidyverse, ggplot2, ggrepel and BiocManager (includes DESeq2 and other packages), with preliminary visualizations done in iDEP (337). miRNA regulatory networks were identified using Qiagen's proprietary analysis suite Ingenuity Pathway Analysis (IPA) by our colleague Dr. Bernard Mari using differentially expressed genes and miRNAs identified in similar methods to our own by Marin Truchi. Enrichments of network targets were quantified using a Fisher's exact test built into PANTHER (338) according to the 2021-02-01 release of the Gene Ontology (GO) Resource.

RIP-seq miRNA Network Generation, Target Prediction and Enrichment

miRNAs identified in RIP-seq and RT-qPCR experiments to be associated with Dicer in Flag-Gag transfected cells were used as queries for target entries in MirTarBase v.8.0 and TarBase v.8.0. All entries (low- and high-scoring) for targets associated with these miRNAs were used for visualization and enrichment analysis. miRNA-target interactions were visualized using MirNet 2.0 (339). Enrichments of these targets were quantified using a Fisher's exact test for Biological Processes (BP) GO terms built into PANTHER as above, except that experimentally validated targets were queried against the background of human miRNA targets listed in MirTarBase v.8.0 and TarBase v.8.0 databases. Type 1 error from multiple testing of BP terms was prevented using Bonferroni correction. miRNA targets were predicted using online prediction databases miRDB and TargetScan release 7.2, which use distinct data-informed algorithms to predict and score miRNA-target pairings (340, 341).

Plasmid Design and Generation

The HIV-1 molecular clone pNL4-3 was cloned in 1986 (342), while pNLXX (pNL4-3 with no Gag expression) was first described in 2002 (343). pCI-Neo-Flag and pCI-Neo-Flag-Gag were kindly provided by Dr. A. Mouland's lab (344). The generation of psiRNA-U6GFP::Zeo

from the U6 RNA Pol III promoter in pSIREN-shuttle vector (Clontech) and psiRNA-7SKGFP::Zeo (InvivoGen) and the creation of the nonsense negative control psiRNA-U6GFP::Zeo-shNS and HIV-targeting shRNA vectors -shLDR4 and -shPol247 were previously described by colleagues in our lab (345, 346). To generate psiRNA-U6GFP::Zeo-shAFF4 and -sh143, complementary oligonucleotides (Table 3, sets 1 & 2) were annealed (1.25 µM each in 75 mM NaCl, 40 µL, 2 min at 80 °C, cooled slowly to 37 °C) and ligated into Bbs1-digested psiRNA-U6GFP::Zeo. The resulting plasmid (psiRNA-U6GFP::Zeo-shAFF4) was confirmed by sequencing using the OL408 primer located in the downstream CMV-HTLV promoter (Table 4). pEGFP-C1-AFF4_3'UTR- plasmids "-Blank" and "-1-2-3" were generated using pEGFP-C1 (Clontech). Complementary oligonucleotides (Table 3, sets 3 & 4) were annealed in respective pairs as done for the psiRNA inserts, then ligated into XhoI and KpnI digested pEGFP-C1. The resulting constructs were confirmed by sequencing using a primer located in the EGFP ORF (Table 4). The generation of pLT3R-TetONE is depicted in Figure 17C, using oligonucleotides listed in Tables 3, 4 and 5, LT3REVIN from Dr. J. Zuber's lab (Addgene plasmid # 111175) (254), pLVX-TetONE-Puro (a kind gift from Dr. R. Lin's lab) and Q5 Hot Start High-Fidelity 2X Master Mix in conditions where PCR was needed. Successful cloning of each step in plasmid generation were confirmed by sequencing using pLT3R MCS and/or pLT3R miR-E sequencing primers.

Table 3: Cloning oligonucleotides (A = annealed, O = overlapping PCR, T = template for PCR)

Name	Reaction	Set	Sequence
FW shNS psiRNA insert	A	1	ACCTCGTACCGCACGTCATTCGTATCCTCGAGCATACGAATGACGTGC GGTACTTT
RV shNS psiRNA insert	A	1	CAAAAAAGTACCGCACGTCATTCGTATGCTCGAGGATACGAATGACGT GCGGTACG
FW shAFF4 psiRNA insert	A	2	ACCTCGCACCAGTCTAAATCTATGTTCTCGAGAACATAGATTTAGACT GGTGCTTT
RV shAFF4 psiRNA insert	A	2	CAAAAAAGCACCAGTCTAAATCTATGTTCTCGAGAACATAGATTTAGA CTGGTGCG
FW AFF4 3' no target	A	3	TCGAGCATAATGATAATCCAAAGCGATCATGTCAGTTGGCCCTTTAAT ATTTCCAATGTGAGGTAC
RV AFF4 3' no target	A	3	CTCACATTGGAAATATTAAAGGGCCAACTGACATGATCGCTTTGGATT ATCATTATGC
FW AFF4 3' 3-site	A	4	TCGAGCATAATGATATGTACTATGAAATGTGTCTGATTATATTTTCTCT TTAAAACTGTGTCAATTTCCCCCCTCCCTCCTCAATAGGTGTCCGGTAC
RV AFF4 3' 3-site	A	4	CGGACACCTATTGAGGAGGGAGGGGGGAAATTGACACAGTTTTAAAG AGAAAATATAATCAGACACATTTCATAGTACATATCATTATGC
FW miR-29a full	O + T	8	GGCAGCCTCGAGAAGGTATATTGCTGTTGACAGTGAGCATGACTGATT TCTTTTGGTGTTCAGAGTCAATATAATTTTCTA
RV miR-29a full	O + T	8	CCGACTGAATTCTAGCCCCTTGAAGTCCGAGGCAGTAGGCATAACCGA TTTCAGATGGTGCTAGAAAATTATATTGACTCT
FW miR-642a full	O + T	9	GGCAGCCTCGAGAAGGTATATTGCTGATCTGAGTTGGGAGGGTCCCTC TCCAAATGTGTCTTGGGGTGGGGGATCAAGAC
RV miR-642a full	O + T	9	CCGACTGAATTCTAGCCCCTTGAAGTCCGGCCGAGTTGGGAGGTTCCC TCTCCAAATGTGTCTTGATCCCCCACCCCAAG

FW shRNAmiR-29a-3p	O + T	10	GGCAGCCTCGAGAAGGTATATTGCTGTTGACAGTGAGCGTAACCGATTTCAGATGGTGCTATAGTGAAGCCACAGATGTA
RV shRNAmiR-29a-3p	O + T	10	CCGACTGAATTCTAGCCCCTTGAAGTCCGAGGCAGTAGGCATAACCGATTTCAGATGGTGCTATACATCTGTGGCTTCACTA
FW sh143 psiRNA insert	A	11	ACCTCGTTAGTACCAGTTGAACCACTCGAGTGGTTCAACTGGTACTAACTTT
RV sh143 psiRNA insert	A	11	CAAAAAAGTTAGTACCAGTTGAACCACTCGAGTGGTTCAACTGGTACTAACG
FW NeoR removal cloner	A	12	GTACACCGCTCCCGATTCGCAGCGCATCGCCTTCTATGCA
RV NeoR removal cloner	A	12	TAGAAGGCGATGCGCTGCGAATCGGGAGCGGT
miR29a-template	T	13	TGCTGTTGACAGTGAGCGATGACTGATTTCTTTTGGTGTTCAGAGTCAATATAATTTTCTAGCACCATCTGAAATCGGTTATTGCCTACTGCCTCGGA
miR642a-template	T	14	TGCTGTTGACAGTGAGCGGGAGGGTCCCTCTCCAAATGTGTCTTGGGGTGGGGGATCAAGACACATTTGGAGAGGGAACCTCCTGCCTACTGCCTCGGA

Table 4: Oligonucleotides used for sequencing

Primer	Target	Sequence
OL408	psiRNA-U6GFP::Zeo shRNA CS	GCGTTACTATGGGAACATAC
pEGFP_C1MCS seq prim F	pEGFP-C1 3'UTR MCS	TCACATGGTCCTGCTGGAGTT
pLT3R MCS seq prim F	pLT3REVIN PGK MCS	TTTGCTCCTTCGCTTTCTGG
pLT3R miR-E seq prim F	pLT3REVIN miR-E CS	TACTTTACAGAATCGTTGCCTGC

Table 5: Oligonucleotide primers used for cloning PCRs

Primer	Templates	Sequence
miR-E fw XhoI	Cloning sets 8-10	GGCAGCCTCGAGAAGGTATA
miR-E rv EcoRI	Cloning sets 8-10	CCGACTGAATTCTAGCCCCT
pLT3R PGK fw	pLT3REVIN	AATAGCAGCTTTGCTCCTTCGCTTTCTGGGCTCAGA
pLT3R PGK rv	pLT3REVIN	TTGTACAGCTAGCTAGTCGACTTGGGCTGCAGGTCGAAA
TetON3G fw SalI	pLVX-TetOne-Puro	TATGCGTCGACACCATGTCTAGACTGGAC
TetON3G rv NheI	pLVX-TetOne-Puro	TATTATGCTAGCATTCGCGCGTTTACCCGG
miRE-Xho-fw	Cloning sets 12-13	TGAACTCGAGAAGGTATATTGCTGTTGACAGTGAGCG
miRE-EcoOligo-rv	Cloning sets 12-13	TCTCGAATTCTAGCCCCTTGAAGTCCGAGGCAGTAGGC

Cell Viability

The WST-1 assay was used to check cell viability in response to different antiviral miRNA sequences. This colorimetric assay measures the activity of NAD(P)H-dependent cellular oxidoreductase enzymes to reduce the tetrazolium WST-1 salt to the insoluble dye Formazan, which is taken as a quantifiable correlate for proliferating live cell count. HEK 293T cells were incubated in 96-well plates with miRNAs for 72 h, after which they were exposed to WST-1 according to the manufacturer's protocol. The Formazan absorbance of each sample was measured at a 450 nm wavelength using a Synergy 4 (BioTek) microplate spectrophotometer, while a control reading at 690 nm was used to control microplate imperfections. Absorbance values were normalized to respective nonsense controls.

Chapter 3: Results

Aim 1: Identifying Networks Associated with Blocks in Virus Expression during Latency

Preamble

As described in section 1.2c, the fate of an HIV-1 provirus is regulated at multiple different steps before and after transcription initiation. The Tat/TAR feedback loop has an especially important influence on regulation, as it specifically amplifies transcription at the HIV-1 promoter through several distinct mechanisms. Most researched models of latency include active Tat/TAR regulatory axes, complicating any attempts to distinguish between agents that affect pre- or post-initiation regulatory events. To study individual steps involved in latency maintenance or reversal, our collaborator Dr. Alan Cochrane from the Department of Molecular Genetics at the University of Toronto designed and generated a modified HIV-1 genome that is independent of Tat/TAR transactivation and preferentially develops a latent phenotype in lymphocytes and other cell types, called HIV-1 GagzipGFP (Figure 5A). This provirus was generated by modifying a previously developed conditionally replicating virus under the control of tetracycline-responsive promoter sequences (TetO) to contain a GFP reporter that could easily be detected in flow cytometry experiments (347). The modified HIV-1 promoter is permissive to extremely low transcription in the absence of Dox, which is sufficient to produce basal levels of the reverse tetracycline-controlled transactivator (rtTA) transcription factor, transcribed in place of the viral *nef*. In Dox-treated cells, the antibiotic binds to rtTA, activating the transcription factor to associate with TetO promoter elements present in the modified HIV-1 promoter to stimulate basal epigenetic and regulatory changes at the promoter that favour basal transcription of vRNAs and GFP without regulating elongation (Figure 5B). The Cochrane lab generated cell lines latently infected with GagzipGFP by creating Vesicular Stomatitis Virus glycoprotein (VSV-G) pseudotyped particles carrying the defective GagzipGFP genome and transducing these into $CD4^+$ CCRF-CEM (CEM-T4) lymphoblasts and THP-1 monocytes before sorting during active infection for GFP expression (Figure 5C). The genomic integration sites of transduced cells chosen for cell line characterization were not assessed.

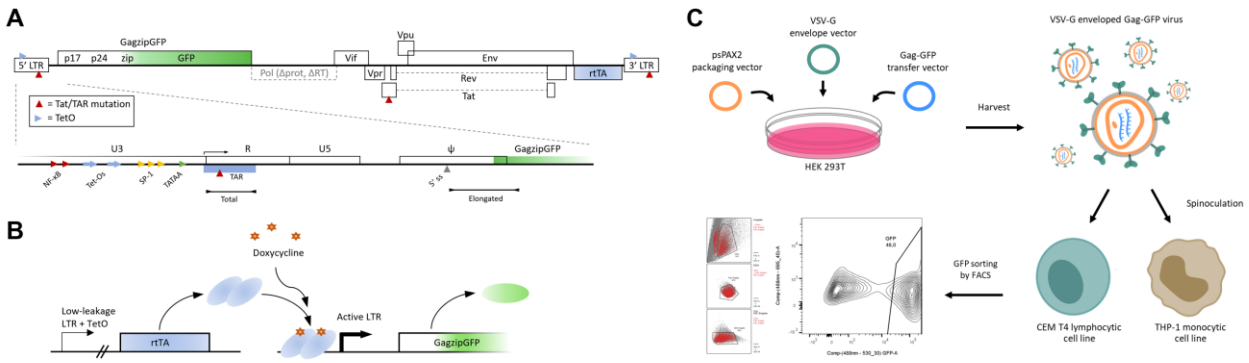


Figure 5: The Tat/TAR-independent rtTA GagzipGFP virus. A) Schematic of the virus genome with indicated modifications, promoter elements and primers. **B)** Mechanism of induction by Doxycycline at the GagzipGFP promoter. **C)** Generation of HIV-1 GagzipGFP cell lines.

A Model for Testing post-Initiation Events in HIV-1 Transcription

This model was engineered to study post-initiation events in latency reversal, so we first needed to confirm whether basal transcription of the model was regulated by doxycycline alone. After selection, we confirmed the conditional doxycycline dependency of these HIV-1 GagzipGFP-infected cell lines using Western blots (WB) for Gag and GFP proteins in both cell lines following induction by 72 h of Dox exposure (Figure 6A). Conditional expression was further tested in response to LRAs with various modes of action in the presence or absence of Dox using WBs. We used vorinostat (SAHA), a HDACi, or chaetocin, a HMTi, to open chromatin at the TSS (165, 348, 349). We released P-TEFb from the 7SK snRNP complex using JQ1, a bromodomain and extraterminal inhibitor, or hexamethylene bisacetamide (HMBA), a P-TEFb agonist (126, 166, 167, 188). We also tested the transcriptional activators disulfiram, an agonist of protein kinase B (Akt) phosphorylation and signaling, and the protein kinase C agonist prostratin, to induce nuclear translocation of NF-κB and activation of the HIV-1 promoter and T-cell proliferation signals (168, 169). Prostratin and SAHA are also known to stimulate CycT1 expression and their recorded HIV-1 reactivation effects rely in part on Tat (127, 315). Drugs were exposed in GagzipGFP lymphocytes for 3.5 days, monocytes for 2.5 days and monocyte-derived macrophages (MDMs) for 2.4 days using concentrations listed in the Methods section. The choice of the concentration for each LRA as well as the exposure times for each cell line were determined by lab colleagues Dr. Elodie Rance and Craig McCullough as described in our forthcoming paper (Rance E, et al. under review). GagzipGFP lymphocytes were reactivated most strongly by JQ1, HMBA and SAHA with a moderate reactivation by prostratin, but these conditions showed no reactivation in

the absence of Dox (Figure 6B). GagzipGFP monocytes and MDM were reactivated most by HMBA, SAHA and prostratin, and JQ1, HMBA, SAHA and prostratin, respectively, but again showed no reactivation in the absence of Dox (not shown).

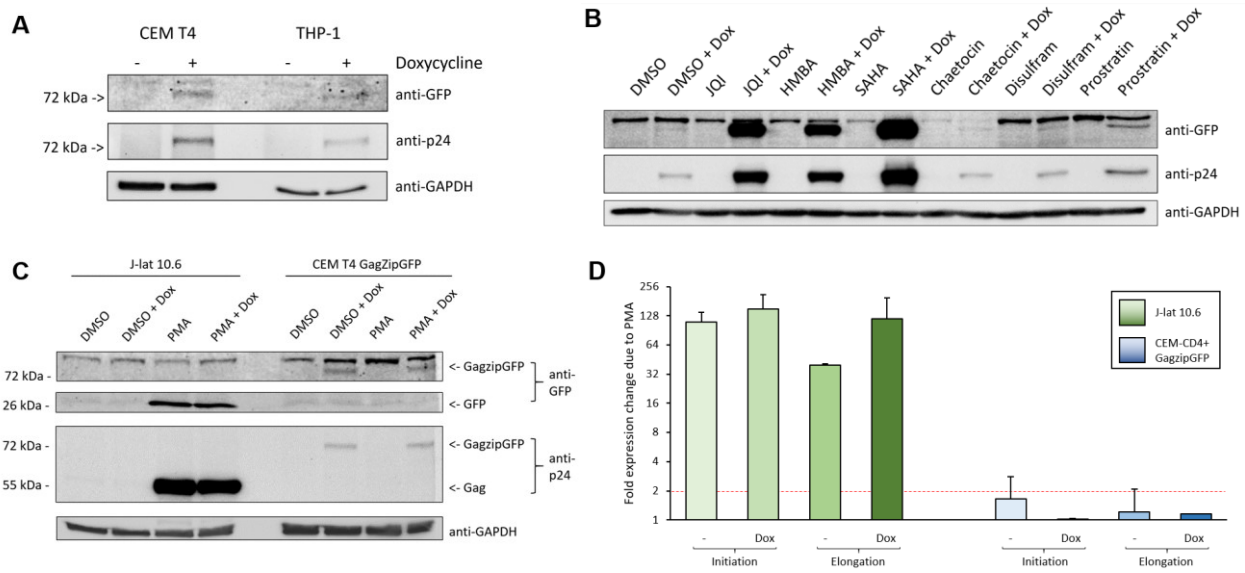


Figure 6: Characterization of GagzipGFP cell lines. A) CEM T4 and THP-1 HIV-1 GagzipGFP cells were incubated for 72 h in the presence (+) or absence (-) of Dox. 70 µg of cell extracts were fractionated on SDS-PAGE gels. Shown are Western blots with anti-GFP, anti-p24 and anti-GAPDH antibodies. **B)** LRAs alone do not induce HIV-1 GagzipGFP expression in CEM T4 lymphocytes. CEM T4 HIV-1 GagzipGFP were incubated in the presence of Dox when indicated and/or the LRA at the concentration indicated in the methods section for 3.5 days. 30 µg of cell extracts were fractionated on an SDS-PAGE and blotted as in A. Shown is a representative experiment among three. **C, D)** Latent HIV expression is basally regulated by Dox and not T-cell signaling in CEM T4, whereas it is regulated by T-cell signaling and not by Dox in J-lat cells. **C)** J-Lat 10.6 and CEM T4 HIV-1 GagzipGFP were incubated with Dox for 72 h and 20 ng/mL (32.4 nM) PMA for 24 h. 70 µg of cell extracts were fractionated on an SDS-PAGE gel and as in A. **D)** RT-qPCR of HIV-1 transcripts. CEM T4 HIV-1 GagzipGFP and J-Lat cells were incubated for 24 h with Dox and PMA. RNA from each well was extracted and subjected to RT-qPCR using either a total transcript primer or a primer for elongated transcripts. Shown is the fold change in expression of the product of each primer in each condition, normalized to the expression of β-actin. Numbers are averaged from experiments performed in triplicate ± SEM, excluding outliers. A horizontal bar is used to indicate a fold change cut-off of 2.

To determine if our lymphocytes can exhibit transcription initiation and elongation at the GagzipGFP promoter independently from Dox like other latency models, we compared its activation by phorbol myristate acetate (PMA), a phorbol ester in the same class as prostratin and an activator of NF-κB signaling, to the J-Lat 10.6 cell line that has an intact HIV-1 promoter and is well described in HIV-1 latency literature (350-352). Dox was added to both cell lines for 48 h,

followed by PMA for an additional 24 h, amounting to 72 h in the presence of Dox before cell extracts were collected for WB. PMA clearly induced the expression of GFP and Gag in J-Lat cells, while Dox induced a weak reactivation of GagzipGFP lymphocytes, but showed no noticeable additional reactivation with the help of PMA, suggesting a weak response to NF-κB (Figure 6C). To assess PMA activation with a minimal contribution by Dox, RT-qPCR was performed on the same cells induced for only 24 h with both Dox and PMA. Primer pairs targeting promoter proximal regions before and after transcription pausing were used to assess transcription initiation and elongation (Figure 5A). PMA strongly induced transcription initiation and elongation in J-Lat cells but showed a minimal effect on both transcriptional steps in GagzipGFP lymphocytes, further confirming a poorly reactive NF-κB site in the promoter (Figure 6D). Collaborators of ours then treated this model to drugs as in Figure 6B and used droplet digital PCR on vRNA regions before and after the HIV-1 transcriptional pause site. These data showed that JQ1, HMBA and SAHA increased the ratio of elongated transcripts to initiated transcripts with respect to Dox treatments in lymphocytes, while prostratin stimulated a moderate increase in this ratio, suggesting that the above drugs reactivated GagzipGFP proviruses in large part through steps involved in transcription elongation (Rance E, et al. under review).

RNA-seq and Regulatory Networks Associated with Latency

Once we confirmed that the above model can be used to understand post-initiation events in HIV-1 transcription without the influence of Tat/TAR feedback loops, we decided to profile host transcriptional changes and regulatory networks implicated in later steps in virus reactivation by sequencing the long and short RNA transcriptomes in GagzipGFP CEM T4 cells reactivated or not reactivated by SAHA and/or prostratin. Cells were given no treatment (Mock) or Dox in combination with SAHA and/or prostratin (Figure 7A) for 3.5 days. Cells were then sorted by FACS into reactivated and non-reactivated populations, showing distinct levels of reactivation between drug treatments as above (Figure 7B, C). For each collected condition, long ribosome-depleted transcriptomes were sequenced as paired-end 125 bp reads and processed as in Figure 7D, while short RNAs were sequenced over fifty sequencing cycles and processed as in Figure 7E. We performed the following analysis on a first sequencing replicate while we completed collection for a second replicate for each condition not yet analyzed in this thesis.

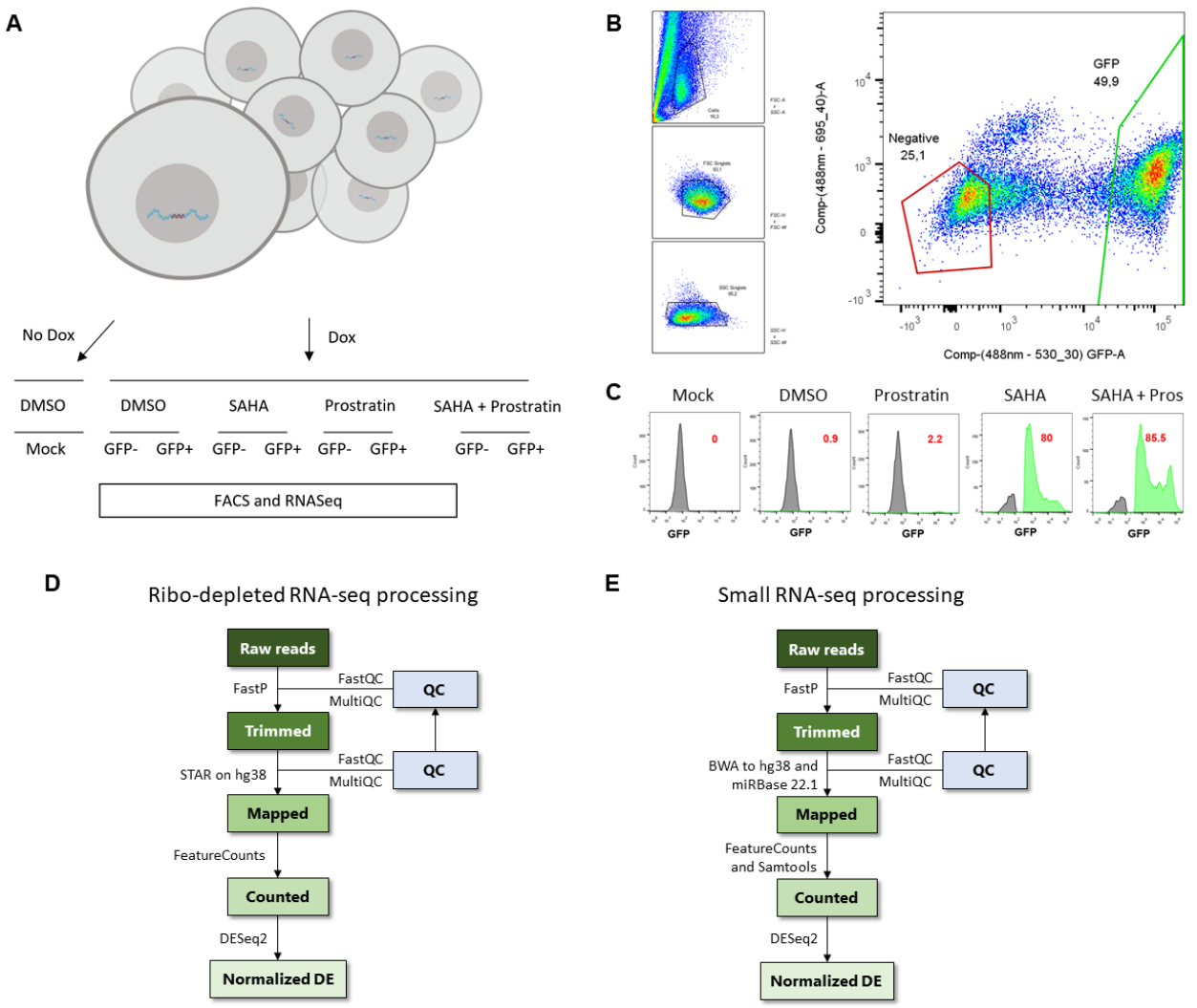
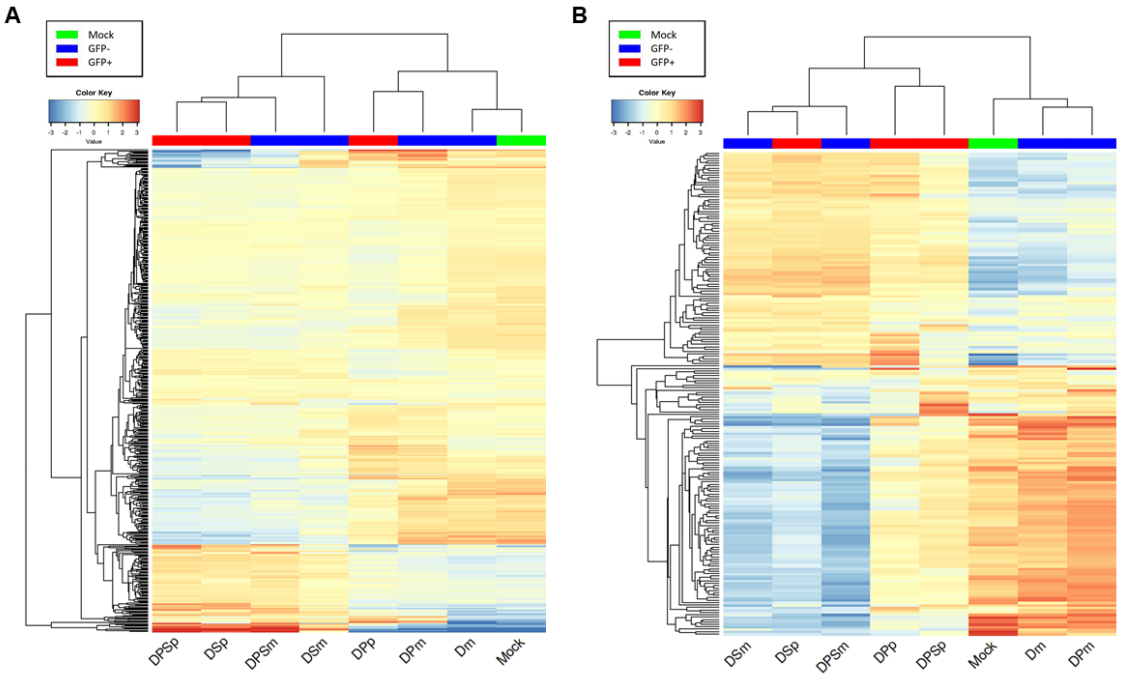
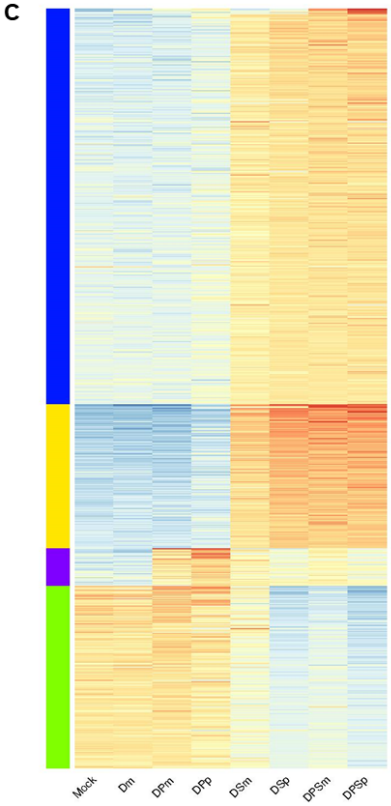


Figure 7: Preparation of RNA-seq data from reactivated CEM T4 GagzipGFP cells. A) Conditions used to generate the RNA-Seq samples. CEM T4 GagzipGFP cells were exposed to no Dox or Dox in combination with the SAHA and/or prostratin for 3.5 days. Treatments sequenced were: Mock, Dox DMSO GFP^- (Dm), Dox SAHA GFP^- (DSm) and GFP^+ (DSp), Dox prostratin GFP^- (DPm) and GFP^+ (DPp), and Dox SAHA prostratin GFP^- (DPSm) and GFP^+ (DPSp). **B)** Each group of cells exposed to a drug combination was sorted by FACS into GFP positive (reactivated) or GFP negative (non-reactivated) sub-populations, from which RNA was isolated. **C)** Drug combinations led to more or less reactivated cells as percentages of whole populations (red). Figure 7C courtesy of Dr. Elodie Rance. **D)** Workflow used for processing of ribo-depleted paired-end RNA transcriptomes **E)** Workflow used for processing of small RNA transcriptomes.

Read qualities, read counts and mapping qualities for both long and short RNA sequencing datasets were consistent across all conditions. Phred quality scores were consistently above 35 in central regions of reads for both 125 bp and 50 bp datasets, while the frequencies of mapped-to-total reads deviated by less than 5% between conditions for 125 bp reads. Once processed and





D

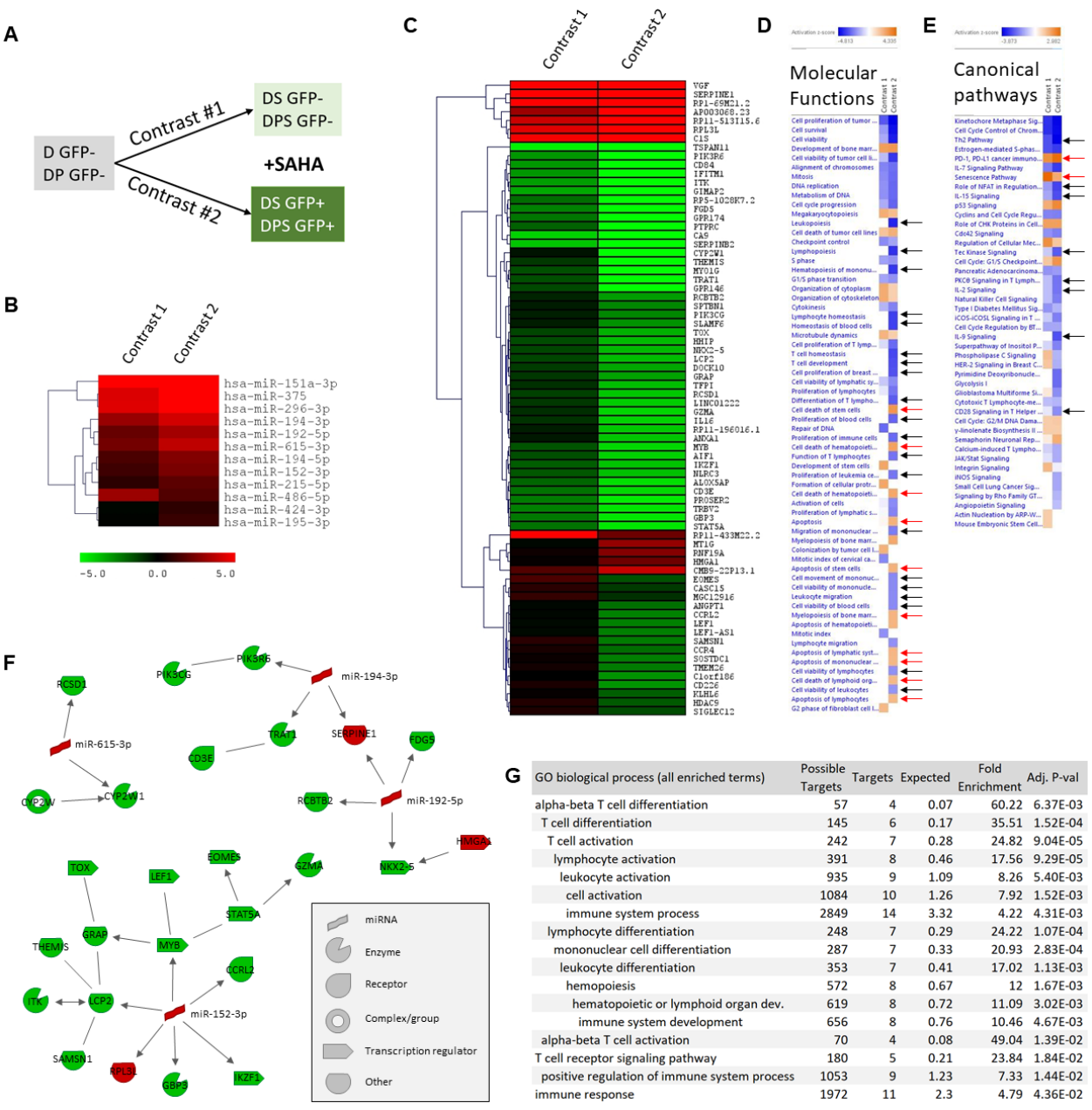
Cluster	adj.Pval	nGenes	Pathway
A (N=1032)	3.57E-05	79	Chemical homeostasis
	3.57E-05	61	Ion homeostasis
	3.57E-05	211	Regulation of biological quality
	5.94E-05	68	Cellular homeostasis
	5.94E-05	60	Cellular chemical homeostasis
	5.94E-05	50	Muscle structure development
	6.89E-05	51	Cellular cation homeostasis
	6.89E-05	55	Cation homeostasis
	1.29E-04	116	Movement of cell or subcellular component
	1.74E-04	35	Muscle organ development
B (N=375)	9.65E-07	69	Nervous system development
	9.65E-07	75	Anatomical structure morphogenesis
	4.35E-06	52	Neurogenesis
	6.58E-06	60	Movement of cell or subcellular component
	1.70E-05	48	Generation of neurons
	2.68E-05	37	Cell morphogenesis
	2.91E-05	39	Cellular component morphogenesis
	4.78E-05	53	Locomotion
	5.47E-05	43	Neuron differentiation
	6.57E-05	58	Cell development
C (N=122)	1.84E-04	34	Regulation of multicellular organismal process
	5.67E-04	27	Response to external stimulus
	5.67E-04	32	Regulation of signal transduction
	5.67E-04	10	Lymphocyte differentiation
	5.67E-04	33	Immune system process
	5.67E-04	12	Leukocyte differentiation
	5.67E-04	39	Regulation of response to stimulus
	5.67E-04	27	Positive regulation of response to stimulus
	5.67E-04	17	Anatomical structure formation involved in morphogenesis
	6.27E-04	5	B cell receptor signaling pathway
D (N=471)	1.48E-13	122	Immune system process
	4.43E-10	92	Immune response
	4.43E-10	53	Mitotic cell cycle
	7.99E-10	48	Mitotic cell cycle process
	1.40E-09	61	Leukocyte activation
	1.40E-09	43	Lymphocyte activation
	1.68E-09	26	Mitotic nuclear division
	7.62E-09	56	Chromosome organization
	1.25E-08	72	Cell cycle
	1.25E-08	63	Cell activation

Figure 8: Global trends in treatment-related and reactivation-related transcriptomes. A, B) Hierarchical clustering of sample sets based on Euclidean distance in the vector space of **A)** the 500 most variable among the 1000 most expressed genes, and **B)** the 200 most variably expressed miRNAs. **C)** K-means clustering of the 2000 most differentially expressed genes into four clusters. **D)** GO (BP) terms enriched in the four clusters in C, sorted by adjusted P-value.

normalized, all eight conditions of long RNA and short RNA transcriptomes were compared to determine the similarity and variance between transcriptomes as determined by drug exposures and sorting. When hierarchically clustered according to their Euclidean distances in a matrix of the top 500 most variably expressed genes across conditions among the 1000 most expressed genes in all conditions; Mock and Dox DMSO GFP^- (Dm) conditions were highly similar, as were Dox prostratin GFP^- (DPm) and Dox prostratin GFP^+ (DPp), although DPp lacked some expression markers found commonly amongst Mock, Dm and DPm (Figure 8A). Interestingly, all GFP^+ and GFP^- conditions containing SAHA were clustered together while DPp was more similar to its GFP^- counterpart than to the GFP^+ condition containing SAHA and prostratin (DPSp), suggesting that SAHA has a strong and significant signature on long cellular transcriptomes not matched by prostratin. Hierarchical clustering of an miRNA expression matrix again showed a clustering between Mock, Dm and DPm as well as a SAHA signature common to $GFP^{+/-}$ SAHA-treated conditions, although interestingly, DPSp and DPp clustered together, separately from the miRNA markers common to the other SAHA-treated conditions (Figure 8B). When clustering the 2000 most variable genes across conditions into four bins using a K-means algorithm, the first two clusters, A and B, appeared to be weakly and strongly affected by the presence of SAHA, respectively (Figure 8C). Meanwhile, the third cluster (C) appeared to be upregulated in all prostratin and SAHA-treated cells but not in the Mock and Dm controls. The final cluster (D) appeared to be differentially expressed between pairs of GFP^- and GFP^+ conditions. To determine whether these clusters mapped to gene sets involved in specific cellular processes, we explored the biological process gene ontologies enriched in each of the four clusters. These data showed that genes in cluster A were largely involved in processes related to homeostasis, genes in cluster B were largely involved in morphogenesis and neuron differentiation, genes in cluster C were involved in signal transduction and immune differentiation, while genes in cluster D were highly enriched in immune system processes such as activation of lymphocytes (Figure 8D).

We next aimed to identify sncRNAs and mRNAs differentially expressed between non-reactivated and reactivated cells. As treatment by SAHA was a more dominant marker on the cellular transcriptome than was the presence of prostratin or the level of GagzipGFP reactivation in this first replicate, we restricted ourselves to the analysis of genes and sncRNAs stimulated by SAHA that differed between non-reactivated and reactivated populations. For this analysis, we compared three separate groups of conditions: controls (Dm and DPm), non-reactivated SAHA

(DSm and DPSm), and reactivated with SAHA (DSp and DPSp). Comparisons were made between the controls and either SAHA-treated group to determine markers of the SAHA signature common to both comparisons and the markers of reactivated populations differentially expressed between SAHA-treated GFP^+ and GFP^- conditions (Figure 9A). Although no miRNAs were significantly differentially expressed between SAHA GFP^- and GFP^+ conditions in this first replicate, a distinct signature of miRNAs were differentially expressed between the control group and the two SAHA treated groups, among which several miRNAs including miR-192-5p, miR-194-3p, miR-615-3p and miR-152-3p were heightened in reactivated cells as compared to non-reactivated cells (Figure 9B). Gene expression comparisons showed a similar 'SAHA signature' in both comparisons with respect to the controls, but most of the differentially expressed genes identified in these contrasts were further downregulated in reactivated cells with respect to their non-reactivated counterparts (Figure 9C). We then explored the molecular functions and canonical pathways enriched among genes differentially expressed between the two contrasts to explore the cellular functions dysregulated during SAHA exposure and reactivation. Interestingly, reactivated cell transcriptomes showed deficits in functions and pathways associated with immune system activation, while showing an increased expression of genes involved in senescence, anergy and controlled cell death (Figure 9D, E). To assess whether any miRNA regulatory networks were involved in the dysregulation of genes seen in SAHA-mediated reactivation conditions, our collaborators at the IPMC in France performed miRNA Target Filter analysis on the miRNAs and mRNAs differentially expressed in Figure 9B and 9C according to miRNA-target interactions annotated in the proprietary Ingenuity Pathway Analysis knowledgebase. Regulatory networks whose miRNAs were significantly upregulated and whose targets were significantly reduced in reactivated cells were identified for miR-152-3p, miR-194-3p, miR-192-3p and miR-615-3p (Figure 9F). The regulated targets of these miRNA networks were enriched in immune cell activation processes (Figure 9G).



G

GO biological process (all enriched terms)	Possible Targets	Targets	Expected	Fold Enrichment	Adj. P-val
alpha-beta T cell differentiation	57	4	0.07	60.22	6.37E-03
T cell differentiation	145	6	0.17	35.51	1.52E-04
T cell activation	242	7	0.28	24.82	9.04E-05
lymphocyte activation	391	8	0.46	17.56	9.29E-05
leukocyte activation	935	9	1.09	8.26	5.40E-03
cell activation	1084	10	1.26	7.92	1.52E-03
immune system process	2849	14	3.32	4.22	4.31E-03
lymphocyte differentiation	248	7	0.29	24.22	1.07E-04
mononuclear cell differentiation	287	7	0.33	20.93	2.83E-04
leukocyte differentiation	353	7	0.41	17.02	1.13E-03
hemopoiesis	572	8	0.67	12	1.67E-03
hematopoietic or lymphoid organ dev.	619	8	0.72	11.09	3.02E-03
immune system development	656	8	0.76	10.46	4.67E-03
alpha-beta T cell activation	70	4	0.08	49.04	1.39E-02
T cell receptor signaling pathway	180	5	0.21	23.84	1.84E-02
positive regulation of immune system process	1053	9	1.23	7.33	1.44E-02
immune response	1972	11	2.3	4.79	4.36E-02

Figure 9: Enriched molecular functions and regulation networks in reactivated cells. A) Groups compared for contrasts and IPA. **B)** Highly differentially expressed miRNAs in both contrasts, expressed as Log2 fold changes from the control group. **C)** Expression of highly differentially expressed genes in contrasts, expressed as in B. **D)** Molecular functions differentially expressed in gene contrasts (annotated according to Qiagen's IPA annotation database). Black arrows point to downregulated functions associated with the immune system. Red arrows point to upregulated markers of cell death. **E)** Gene enrichments in IPA's canonical pathways database as in D. **F)** Networks of miRNAs-target pairs in networks differentially expressed between the two contrasted groups in B and C. **G)** Enriched GO biological processes in target genes out of complete human genome using (Fisher's exact test with Bonferroni correction for multiple testing). Figures 9B and C were generated by Marin Truchi, while Figures 9D-F are courtesy of Dr. Bernard Mari.

Aim 2: Determining the RNA-Mediated Downstream Effects of an Interaction between HIV-1 Gag and the RNAi Protein Dicer

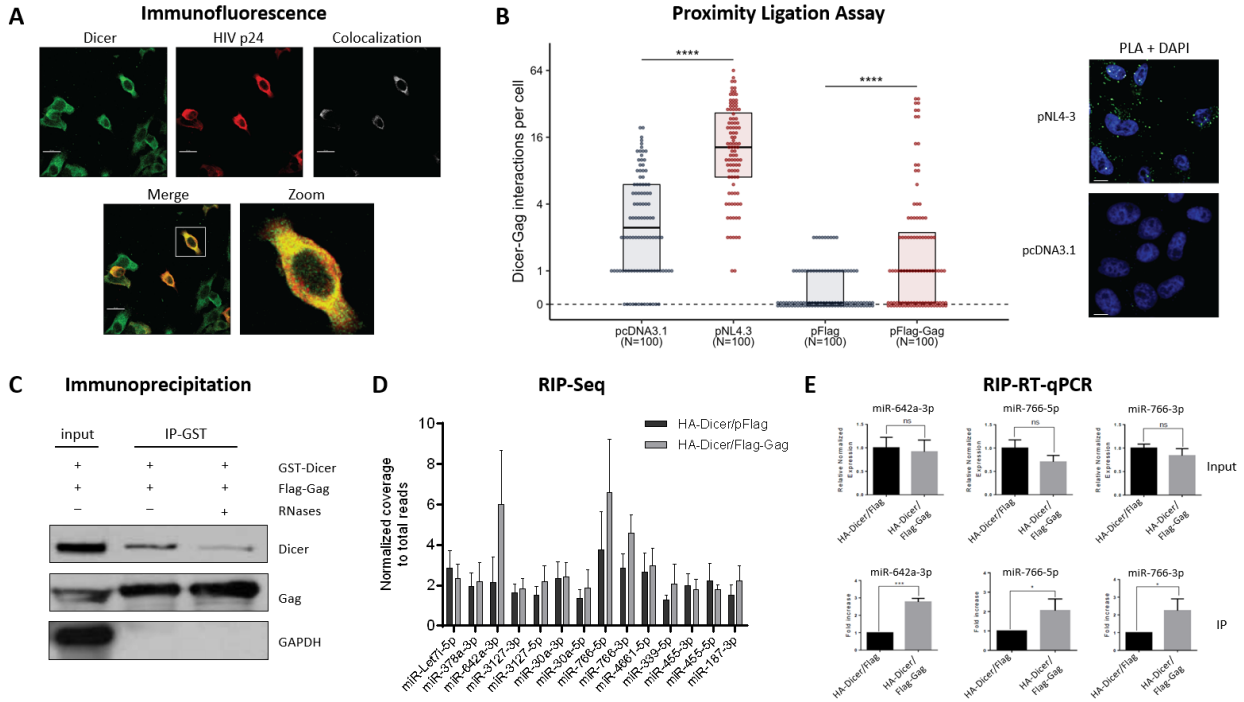


Figure 10: HIV-1 Gag and Dicer interact, recruiting specific ncRNAs in the process. A) HeLa cells were transfected with 0.2 µg of pNL4-3, fixed at 48h and stained with mouse anti-Dicer 13D6 (green) and rabbit anti-p24 (red). A 20 µm scale is shown at the bottom left of each image. Digitally zoomed images of the merged channels are shown on the far right. A colocalization channel was built using Imaris software and is displayed in the third lane. **B)** Proximity ligation assay scatter plot from HeLa cells transfected with Gag-containing plasmids pNL4-3 ($p < 0.0001$) or Flag-Gag ($p < 0.0001$). Representative images shown on right. **C)** The Gag-Dicer interaction does not require RNA. HEK 293T cells were co-transfected with GST-Dicer/Flag-Gag. RNase treatment was added during the immunoprecipitation. **D)** The presence of Gag in cells results in higher binding of specific miRNAs to Dicer. HEK 293T cells were transfected with HA-Dicer and Flag or Flag-Gag. HA was then immunoprecipitated from lysates and bound RNAs were sequenced. **E)** RNA immunoprecipitation as in Figure 10D, followed by RT-qPCR showed that hsa-miR-642a-3p, hsa-miR-766-5p and hsa-miR-766-3p are more bound to Dicer in Gag-transfected cells than in mock transfected cells. Input is cell lysates without HA immunoprecipitation. See section "Preface and Author Contributions" for detailed attributions.

Preamble

In earlier work performed in HeLa and HEK 293T cells, our lab identified through immunofluorescence that $p55^{Gag}$ co-localizes with Dicer (Figure 10A), while RNAi remains functional in cells affected by HIV-1 and the cellular localizations of miRNA biogenesis proteins are unaffected by the presence of HIV-1 expression (not shown). Proximity Ligation Assays

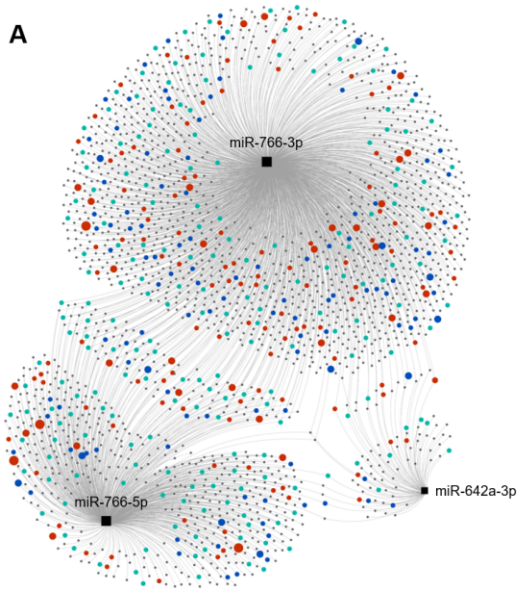
against both proteins confirmed that Dicer and Gag come into close (>40nm) contact with each other in cells (Figure 10B), while Immunoprecipitations (IP) of Dicer in the presence or absence of RNAses confirmed that this interaction does not rely on an RNA intermediate (Figure 10C). In substrate cleavage experiments, we saw that the presence of Gag in cells had no effect on the catalytic activity of Dicer for a well described canonical pre-miRNA, pre-let7c, and for a pre-miRNA known to be de-regulated during HIV-1 expression, pre-miR-29a (not shown). We therefore wondered whether this interaction might be loading or sequestering specific RNAs on Dicer rather than inhibiting the enzyme's function for all miRNAs. IPs of Dicer in the presence or absence of Gag followed by RNA-seq (RIP-seq) showed increased binding of specific ncRNAs to Dicer in cells expressing Gag (Figure 10D). These results were confirmed in Dicer IPs followed by quantitative reverse-transcription PCR (RT-qPCR) (Figure 10E), showing that hsa-miR-642a-3p (herein referred to as miR-642a) and both strands of hsa-miR-766 were specifically enriched on Dicer in cells transfected by Flag-Gag. We thus hypothesized that the enrichment of these specific miRNAs on Dicer in some way regulates HIV-1 expression or latency.

miRNAs Enriched on Dicer Converge on Specific Targets

We first explored the targets of our three miRNAs in case they might relate to processes that regulate HIV expression. We queried MirTarBase v.8.0 and TarBase v.8.0, two independently curated databases of miRNA-RNA target pairs identified in experiments, to identify targets of the enriched miRNA strands. We plotted this interaction network using mirNet 2.0 and saw that a significant percentage (30.7%) of the genes targeted by miR-642a were also shared with one or both strands of miR-766 (Figure 11A). Using a Fisher's Exact test against a background of all identified human miRNA targets in MirTarBase v.8.0 and TarBase v.8.0, we explored enriched Gene Ontology terms for biological processes among the target genes of our three miRNA strands (Fisher's exact test with Bonferroni correction) (Figure 11B). Among target biological processes, 'intracellular transport of virus' (GO:0075733), a daughter term of 'viral process' (GO: 0016032), was the most enriched term. Another enriched daughter term of 'viral process', 'viral gene expression' (GO:0019080), retains little overlap with the most enriched term, suggesting that HDFs involved in different stages of virus replication cycles in humans are conserved targets of these three miRNAs. Other enriched functions of the complete set of curated target genes are associated with multiple processes involved in the regulation of cellular replication by cell cycle

checkpoints, and involved in gene expression regulation during epigenetic remodeling, transcription or translation. We performed the same enrichment analysis on genes in the network that were shared targets of more than one miRNA (degree >1), resulting in three terms with more than two-fold enrichment (Figure 11C). These three GO terms were associated with distinct events in gene expression regulation. Of note, miR-766-5p and miR-766-3p contributed more targets to both the complete target set and the higher-degree target set than did miR-642a-3p, indicating that these analyses may have been biased in favour of the targets of the former two miRNAs.

A



B

GO biological processes	Possible Targets	Targets	Expected	Fold Enrichment	Adj. P-val
intracellular transport of virus	54	23	6.43	3.58	2.65E-02
mitotic G1 DNA damage checkpoint	66	26	7.85	3.31	1.96E-02
transport of virus	61	24	7.26	3.31	4.86E-02
mitotic G1/S transition checkpoint	67	26	7.97	3.26	2.45E-02
G1 DNA damage checkpoint	67	26	7.97	3.26	2.45E-02
DNA damage response, signal transduction by p53 c.m.	79	30	9.40	3.19	6.24E-03
signal transduction in response to DNA damage	102	38	12.14	3.13	3.25E-04
signal transduction involved in DNA damage checkpoint	73	27	8.69	3.11	3.30E-02
signal transduction involved in DNA integrity checkpoint	73	27	8.69	3.11	3.30E-02
signal transduction involved in cell cycle checkpoint	76	28	9.04	3.10	2.33E-02
signal transduction by p53 class mediator	118	38	14.04	2.71	1.07E-02
DNA integrity checkpoint	148	43	17.61	2.44	2.15E-02
DNA damage checkpoint	138	40	16.42	2.44	3.51E-02
viral gene expression	172	47	20.47	2.30	2.16E-02
regulation of mRNA catabolic process	205	53	24.39	2.17	1.96E-02
negative regulation of mitotic cell cycle phase transition	223	56	26.53	2.11	2.69E-02
negative regulation of cell cycle phase transition	238	59	28.32	2.08	2.12E-02
nuclear transport	259	63	30.82	2.04	1.43E-02
nucleocytoplasmic transport	256	62	30.46	2.04	1.96E-02
regulation of mRNA metabolic process	332	80	39.50	2.03	9.20E-04
covalent chromatin modification	351	84	41.77	2.01	5.38E-04
histone modification	347	83	41.29	2.01	7.05E-04

C

GO biological processes	Possible Targets	Targets	Expected	Fold Enrichment	Adj. P-val
posttranscriptional regulation of gene expression	537	18	4.86	3.70	2.36E-02
chromatin organization	647	20	5.86	3.41	2.10E-02
regulation of transcription by RNA polymerase II	2478	47	22.45	2.09	5.16E-03

Figure 11: miRNAs enriched on Gag-Dicer target genes involved in virus-regulating functions. Curated miRNA-gene interactions on MirTarBase v.8.0 and TarBase v.8.0. **A)** Network visualized and annotated using MirNet 2.0 (339). Circles represent genes, squares represent miRNAs and edges represent curated interactions. Red nodes represent genes associated with viral processes, blue nodes are involved in the regulation of cell cycle checkpoints, cyan/green nodes are involved in gene expression regulation. Nodes with multiple annotated functions are larger. **B)** Enriched GO BP annotations in all experimentally validated target genes, sorted by fold enrichment >2.00 (Fisher's exact test with Bonferroni correction). Biological process font colours as in A. **C)** Enriched GO BP annotations for all nodes in A with degree >1, sorted as in B.

miR642a-3p Targets AFF4 mRNAs and Inhibits HIV-1 Expression

We then chose to explore in depth one specific interaction that we thought might be especially relevant to HIV-1 processes. For this, we explored the interaction between miR-642a and the 3' UTR of mRNAs encoding AFF4, a key player in HIV-1 regulation through the SEC (section 1.2c). To validate this interaction, we first verified that the miR-642a-AFF4 entries

identified in high-throughput experiments in the above curated databases were not false-positive entries. We used TargetScan release 7.2, which is based on a stepwise weighted regression model of known features that regulate miRNA-mRNA target pairing in humans (341), to output a context score of predicted interactions at the AFF4 3'UTR according to the known heuristics of human miRNA binding. Here, miR-642a had the second most favourable cumulative weighted context score for regulation of AFF4 mRNAs of all miRNAs listed on TargetScan (Table 6). This is significant, as miR-642a is only conserved among simian primates, which negatively affects its context score with respect to more evolutionarily conserved miRNAs (341). miRDB is a less constrained algorithm founded upon experimental data fed into a support vector machine for a less biased target score that has no conservation input (340). Similarly, AFF4 was among the five most favourably predicted mRNA targets of miR-642a in the miRDB database (Table 7).

Table 6: TargetScan prediction of human miRNA families with highest context ++ scores for targeting the 3' UTR of AFF4

miRNA family	Conserved sites	Poorly conserved sites	6mer sites	Cumulative weighted context++	Total context++ score
miR-193-3p	0	3	2	-0.69	-0.72
miR-642-3p	0	4	2	-0.52	-0.53
miR-27-3p	2	0	0	-0.46	-0.48
miR-151-3p	0	3	0	-0.44	-0.46
miR-361-5p	0	4	3	-0.41	-0.42
miR-505-3p	1	2	2	-0.4	-0.42
miR-802	0	2	1	-0.15	-0.41
miR-140-3p	1	1	0	-0.36	-0.37
…	…	…	…	…	…
miR-766-5p	0	1	0	-0.15	-0.15

Table 7: Top-ranked miRDB mRNA prediction targets of hsa-miR-642a-3p

Target Rank	Target Score	Gene Symbol	Gene Description
1	99	ZNF449	zinc finger protein 449
2	99	AGO4	Ago RISC catalytic component 4
3	98	NCOA7	nuclear receptor coactivator 7
4	**98**	**AFF4**	**AF4/FMR2 family member 4**
5	98	DIP2C	disco interacting prot. 2 homolog C
6	97	TMEM116	transmembrane protein 116
7	97	PABPN1	poly(A) binding protein nuclear 1
8	96	PCDH18	protocadherin 18
9	96	VGLL3	vestigial like family member 3
10	95	SPESP1	sperm equatorial segment protein 1

Once we were confident that AFF4 was a likely target of our miRNA, we started *in vitro* experiments by cloning the three first predicted 7mer miR-642a target sites from the AFF4 3'UTR alongside their 3' supplementary pairing sequences into the 3'UTR of EGFP in pEGFP-C1 (Figure 12A). EGFP was used here to have a clear read-out of miR-642a regulation at these sites in a gene reporter assay with an miR-642a mimic and antimiR targeting miR-642a. When co-transfected, with the reporter plasmid, miR-642a regulated the cloned GFP 3'UTR but not a control UTR containing GC-matched non-target sites from AFF4's 3'UTR (Figure 12B), showing that the predicted sites in AFF4's 3' UTR are indeed targets of miR-642a. The antimiR increased expression of the GFP reporter, showing that there is some endogenous activity of miR-642a in our cellular model. I then developed a positive control for AFF4 knockdown in cells by cloning an shRNA directly targeting the primary open reading frame of AFF4 transcripts into a psiRNA-U6 RNA Pol III expression plasmid. RT-qPCR of endogenous AFF4 mRNA in HEK 293T cells

showed similar levels of RNA interference by the positive knockdown control plasmid and the miR-642a mimic at standard miRNA mimic transfection concentrations (Figure 12C), supporting the hypothesis that miR-642a indeed targets AFF4 transcripts. We then ran a set of WBs for AFF4 in cells treated or absent of overexpressed HA-tagged AFF4 in combination with shAFF4. This assay confirmed the specificity of our AFF4 antibody (Figure 12D), which was used to quantify AFF4 expression in miRNA-treated cells, demonstrating that miR-642a induced a reduction of AFF4 expression in cells with respect to a nonsense miRNA control (Figure 12E).

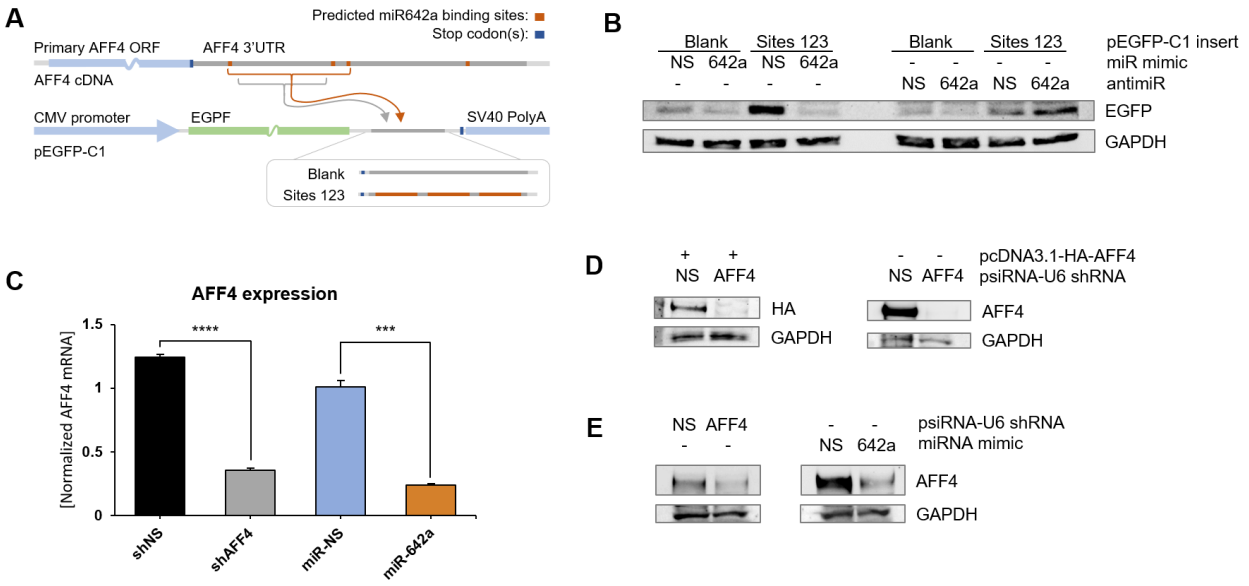


Figure 12: hsa-miR-642a targets AFF4 mRNA. A) Schematic showing the location of three TargetScan predicted miR-642a target sites in the 3'UTR of AFF4 cDNAs that were cloned into the 3'UTR of EGFP in pEGFP-C1 after an inserted Stop codon. **B)** 100 µg of whole-cell extracts from HEK 293T cells transfected with miRNA mimics or antimiRs for 48 h followed by 0.25 µg pEGFP-C1 for 24 h, were subjected to SDS-PAGE and blotted using anti-GFP and anti-GAPDH antibodies. **C)** RT-qPCR of AFF4 mRNA from HEK 293T cells transfected with psiRNAU6-shNS or psiRNAU6-shAFF4 alongside miR-NS or miR-642a miRNA mimics. Cells were treated for 48 h and their RNA was harvested for reverse transcription and qPCR using primers against AFF4 cDNA, normalized to Actin. Data are represented as means from three independent experiments +/- SEM. Unpaired two-tailed T-tests were performed using GraphPad Prism ($p(shNS/shAFF4) < 0.0001$, $p(miR\text{-}NS/miR\text{-}642a) = 0.0002$). Data for Figure 12C provided by Dr. Sergio P. Alpuche Lazcano. **D)** Western Blots of RIPA buffer extracts taken from HEK 293T cells transfected with pcDNA3.1-HA-AFF4 (0.25 µg/mL) or not, and psiRNA-U6 (-shNS or -shAFF4) (1.0 µg/mL) for 24 h. Wells were loaded identically with 35 µg of whole cell extracts and blotted differentially with anti-HA or anti-AFF4 antibodies. pcDNA3.1-HA-AFF4 does not contain the 3'UTR of AFF4 cDNAs and is not down-regulated by miR-642a (not shown). **E)** Cells were treated with miRNA mimics miR-NS or miR-642a for 72 h or psiRNA-U6 (-shNS or -shAFF4) (1.0 µg/mL) for 24 h. Wells were loaded with 100 µg of whole cell extracts and blotted using anti-AFF4 and anti-GAPDH antibodies.

Next, to confirm the predicted downstream effect of AFF4 downregulation on HIV expression, shAFF4, miR-642a and antimiR-642a were co-transfected or transfected sequentially with the HIV-1 molecular clone pNL4-3. In RT-qPCR and WB experiments, both shAFF4 and miR-642a downregulated expression of vRNAs and HIV proteins, respectively (Figure 13). In a WB, the antimiR targeting endogenous miR-642a increased viral protein expression (Figure 13B).

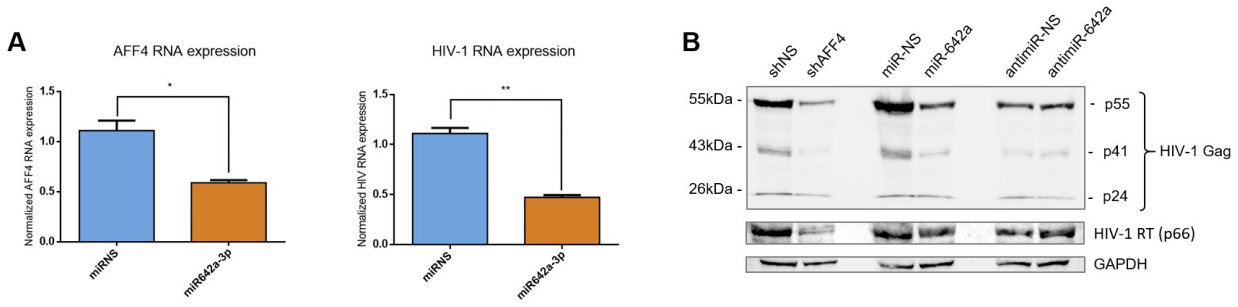


Figure 13: hsa-miR-642a-3p reduces HIV-1 expression. A) RT-qPCR of AFF4 mRNA and HIV-1 transcripts from HEK 293T cells transfected with miR-NS or miR-642a for 48 h then transfected with HIV pNL4-3 for an additional 24 h. RNA was harvested for reverse transcription and qPCR using primers against AFF4 cDNA and elongated HIV-1 transcripts (LTR3). Cq values are normalized as in Figure 12C. Data are represented as means from three independent experiments +/- SEM. Unpaired two-tailed T-tests were performed using GraphPad Prism ($p<0.05$ = *, $p<0.01$ = **). Figure 13a courtesy of Dr. Sergio P. Alpuche-Lazcano. **B)** 150 µg of whole-cell extracts from HEK 293T cells transfected with miRNA mimics or antimiRs for 48 h followed by 0.5 µg pNL4-3 for 24 h or co-transfection with 1 µg psiRNA-U6-(shNS or shAFF4), were subjected to SDS-PAGE and blotted using anti-p24, anti-RT and anti-GAPDH antibodies.

miR642a-3p Function is Counteracted in Gag Expressing Cells

To determine whether Gag enriches miR-642a on Dicer to increase loading of the miRNA into active miRISCs or to inhibit loading of the miRNA into Ago, cells were transfected with shAFF4, Flag or Flag-Gag for AFF4 expression to be quantified by RT-qPCR (Figure 14A). Transfected cells had a higher AFF4 expression than a no transfection control and shAFF4-transfected cells, but there was sufficient variance among replicates that the perceived increase in AFF4 in Flag-Gag with respect to Flag did not meet standard significance cut-offs ($p = 0.0685$). Similarly, a set of WBs from cells transfected with Flag, Flag-Gag, pNL4-3 and a pNL4-3 clone lacking Gag expression (pNLXX) showed higher AFF4 expression in Flag-Gag and pNL4-3 conditions than in conditions lacking Gag (Figure 14B, C). RT-qPCR of cells transfected sequentially with both the miRNA and one of the two molecular clones of HIV-1 (pNL4-3 or pNLXX) showed significant reductions in AFF4 and elongated HIV-1 transcripts by miR-642a for

both HIV-1 clones (Figure 14D). However, for both quantified transcripts, pNL4-3 conditions expressing Gag appeared to reduce this miRNA-mediated effect, suggesting a weakened effect of miR-642a in Gag expressing cells.

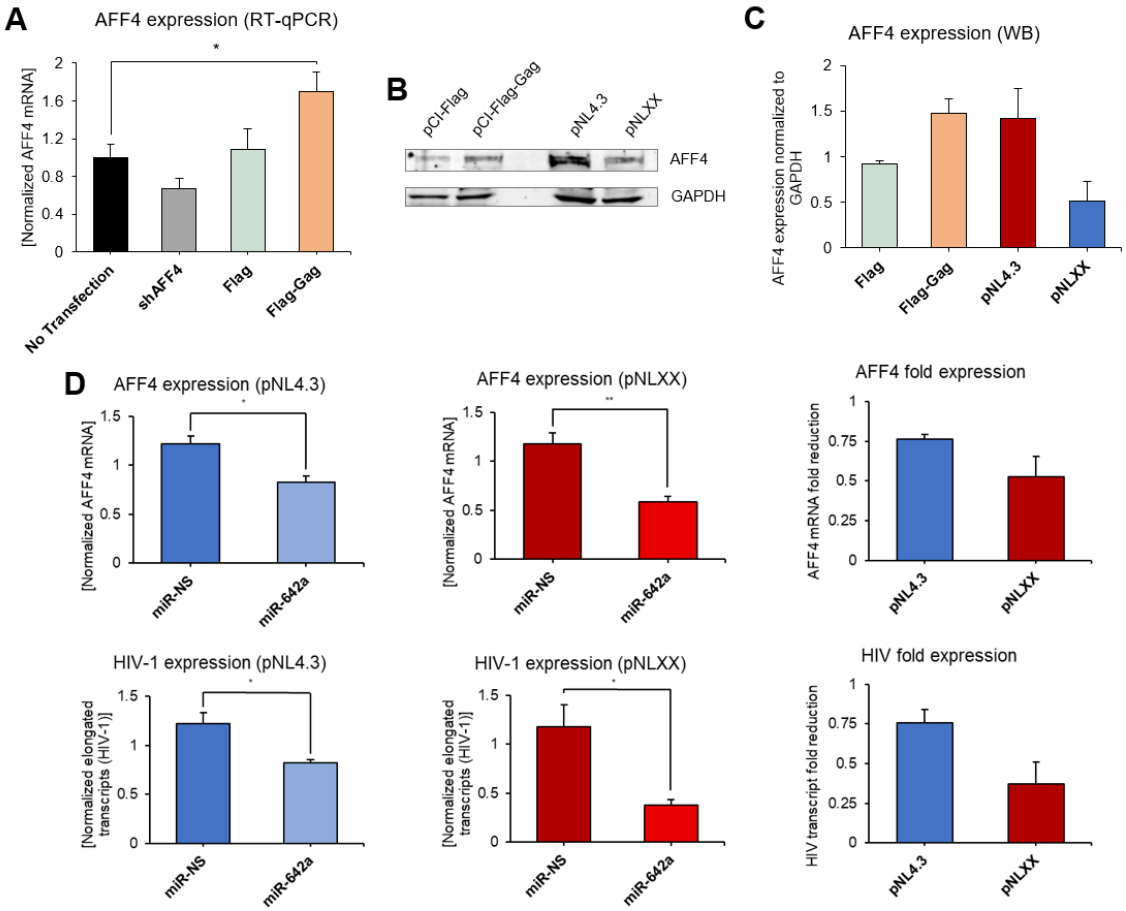


Figure 14: Gag increases AFF4 expression. A) RT-qPCR of AFF4 mRNA from HEK 293T cells transfected with psiRNAU6-shAFF4, pCI-Flag or pCI-Flag-Gag. Cells were treated for 48 h and their RNA was harvested for reverse transcription and qPCR using primers against AFF4 cDNA as in Figure 12C. Data are represented as means from five independent experiments +/- SEM. An ANOVA ($p=0.0046$) and a subsequent Dunnett's multiple comparisons test (p(no tranfection/Flag-Gag)=0.0262, p(Flag/Flag-Gag)= 0.0685 (ns)), were performed using GraphPad Prism ($p<0.05$ = *). **B, C)** 100 µg of whole-cell extracts from HEK 293T cells transfected with 1 µg/mL pCI-Flag, pCI-Flag-Gag, pNL4-3 or pNLXX (no Gag) for 48 h, were subjected to SDS-PAGE blotted using anti-AFF4 and anti-GAPDH antibodies. **B)** Representative WB of three. **C)** AFF4 band intensity was quantified for each blot using ImageJ software and normalized to GAPDH band intensity for corresponding lanes. Data are represented as means from three independent experiments +/- SEM. **D)** RT-qPCR of AFF4 mRNA and elongated HIV-1 transcripts from HEK 293T cells transfected miR mimics for 48 h and pNL4-3 or pNLXX for an additional 24 h. RNA was harvested for reverse transcription and qPCR using primers against AFF4 cDNA and elongated HIV-1 transcripts (LTR3). Data are represented as means from three independent experiments +/- SEM. Unpaired two-tailed T-tests were performed using GraphPad Prism ($p<0.05$ = *, $p<0.01$ = **). Raw data for Figure 14D were collected in large part by Dr. Sergio P. Alpuche Lazcano.

Aim 3: Developing an HIV-1 Locking Strategy Using ncRNAs

Preamble

One potential route towards a successful B&L intervention is the long-term suppression of pathways involved in latency reversion. Although assays have been developed to test candidate RNAi LPAs by acute LRA stimulation events (197-199), there is to our knowledge no published protocol for quantifying the long-term locking potential of single or multiple ncRNAs in cells. For this purpose, we made preliminary assessments of the efficacy of antiviral miRNAs and shRNAs identified in the literature review and in our own experiments for their variable effects on HIV-1 expression, cellular toxicity and inhibition of latency reversion, then proceeded to design a novel testing protocol to evaluate the efficacy, toxicity and long-term locking potential of short antiviral RNAs for medium-throughput RNA testing.

Identifying RNA Candidates for Combined Locking Therapies

We first assessed the efficacy of miRNAs already known to target a range of pathways involved in HIV-1 transcription and translation (miR-29a-3p and miR-155-5p) (section 2.3c) alongside the miRNAs discovered in Aim 2 of this thesis, to determine whether these RNAs would be good candidates for comparison in later optimisations of a medium-throughput assay using a standard vector. To determine whether the above miRNAs demonstrated any antiviral effects on HIV-1 expression, miRNA mimics were transfected into HEK 293T cells for 48 h, followed by transfection with 500 ng/μL pNL4-3 and incubation for an additional 24 h. Cell extracts were subjected to WBs against Gag, showing that positive controls miR-29a-3p and miR-155-5p reduced HIV-1 Gag expression with respect to a nonsense miRNA control, while miR-642a-3p and miR-766-3p unexpectedly showed more substantial reductions in virus expression (Figure 15A, B). We next assessed viability of HEK 293T cells transfected with each miRNA by WST-1 assay to determine whether there was any toxicity associated with the effects induced by the miRNA sequences. No significant difference in cell viability was seen between groups (single factor ANOVA, $p = 0.4945$) (Figure 15C). Finally, we explored the ability for each miRNA to inhibit HIV-1 reactivation from a latent state. J-Lat 10.6 cells were transfected with miRNAs and were then reactivated with PMA. WBs of these cell lysates showed a moderate inhibition of PMA-stimulated reactivation by miR-155-5p and miR-766-3p but showed no difference in reactivation levels between the nonsense control miRNA and miR-29a-3p or miR-642a-3p (Figure 15D).

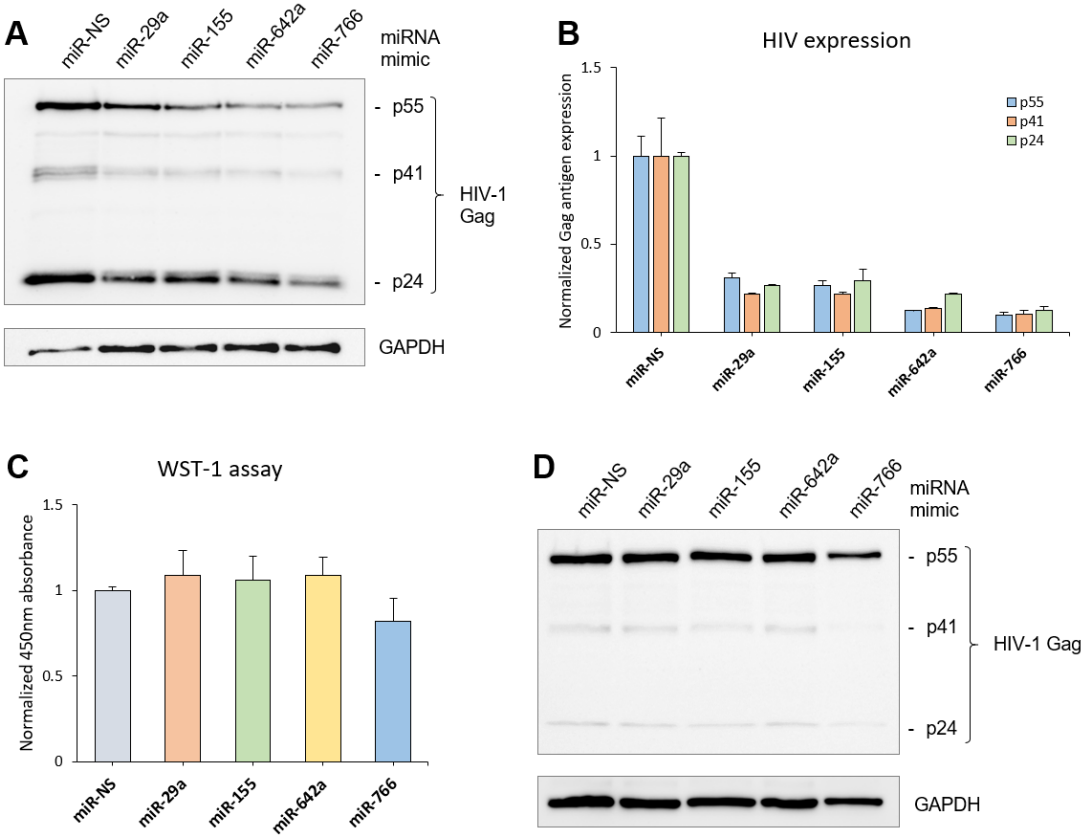


Figure 15: Effects of selected miRNAs on HIV expression, toxicity and latency. A) 30 µg of whole-cell extracts from HEK 293Ts transfected with miRNA mimics for 48 h, followed by 0.5 µg/mL pNL4-3 for 24 h, were subjected to SDS-PAGE and blotted using anti-p24 and anti-GAPDH antibodies. Representative WB of two. **B)** Gag band intensity was quantified for each blot and each band using ImageJ software and normalized to GAPDH band intensity for corresponding lanes. Data are represented as means from two independent experiments +/- SEM. **C)** WST-1 assay on HEK 293T cells treated with miRNAs for 48h. Data are represented as means normalized to miR-NS absorbance from three independent experiments +/- SEM. **D)** 30 µg of whole-cell extracts from J-Lat 10.6 cells transfected with miRNA mimics for 48 h, followed by reactivation with 20 ng/µL PMA for 24 h, were subjected to SDS-PAGE and blotted as in A.

To similarly make preliminary assessments of candidate shRNA constructs, sh143 that is known to inhibit HIV-1 reactivation from latency by targeting the HIV-1 promoter for TGS (292, 295) and shLdr4 and shPol247 that are known to regulate HIV-1 expression and replication by targeting conserved sites in the HIV-1 genome (346), were tested against active HIV-1 expression. None of these candidates demonstrated a robust inhibition of HIV-1 protein expression during 2-day HEK 293T co-transfections of 500 ng/µL of the shRNA construct and 500 ng/µL of pNL4-3 (single factor ANOVA, $p > 0.1$ for each of three bands), although shLDR4 and shPol247 appeared to moderately affect HIV expression (Figure 16A, B). This result could not be explained by

differences in construct toxicity measured by a WST-1 assay (single factor ANOVA, p = 0.5074) (Figure 16C). Although we used different techniques and readouts for HIV-1 inhibition than previous publications, these results demonstrate the need for a uniform testing protocol to assess shRNAs and miRNAs for their activities against HIV-1 expression and latency reactivation.

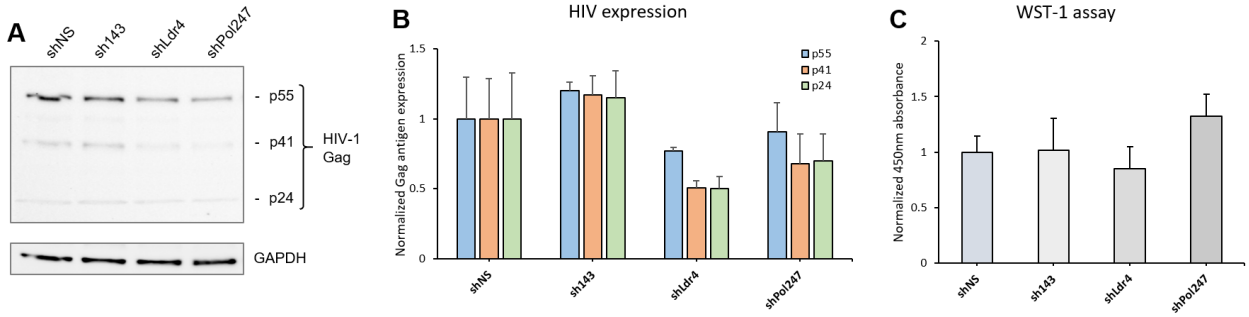


Figure 16: Effects of selected shRNAs on HIV expression and toxicity. A) 30 µg of whole-cell extracts from HEK 293Ts co-transfected with 0.5 µg shRNAs in psiRNA-U6 plasmids and 0.5 µg/mL pNL4-3 for 48 h, were subjected to SDS-PAGE blotted using anti-p24 and anti-GAPDH antibodies. Representative WB of three. **B)** Gag band intensity was quantified for each blot and each band as in Figure 15B. Data are represented as means from 3 independent experiments +/- SEM. **C)** WST-1 assay on HEK 293T cells transfected with shRNAs for 48 h. Data are represented as means normalized to shNS absorbance from three independent experiments +/- SEM.

Developing a Platform for Latency Testing

With the help of Dr. Ian Tietjen, a collaborator from the Wistar Institute, I developed a novel testing protocol to make unbiased assessments of the LPA potential of any miRNA or shRNA in parallel (Figure 17A). In this proposed experiment, sncRNAs of interest are cloned into an all-in-one Dox-responsive miRNA/shRNAmiR expression cassette for conditional expression in multiple cell lines (Figure 17B). Acute antiviral activity is first assessed transiently in HEK 293T cells by co-transfection of the ncRNA expression plasmid with pNL4-3 in the presence or absence of Dox, with readouts of a red fluorescence reporter for miRNA expression and viral proteins for viral expression (Figure 17A.1). sncRNAs that demonstrate inhibitory effects against active HIV-1 expression can then be prioritised. Selected antiviral sncRNAs are next co-transfected with packaging and envelope vectors to generate VSV-G coated lentiviral vectors to integrate these all-in-one expression cassettes into T-cells. Lentiviral constructs are then transduced into actively replicating $CD4^+$ T-cells to assess the efficacy of RNAi activity against infection and replication of HIV-1 viruses (Figure 17A.2). This secondary endpoint can also be used to filter candidate sncRNAs for their efficacy before committing to assessments of viral locking potential. To assess locking potential, VSV-G coated conditional sncRNA expression

cassettes are transduced into J-Lat 10.6 cells or another well characterized latent cell model for a preliminary assessment of conditional reporter expression and cell viability in the presence and absence of Dox. sncRNAs are induced over a period of 20 days, during which fractions are frozen down at regular intervals for later toxicity and virus expression assays. After 20 days of induction, cells from each condition are split into three groups: one group is shocked using PMA, the second continues to be exposed to sncRNA overexpression, and the third continues to be grown without sncRNA induction (Figure 17A.3). At regular intervals, fractions of the most promising candidates from the latter two groups are shocked using the same LRAs to determine the effects of sncRNA continuation/discontinuation on latency reversion.

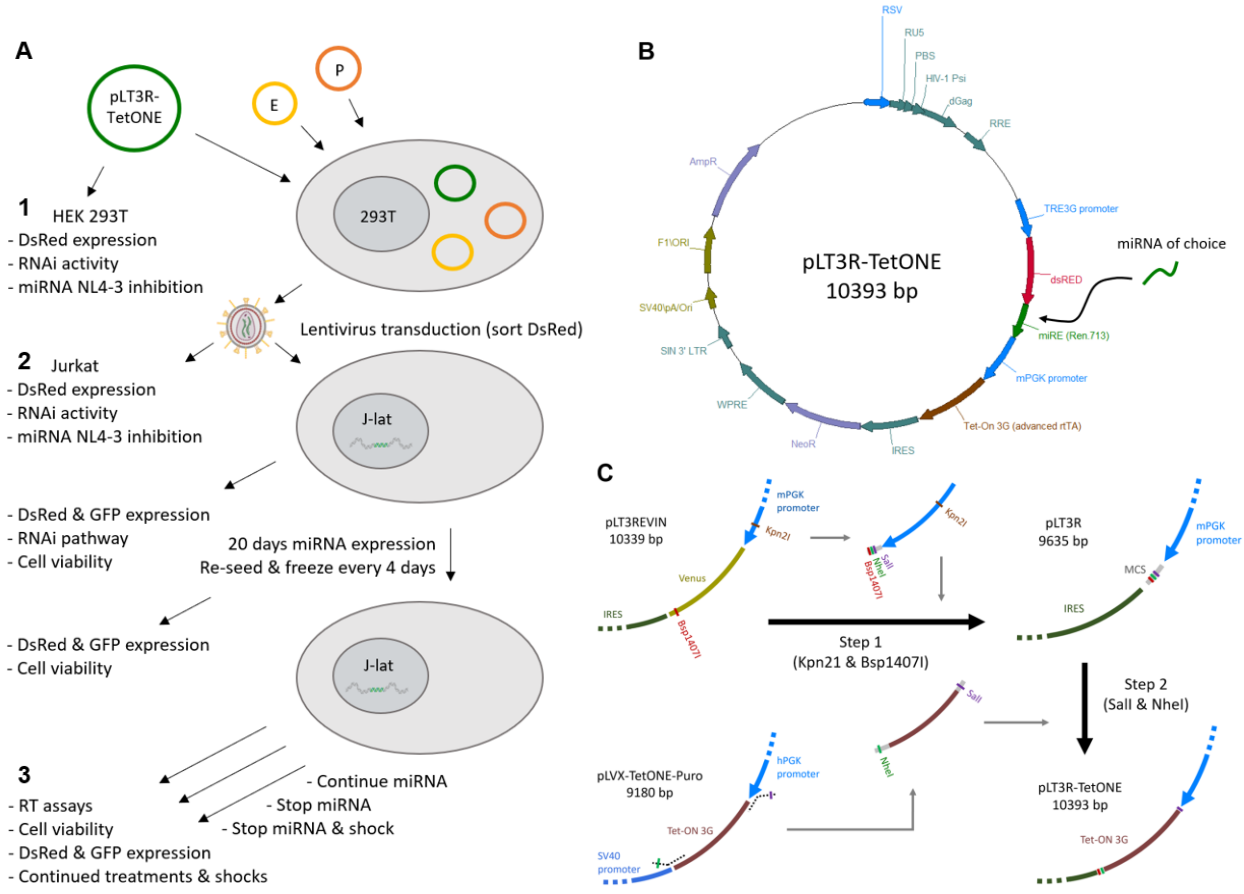


Figure 17: Latency testing protocol using pLT3R-TetONE. A) Schematic of the proposed latency testing protocol using inducible miRNA/shRNA expression, with primary endpoints testing (1) inhibition of HIV-1 expression, (2) inhibition of HIV-1 replication, and (3) short and long-term effects on latency reactivation. Protocol designed in collaboration with Dr. Ian Tietjen. **B)** pLT3R-TetONE. **C)** Workflow used to clone the inducible pLT3R-TetONE (bottom right) from pLT3REVIN (top left) in two cloning steps (bold arrows).

The first necessary element in the above protocol is a plasmid that can be stably integrated into target cells and can express ncRNAs of choice in an inducible manner. For this, we designed a lentiviral plasmid entitled pLT3R-TetONE that constitutively expresses an advanced rtTA protein called Tet-ON 3G, which allows for the conditional expression of a separate tetracycline-responsive cassette that includes a DsRED reporter and an miRNA "miR-E" expression backbone that can be cloned by chosen shRNA or miRNA sequences to induce strong and uniform sncRNA expression by RNA Pol II (254). The backbone of this plasmid including the Tet-responsive miR-E expression cassette were modified from pLT3REVIN (Figure 17C) (254). Briefly, the *Venus* gene on pLT3REVIN was replaced between restriction enzymes Kpn2I and Bsp1402I with a multiple cloning site (MCS) containing SalI and NheI. Tet-On 3G was then amplified from pLVX-TetONE-Puro and inserted into the newly created SalI-NheI MCS downstream of a PGK promoter to obtain pLT3R-TetONE. Ligations were performed in low-temperature conditions for optimal annealing and ligation of unstable substrates and cloning was performed in MAX Efficiency Stbl2 competent cells (Thermo Fisher) specifically designed for cloning unstable substrates. Although, each cloning step required multiple attempts with optimised conditions to proceed, we successfully generated pLT3R-TetONE. The completed plasmid was sequenced at both the MCS containing Tet-ON 3G and the miR-E site to confirm that the miR-E backbone could be cloned between restriction sites XhoI and EcoRI by different potentially antiviral sncRNAs.

After verifying that the miRNA expression site had intact XhoI and EcoRI restriction sites, we attempted to clone experimental miR-E constructs into this site. To express miRNAs to be processed by the Microprocessor as per their genomic forms, cloning was first attempted using overlapping PCR of miRNAs 29a and 642a using their full canonical stem loops (Table 3 oligonucleotide sets 8 & 9) listed on miRbase release 22.1 (353) followed by a templated PCR using cloning primers for miR-E (Table 5). At the same time, the processed RISC-loaded sequence for miR-29a was cloned in the same fashion as an shRNAmiR construct in case canonical stem loops were processed less efficiently (Table 3 oligonucleotide set 10). After multiple unsuccessful attempts using modified buffers or ligation conditions, we removed the plasmid's internal ribosome entry site (IRES) and neomycin/kanamycin/G418 resistance (NeoR) genes between Bsp1407I and NsiI using oligo set 12 (Table 3) to reduce the size of the plasmid and make it easier to work with in cells and obtain pLT3R-TetONE-ΔNeoR. We additionally attempted using oligonucleotides 13 & 14 (Table 3), primer pairs (Table 5) and matched conditions from the initial

publication by Fellman et al. that described the generation and shRNAmiR cloning of LT3REVIN to no avail (254). To date, optimal cloning conditions have not yet been found to allow the insertion of new sncRNA constructs into miR-E sites of pLT3R-TetONE-ΔNeoR, pLT3R-TetONE, or the unmodified pLT3REVIN copy supplied by the Zuber lab, so we could not proceed with our testing protocol as planned during the time limit of this thesis. Additional cloning strategies are currently ongoing.

Chapter 4: Discussion

Identifying Networks Associated with Blocks in Virus Expression during Latency

Much research into Block & Lock strategies against HIV-1 has focused on inhibiting initiation of HIV-1 transcription. However, growing evidence is supporting the new paradigm that post-initiation events play a dominant role in the regulation of latency reversal (section 1.2d). In Aim 1 of this thesis, we sought to identify host genes and ncRNA regulatory pathways that are modulated during reactivation of HIV-1 through post-initiation events.

Most researched models of latency include active Tat/TAR regulatory axes, complicating attempts to distinguish between agents that affect pre- or post-initiation regulatory events. To determine the factors associated with individual steps in latency reversal, we needed to first identify a model that could be used to exclusively study post-initiation events in HIV-1 regulation with an easily sorted read-out for reactivation. With the help of collaborators, our lab developed and characterized a novel Tat/TAR deficient model for HIV-1 latency in lymphocytes and monocytes. Using a variety of differently acting LRAs in the presence or absence of Dox, we confirmed that reactivation of this model could not be stimulated in the absence of Dox, so the model was indeed dependent on transcription initiation by the Dox-responsive rtTA transcription factor. We next verified whether we had successfully disrupted transcription initiation amplification events by T-cell activation, canonical NF-κB signaling and Tat-mediated transactivation at the GagzipGFP promoter. By comparing this model to the commonly referenced latency model J-Lat 10.6, we showed that transcription regulation by NF-κB signaling was largely inhibited at an mRNA level and undetectable at a protein level. We worked with collaborators to confirm using RT-ddPCR that the drugs we found to strongly reactivate GagzipGFP proviruses indeed act at the level of transcription elongation rather than initiation.

Once characterized, we reactivated this model using two different LRA classes and collected whole cell RNAs from populations of cells sorted for their expression of the recombinant GagzipGFP protein. rRNA depleted transcriptomes were sequenced in parallel with short RNA transcriptomes from each sorted population. We showed that reactivations of provirus expression were associated largely with unique transcriptome signatures for each drug treatment, but that specific long and short transcription modules were associated with reactivation no matter the drug combination. We noted that SAHA played an outsized role on the mRNA transcriptome, which

we expected to see due to the drug's non-specific HDACi activity. Unexpectedly however, prostratin appeared to have a greater effect on the miRNA transcriptome of reactivated cells. Several biological mechanisms might explain this finding, such as an enrichment of NF-κB binding sites in the promoters of pri-miRNA cassettes involved in post-initiation events in HIV-1 reactivation, but these findings might also be the result of batch effects in miRNA sequencing and so will have to be explored more in depth during the analysis of an additional sequencing replicate. We then showed that a gene cluster that differed between non-reactivated and reactivated populations for each drug combination tested were enriched in immune cell functions, supporting the importance of immune signaling cascades and functions in HIV-1 reactivation.

With the help of collaborators, we found enriched miRNAs, genes and gene functions that were co-regulated with a reactivation of HIV-1 elongation in SAHA-treated cells. These comparisons confirmed that SAHA treatment imprinted a specific signature on the cellular transcriptome and that GagzipGFP reactivation by SAHA relied largely on the amplification of this signal, possibly through increased drug penetrance and activity in these cells or by amplified stimulation of specific HDFs involved in HIV-1 reactivation. Genes decreased or amplified in the SAHA signature were involved in immune system activation and development or anergy and apoptosis. We then identified three RNAi regulatory networks whose four miRNAs were upregulated in reactivated cells and whose target genes were active in non-reactivated cells from the same SAHA-treated populations. The mRNAs targeted in the three regulatory networks were again enriched in functions associated with immune cell regulation, highlighting the importance of the immune regulation axis in GagzipGFP reactivation. As none of the four miRNAs identified in this analysis nor their regulatory networks have previously been identified in experiments tied to HIV-1 latency, this study may have identified novel regulators of HIV-1 reactivation. It is important however to caution that host factors and networks identified in these analyses were found to be co-regulated with HIV-1 expression, but this correlation does not imply causation and may rather be the downstream result of drug-induced regulatory programs that stimulate HIV-1 elongation through different pathway nodes. Also, this preliminary analysis was performed on single biological replicates for each drug treatment and bias may have been incorporated into these analyses by uncontrolled biological fluctuations or sequencing lane heterogeneities. A second replicate for every condition enumerated in this thesis has been collected and will be sequenced shortly to determine the reproducibility of our findings. Although further research is needed to

confirm the validity and reproducibility of these newly identified genes and RNAi regulatory networks, we suggest that approaches targeting similarly identified regulatory systems may be worth exploring in future Block & Lock studies.

HIV-1 Gag Inhibits the Function of Endogenous Antiviral miRNAs

We recently discovered that $p55^{Gag}$ interacts with Dicer in the cell and that this interaction leads to the specific enrichment of three miRNAs on Dicer in Gag-transfected cells without affecting their levels of expression. To our knowledge, no post-transcriptional sequence-specific recruitment of miRNAs to the RNAi pathway has previously been reported for a human virus, suggesting that this recruitment represents a novel post-transcriptional regulatory mechanism of specific PTGS effectors. As the RNAi pathway is known to regulate most human protein coding genes and HIV-1 is an obligate intracellular pathogen that requires host proteins to complete its replication cycle, we hypothesized that the Gag-Dicer interaction plays a role in tuning HIV-1 expression or latency by modulating the efficacy of these miRNAs.

To answer this hypothesis, we first explored targets shared by these identified miRNAs. We found in curated experimental miRNA-target interaction databases that 30.7% of the genes targeted by miR-642a-3p were also targeted by miR-766-5p and/or miR-766-3p, suggesting that the former miRNA may share similar regulatory functions to latter two which are co-regulated in cells. We therefore explored functional enrichments of the targets of these three miRNAs as a set and found that target genes were often annotated as HDFs of viral processes, regulators of cell cycle checkpoints and regulators of gene expression. Regulators of distinct stages of gene expression were similarly enriched for mRNA targets shared between miRNAs (degree >1). These data suggested that the selected enrichment of these three miRNAs on Gag-bound Dicer may modulate the expression of critical factors in HIV replication; namely direct acting HDFs that are known to support events in viral replication cycles, checkpoints in the cell cycle that might inhibit HIV replication through cell proliferation, and regulators of epigenetic remodeling, transcription and translation, which are known to critically regulate HIV provirus fate.

In the above network, miR-766-3p and miR-766-5p share the same pre-miRNA stem loop, many of the same gene targets and similar annotation frequencies of the above cellular functions with each other, while each targeting a large list of human genes. miR-642a has a much more limited target set but shares similar functions to the former two miRNAs. miR-642a would thus be

a simpler miRNA to study in a hypothesis-driven analysis of a chosen miRNA-mRNA target pair that might represent the downstream effects of the Gag-Dicer interaction. With this in mind, we decided to explore one miR-642a-target interaction in depth using direct *in vitro* techniques. From the above network, we chose to study an interaction between miR-642a and AFF4 because the latter had already been identified as a key regulator of HIV-1 expression and latency reactivation and therefore may have been a selecting factor in the evolution of Gag in support of an miRNA-specific Gag-Dicer interaction (section 1.2c) (108, 135).

While curated experimental databases are useful for exploring the scope of miRNA regulatory networks that can be seen in cells, they sometimes contain false positive target pair entries from high-throughput sequencing experiments. All listed experiments in miRTarBase and TarBase that identified an interaction between miR-642a and AFF4 involved high-throughput sequencing, so the validity of this proposed interaction was not yet certain. We therefore verified the miR-642a-AFF4 interaction using two distinct algorithms that model and score interactions between miRNAs and target sequences in cellular mRNAs based on known rules of human miRNA-target interactions. Both prediction tools scored this interaction highly and predicted as many as six functional miR-642a targets in the 3'UTR of AFF4, confirming that AFF4 was most likely a target of miR-642a and would thus be worth exploring in further detail *in vitro*.

The gold-standard for confirming the functional regulation of an mRNA by an miRNA is to demonstrate a direct inverse correlation between the abundance of the miRNA and its proposed target in cells via low-throughput experiments. To confirm that miR-642a interacts with AFF4 mRNAs in cells, predicted miR-642a target sequences in the 3'UTR of AFF4 were used in a gene reporter assay in cells transfected by the miRNA mimic, which showed that miR-642a indeed downregulated the reporter's expression. It is important to note here that miRNA targets that are in close proximity to each other can cooperate to increase the efficacy of silencing by miRISCs (219). As we concatenated three predicted miR-642a target sites from the AFF4 3'UTR within a 100 bp stretch for this gene reporter assay, this assay could only confirm that these predicted sequences in the AFF4 3'UTR are bound by miR-642a-RISC in the cell. To then determine whether miR-642a expression would be sufficient to inhibit AFF4 mRNAs, we sought to quantify levels of endogenous AFF4 in conditions where miR-642a was over- or under-expressed. RT-qPCR showed similar levels of endogenous AFF4 mRNA regulation by a miR-642a mimic and

the shAFF4 knockdown control, while a WB showed similar results at a protein level, confirming unequivocally that miR-642a regulates AFF4 expression in human cells.

We next tested whether AFF4 knockdown by shAFF4 and AFF4 regulation by miR-642a have any effects on HIV-1 expression. shAFF4 and miR-642a inhibited HIV-1 expression in both RT-qPCR and WB experiments, while the inhibition of endogenous miR-642a by an antimiR increased expression of HIV-1 proteins. These data confirmed that a knockdown of AFF4 can result in a reduction of HIV-1 expression and showed that miR-642a similarly inhibits HIV-1. Considering the absence of miR-642a seed targets in the NL4-3 HIV-1 genome and the above finding that miR-642a regulates AFF4 expression through multiple seed targets, we can infer that miR-642a's negative effect on HIV-1 works indirectly at least in part through AFF4 repression.

Although we had confirmed that miR-642a indeed regulates AFF4 and has a negative effect on HIV-1 expression, it remained unclear whether the increased occupancy of miR-642a on Gag-bound Dicer has any effect on miR-642a's activity in cells. RT-qPCR and WB assays in cells expressing Gag showed that Gag increases AFF4 mRNA and protein expression. RT-qPCRs further demonstrated that transfection with an HIV-1 clone that expresses Gag can overcome reductions in AFF4 and HIV-1 expression that are stimulated by miR-642a transfection. These data demonstrate collectively that HIV-1 Gag can disinhibit AFF4 expression and promote HIV-1 expression, a finding not previously shown in the literature.

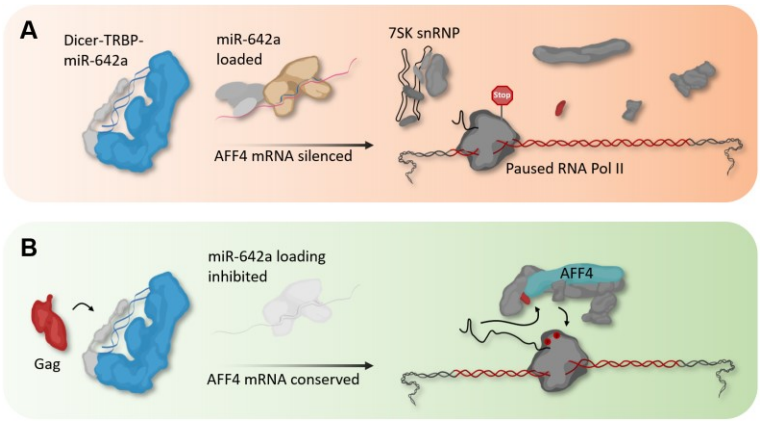


Figure 18: Proposed model: Gag sequesters miR-642a and supports HIV-1 expression. A) In the absence of Gag, miR-642a is processed by Dicer and loaded onto RISC, maintaining low levels of SEC assembly and HIV-1 elongation. SEC members as in Figure 3B. **B)** Above a threshold of Gag expression, miR-642a is sequestered on the Gag-Dicer complex and SECs can form to elongate vRNAs.

In this aim, we showed that Gag binds to Dicer and increases the Dicer occupancy of three specific miRNAs that target genes involved in virus and gene expression regulation pathways. We showed that miR-642a can directly reduce AFF4 expression and indirectly inhibit HIV-1 expression. Finally, we showed that Gag disinhibits AFF4 expression and resultingly increases HIV-1 expression. We propose that in interacting with Dicer, Gag may be preventing or stalling

the processing of miR-642a-3p into an effective mature miRNA (Figure 18), which would be consistent with the increased post-transcriptional occupancy of miR-642a-3p on Dicer. Such an activity has not previously been reported for any other viral protein, but has been shown for the cellular RNA-binding protein Lin28a, which interferes selectively with the biogenesis of miR-Let7 by binding to the miRNA's terminal loop during Dicer processing (354, 355), and which shares homology with a conserved Zinc-knuckle domain in Gag's nucleocapsid substructure (356).

Although these data point towards a promising model for explaining an interaction between Gag and Dicer that sequesters specific miRNAs on the complex, further experiments will be needed to confirm this model and to determine its importance in HIV-1 infected cells. A targeted look at the abundance of miR-642a-loaded RISCs in HIV-1 expressing T-cells will be needed to confirm the suggested model that the Gag-Dicer interaction sequesters miR-642a from effective AFF4 inhibition. To determine whether Gag increases HIV expression primarily by inhibiting miR-642a and increasing AFF4 or whether Gag effectuates this change through other redundant mechanisms, Gag and HIV-1 will need to be expressed and assessed in cells that lack miR-642a expression. Further research will also be needed to confirm our findings in physiologically relevant conditions; our work was focused in HEK 293T and HeLa cell lines, which are easy to work with for experimental manipulations and broadly reflect conditions in other human cells but are not identical to human T lymphocytes. The above findings will need to be replicated in primary cells derived from people living with HIV before any generalizations can be made. More broadly, it is not yet clear how HIV-1 Gag interacts with Dicer and how this interaction leads to the post-transcriptional enrichment of specific miRNA sequences on the complex. Future phylogenetic analyses of HIV-1 variants will be useful to define evolutionary constraints on Gag that define this interaction and whether this interaction had a strong or dilute effect on the transmissibility of the virus, while structural analyses will better explain the domains, cofactors and steps involved in this interaction, which may define new target moieties for antiviral therapies.

Comparing the Locking Potentials of sncRNAs

Latency is an ill-defined and diverse combination of cell states (section 1.2d). For a Block & Lock intervention to be successful at achieving its aim of a continued suppression of the viral reservoir in the absence of drugs, it will need to be successful at inhibiting events in latency reversal that may be triggered by strong external environmental cues (ex. drugs, stress or T-cell

activation) or stochastic fluctuations in the strength of other inhibitory regulatory networks. Studies into the *in vitro* efficacy of an LPA thus require assessments of the drug's ability to inhibit acute drug-mediated reactivation events in addition to it's ability to maintain a cellular environment that is not permissive to low-level translation or minor variations in the cellular regulatory environment that can lead to virus expression feedback loops once cART is paused.

Several shRNAs and miRNAs have been suggested as candidates for B&L therapies for their abilities to inhibit the virus directly or to modulate the expression of host pathway members that contribute to a reactivation-permissive cell state (section 2.3c). Although multiple candidate RNAi molecules have been researched to limit HIV-1 reactivation, few studies have compared the top candidates side-by-side for their efficacies against reactivation. Furthermore, no studies that have tested shRNAs and miRNAs against latency reactivation have gone past the step of measuring locking potentials against drug-mediated reactivation. Before significant investments are to be made into B&L RNAi therapies, candidate RNAi effectors will need to be compared in an unbiased manner in a single model system. In doing so, it will be important to quantify several measures of drug effects, including the abilities of each candidate to limit both acute and stochastic events in latency reactivation. With these constraints in mind, we designed a novel protocol that can be used to score shRNAs and miRNAs together in a single experiment with four primary scoring endpoints that will all be factors in the assessment of candidate B&L RNAi and ncRNA drugs.

Before we could jump into the development of our assay, we began with a pilot experiment to see whether miRNAs could coordinate similar levels of antiviral activities to shRNAs, and to define a set of baseline comparisons of strong and weak inhibitors that we could then use to optimise a novel locking assay. We compared the most cited candidate B&L RNAi substrates for their abilities to inhibit HIV-1 expression and drug-mediated latency reversal in addition to their cellular cytotoxicities. In our preliminary analysis, we included miR-29a and miR-155 as the most studied miRNA candidates in B&L therapies, alongside sh143 (involved in TGS against the HIV-1 promoter), and shLDR4 & shPol247 (involved in PTGS of conserved regions in the viral genome) (292, 295, 308, 312, 313, 346). We also included miR-642a and miR-766, which we have shown target several systems involved in virus replication/reactivation and can directly dampen the expression of cellular factors necessary for reactivation (Figures 11 & 12).

This preliminary analysis of miRNA candidates interestingly showed that miR-642a and miR-766 were more potent inhibitors of HIV-1 expression than the positive controls miR-29a and

miR-155. These data bolster the proposition in Aim 2 that the Gag-Dicer interaction may be a critical regulator of the HIV-1 replication cycle, while supporting the use of these miRNA candidates in locking assay optimisations because of their wide range of antiviral activities. No significant difference in HEK 293T cell viability was seen between miRNA treatments, supporting the validity of the above findings. We then explored the ability for each miRNA to inhibit HIV-1 reactivation from a latent state. miR-155 and more notably miR-766 appeared to moderately inhibit PMA-stimulated latency reactivation, while miR-29a and miR-642a had no effect on reactivation levels. Lymphocytes are notoriously difficult to transfect, so it is possible that miRNA levels in this last experiment were not sufficient to be biologically significant. Although further replications of this experiment in the same and other models of latency will be needed, these data support the use of these miRNAs for the optimisation of a B&L testing protocol, as there appears to be a noticeable variability between miRNA conditions.

shRNA candidates were similarly tested in preliminary assessments. These data showed no notable variability in cellular viability between conditions, but also surprisingly demonstrated that none of the three proposed candidates significantly inhibited HIV-1 protein expression over 2-day co-transfection experiments. Although these results stand in contrast with previous publications that identified sh143, shLDR4 and shPol247 as antiviral candidates, our experiments differed from these previous experiments in several procedural ways and thus do not repudiate previous reports (292, 295, 346). Nevertheless, further candidates and testing conditions will have to be screened in order to establish a suitable level of background variability in anti-HIV-1 candidate activity for the B&L assay to be optimised as a valid measure of LPA potential. We excluded shRNAs targeting HDFs in our preliminary analysis because direct knockdown of cellular genes involved in multiple host processes may result in unpredictable cellular phenotypes, while miRNAs that are already present in the cell and shRNAs that specifically target exogenous sequences are likely to be better tolerated (196, 256). shRNA constructs that are expressed at low levels or that like miRNAs do not have complete complementarity to their target genes might possibly be used in the future to selectively sculpt the transcriptome to become a less favourable environment for virus replication without completely knocking down essential genes. However, HDF regulatory networks that are identified in studies like those in Aims 1 & 2 of this thesis are presently more likely to be effectively used to refine candidate lists for small molecules and aptamers that target HIV-promoting HDF domains, rather than to be targeted for translational regulation.

As our B&L scoring assay required consistent conditional long-term expression of RNAi substrates in several cell types, our protocol demanded the parallel development of a versatile vector that could express shRNAs and miRNAs at equal levels. Our ideal vector would therefore need to be lentiviral to be delivered for consistent expression over long experiments, it would need to have a different fluorescence marker than the current cellular models of HIV-1 latency (most often GFP) and it would need to have a miRNA backbone for RNA Pol II expression of miRNAs or shRNAmiRs under the control of a conditional all-in-one expression system. We conceived the pLT3R-TetONE plasmid for this purpose (Figure 17). Although we were able to create pLT3R-TetONE, time constraints did not allow us to insert shRNAs and miRNAs into the miR-E cloning site. This has delayed the implementation of our candidate testing system until after the writing of this thesis. The most promising candidates from our preliminary analysis will be tested soon.

Overview & Future Directions

In this thesis, we explored three aims which collectively built towards the larger goals of better understanding host-virus interactions involved in the control of HIV-1 expression and better informing the development of future antiviral RNA therapies.

In our first aim, we characterized a new model of HIV-1 latency which can be used to study the host transcriptome specifically associated events in latency reactivation that follow vRNA transcriptional initiation. This first aim identified global gene and miRNA transcriptional changes that were associated with latency maintenance or reactivation that were either generalizable or specific to the drug used for reactivation. Our analyses identified specific gene functions that were modulated in cells for which the virus was reactivated and additionally pointed towards RNAi regulatory networks that might govern the cellular environment in order to affect the permissiveness to post-initiation events in reactivation. As mentioned above, further research will be needed to confirm the validity of these findings before we can begin an in-depth analysis of these regulatory networks. Nevertheless, this and similar studies are critical for painting a more granular portrait of the regulatory networks that maintain latency at different levels.

We next explored an interaction between HIV-1 Gag and the RNAi endoribonuclease Dicer. Although it remains controversial whether HIV-1 is globally negatively affected by the functionality of RNAi in patients (276, 301, 311, 357), it appears that the virus has evolved to interact with Dicer in order to modulate the functions of a few miRNAs that might play outsized

roles in the regulation of HDFs involved in the viral replication cycle. We showed that Gag increased the Dicer occupancy of three miRNAs that targeted genes involved in processes necessary for provirus expression, propagation and replication. We further showed that Gag nullified the effects of miR-642a on AFF4, thereby increasing HIV-1 expression; this suggests a possible evolutionary rationale for this sequence-specific enrichment of miRNAs on Dicer. As miRNAs are key regulators of cellular homeostasis and innate immune signaling in cells (205, 358), it may be more advantageous for the virus to specifically target this small list of antiviral miRNAs post-transcriptionally than to encode global RNAi silencing suppressors that might trigger immune activation or aggravate cellular toxicity. Although further research will be needed to explain the evolutionary importance and structural mechanism of this interaction, we proposed from these findings that miR-642a and either strand of miR-766 would nevertheless be worthy candidates to include in a search for new Block & Lock therapies.

We finally sought to develop a protocol to standardise measurements and rankings of the acute and long-term efficacies of antiviral RNAs that are to be combined in B&L therapies. We identified several candidate RNAs that regulate HIV-1 expression either directly or through host factors and interestingly found that the miRNAs identified in Aim 2 were more effective at inhibiting active HIV-1 expression than the most promising miRNA candidates proposed in the literature. We then designed and began the development of our B&L testing protocol that will be optimised using the above candidates. The engineered vector will help future studies in making direct unbiased rankings of the most effective candidate RNAs for B&L therapies.

In order to appropriately prioritise the future directions of our research, it is important to consider the intended uses of an oligonucleotide B&L cure and to accordingly evaluate discoveries of antiviral RNAs within this context. Several novel gene therapy approaches exist, but different approaches would be more appropriate than others in the context of an HIV cure. RNA gene therapies can be delivered to cells *ex vivo* and returned into patients as HSCTs, but these approaches are presently highly costly and expertise-dependent, which may limit their use for the majority of people who live with HIV in developing nations. RNA therapies that can be delivered systemically are less dependent on expertise, can be effective in RNAi applications [ex. patisiran (Onpattro)], and have gained widespread public acceptance with the recent developments of Moderna's mRNA-1273 vaccine and Pfizer-BioNTech's BNT162b2 vaccine, but face different challenges for targeting HIV-1. To eliminate the need for repeated drug exposures, systemic

oligonucleotide therapies will likely need to be expressed long-term in reservoir cells. Synthetic siRNAs or miRNAs may become attractive therapies if nanoparticles can be formulated to introduce multiple RNAs into reservoirs in a single treatment and to extend treatment half-lives. Pre-fabricated sncRNAs may thus be used in the near future in an HIV locking cure in parallel with long-lasting viral vectors to offer patients better and individualized treatment options.

It might be worth considering a hypothetical construct in order to better visualize the challenges that need to be faced in the coming years of B&L cure research. Here, we may consider an AAV vector that includes a lymphocyte- or HIV Tat-specific RNA Pol II promoter and an array of multiplexed antiviral sncRNAs (Figure 19). In this system, we might imagine including an shRNAmiR that directly downregulates CCR5 to prevent reservoir propagation. We might also imagine including an shRNAmiR that targets conserved regions of the HIV-1 promoter for TGS, such as sh143 or shPromA (241, 292, 295). Decoys for functional RNA elements in the HIV genome such as TAR or RRE may help dilute the effects of Tat and Rev on viral expression, while small RNA aptamers that inhibit Tat-P-TEFb binding or other host-virus interactions might prevent other latency reactivation events (195). When used in combination, shRNAs that directly target conserved and accessible regions in the HIV-1 genome can effectively delay viral rebound and so could also be included in this hypothetical polycistron (346). Finally, miRNAs such as miR-642a might also be incorporated into the AAV if they can downregulate networks of host factors that promote viral reactivation without causing undue cytotoxicity.

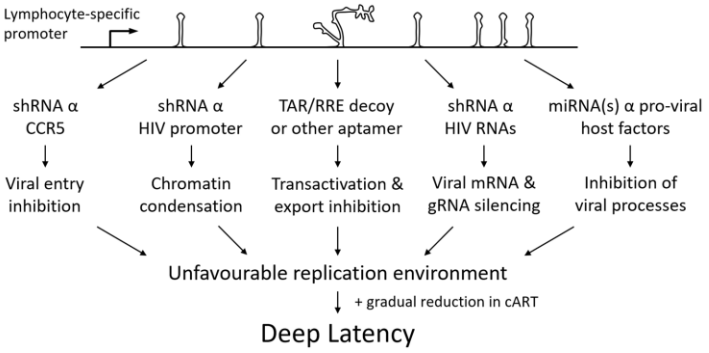


Figure 19: A hypothetical curative combination therapy. A vector system representing operative segments and modes of action that can be concatenated in a multivalent gene therapy for a possible HIV-1 functional cure.

In order to successfully prevent viral escape from a heterogeneous reservoir, a curative gene therapy will need to exploit multiple types of molecules that target different stages of the viral replication cycle. To expand the repertoire of antiviral RNAs that can be used in therapies, more needs to be known about host regulatory networks that determine the latent or reactivated fates of HIV-1 proviruses. Meanwhile, it remains unclear which antiviral RNAs in the literature

should be combined in multivalent curative approaches, because no efforts have yet been made to systematically evaluate B&L efficacy. Going forward, there is a need for further in-depth examinations of host-HIV interactions and more direct comparisons between antiviral RNAs.

Conclusion

In this thesis, we characterized a new cell model of HIV-1 latency that can be used to study events in HIV-1 reactivation that are independent of transcription initiation, which we then used to identify global transcriptome signatures, specific mRNAs and novel miRNA regulatory networks that are associated with these later stages of reactivation. We then explored and explained *in silico* and *in vitro* an interaction between HIV-1 and a critical member in RNAi functionality, which we identify as a novel post-transcriptional mechanism by which mammalian viruses are capable of selectively modulating the cellular regulatory environment, and which we propose is an evolved mechanism by which HIV-1 is able to indirectly overcome host limitations to viral replication. We finally identified functional RNAs that demonstrate variable effects on viral expression and acute reactivation from latency, and began the development of a protocol for testing identified ncRNAs for their potential to hinder HIV-1 reactivation in a Block & Lock approach. While each of these aims require further development, insights gained from their combined efforts may contribute to a better understanding of host pathways involved in latency and to new effector molecules for the development of an HIV-1 cure.

RNAi and other inhibitory RNAs can theoretically target every known regulator of HIV-1 infection, integration, latency disruption and replication. Improved stability, toxicity and immune evasion chemistries of nucleic acid delivery systems have shown great promise in recent clinical trials, pointing towards a new age of precision oligonucleotide therapeutics. Due to a rapidly growing understanding of the mechanisms by which therapeutic RNAs can maintain or reverse HIV-1 latency, combined oligonucleotide therapies may soon develop into a *bona fide* HIV cure.

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