Exploring Cure Strategies for Viruses

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Abstract

Viruses are an ongoing and evolving threat to public health. Although Human Immunodeficiency Virus 1 (HIV-1) has been widespread for decades, the virus was only first identified in the early 1980s. Zika virus (ZIKV), on the other hand, was identified in the late 1940s, but was only brought to the public eye in recent outbreaks. While these viruses are very distinct, they both lack effective curative strategies.

HIV-1 is treated with antiretroviral drugs, but these drugs are unable to eradicate the virus from latent reservoirs. A proposed strategy for a cure involves reactivating latently infected cells with the use of Latency Reversing Agents (LRA) so that these viruses can be targeted by conventional drugs and/or a strong immune response. For our first aim, we have characterized a new model of HIV-1 latency in CEM lymphocytes, THP-1 monocytes, and THP-1 monocyte-derived macrophages to test the efficacy and toxicity of various LRA. Then, using this model, we have separated reactivated cells from non-reactivated cells and isolated the corresponding RNA, which has been sent for RNA sequencing to build a differential expression pattern of mRNA, miRNA, and long non-coding RNAs. These RNAs will be studied for their implication in the maintenance or disruption of HIV-1 latency.

While ZIKV is generally associated with non-specific symptoms, recent outbreaks have implicated the virus in the development of Guillain-Barré syndrome in adults and microcephaly in babies born from infected mothers. Furthermore, several of these outbreaks have infected large portions of the population. This would suggest that recent mutations in the virus have led to increased pathogenicity and spread. Furthermore, there is currently no approved vaccine or treatment for this virus. Small interfering (si)RNAs have been used to inhibit the replication of many viruses. Recently, the delivery of a siRNA targeting Ebola virus has been shown to be

protective against the virus in rhesus macaques, but there has been no siRNA developed against ZIKV. In the second aim of this project, we have studied two strains of ZIKV: a Brazilian strain from a recent outbreak, and a Thai strain whose lineage is distinct from those associated with neurological complications. We compare their cytopathic effect and ability to generate viral RNA and viral titers. Next, we have designed and are in the process of validating siRNA(s) targeting the ZIKV genome that could be used in to prevent the spread of the virus.

Résumé

Les virus constituent une menace permanente et en constante évolution. Bien que le virus de l'immunodéficience humaine 1 (VIH-1) soit répandu depuis des décennies, le virus n'a été identifié que pour la première fois au début des années 1980. D'autre part, le virus Zika (ZIKV) a été identifié à la fin des années 1940, mais il n'a été révélé au public que lors des récentes épidémies. Bien que ces virus soient très distincts, leur point commun est le manque de traitements curatifs efficaces.

L'infection au VIH-1 est traitée avec des médicaments antirétroviraux, mais ces derniers sont incapables d'éradiquer le virus des réservoirs latents. Une stratégie proposée pour une guérison consiste à réactiver l'expression du VIH-1 par les cellules infectées de manière latente et de cibler et tuer ces cellules par des médicaments conventionnels et / ou une forte réponse immunitaire. Ces cellules latentes sont réactivées à l'aide d'agents de réversion de la latence appelés LRA. Pour notre premier objectif, nous avons caractérisé l'efficacité et la toxicité de divers LRA dans un nouveau modèle de la latence du VIH-1 à la fois dans les lymphocytes CEM, les monocytes THP-1 et les macrophages dérivés des monocytes THP-1. Ensuite, en utilisant ce modèle, nous avons séparé les cellules réactivées des cellules non réactivées et isolé l'ARN total de ces deux populations cellulaires. Ces ARN ont été envoyé pour séquençage (RNA-seq) afin d'obtenir un profil d'expression différentielle des ARNm, des microARN et des longs ARN non codants. Ces ARN seront étudiés pour leur implication dans le maintien ou la perturbation de la latence du VIH-1.

Alors que le ZIKV est généralement associé à des symptômes non spécifiques, des épidémies récentes ont impliqué le virus dans le développement du syndrome de Guillain-Barré chez l'adulte et la microcéphalie chez les bébés nés de mères infectées. De plus, certaines de

ces épidémies ont infecté de larges portions de la population. Ceci suggère que des mutations récentes du virus ont conduit à augmenter sa pathogénicité et sa propagation. En outre, il n'existe actuellement aucun vaccin ou traitement approuvé pour ce virus. De petits ARN interférents (siARN) ont été utilisés pour inhiber la réplication de nombreux virus. Récemment, l'administration d'un siARN ciblant le virus Ebola a conféré une protection contre le virus chez les macaques rhésus, cependant aucun siARN n'a été développé contre le virus Zika. Dans La deuxième partie de ce projet, nous avons étudié deux souches de ZIKV: une souche brésilienne issue d'une épidémie récente, et une souche thaïlandaise dont la lignée est distincte de celles associées à des complications neurologiques. Nous avons comparé leurs effets cytopathiques et leurs capacités à générer de l'ARN viral et des virus infectieux. Par la suite, nous avons conçu et sommes toujours en train de valider des siARN ciblant le génome du ZIKV qui pourraient prévenir la propagation du virus.

Acknowledgments

After interviewing with several labs for my B.Sc. honours project – which would later become my B.Sc. – M.Sc. fast track project – I found myself in the 5th floor conference room of the Lady Davis Institute with Drs. Gatignol, Rance, and Scarborough, and lab manager Aicha Daher. I was touched that all of these fantastic researchers had taken time out of their busy day to share their work with me. It showed me what a collegial environment and positive learning atmosphere Dr. Gatignol had set up in her lab, and I was eager to be a part of it. Fast-forward two years and I am deeply saddened to be leaving the lab, but ever so grateful to have been a part of it and to have learned so much from each of its members.

First off, I would like to thank Anne for all of her support; in the lab, in my coursework, and outside of my degrees. She always encouraged me to attend conferences, apply for scholarships, and pursue my research interests. Indeed, she was deeply involved in every aspect of my project, by helping me interpret results and plan future experiments, or by editing all of my work multiple times, to make sure I got the most out of this M.Sc. She truly does go above and beyond for all of her students. With over 20 years of experience working with HIV, her on-going enthusiasm for scientific research inspires me to pursue my own passions. I hope to be as dedicated to my future career as she is towards hers.

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I really did benefit from each member of the Gatignol lab, both past and present. Sergio and I had the exciting opportunity to begin working on Zika together. I thoroughly enjoyed my time working with him because of his passion for science, his intellectual curiosity and his vision. I am very excited to see where his ambitions will take him. Rob played a great advisory role in many of my projects in the lab. I will always look up to him for his kindness, his open-mindedness and his ability to communicate science. Aicha is one of the most generous and patient people I have ever met. She was always willing to lend me a hand in all of my experiments, whether she had the expertise or not – although she often did. Olivier was always eager to teach me protocols, lend me reagents, or show me to latest viral video. I will never forget the great HQ Trivia team of me, Aicha, Rob, Roman, and Ryan. We may not have won any money, but I will always cherish those very important 3:00PM "lab meetings". While it is unfortunate that they will no longer be my colleagues, I am privileged to call them my friends.

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Finally, I would like to thank my parents. I could probably write another 27,000 words on all that they have taught me and given me that has made this – and all of my other accomplishments – possible. The easiest way to summarize is "I love you guys".

Preface and Author Contributions

This thesis was written under the supervision of Dr. Anne Gatignol. The thesis in its entirety was extensively reviewed by Dr. Gatignol and Dr. Elodie Rance. The Abstract, Résumé, Introduction Material and Methods, Results, and Discussion were edited by Drs. Gatignol and Rance.

For project 1 on HIV-1, all of the work presented was performed in conjunction with Dr. Elodie Rance. While the experiments were performed by Dr. Rance and me, the majority of the figures were prepared by Dr. Rance to be included in our manuscript, which has been submitted for publication, as indicated under each figure.

For project 2 on Zika Virus, the work presented in aim 3 was performed in conjunction with Sergio Alpuche. Data collection, analysis and preparation of figures was completed by Mr. Alpuche and me, to be included in our manuscript which has been published, as indicated under each figure. For aim 4, Drs. Rob Scarborough, Gatignol, and Rance as well as Mr. Alpuche provided extensive guidance on the project. All data collection, analysis, and preparation of figures was performed by me.

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List of Abbreviations

Aa: Amino Acid

AIDS: Acquired Immunodeficiency Syndrome

APOBEC3G: Apolipoprotein B mRNA editing enzyme

ART: Antiretroviral Therapy

ARV: Antiretroviral

BET: Bromodomain and extraterminal domain

BSA: Bovine Serum Albumin

BST-2: bone marrow stromal antigen 2, also known as Tetherin

C: ZIKV Capsid

CA: HIV Capsid

cART: combination ART

CCR5: C-C chemokine receptor type 5

CD4: cluster of differentiation 4

cDNA: complementary DNA

CMC: carboxymethylcellulose

CT: Cycle threshold

CXCR4: C-X-C chemokine receptor type 4

DENV: Dengue Virus

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

Dox: Doxycycline

dsRNA: double stranded RNA

E: ZIKV Envelope

EDTA: Ethylenediaminetetraacetic acid

EMEM: Eagle's Minimum Essential Medium

Env: HIV Envelope

FBS: Fetal Bovine Serum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GBS: Guillain-Barré Syndrome

GFP: Green Fluorescent Protein

Gp: glycoprotein

HDAC: Histone Deacetylase

HIV: Human Immunodeficiency Virus

HMBA: Hexamethylene bisacetamide

IFN: Interferon

IN: HIV Integrase

IκB: inhibitor of κB

JAK: Janus kinase

JEV: Japanese Encephalitis Virus

lncRNA: long non-coding RNA

LRA: Latency Reversing Agent

LTR: Long terminal repeats

MA: HIV Matrix

MDM: Monocyte-derived macrophages

MHC: Major Histocompatibility Complex

miRNA: micro RNA

MOI: Multiplicity of Infection

mRNA: messenger RNA

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NaCl: Sodium Chloride

NC: HIV Nucleocapsid

Nef: HIV Negative regulatory factor

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

NNRTI: Non-nucleoside reverse-transcriptase inhibitors

NP40: nonyl phenoxypolyethoxylethanol

NRTI: Nucleoside reverse-transcriptase inhibitors

NS1: ZIKV non-structural 1 protein

NS2A: ZIKV non-structural 2A protein

NS2B: ZIKV non-structural 2B protein

NS3: ZIKV non-structural 3 protein

NS4A: ZIKV non-structural 4A protein

NS4B: ZIKV non-structural 4B protein

NS5: ZIKV non-structural 5 protein

p-TEFb: positive transcription elongation factor b

PBS: Phosphate-buffered saline

PFA: Paraformaldehyde

PKC: Protein kinase C

PMA: Phorbol 12-myristate 13-acetate

PPIA: Peptidylprolyl isomerase A

PrM: ZIKV precursor of protein M

qRT-PCR: quantitative real-time reverse transcription polymerase chain reaction

Rev: HIV Regulator of viral expression

RISC: RNA induced silencing complex

RNA: Ribonucleic acid

RPMI: Roswell Park Memorial Institute medium

RRE: Rev response element

RT: HIV Reverse Transcriptase

SAHA: suberanilohydroxamic acid, also known as Vorinostat

shRNA: short hairpin RNA

siRNA: small interfering RNA

SIV: Simian Immunodeficiency Virus

snRNP: small nuclear ribonucleoproteins

STAT: signal transducer and activator of transcription

TAR: Trans-Activation Response element

Tat: Trans-activator of transcription

TBP: TATA-binding protein

TBS-T: Tris-buffered saline 1% Tween-20

TLR: toll-like receptor

TRBP: TAR RNA binding protein

UTR: untranslated region

Vif: HIV viral infectivity factor

Vpr: HIV viral protein R

Vpu: HIV-1 viral protein U

vRNA: viral RNA

WNV: West Nile Virus

WST-1: Water-soluble tetrazolium salt 1

YFV: Yellow Fever Virus

ZIKV: Zika Virus

ZIKV BRZ: ZIKV Brazilian strain, HS-2015-BA-01 (Genbank accession KX520666.1)

ZIKV THAI: ZIKV Thai strain, PLCal_ZV (Genbank accession KF993678.1)

Introduction

1) HIV

1.1 HIV-1 epidemic

According to the World Health Organization there were 36.7 million [30.8-42.9 million] worldwide living with Human Immunodeficiency Virus by the end of 2016, with an estimated 1.8 million new infections occurring that same year [1]. Endemic regions include Sub-Saharan Africa, where 1 in 25 adults are HIV-1 positive, accounting for 70% of the global burden (www.who.int). Although infection rates are much lower in North America, the Government of Canada estimated that 75,500 Canadians were living with HIV in 2014[2].

1.2 History

HIV was first discovered in 1983 [3] in patients afflicted with Acquired Immune Deficiency Syndrome (AIDS), which was characterized 2 years earlier [4]. Studies suggest that HIV originated from multiple zoonotic transmission of Simian Immunodeficiency Virus (SIV), from monkeys to humans in West/Central Africa [5], where primates are often butchered for bushmeat. The multiple transmission gave rise to HIV-1 and HIV-2. However, it has been noted that HIV-2 is less pathogenic [6] than HIV-1. Furthermore, HIV-2 represents a much smaller portion of the global HIV burden, infecting approximately 2 million people in West Africa [7]. HIV-1 is responsible for the HIV pandemic, and is divided into 4 groups, M, N, O, and P, with group M being the predominant strain circulating around the globe [8]. Group M can then be subdivided into subtypes A-K as well as other circulating recombinant forms. While subtype B is the most prevalent strain in the Americas and Western Europe, it only accounts for 12% of global

infections. Subtype C is present in many African countries and represents approximately 50% of the global burden[9]. However, most HIV-1 research is conducted on subtype B, and less is known about subtype C.

1.3 AIDS

If left untreated, HIV-1 can lead to AIDS, which is characterized by a progressive failure of the immune system, which increases the occurrence of infection and cancer, leading to premature death [10]. In the absence of treatment, roughly half of HIV infected patients will develop AIDS within 10 years. HIV-1 infects and kills cells of the immune system[11], which are important for fighting off infection. Therefore, opportunistic infections, which are usually controlled by the immune system, can take hold. This includes many bacteria, viruses, and fungi [12]. Furthermore, since AIDS results in a greater number of viral infections, AIDS is also associated with an increase in various viral-induced cancers. These include Kaposi's sarcoma and certain lymphomas, as a result of human herpes virus 8 infection[13,14], as well as cervical cancer, as a result of human papilloma virus infection[15].

1.4 Replication cycle

HIV-1 infects mainly $CD4^+$ T lymphocytes by binding of the viral gp120 spike protein to its receptor CD4 and co-receptor CXCR4[16,17] (figure 1). HIV-1 also infects myeloid cells such as macrophages using CCR5 as a co-receptor[11,18,19]. Upon entry into the cell, the HIV-1 Reverse Transcriptase (RT) will reverse transcribe viral RNA into DNA [20]. Following the production of viral DNA, viral Integrase (IN) will integrate the viral DNA into the genome of the host [21]. This will lead to one of two important outcomes; either the proviral DNA is expressed and there is

productive replication of the virus, or there is no expression and the virus remains in a state of latency.

The proviral DNA is transcribed from the promoter in the HIV-1 long terminal repeat (LTR). In the early phase, a small amount of full-length transcript is produced, doubly-spliced, and exported to the cytoplasm where it is translated into Tat, Rev, and Nef. Tat and Rev translocate to the nucleus in the late phase. In the case of a productive infection, transcription is enhanced by the viral protein Tat [22]. Tat will recruit Cyclin T1 and Cyclin-dependent kinase-9 and the complex will bind the Trans-activation response (TAR) RNA, to enhance both transcription initiation and elongation[23]. A large number of transcripts are then produced. Rev binds to a specific region in the Env RNA called the Rev Response Element (RRE) and allows the

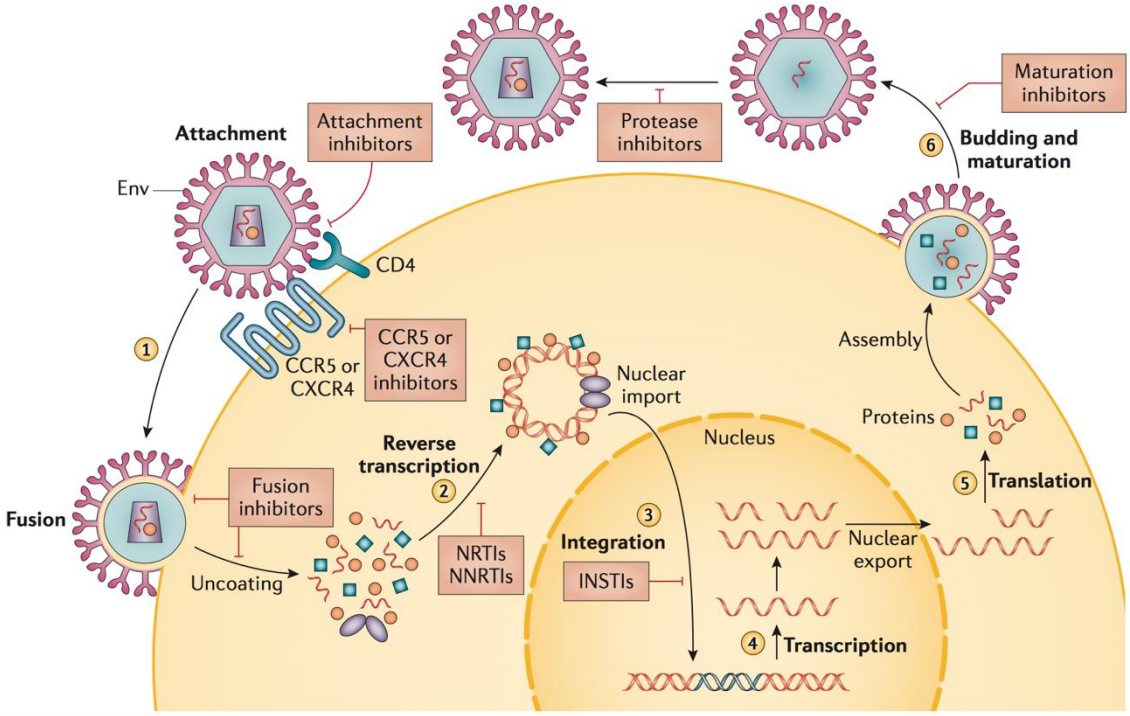


Figure 1: HIV Replication Cycle: HIV enters the cell by binding to its receptor (CD4) and co-receptor (CCR5 or CXCR4). Upon entry the virus is uncoated and reverse transcription of the viruses –ssRNA genome begins. The genome is then transported to the nucleus where it can be integrated into the host cell genome. Then, transcription of the viral RNA is carried out by host cell transcription machinery, and the singly or double spliced RNAs are transported out of the nucleus where translation begins. Again, the virus uses host machinery to translate viral proteins, which assemble and bud from the cell surface. Image from [17]

nuclear export of the unsliced and singly spliced RNA. The full-length transcript is translated into the Gag and Gag-Pol HIV-1 proteins and is also used as new HIV-1 RNA genomes. Other viral proteins, such as Vif, Vpr, Vpu, Env, are derived from singly-spliced HIV-1 RNAs[24]. Full length viral RNA and structural proteins assemble and migrate to the plasma membrane, where immature particles bud from the cell. Maturation occurs after the viral Protease (PR) has cleaved Gag and Gag-Pol. Gag is cleaved into the structural proteins Matrix (MA), Capsid (CA) and Nucleocapsid (NC), while Gag-Pol also gives rise to the PR, RT and IN proteins. In the mature virion, NC protects the viral genome, which, along with other viral proteins are encased by CA. These viral particles are surrounded by MA. A host-derived lipid membrane surrounds the virus with Env, gp120 and gp41 at their surface.

Although not absolutely required for the HIV-1 replication cycle, auxiliary viral proteins Nef, Vif, Vpr, and Vpu are critical to counteract cellular restriction factors. Nef is able to down-regulate expression of CD4 on the surface of infected cells, which aids in the release of new viral particles from the host cell surface[25]. It has also been shown to help the virus evade innate immune recognition by down-regulating the expression of the major histocompatibility complex 1 and 2 (MHC I and MHC II) [26,27]. Vif directs the polyubiquitination of APOBEC3G, a HIV-1 restriction factor. APOBEC3G is a cytidine deaminase that catalyzes the conversion of cytidine to uridine. Vpu also directs the polyubiquitination of a host cell restriction factor. Its target is bone marrow stromal cell antigen 2 (BST-2), also known as tetherin, which prevents virus release at the host cell surface[28].

1.5 Mechanisms of Latency

Most productively infected cells will be killed by the virus' cytopathic effect. In the case of a latent infection, the proviral DNA remains integrated in the genome of the host. These cells can remain latent for the rest of their life, or they can reactivate and begin producing infectious virus. The cells harboring transcriptionally silent, but replication competent provirus are termed reservoir cells[29]. While the major reservoir for HIV-1 is resting $CD4^+$ T lymphocytes [30-33], other potential reservoirs include hematopoietic progenitor cells [34,35], dendritic cells [36], thymocytes [37], microglial cells [38], and monocyte/macrophages [39-42].

Latency can be maintained via several different mechanisms (figure 2). Firstly, epigenetic silencing has also been implicated in latency. Gene expression in humans is largely influenced by the state of chromatin condensation, whereby lightly packaged chromatin, or euchromatin, is more easily accessible to transcription factors and transcription machinery than compact heterochromatin[43]. Modifications to histones such as acetylation and methylation can alter chromatin packaging by the recruitment or repression of certain proteins[44]. Histone acetyl transferases (HAT) will transfer an acetyl group to lysine residues of histone tails, resulting in increased transcription. Meanwhile, histone deacetylases (HDAC) remove these acetyl groups and reduce transcription[45,46]. Chromatin remodeling via histone acetylation has been shown to impact HIV-1 transcription[47]. HDACs recruited to the HIV-1 LTR have been shown to help maintain latency[48]. Also, both CpG and histone methylation have been shown to promote latency [49,50]. Histone methylation results in chromatin condensation, and thus histone methyltransferases (HMT) are able to silence HIV-1 provirus[51-53]. Methylation of the CpG islands of DNA can recruit HDACs and silence HIV-1 transcription[54]. In fact, DNA methylation of HIV-1 promoter and enhancer regions is higher in latently infected cells of patients with undetectable

virus compared to those with detectable virus[55]. Finally, the availability of transcription factors, both enhancers and negative regulators can affect whether the integrated viral DNA will be transcribed. Positive factors include p-TEFb [56] and NFκB [57].

Cellular miRNAs have also been shown to influence many aspects of the virus replication cycle. A study in monocyte and macrophages showed that the suppression of miRNA-28, miRNA-150, miRNA-223, and miRNA-382 facilitated infectivity of these cells, whereas their overexpression inhibited replication of the virus[58]. The expression of cellular miRNAs have also been implicated in HIV-1 latency. In fact, those same 5 miRNAs (miRNA-28, miRNA-150, miRNA-223, and miRNA-382) were shown to contribute to HIV-1 latency in resting CD4+ T-cells[59]. As mentioned above, Cyclin T1 is an important part of the HIV-1 replication cycle. Cyclin T1 is itself

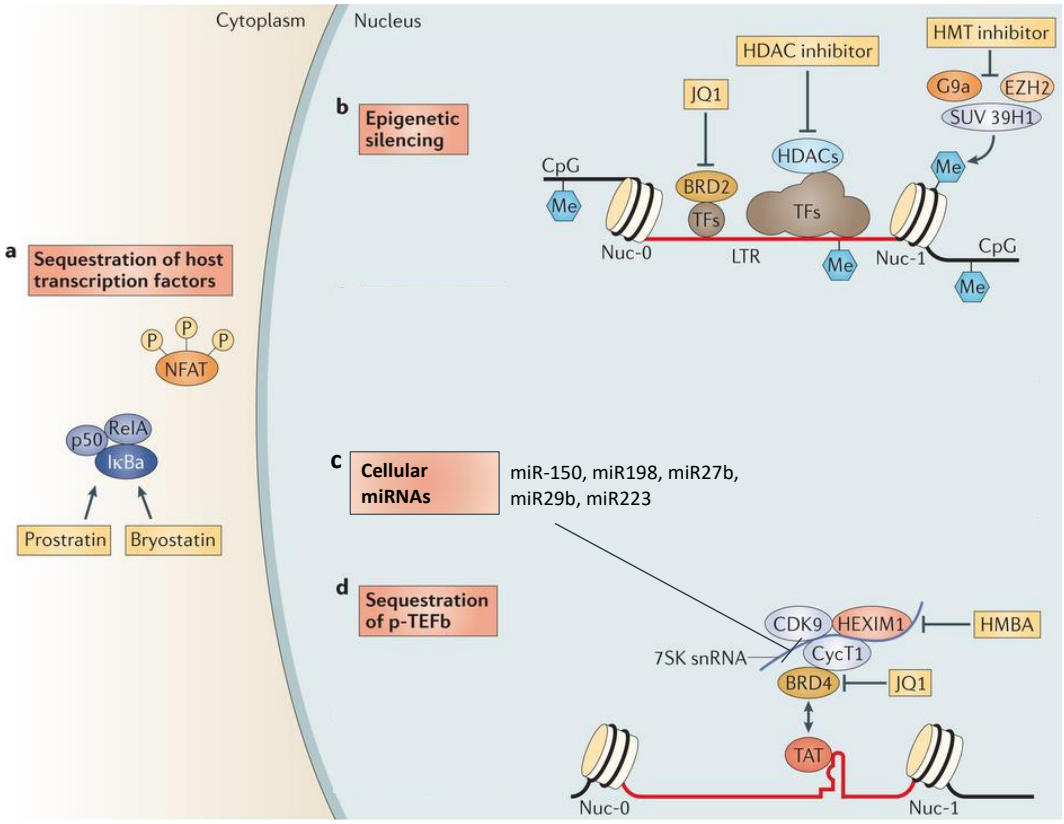


Figure 2: HIV-1 Latency Mechanisms. HIV-1 is maintained in latent viral reservoirs. In these cells, a number of host factors contribute to supressing transcription of the integrated provirus, including a) sequestering host transcription factors, b) epigenetic silencing, c) cellular miRNAs, d) sequestration of p-TEFb
Adapted from [29]

regulated by miRNA-198, and this miRNA has been shown to inhibit HIV-1 gene expression[60]. miRNA 29a and 29b have been shown to inhibit HIV-1 Nef expression, and thus suppress viral replication[61]. Another study showed that expression of the cluster of miRNA-17/92 decreased during HIV-1 infection[62]. Encoded by this cluster are miRNA-17-(5p/3p), miRNA-18, miRNA-19a, miRNA-20a, miRNA-19b-1 and miRNA-92-1. miRNA-17-5p and miRNA-20a target a histone acetyltransferase, and therefore might affect HIV-1 latency similarly to the use of HDACi. The use of miRNA inhibitor has already been shown to be effective against other viruses. miRNA-122 is a cellular miRNA essential to the Hepatitis C virus replication cycle and is inhibited by Miravirsen. Clinical trials with this inhibitor have yielded undetectable virus levels in patients[63].

1.6 Antiretroviral Therapy

The dawn of antiretroviral therapy (ART) began with the first antiretroviral drug (ARV) approved for use in 1988: Zidovudine [64], a nucleoside reverse transcriptase inhibitor (NRTI). Use of this drug improved survival and slowed disease progression, however, it did not stop $CD4^+$ T cell decline. Another big step in HIV-1 treatment occurred in 1996 with the introduction of protease inhibitors. However, monotherapy ultimately lead to the emergence of resistant viruses. The high infectivity [65] and error prone replication of the viral RT [66] means that infected individuals harbor a heterogenous pool of virus [67]. As a result, it is believed that patients will have a unique copy of the virus that has any viable mutation [68]. As early as 1993, resistance to Zidovudine had been documented [69]. Further research also noted resistance to protease inhibitors [70]. While the use of a single drug was unable to sustain virologic suppression, the use of combination ART (cART) managed to dramatically reduce HIV-1 RNA, improve immune function [71], and decrease HIV-1 related morbidity and mortality [72].

The current standard is to give 3 drugs, 2 NRTI and 1 other drug, such as a protease or integrase inhibitor, called cART. Furthermore, with over 2 dozen ARVs now approved by the FDA, drug regimens can be tailored to individual based on efficacy, side effects, drug resistance, etc. We now have drugs that can block HIV-1 at almost every step of its life cycle: enfuvirtide can block membrane fusion [73]. Once inside the cell, it can be blocked by lamivudine [74], a NRTI, or Efavirenz [75], a non-nucleoside reverse transcriptase inhibitor (NNRTI). Furthermore, we can prevent integration of the provirus into the host genome by use of integrase inhibitors such as dolutegravir [76]. Finally, we can block virus maturation through inhibiting the viral protease with darunavir [77]. One of the goals of ART is to keep plasma viral load below 50 copies/ml, which is considered a suppressed infection. Although a higher viral load has been associated with virologic relapse [78], the restoration of the immune system can still occur in these conditions [79]. Epidemiological studies suggest that HIV-1 subtype may dictate progression to AIDS in ART naïve populations[80]. Thus, since most ARV are developed in subtype B populations, the treatments could be suboptimal for people infected with other subtypes. Indeed, in ART-experienced populations, research suggests that subtype C patients had a greater incidence of virological failure than subtype B[81].

To reduce viral load in the plasma, the drug concentration of ART must be high enough in the plasma to inhibit the virus' replication and spread. Due to the long list of ART side effects, dosing must also be optimized to decrease toxicity. However, there are sites within the body where ART concentrations do not reach optimal levels. The lymphatic tissues, namely the lymph nodes, gut associated lymphoid tissue and rectal associated lymphoid tissue, have all been shown to have lower ART concentration than the peripheral blood mononuclear cells [82]. Lower drug concentrations may allow the virus to replicate at low levels [83]. In fact, in patients undergoing

cART, deep sequencing of blood and lymphoid tissue samples revealed that there were nucleotide substitutions occurring at a constant rate and that the blood and lymphoid tissue samples were diverging at a similar rate [84]. These findings suggest that persistent replication in the lymphoid tissue is contributing to the maintenance of HIV-1 sanctuaries. However, further analysis showed that while the virus is replicating, it does not seem to be evolving in response to any selective pressure. This supports the theory that ARV concentrations in certain body compartments may be inadequate to fully suppress viral replication. They also showed that there is trafficking of the virus between the plasma, peripheral blood mononuclear cells, and the lymph nodes. In particular, there was significant movement from the lymph nodes to the blood. They showed that haplotypes derived from replication in the lymph nodes give rise to the haplotypes observed in the blood, whereas there was little to no evidence of replication occurring in the blood. Therefore, these data suggest that ongoing replication in the lymphatic tissue replenishes the viral reservoir.

For ARV resistance to develop, the virus must be exposed to the drug, a selective pressure, but in a concentration that is unable to completely inhibit viral replication. There are a couple of cases where this can happen. Firstly, the virus can carry with it some intrinsic level of resistance to certain ARV. This is seen in HIV-1, where the high genetic diversity of the virus has led to the evolution of many different subtypes around the globe [85]. These different subtypes show different resistance patterns to certain ARV [86]. Therefore, while the concentration of a certain ARV may be high enough to inhibit one subtype, this cannot be generalized for all HIV-1 subtypes. For example, in a study looking at single dose nevirapine to prevent mother to child transmission, there was a greater occurrence of resistance in women with subtype C compared to D [87], and greater in subtype D compared to A [88].

Another situation where resistance can emerge is poor drug adherence. In this case, the drug is present long enough and in high enough concentration to lead to selection, but not long enough or in low enough concentration to allow the virus to continue replicating. The greatest risk is to those whose adherence is between 70-89% [89]. Interestingly, it has been shown that low levels of adherence are associated with lower prevalence of drug resistant mutations at virologic failure [90]. A possible explanation for this phenomena is that the drug concentration is too low to provide a selective pressure, since resistance mutations often carry with them an associated fitness loss [91].

1.7 Why we need a cure

cART decreases AIDS-related morbidity and mortality [92]. However, many patients on cART experience associated pathologies, such as cardiovascular and neurological diseases, liver and kidney failure, and cancer [93]. Furthermore, this treatment does not eliminate cells that are latently infected with integrated provirus [94,95]. Therefore, cART is not curative because it does not eliminate viral reservoirs, turning HIV-1 infection into a chronic disease. Furthermore, as patients need to use cART for the rest of their lives, the cost of treatment is high [96]. In addition, both the short-term and long-term side effects of cART make adherence to the regimen difficult for some [97]. Another factor affecting drug adherence is the incomplete access to drugs in endemic areas of Sub-Saharan Africa [98]. Finally, the emergence of resistant HIV-1 strains presents the possibility that these drugs may become ineffective[99]. These factors highlight the need for the development of a cure for HIV-1.

1.8 Strategies towards a Functional HIV-1 cure

A functional cure for HIV-1 would involve a strategy that would prevent the virus from replicating, while allowing patients to discontinue administration of cART. Timothy Brown, also known as the Berlin Patient, is the only patient to be cured of HIV-1 so far. In order to treat his myeloid leukemia, he received an allogenic bone marrow transplant from a HLA-matched donor who was homozygous for a mutation in the CCR5 HIV-1 co-receptor ($CCR5^{\Delta 32/\Delta 32}$) in 2007 [100]. The $CCR5^{\Delta 32/\Delta 32}$ inhibits binding to the transplanted hematopoietic cells, therefore the virus is no longer able to propagate and damage his immune system. While he might still have some reservoir cells infected with the virus, researchers have been unable to detect any viremia 10 years after discontinuing cART [101]. However, the risks and the costs associated with hematopoietic stem cell transplants, as well as the limited number of $CCR5^{\Delta 32/\Delta 32}$ donors makes this approach impractical as a large-scale treatment. Another approach has been to start patients on cART as soon as possible. This was attempted with a patient termed the "Mississippi baby", an infant born from an infected mother who began receiving cART within 30 hours of being born. After 18 months, cART was discontinued and the baby had no detectable virus in the 12 months following [102]. However, 27 months after stopping cART, the child experienced a virological rebound [103]. More interestingly, a cohort of 14 post-treatment HIV-1 controllers in the "VISCONTI cohort" have a long-term virological remission, indicating that early treatment could reduce the viral reservoir and induce long-term remission without cART[104]. There was another report of a young woman that has a 12-year remission following treatment interruption where cART was initiated early in the infection[105]. However, this approach is not appropriate for patients already infected and treatment interruption cannot be recommended.

A new approach towards a functional cure involves inhibiting transcription of the virus, thereby inducing a state of "deep latency", whereby latently infected cells will not reactivate to

generate new viral particles. Cortistatin A is a molecule that is able to specifically inhibit Tat, the virus' transactivator [106]. However, *in vivo* suppression of HIV-1 rebound only lasted one week, indicating that more effective compounds are needed to "lock" the virus in an inactive state[107].

Finally, gene therapy has been explored as a possible functional cure for HIV. Owing to the success of the Berlin Patient, many groups have attempted homologous cell transplant after CCR5 modification[108-110]. Others have also attempted to express a dominant-negative HIV-1 REV protein[111]. However, a limitation of expressing foreign proteins inside human cells would be the possibility of activating an immune response. Another avenue of gene therapy is the expression of small anti-HIV-1 RNAs. Firstly, it is possible to introduce decoy RNAs that will bind HIV-1 regulatory proteins Tat and Rev by mimicking the TAR and Rev response element (RRE)[111,112]. Another possibility is to use short hairpin RNAs (shRNAs) and small interfering RNAs (siRNAs) against HIV-1 sequences to target them to the RNA interference pathway[113,114], described later. Limitations of this work include the ability of these small RNAs to saturate components of the RNA-Induced Silencing Complex (RISC), and possibility of triggering an ds-RNA mediated innate immune response[115]. Furthermore, the major reservoir of HIV-1, $CD4^+$ T lymphocytes[116], are widely distributed throughout the host, making delivery to all of these cells difficult.

1.9 Strategies toward a sterilizing HIV-1 cure

A sterilizing cure would involve a strategy that would eradicate all virus from the body, thus eliminating the need for cART. Establishment of viral reservoirs represents the main source of viral persistence and a major barrier to a sterilizing cure [117]. One proposed strategy for a potential cure involves reactivating the latently infected cells, which would then allow them to

be targeted by conventional drugs or by a strong immune response [118]. This approach has been termed the "shock and kill" strategy. While lymphocytes have been largely studied as viral reservoirs, evidence indicates that myeloid cells such as macrophages contribute to HIV-1 persistence [119], but the mechanism of HIV-1 latency in these cells is not well understood. Mechanisms of latency occur at the level of chromatin compaction, transcriptional, and post-transcriptional regulation [120]. Therefore, drugs that target these pathways could be investigated as potential Latency Reversing Agents (LRA).

1.10 Latency Reversing Agents

Epigenetic controllers that increase transcription have been explored as LRAs. One class of epigenetic controllers are histone deacetylase inhibitors (HDACi), which leads to the accumulation of acetylated histones, which increases transcription of surrounding DNA [121]. Examples of HDACi include Suberanilohydroxamic acid (SAHA)[122], panobinostat[123], romidepsin[124]. While these drugs have been approved to treat cutaneous T-cell lymphoma[125,126] and multiple myeloma[127], they have also been tested as LRAs with HIV-1[128]. SAHA has been shown to disrupt latency in patients on cART [129]. Like histone acetylation, histone methylation can also silence transcription and therefore the use of Histone Methyltransferase inhibitors such as Chaetocin could also induce transcription [130].

Positive Transcription Elongation Regulator b (P-TEFb) is composed of Cyclin T1 associated to cyclin dependant kinase 9 and regulates transcriptional elongation of RNA polymerase Ⅱ. P-TEFb is itself regulated by associating with the 7SK small ribonuclear protein (snRNP) complex, which sequesters the complex in an inactive form, thus preventing transcriptional elongation [131]. JQ1, a bromodomain and extraterminal inhibitor, induces the

release of P-TEFb from the 7SK snRNP, which will in turn increase transcriptional elongation [132]. Disulfiram and Hexamethylene bisacetamide (HMBA) stimulate the Akt pathway, which will also induce the release of P-TEFb [133,134].

Protein kinase C (PKC) agonists activate transcription through the NF-κB pathway. PKC will phosphorylate NF-κB inhibitor, IκB, leading to its degradation and releasing NF-κB for nuclear translocation [135]. Prostratin[136], Bryostatin[137], and Ingenol[138] are all PKC agonists that modulate HIV-1 latency.

Latency can also be maintained through methylation of CpG islands in the HIV-1 LTR [139], and thus DNA methyltransferase inhibitors could also be explored as LRA. In terms of post-transcriptional mechanisms of latency, nuclear export/retention of HIV-1 transcripts via interference with exportin 1 can also help maintain latency. Furthermore, the activity of certain miRNAs have been shown to modify HIV-1 expression by targeting essential cellular factors for the virus [140] or by inhibiting expression of the virus itself by associating with its 3'UTR [59]. The expression of certain miRNAs has been associated with viral progression or inhibition in HIV-infected lymphocytes [141,142]. This suggests a role of miRNAs in the regulation of the viral replication cycle, and potentially in latency establishment or disruption. Although the role of miRNAs have been studied in latently infected lymphocytes [59], very few analyses have been performed in monocytes and macrophages infected with HIV-1. The LRAs previously described could be used to study differential miRNA expression patterns that occur when latently infected cells are reactivated, leading to the discovery of new factors involved in latency, or markers for latently infected cells.

1.11 Models to Study HIV-1 Latency

There are a number of *in vivo* and *in vitro* models of HIV-1. While both types present several drawbacks, the information generated from these models has been paramount to our understanding of the virus. *In vivo* models are often set up in humanized mice and non-human primates such as rhesus macaques. While these models better represent the heterogeneity of HIV infection and latency, they are both much more expensive than *in vitro* models and not suitable for the testing a wide range of substances as HIV-1 drugs or LRAs. Furthermore, many mouse models are limited by the development of graft-versus-host disease, while non-human primate models bear an additional financial and ethical cost. These animal models are well-suited for pre-clinical testing, but the discovery and initial characterization of new treatments is often performed *in vitro*.

Primary cell models of HIV-1 latency have been set up in $CD4^+$ T cells [143-145]. While these models are perhaps more physiologically relevant than immortalized cell lines, they are limited by the small fraction of latently infected cells. This is compounded by the fact that these models include high background from cells that are uninfected or infected with deficient provirus. Finally, there are HIV-1 models that have been set up in immortalized cell lines. This includes, but is not limited to, T-lymphocytic clones such as Jurkat [146] and ACH-2 [147], as well as monocytic clones such as U1 [148] . The advantage of using these cell lines is that they are easy to work with, faster to grow, and are much better suited for drug/small molecule screens. In this thesis, we use a new HIV-1 latency model in CEM lymphocytes, THP-1 monocytes, and THP-1 monocytes-derived macrophages (MDM).

1.12 HIV-1 Latency Model in CEM Lymphocytes, THP-1 monocytes, and THP-1 MDM

The laboratory of Dr. Alan Cochrane (University of Toronto) has developed a model, in which the integrated HIV-1 Tat-TAR transcriptional axis is replaced with a tetracycline induction system so that cells will only be reactivated in the presence of doxycycline (Dox), as initially developed by Das et al [149]. Therefore, infected cells can be reactivated by the addition of Dox and various LRA (figure 3). The virus is also mutated in the Protease and RT genes, thus it is non-infectious. This presents the possibility for the safe study of HIV-1 transcription and post-transcriptional mechanisms. The virus has been further engineered to express a Green Fluorescence Protein (GFP)-gag fusion protein by Dr. A Cochrane (figure 3). This allows the cells to be isolated by cell sorting, as reactivated cells will be GFP+, while non-reactivated cells will be GFP-. The lab of Dr. Cochrane has inserted this modified virus in CEM lymphocytes, and THP-1 monocytes and selected for stable clones. Our lab has expanded this model to study macrophages, by inducing the monocytic THP1 cells to differentiate into macrophages[150].

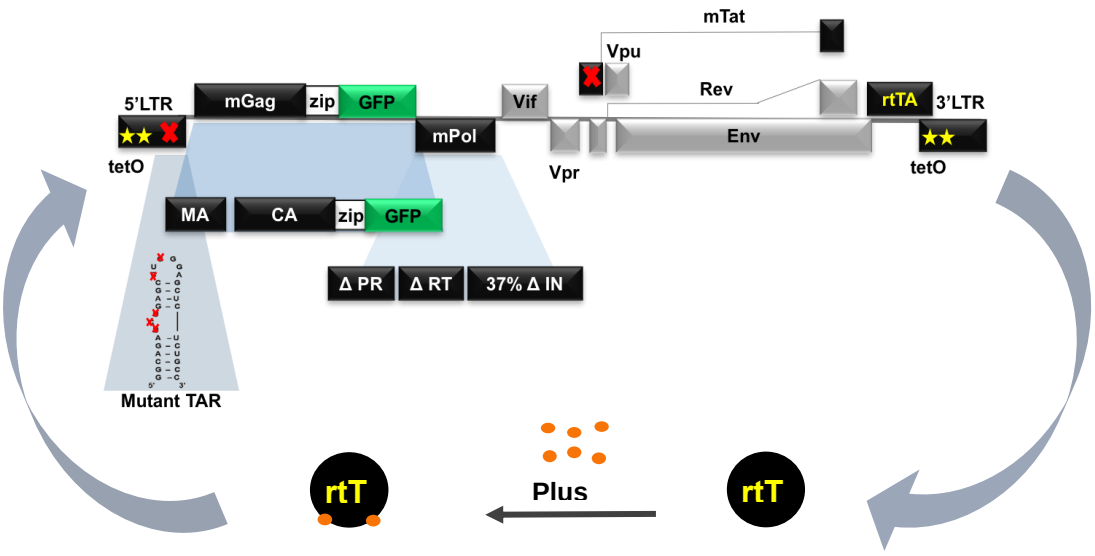


Figure 3: HIV-1 GagZipGFP model. Integrated virus is mutated in Protease (PR) and Reverse Transcriptase (RT). Tat-TAR transcriptional axis replaced by Tet-on/Tet-off dox inducible transcription.
Image by Dr. Elodie Rance [150]

2) Zika Virus

2.1 History

Zika Virus (ZIKV) was first discovered in a rhesus monkey in 1947 in the Zika forest of Uganda[151]. Research was being conducted to monitor the vector for the sylvatic cycle of Yellow Fever virus, which ended up identifying 7 new viruses, including ZIKV[152]. This strain has since become the prototype strain for ZIKV and was named ZIKV MR 766. The virus was then subsequently isolated from an *Aedes albopictus* mosquito in 1948 and the first confirmed human case was in 1954 in a 10 year old Nigerian female[153]. Although data is sparse, research in mosquitoes from second half of the 20th century would suggest that ZIKV is endemic to many African[154,155] and some Asian[156] countries. Very few human cases were reported until a large outbreak occurred in 2007 in Yap Island, a part of the Federated States of Micronesia. An estimated 5,000 people of the 7,000 residents over 3 years of age were infected, which represents about 72% of the population[157]. The next significant outbreak occurred in French Polynesia in 2013. During the 21-week long outbreak, 855 patients presenting symptoms of ZIKV infection were tested for ZIKV RNA, 392 were positive[158]. Initial estimates of the number of cases of Zika fever was about 30,000 or 11.5% of the population [159], however later serosurveys suggest that up to 66% of the population may have been infected[160]. As will be discussed below, not all ZIKV infections are symptomatic, which would explain the discrepancy between the two numbers. From there the virus spread to other Pacific islands, where smaller outbreaks occurred[161]. Then in May of the 2015, the first native case of ZIKV was confirmed in Brazil, in the state of Bahia[162]. At this time, ZIKV cases were also confirmed in 14 other countries in the Americas. By 2016, another 6 counties were added to the list[163]. Estimates for the America's

Figure 4: Spread of ZIKV. After its discovery in 1947, the virus spread eastward towards Southern Asia, eventually reaching Yap Island in 2007, where a large outbreak occurred that infected approximately 72% of the population. From there, the virus spread to other Pacific Islands, including French Polynesia, where another large outbreak occurred, infecting 66% of the population. From there, smaller outbreaks occurred in the Pacific before the virus reached Brazil in 2015, marking the beginning of the outbreak in the Americas. Adapted from Washington Post article: What is Zika?

outbreak range from 500,000 to over 1 million, however the number of confirmed cases is 222,477 as of January 4th, 2018[164].The suspected spread of the virus from Uganda to Brazil and the Americas is outlined in figure 4.

2.2 Classification

ZIKV is a member of the *Flaviviridae* family and the genus *flavivirus*, which encompass other human pathogens such as Dengue (DENV), Yellow Fever (YFV), and West Nile (WNV) viruses. Another thing ZIKV has in common with these viruses is that they are all arboviruses, a term generated from the contraction of arthropod-borne virus. Arthropods include insects such as mosquitoes and ticks. For the most part, arboviruses are maintained in non-human animal species and humans are a dead end host[165], with the exception of the viruses mentioned above[166]. Phylogenetic analyses have separated ZIKV into two major lineages, African and Asian[167], the former can be subdivided into East and West Africa[168]. These strains are still quite

similar, with 88.9% nucleotide identity between the original MR 766 strain and the Yap strain from 2007[168]. Amongst the Asian strains are those from the outbreaks just mentioned in Yap, French Polynesia and the Americas. The French Polynesian strain shares 99% nucleotide identity with the Brazilian strain[169]. We will be working with both a Thai (ZIKV THAI) and Brazilian (ZIKV BRZ) strain. As seen in figure 5, the Thai strain branches off just before the French Polynesian strain that was first associated with neurological complications.

Figure 5: ZIKV phylogeny. ZIKV is separated into an African and Asian lineage. The strains we will be working with are a Thai strain (ZIKV THAI) and a Brazilian strain (ZIKV BRZ). The ZIKV THAI lineage branches off earlier than that of the ZIKV BRZ. ZIKV THAI also branches off before the French Polynesian strains where the virus was first associated with neurological complications.
Image adapted from [167]

2.3 Pathogenesis

Symptoms of Zika fever are non-specific and can often be confused with other related flaviviruses. The most common symptoms are fever, rash,

arthritis, arthralgia, myalgia, conjunctivitis and fatigue[153,159,170,171]. After the outbreak in French Polynesia, serosurveys suggested that anywhere from 50-66% of the population had been infected[160], but the estimated number of cases of Zika fever was only 11.5 % of the population[172], which would suggest the ratio of symptomatic to asymptomatic is about 1:5 or 1:6.

2.4 Neurological complications

What is most concerning about this virus is the neurological conditions it has been associated with in recent outbreaks. Guillain-Barré Syndrome (GBS) is defined as rapid onset muscle weakness resulting from an autoimmune attack of the peripheral nerves[173]. During the acute phase, 15% of patients develop weakness of the breathing muscles, requiring mechanical ventilation. In French Polynesia, the average number of GBS cases was 5 per year between 2009-2012[174]. However, coinciding with the ZIKV outbreak in 2013, French Polynesia saw 42 cases, all of whom had experienced a "zika-like syndrome"[175]. Countries in the Americas also saw an increase in the number of GBS cases when ZIKV arrived in 2015, however the fold increase was much less dramatic[176].

Microcephaly is a condition where the brain does not develop fully resulting in babies being born with smaller heads. This usually results in intellectual disability and poor motor function of the child. In Brazil, the annual incidence of microcephaly was about 150-200 cases between 2010-2014[177]. However, coinciding with the ZIKV outbreak, Brazil saw 4783 cases between October 2015 and January 2016[178]. Studies have detected ZIKV RNA in the amniotic fluid of pregnant women who gave birth to the microcephalic babies[179], as well as ZIKV IgM in cerebrospinal fluid of the babies[180]. A retrospective study in French Polynesia also showed an

increase in the number of congenital central nervous system malformations - which includes microcephaly - during the ZIKV outbreak, from 1 case per year to 17 in 2013[181].

2.5 Genome organization and function

Zika is a single stranded positive sense RNA virus. It encodes a single polyprotein which is then cleaved by host and viral proteases into 3 structural proteins and seven non-structural proteins (figure 6). Although few analyses have been done with ZIKV, given its similarity to other flaviviruses, the role of the various proteins are likely also similar. In DENV, the C protein forms the core of the mature virus particle. It is important for virus budding and gathering viral RNA inside the nucleocapsid[182]. The PrM protein acts as a chaperone for the E protein, which forms the viral envelope and is needed for binding to host cell surface receptors, as shown in studies of DENV[182,183]. Interestingly, an amino acid change in the PrM protein of ZIKV plays a role in the induction of microcephaly in fetal mice born from infected mothers[184]. The NS1 protein has been implicated in immune evasion[185,186], pathogenesis and viral replication[186,187] in WNV. In DENV, NS2A is a component of the viral replication complex[188]. Studies in mice infected with ZIKV have also suggested that it reduces proliferation, increases differentiation, and causes adherent positioning of immature neurons[189]. NS2B on the other hand is a cofactor for the NS3[190], the viral serine protease. NS3, in association with NS2B, cleaves the polyprotein at the following sites: C-PrM, NS2A-NS2B, NS2B-NS3, NS3-NS4A, NS4A-2K and NS4B-NS5[191-193].

Figure 6: ZIKV genome organization. The ZIKV genome is translated into a single polyprotein and the cleaved to form 3 structural (C, PrM, E) and 7 non-structural proteins (NS1, NS2A, NS2A, NS3, NS4A, NS4B,NS5).

The NS3 also has RNA helicase activity which is regulated by the NS4A[194,195]. NS4B has been shown to inhibit interferon signalling in DENV through its activity on STAT1 (phosphorylation) and STAT2 (translocation)[196]. Finally, the NS5 is the virus' RNA-dependant RNA polymerase. It is also responsible for transferring a guanyl group to the 5' RNA to form a RNA cap stucture[197]. It has also been shown in WNV that the NS5 is able to inhibit the interferon pathway by blocking the JAK and STAT pathway[198].

2.6 Replication cycle

As mentioned above, the virus is transmitted mainly via mosquitoes. While the virus was first isolated from *Aedes africanus*, *Aedes albopictus* and *Aedes aegpyti* are believed to be the principal vectors in the most recent outbreaks, since they are both present in these regions[199] and have experimentally been shown to be able to transmit ZIKV[200,201]. The virus is maintained via both a sylvatic cycle in monkeys and an urban cycle in humans[202] (figure 7). In addition, sexual transmission[203] and transmission via blood transfusion[204] have been observed in humans. Finally, there is also an important and clinically relevant vertical transmission from mother to baby in utero.

ZIKV has been shown to be able to infect human dermal fibroblasts, epidermal keratinocytes, and immature dendritic cells, through binding to DC-SIGN, AXL, Tyro3, and TIM-

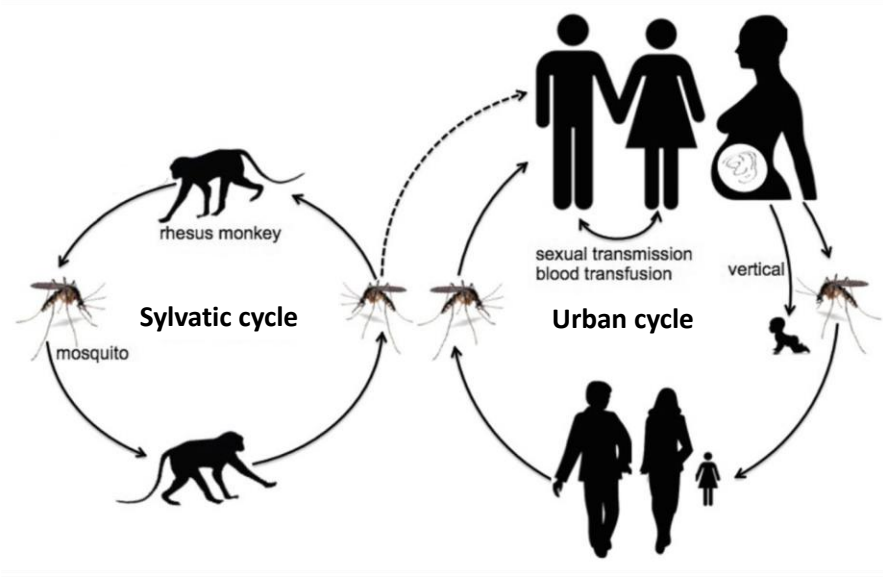


Figure 7: ZIKV Transmission Cycles. ZIKV is maintained in a sylvatic cycle, where mosquitoes take up a blood meal from infected monkeys and transmit to uninfected monkeys. The same cycle exists in humans, however other modes of transmission have been observed in humans: sexual transmission and transmission via blood transfusion have been documented. There is also a vertical transmission from mother to fetus in utero. Adapted from [202]

1[205]. The rest of the viral life cycle resembles that of other flaviviruses[206] (figure 8). Upon receptor binding, mature ZIKV virions enter the host cell via receptor-mediated endocytosis[207]. The viral RNA is released from the viral membrane after endosomal acidification triggers fusion of the viral membrane with the endosomal membrane. The viral genome is translated into a single polyprotein, which is then cleaved by both host and viral proteases. Before the virus can begin replicating its genome, the replication machinery must be assembled since the virus does not package these proteins in the viral particle. Then vRNA translation is switched off so that RNA synthesis may begin. RNA replication occurs on altered cellular membranes[208]. Next, the immature viral particle buds from the endoplasmic reticulum and travels through the trans-Golgi network. Finally, pH changes induce conformational changes and mature virions exit the cell via the secretory pathway[209].

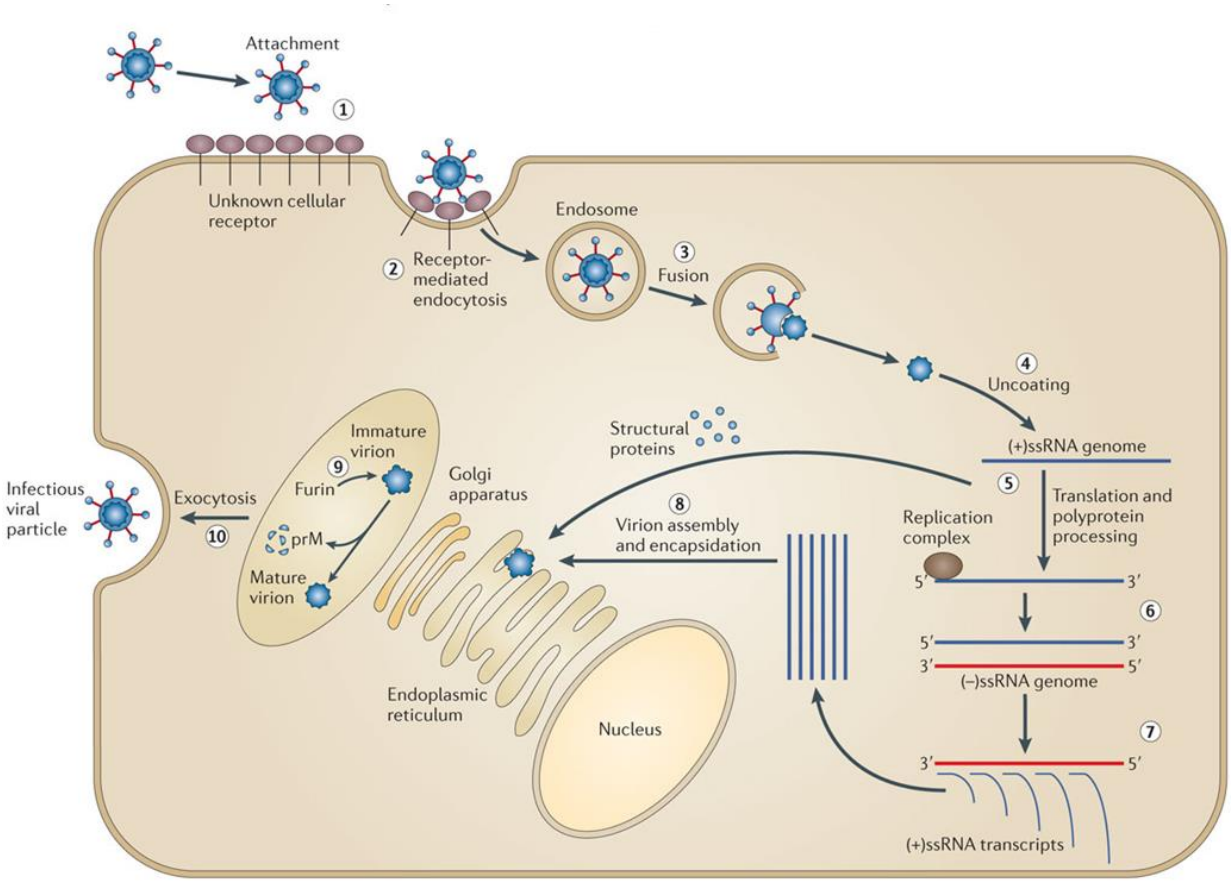


Figure 8: ZIKV Replication cycle. The virus binds to one of its receptors (DC-SIGN, AXL, Tyro3, and TIM-1), and enters the cell via receptor-mediated endocytosis. Endosomal acidification triggers fusion of the viral membrane with the endosomal membrane, resulting in release of the viral particle into the cytoplasm. Host translation machinery then translates the ZIKV polyprotein which is cleaved into both structural and non-structural proteins. The structural proteins assemble on the ER, while several of the non-structural proteins form the viral replication complex to generate progeny genomes. These +ssRNA genomes are then packaged into viral particles, which travel through the secretory pathway. pH changes induce a conformational change resulting in a mature viral particle which is then exocytosed from the host cell.
Image from [196]

2.7 Treatment, Vaccine Perspectives, and Prevention

At the moment there is no specific treatment for ZIKV. Instead, treatment is prescribed for the symptoms described above. Furthermore, there is no vaccine available, although several candidates are being explored[210]. In rhesus monkeys, an inactivated virus induced neutralizing antibodies against the ZIKV E protein, which was protective against 2 different strains of the

virus[211]. Another group was also able to protect against ZIKV infection using a DNA vaccine that expressed both the prM and E proteins in monkeys[212].

As with any mosquito-borne disease, primary prevention protocols include the use of mosquito nets, eliminating mosquito breeding grounds such as sitting water, as well as the use of insecticides. However, the recent ZIKV outbreaks highlight a need for further measures. A novel approach towards vector control is genetic control. Several groups have been able to drastically reduce *A. aegypti* populations, the major vector for ZIKV transmission, on a small scale by releasing males carrying a dominant lethal gene, whereby the offspring are not viable[213,214]. CRISPR-Cas9 technology has also been used in a gene drive strategy that could potentially eliminate *Anopheles gambiae* mosquitoes, the primary vector of malaria[215]. In this technique, a guide RNA binds to the DNA sequence and directs the Cas9 endonuclease to cleave this segment of the mosquito genome. When inserted in genes essential for female fertility, this can confer infertility in progeny mosquitoes. However, these approaches carry a potential environmental impact. The elimination of a species can alter the eco-system in ways that we may not be able to predict. Finally, the ethics of wiping out an entire species should be questioned[216], especially if there is the possibility of alternative approaches.

3) RNA Interference

3.1 Mechanisms of RNA interference

The observation that small, non-coding RNAs could have a regulatory effect on gene expression was first noted in *Caenorhabditis elegans* in 1993[217]. Subsequent research in *C. elegans* showed that small double stranded RNAs (dsRNAs) could potently downregulate gene expression by a mechanism called RNA interference[218]. The RNA interference pathway is a

mechanism of gene regulation mediated by small (21-27nt) dsRNAs called microRNAs (miRNAs)[219]. miRNAs have been shown to regulate many cellular functions such as immune responses, inflammation, and cell growth [220]. The primary-miRNAs are transcribed by RNA polymerase II, then cleaved in the nucleus by enzymes named Drosha and DGCR8, and exported to the cytoplasm as precursor(pre)-miRNAs by the exportin 5 pathway[221]. These pre-miRNAs form imperfectly paired hairpins. The hairpin is then cleaved by an enzyme called Dicer, which is bound to TAR-RNA binding protein (TRBP), resulting in a mature miRNA [222]. The mature miRNAs silence gene expression by associating with the RISC, which is composed of Dicer, TRBP, and Ago2[223]. One of the two strands will be maintained in the RISC, known as the guide strand, which usually binds the 3' untranslated region (UTR) of the target mRNA [224]. The guide strands bind with perfect complementarity in the seed region (position 2-7nt from the miRNA 5' end), however, there can be mismatches in the rest of the sequence[225]. Binding will lead to either repression of translation[226] or degradation of the mRNA via deadenylation[227] and destabilization[228].

miRNAs are conserved across many higher order organisms. In humans, it has been estimated that approximately 60% of our genes could be regulated by miRNAs[229].

The mechanism outlined above is the endogenous RNA interference pathway (figure 9A), however, there also exists an exogenous RNA interference pathway (figure 9B), whereby small interfering RNAs (siRNAs) are also able to down regulate gene expression[230]. siRNAs are often derived from virus or exogenous siRNAs[231]. Exogenous siRNAs can be delivered to individual cells[232], all the way to entire organisms [233]. Furthermore, it is also possible to construct vectors, such as viruses, capable of expressing short hairpin RNAs which are then cleaved to form

siRNAs[233]. In contrast to the miRNAs, siRNAs bind with perfect complementarity to their target mRNA, which promotes cleavage of the mRNA[234].

3.2 Applications of RNA interference

The deregulation of certain miRNAs is associated with diseases including infectious diseases such as HIV-1 [62,235]. The expression of certain miRNAs has been associated with viral progression or inhibition in HIV-infected lymphocytes [141,142]. This suggests a role of miRNAs in the regulation of the viral replication cycle. Furthermore, certain cellular miRNAs have also

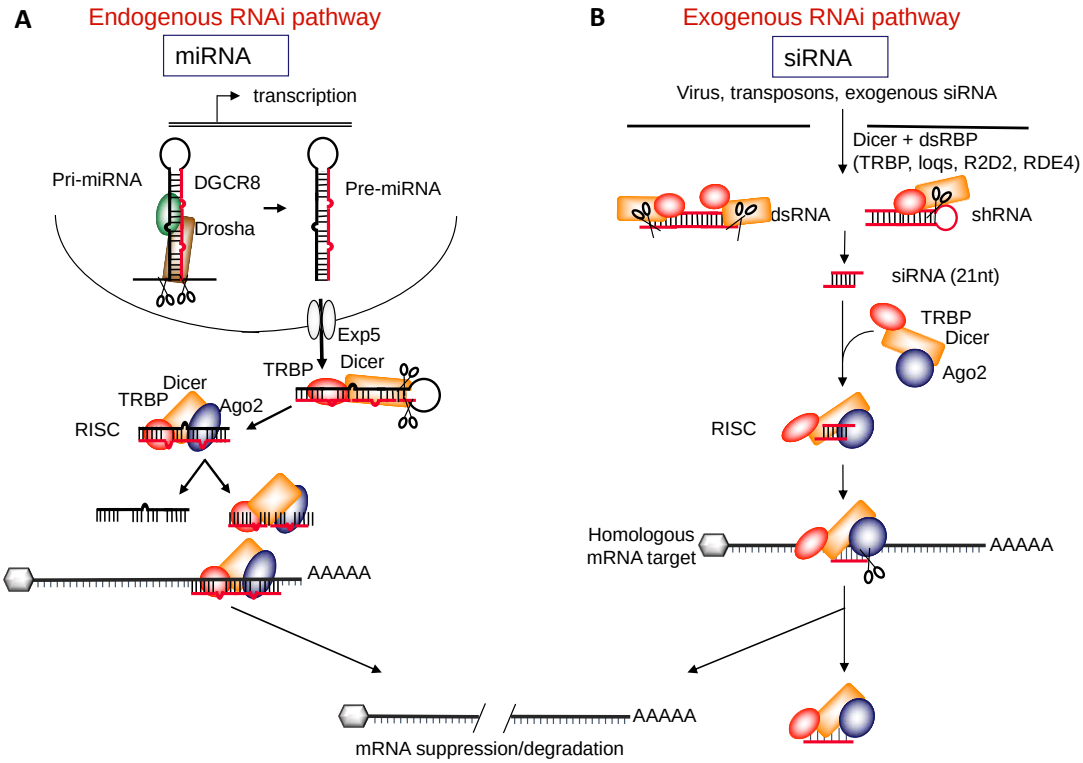


Figure 9: Mechanisms of RNA interference. RNAi has both an endogenous (A) and exogenous (B) pathway. In the endogenous pathways, pri-miRNAs are transcribed and then processed by Drosha to form pre-miRNAs. The pre-miRNAs are exported to the cytoplasm where they associate with proteins of the RISC and are further processed in to mature miRNAs. With the RISC, these miRNA will bind their target mRNA and supress its translation or lead to its degradation. In the exogenous pathways, exogenous siRNA can also associate with the RISC, and bind to their target mRNA leading to its degradation.
Adapted from [230]

been implicated in HIV-1 latency. The miRNA clusters miR-28, miR-125b, miR-150, miR-223 and miR-382 have been found to target the 3' end of HIV-1 mRNAs and are enriched in resting $CD4^+$ T cells over activated $CD4^+$ T cells[59]. By inhibiting the activity of these miRNAs, viral production is increased, which suggests that these miRNAs could play a role in HIV-1 latency. Another study showed that miR-29a can specifically target HIV-1 nef mRNA, and overexpression leads to a decrease in Nef protein expression and virus production[61]. While these miRNAs act directly against the virus, it is also possible for miRNA to target key cellular factors in the virus' replication cycle. miR-198 has been shown to decrease HIV-1 gene expression, through its activity on Cyclin T1[60], an important cellular factor for viral transcription.

siRNAs can then be exploited in a gene therapy strategy against viruses that cause chronic infections [114,236] or directly against viruses causing acute infections [237,238]. Mouse studies have shown that various respiratory viruses can been targeted by siRNA after delivery through the respiratory tract. They include influenza A virus[239], severe acute respiratory syndrome coronavirus[238], and respiratory syncytial virus[240], and similar results have been obtained in monkeys[238] and humans[241]. Recent studies showed that siRNA treatment 3 days post infection was protective against Ebola virus in rhesus monkeys[242]. This highlights the potential for siRNAs to be used as treatment against viruses. Furthermore, several groups have developed siRNAs that are able to inhibit the replication of many flaviviruses. In cell lines, adeno-associated virus vectors have been used to deliver siRNAs that are able to inhibit DENV in a dose dependent manner[243]. siRNA delivery via adenovirus and retrovirus were both effective against Japanese encephalitis virus (JEV) in mice[244]. In fact, one group was able to use a single siRNA, delivered to mice via a lentivirus, was protective against both JEV and WNV by targeting a conserved region

of the E protein[245]. Delivery of siRNAs in humans still needs to be optimized. At the moment, there are clinical trials underway looking at both topical and systemic delivery mechanisms[233].

It may also become possible to use RNA interference to treat chronic infections, such as HIV-1. There are a couple of mechanisms by which this can be accomplished. As previously mentioned, the only patient considered to have been cured of HIV-1 is Timothy Brown, who received a hematopoietic stem cell transplant with a mutant CCR5 gene, one of the co-receptors for HIV-1. Based upon this success, several groups have attempted to use siRNA to suppress CXCR4[246] and CCR5[108] expression. Delivery of these siRNA therapies will be discussed below. Further, cells could also be modified to express siRNAs acting directly against HIV-1. One trial by The City of Hope uses *ex vivo* lentiviral modified CD34+ T cells to express a combination of anti-HIV-1 molecules, including an shRNA against the HIV-1 tat/rev mRNA[247]. Combination approaches are necessary for viruses such as HIV-1, whose error-prone polymerase could lead to viral escape. Furthermore, as HIV-1 is an RNA virus with a complex tertiary structure[248] (figure 10), the siRNA target site must also be accessible. Therefore, in siRNA design, highly conserved and accessible areas of the viral genome must be identified. Our lab has recently identified one such site in the gag region of the HIV-1 genome, with similar or greater

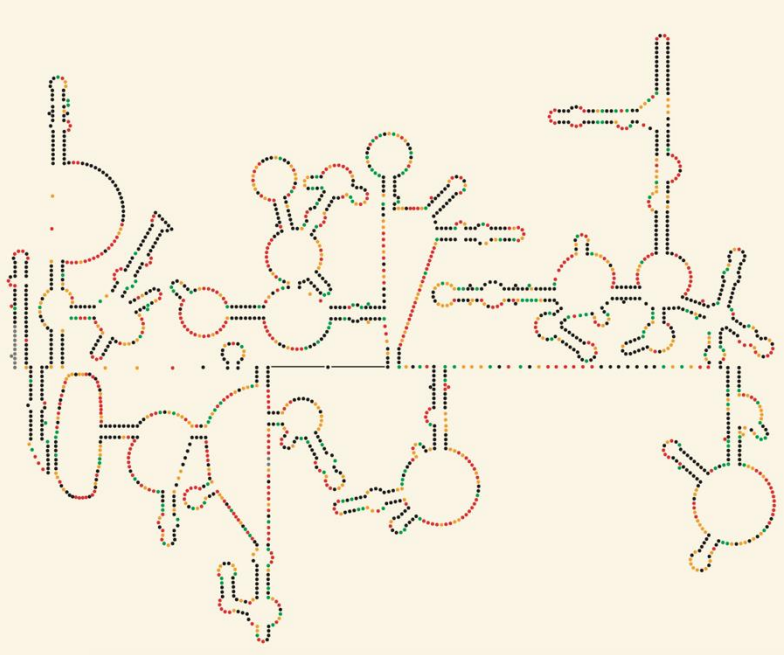


Figure 10: Structure of the HIV-1 RNA genome. The HIV-1 genome is highly structured, making it difficult to target via complementary siRNA inhibition.
Image from [248]

efficacy as the shRNA targeting the tat/rev sequence currently undergoing clinical trials[249]. This research also shows that by varying the length of the siRNA, it is possible to increase to potency of the treatment[114]. Additionally, when designing siRNAs to be used in humans, it is necessary to consider our response to foreign nucleic acids. For example, in vitro synthesis of siRNAs creates 5' triphosphates, which are able to trigger the type 1 interferon (IFN) pathway[250]. Furthermore, blunt-ended siRNAs can lead to IFN production via the retinoic acid inducible gene 1 protein. Both of these can be overcome by chemically synthesized siRNAs, which lack 5' triphosphates and have 5' and 3' overhangs. However, these may still activate the toll-like receptor (TLR) 3 pathway[251].

A potential application for siRNAs targeting arboviruses is the engineering of virus-resistant vectors. In fact, RNAi has been shown to play an important antiviral role in mosquitoes[252] and other arboviruses[253]. Since mosquitoes already use RNAi as an antiviral mechanism, some groups have used RNAi to breed mosquitoes that are genetically resistant to certain viruses, in particular, flaviviruses. In *A. aegypti,* one group was able to express inverted repeats that were complementary to DENV behind a mid-gut specific promoter. In these transgenic mosquitoes, virus replication and transmission were dramatically decreased. Furthermore, the same group later showed that the transgene was stable and did not result in a significant fitness loss[254]. This represents a proof of concept that RNAi could be used to engineer flavivirus-resistance in mosquitoes that could be used in a population replacement strategy to prevent transmission of the virus. This method to end arboviruses transmission would be preferable to the release of mosquitoes carrying dominant lethal genes described above.

Rationale

Project 1:

Combined antiretroviral therapy drugs for HIV-1 are therapeutic but not curative. Establishment of viral reservoirs is a major barrier towards a cure. A proposed strategy for a cure is to reactivate latently infected cells, allowing them to be targeted by conventional drugs and/or a strong immune response. Evidence suggests that miRNAs interact with many components of the HIV-1 replication cycle. Our hypothesis is that miRNAs play a vital role in HIV-1 replication and latency in macrophages by regulation of specific mRNAs. To study HIV-1 latency, a model was set up in lymphocytes, monocytes and MDM with the use of an HIV-1 molecular clone with inducible transcription. The virus was reactivated in macrophages using various latency reversing agents. Next this model was used to isolate miRNAs from reactivated cells to be sequenced. Bioinformatics analysis will identify miRNAs that are up- or downregulated upon reactivation. Selected miRNAs will be analyzed for their involvement in HIV-1 latency in these cell types. They could be susceptibility factors of cells to HIV-1 or factors mediating latency. Cellular factors involved in latency could be exploited in a strategy towards an HIV-1 cure.

Project 2:

Outbreaks of ZIKV in French Polynesia (2013) and Brazil (2015) have associated the virus with Guillain-Barré Syndrome in infected individuals and with microcephaly in babies born from infected mothers. Furthermore, several recent outbreaks have infected large portions of the population. This would suggest that recent mutations in the virus have led to increased pathogenicity and spread. The neurological implications and quick dissemination in recent outbreaks infection highlight the importance of studying this virus. There is currently no

approved vaccine or treatment for this virus. siRNAs have been used to inhibit the replication of many viruses. Recently, the delivery of a siRNA targeting Ebola virus has been shown to be protective against the virus in rhesus macaques, but there has been no siRNA developed against ZIKV. While effective siRNA drug delivery strategies are still being explored in humans, another potential use for siRNAs targeting ZIKV would be to produce and breed ZIKV resistant mosquitoes, which could then be used in a population replacement strategy.

Specific aims:

Project 1:

Aim 1: To set up a model to study HIV-1 latency in lymphocytes, monocytes and macrophages

Aim 2: To use this model to generate a mRNA/miRNA/lncRNA profile after reactivation.

Project 2:

Aim 3: To identify differences between a Brazilian strain from a recent outbreak, and a Thai strain whose lineage is separate from those associated with neurological complications

Aim 4: To design and validate siRNAs that can inhibit ZIKV replication.

Materials and Methods

Cell culture

The GagZipGFP and wildtype CEM and THP-1 cells were maintained in complete RPMI medium: Roswell Park Memorial Institute medium (HyClone™ RPMI 1640) with 10 % fetal bovine serum (FBS) (Hyclone), 2 mM L-glutamine and 50 U/ml Penicillin and 50 µg/ml Streptomycin (Invitrogen).

The HEK-293T cells were maintained in complete DMEM medium: Dulbecco's Modified Eagle's Medium (HyClone DMEM/high Glucose) with 10 % FBS and 50 U/ml Penicillin and 50 µg/ml Streptomycin.

The Vero cells were maintained in Vero DMEM medium: HyClone DMEM/high Glucose with 5 % FBS, 1 % non-essential amino acids (Multicell), 1 % L-glutamine (Multicell), and 50 U/ml Penicillin and 50 µg/ml Streptomycin.

The Neuroblastoma SH-SY5Y cells were maintained in Neuroblastoma medium: DMEM/Ham's F12 50/50 mix (Wisent), with 10% FBS, 50 U/mL Penicillin and 50 µg/mL Streptomycin.

All cell lines were passaged twice per week. Before passage, adherent cells (HEK-293T, SH-SY5Y, and Vero cells) were washed twice with Phosphate Buffer Saline (PBS) (MultiCell) and then trypsinized (MultiCell) for 5 min at 37˚C. Non-adherent cells (THP-1, CEM) were also washed once per week with PBS (5 min, 1200rpm, at room temperature (RT)).

GagZipGFP Monocyte-derived Macrophages (MDM) differentiation

GagZipGFP THP-1 monocytes cells were plated at $2x10^5$ cells/cm^2 in complete RPMI and supplemented with 200 nM phorbol myristate acetate (PMA) (Sigma-Aldrich). Cells are

completely adherent to the plate surface after one day, at which point the medium is changed to complete RPMI without PMA. Macrophages are used 1 week after differentiation.

Latency Reversing Agents (LRA)

CEM, THP-1 monocytes, and THP-1 monocyte-derived macrophages were treated with a combination of 2 µg/ml Doxycycline (Dox) (MultiCell) with the following LRAs: JQ1, HMBA, SAHA, Chaetocin, Disulfiram, and Prostratin at the concentrations indicated in Table 1. The final concentration of Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was adjusted to 0.04 % for each treatment because JQ1, SAHA, Chaetocin, Disulfiram and Prostratin were diluted in this solvent. Dox and HMBA were diluted in PBS.

Table 1: LRA Concentration

LRA	**JQ1**	**hexamethylene bisacetamide (HMBA)**	**Suberoylanilide hydroxamic acid (SAHA)**	**Chaetocin**	**Tetraethylthiuram disulfide (Disulfiram)**	**Prostratin**
Manufacturer	Cayman Chemical	Sigma-Aldrich	Sigma-Aldrich	Cayman Chemical	Sigma-Aldrich	Sigma-Aldrich
Concentration	500 nM	5 mM	4 µM	90 nM	500 nM	1 µM

Immunoblotting

Lysis buffer (Tris HCl 50 nM, NaCl 150 mM, EDTA 5 mM, NP40 1%, Glycerol 10%) containing anti-proteases and anti-phosphatases (Roche, Basel, Switzerland) was used to prepare cell lysates. The amount of protein in each sample was quantified using the Bradford assay. A 10 % denaturing polyacrylamide gel was used to separate 10 µg of total protein. Protein was then transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare, Little Chalfont, United Kingdom). The membranes were blocked with 5 % non-fat dry milk in Tris-buffered saline 0.1 % Tween 20 (TBST) (1 h, at RT, shaking) and were incubated overnight with

the primary antibody (rabbit polyclonal anti-GFP, or mouse serum #856 anti ZIKV E protein, or mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH)) in 3 % BSA-TBST (4˚C, shaking) at the concentrations indicated in Table 2. The membranes were washed 3 times in TBST (10 min, at RT, shaking), then incubated with IgG-horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibody (2h, at RT, shaking). After three washes in TBST (10 min at RT), the bands were visualized treating the membranes with ECL (GE Healthcare) and exposing them with HyBlot CL® autoradiography films (Denville Scientific) for 1, 3, and 5 min. Films were then developed using the X-OMA T 2000A Processor (Kodak). The band intensities were quantified by ImageJ software (MacBiophotonics).

Table 2: Antibodies used for immunoblotting

Antibody	Dilution	Catalogue number	Manufacturer
rabbit polyclonal anti- GFP	1/1000	Sc-8334	Santa Cruz
Mouse #856 serum anti-ZIKV E	1/500	-	MediMabs
mouse monoclonal anti- GAPDH	1/1000	Sc-32233	Santa Cruz
secondary anti-rabbit	1/5000	KP-474-1506	KP Laboratory
Secondary anti-mouse	1/3000	KP-474-1806	KP Laboratory

Cell viability

For the HIV experiments, cell viability was estimated by measuring the metabolism of 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO) [255]. 100 µL of cells ($3.5x10^5$ cells/mL) were plated in a 96-well plate and treated with different LRAs. For each day (one to five post-LRA treatment) cells were incubated for 3 h at 37°C with 20µL of MTT. Blue crystals are formed by the conversion of MTT to formazan, which

is then solubilized by acidified isopropanol. Plates were incubated (15 min, at RT, shaking) and read at 570 nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad, Hercules, CA). Values for LRA treated cells were compared to mock-treated cells. Data are expressed as means ± SEM and cell viability curve was obtained by nonlinear regression analyses (GraphPad Prism Software).

For the ZIKV experiments, cell viability was approximated by the metabolism of the WST-1 Cell Proliferation Reagent (Roche, Germany). $2.5x10^4$ Vero cells, HEK-293T cells, and Neuroblastoma SH-SY5Y cells were seeded in 96 well plates on day 0 and incubated overnight (37˚C, 5% CO^2).

On day 1, the growth medium was removed and cells were washed with PBS. Cells were then infected with either the Brazilian or Asian strain at a MOI of 1, 0.5,0 .1, and 0.01 in 100µl of plain EMEM. The cells were incubated with the virus dilutions for 2h (37˚C, 5% CO_2), and then the virus dilutions were removed and replaced with the respective growth medium.
On day 2 (24h post-infection) and day 3 (48h post-infection), 50µl of DMSO was added to 4 wells of each cell line as a control for cell death. After 15 min incubation (RT), 10µl of WST-1 was added to each well and cells were incubated (37˚C, 5% CO_2) for 1.5h. The cells were then fixed with a solution of NP40 (Sigma-Aldrich) at a final concentration of 1%. The plate was then read by an ELISA Microplate Reader (BioRAD). Absorbance was measured at 450nm (test wavelength) and 690nm (reference wavelength). The reference reading was then subtracted from the test reading. The value of the uninfected cells was set as 100% viability. The viability of each condition was then expressed as a percentage of the uninfected cells for each cell line.

GFP Fluorescence and Flow cytometry analyses

THP-1 MDM GagZipGFP cells were cultured in 96-well plates and treated with different LRAs. The images of bright-field cells and GFP+ cells were captured using the ZOE Fluorescent Cell Imager (Bio-Rad).

To evaluate the percentage of GFP+ cells, cells induced by LRA treatments were washed twice with PBS and collected by trypsinization. The cells were then washed three times with PBS (5 min, 1200 rpm, at RT) and fixed with 4% Paraformaldehyde for 20 min on ice. The cells were washed twice with PBS (5min, 1200rpm, at RT), resuspended in PBS, and filtered through a 70 µm cell strainer (Falcon). The number of GFP+ cells was measured using a LSRFortessa cell analyzer (BD Biosciences) and subsequently analyzed with FlowJo software (BD Biosciences). Cell populations were defined based on forward/sideward scattering. Mock sample (only treated with DMSO) was used to set GFP cell populations.

Cell sorting

For miRNA isolation, monocyte-derived macrophages GagZipGFP cells were treated with either Dox alone, or Dox combined with either SAHA (4µM), or Prostratin (1µM), or SAHA and Prostratin together. 3 days after treatment, the cells were washed twice with PBS and then collected by trypsinization. The cells were washed twice with PBS (5 min, 1200rpm, at RT), then once with PBS-2%FBS (5 min, 1200rpm, at RT), and then resuspended in PBS-2%FBS with 1mM EDTA. The resuspended cells were filtered through a cell strainer (70µM). Cells were then sorted into GFP+ and GFP- fractions by FACSAria Fusion (BD Biosciences), performed by Christian Young (Manager, Lady Davis Institute Flow Cytometry).

miRNA isolation and sequencing

GFP+ cells and GFP- cells were harvested by centrifugation in microtubes (5 min, 5000rpm, at RT) and washed once with PBS. 500µL of TRIzol® Reagent (Ambion) was added to each sample. Cells were lysed by pipetting up and down and the homogenized samples were incubated for 5 min at RT. 100µL of chloroform was added and the tube was shaken vigorously by hand. After a 2 min incubation at RT, the samples were centrifuged (15 min, 12,000rpm, 4˚C). The upper aqueous phase was placed in a new microtube. The miRNAs were then isolated using the miRNeasy Mini kit (QIAGEN). Samples were then submitted to Genome Quebec for RNA-sequencing (RNA-seq).

Zika Virus Strains

The Thai strain, PLCal_ZV (Genbank accession KF993678.1), was isolated in 2013 from a Canadian traveler returning from Thailand and subsequently passaged 4 times in Vero cells[256]. The Brazilian strain, HS-2015-BA-01 (Genbank accession KX520666.1) was isolated in Salvador, Bahia in 2015 and subsequently passaged three times in *Aedes albopictus* C6/36 mosquito cells and once in Vero cells[257].

Zika Virus Amplification

Both strains of ZIKV were amplified by infecting Vero cells. On day 0, $6x10^6$ Vero cells were plated in a T182.5 flask.

On day 1, the growth medium was removed and cells were washed with PBS. Cells were then infected at a MOI of 0.5 in 10ml of plain EMEM (Multicell) and incubated (37˚C, 5% CO^2) for 2h. The infection medium was then removed and replaced with ZIKV infection medium: DMEM with 2% FBS, 1 % non-essential amino acids, 1 % L-glutamine, 50 U/ml Penicillin and 50

µg/ml Streptomycin, and 15mM Hepes buffer (Sigma-Aldrich). Cells were then incubated (37˚C, 5% CO^2) for 48h. On day 3 (2 days post-infection), the supernatant was extracted and filtered through a 0.45µM pore.

Zika Virus Live Cell Imaging

Vero cells were seeded at $3.0x10^5$ cells/ml in 12 well plates. The following day, growth medium was removed and cells were washed with PBS. Then, cells were infected with either ZIKV BRZ or ZIKV THAI at MOI 0.1 in 1ml EMEM for 2h. After incubation, infection medium was replaced with complete DMEM. At 6, 12, 24, and 48h post infection, cells were washed two times with PBS and fixed with 4% PFA for 20 min, then imaged using the ZOE cell imager (Bio-Rad).

Plaque Assay:

Viral titers were measured using the plaque assay. On day 0, $6x10^5$ Vero cells were seeded in 6-well plates and incubated overnight (37˚C, 5% CO_2).

On day 1, 8 1/10 serial dilutions were performed with the filtrate in plain EMEM. The growth medium was removed and cells were washed with PBS. Next, cells were incubated (37˚C, 5% CO_2) with the virus dilutions for 2h. After incubation, the virus dilutions were removed and replaced with CMC: 1.2% carboxymethocellulose (Sigma-Aldrich), 2% FBS, in EMEM.

On day 5 (4 days post-infection), the CMC was removed, and after two washes with PBS, cells were fixed with 2ml of a 4% paraformaldehyde (PFA) solution. The PFA was removed and cells were washed with ddH_2O. Cells were then incubated (RT) with a 0.1% crystal violet

solution for 30 min. The crystal violet solution was rinsed using water and plaques were counted. Viral titers were calculated as follows: $\frac{\#\ of\ plaques}{dilution\ factor\ x\ infection\ volume}$

siRNA design:

The nucleotide sequence of all ZIKV isolates present in GenBank were aligned using Jalview software, and areas of high conservation (greater than 97%) identified. The sequences from these areas were then run through the Dharmacon, Thermofisher, and DesiRM siRNA algorithms, which predict the most potent siRNA against a given sequence. Furthermore, a literature analysis was performed to identify other putative siRNAs against ZIKV [258,259]. 6 siRNAs were selected for further analysis, 3 in the sequences corresponding to the E protein, 1 in the NS1, 1 in the NS5, and 1 in the 3' UTR (Table 3). These 6 siRNAs were synthetized by Dharmacon.

Table 3: Sequences of siRNAs Designed

Target	Strand	Sequence	3' overhang
E(1)	5'-sense-3' 5'-antisense-3'	[redacted]	[redacted]
NS1	5'-sense-3' 5'-antisense-3'	[redacted]	[redacted]
E(2)	5'-sense-3' 5'-antisense-3'	[redacted]	[redacted]
NS5	5'-sense-3' 5'-antisense-3'	[redacted]	[redacted]
E(3)	5'-sense-3' 5'-antisense-3'	[redacted]	[redacted]
3'UTR	5'-sense-3' 5'-antisense-3'	[redacted]	[redacted]

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Using Trizol (Life Technologies), RNA was extracted from Vero, HEK-293T, and SH-SY5Y cells that were either infected or mock infected at MOI of 0.01, 0.1, 0.5 and 1, 24 post infection. This RNA was then purified using the miRNeasy kit (QIAGEN). 1ug of RNA was used to synthesize cDNA with Superscript II according to the manufacturer's protocol (Invitrogen). A standard curve was generated using serial dilutions of the cDNA from infected cells, where 1/60 was found to be the optimal dilution to reach desired cycle threshold values (CT). Samples were loaded in quadruplicates, where for each well, 8µl BrightGreen qPCR MasterMix-Low ROX was mixed with 1µl of diluted cDNA and 1µl of the primers. For ZIKV, the primers used were: forward 5'-CAAAAGGAGGCCCTGGTCAT-3', reverse 5'-ATGAAAGACGTCCACCCCAC-3' (92 bp product). The controls used were TATA-box binding protein (TBP) with primers: forward 5'-TGCACAGGAGCCAAGAGTGAA-3' and reverse 5'-CACATCACAGCTCCCCACCA-3' (132 bp product), and Peptidylprolyl isomerase A (PPIA) with primers: forward 5'-AGACAAGGTCCCAAAGAC-3', reverse 5'-ACCACCCTGACACATAAA-3' (118 bp product). Using the Bio-Rad CFX96, the qPCR was run as follows: 95 °C for 5 min followed by 50 cycles of 95 °C for 10s, 62 °C for 15s, 72 °C for 5s. Finally, one cycle of 65 °C for 5s and one of 95 °C for 5s. The accompanying Bio-Rad CFX software was used to analyze the data.

Antibody development

The sequences for the E protein and C protein for both ZIKV BRZ and ZIKV THAI were sent to MediMabs, who predicted the most immunogenic peptides derived from these sequences (Table 4). The peptides were synthesized, and 6 mice were immunized with both the

E and C peptides. Serum from these mice was extracted and blotted against protein extract from both ZIKV BRZ and ZIKV THAI (see results).

Table 4: Sequences of peptides mice were immunized against

Protein	Sequence
C	KRGVARVSPFGGLKR
E	GEAYLDKQSDTQYV

siRNA transfection

$2.5x10^4$ Vero cells were plated in 96 well plate and incubated overnight (37˚C, 5% CO_2). Then, cell were transfected with 200nm of either si-E1, si-E2, si-E3, si-NS1, si-NS5, or si-3UTR (table 3) using Dharmafect (Dharmacon), or Lipofectamine (Thermo Fisher) as indicated by the manufacturer. Si-non-sense and si-gag1498 were also used as controls. Plates were then incubated for 4h (37˚C, 5% CO_2). Next, cells were washed with PBS and infected with ZIKV BRZ at MOI 0.01 in EMEM. 2h post infection, infection media was replaced with Vero media. Cells were then incubated for 24h (37˚C, 5% CO_2) to be assayed by Western Blot, RT-qPCR and plaque assay, or 48h to be assayed by WST-1.

Results

I) Model to study HIV-1 latency and reactivation

Time course of HIV-1 reactivation in CEM lymphocytes, THP-1 monocytes, and THP-1 MDM following LRA treatment

HIV-1 provirus reactivation in the latency model was measured in CEM lymphocytes, THP-1 monocytes, and THP-1 MDM after treatment with a combination of 2 µg/ml Dox with either JQ1, HMBA, SAHA, Chaetocin, Disulfiram, and Prostratin (figure 11). Cell lysates were examined by Western blot using an antibody against GFP, which will detect the GagZipGFP fusion protein expressed by cells with active transcription. We see the band intensity from day 1 to day 5 (figure 12). Reactivation is compared to the mock (without Dox) as well as Dox alone. The bands are normalized to GAPDH as a marker of the relative amount of protein loaded. The intensity of the GAPDH band is consistent in each LRA treatment, but not necessarily across treatments. The results of the Western blot analysis were quantified to compare each treatment. The GagZipGFP band intensity was measured and normalized to GAPDH and the

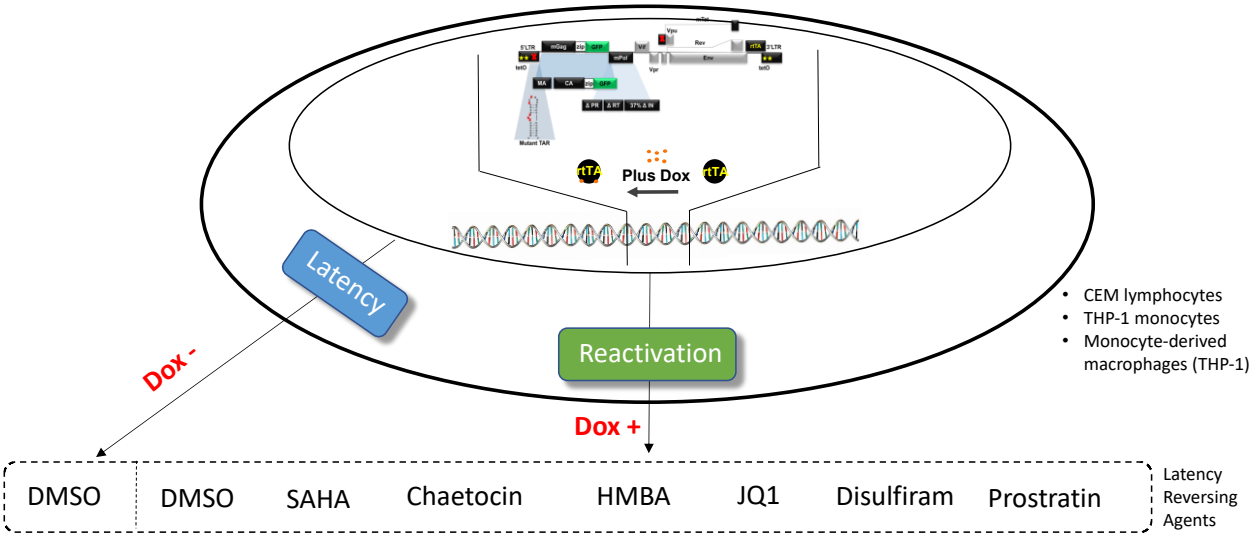


Figure 11: LRA treatment of monocyte-derived macrophages (THP-1). THP-1 GagZipGFP monocyte-derived macrophages were treated with 2 µg/ml of Dox alone or Dox with either 500 nM JQ1, 5nM HMBA, 4µM SAHA, 90nM Chaetocin, 500nM Disulfiram, or 1µM Prostratin up to 5 days.

amounts were plotted on 3 different graphs corresponding to the 3 cell types (figure 13). In all 3 cell types, SAHA was the most potent reactivator. However, the pattern of reactivation varied between cell lines. For SAHA, the lymphocytes peaked at day 5, while the monocytes and MDM

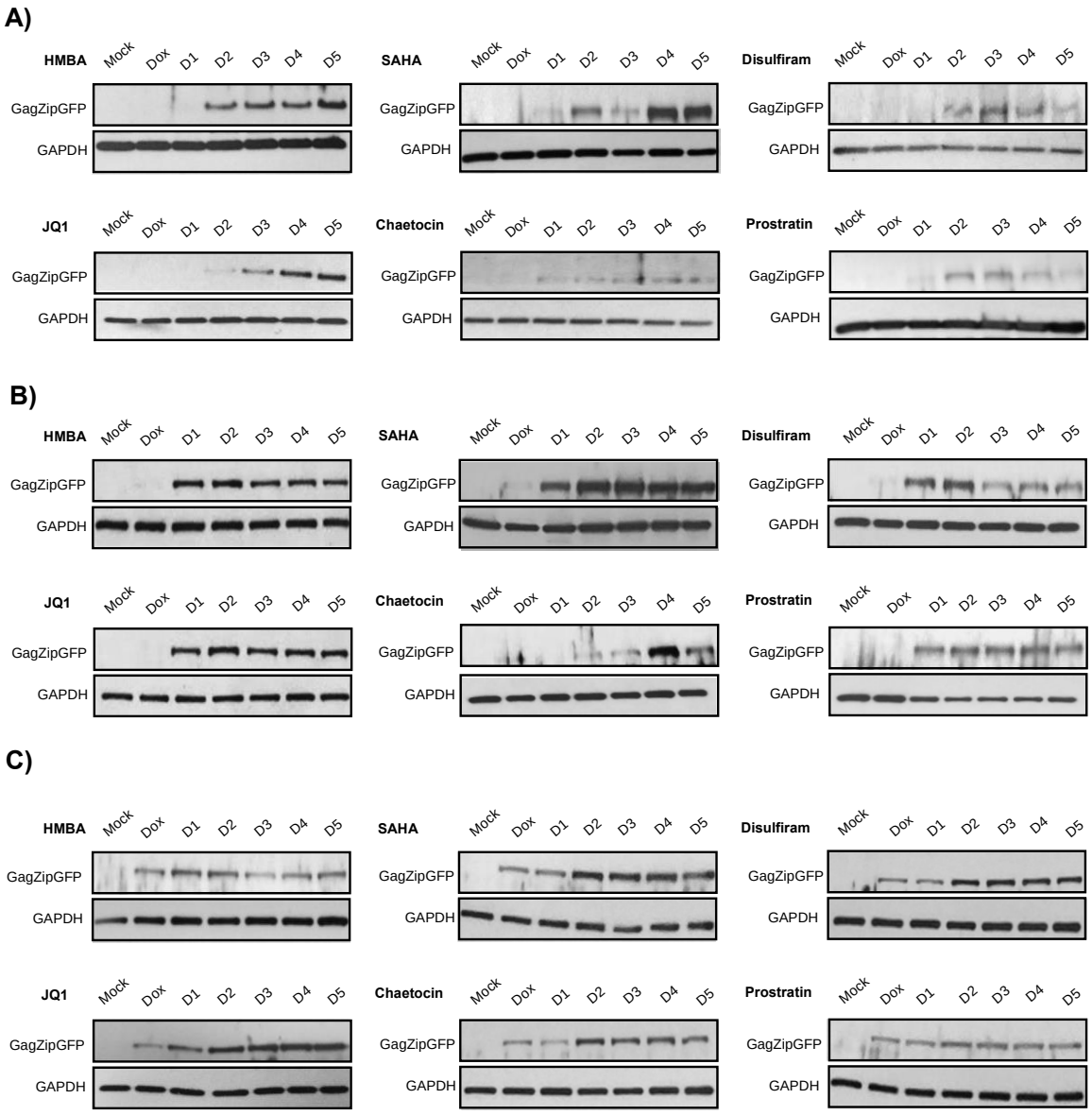


Figure 12: LRA treatments reactivate HIV-1. Time course reactivation of HIV-1 latency model in CEM lymphocytes (A), THP-1 monocytes (B) and THP-1 MDM (C) GagZipGFP cells were treated with 2 µg/ml of Dox alone or combined with either 500 nM JQ1, 5nM HMBA, 4µM SAHA, 90nM Chaetocin, 500nM Disulfiram, or 1µM Prostratin up to 5 days. Western blot analysis, showing the expression of GagZipGFP on top and GAPDH on the bottom (done jointly with Dr. Elodie Rance). Figure adapted from [150]

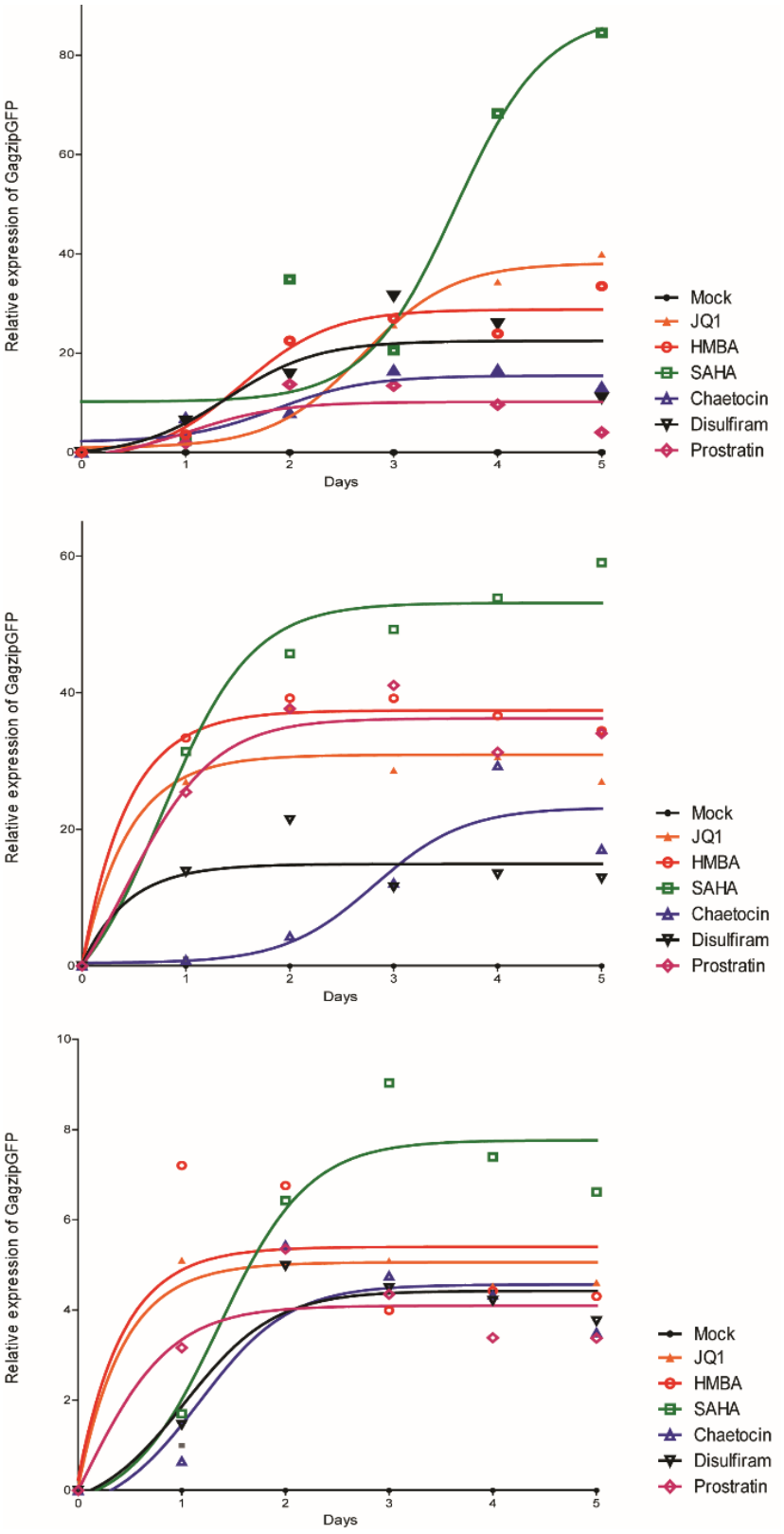


Figure 13: Graphical representation of HIV-1 reactivation by each LRA. CEM lymphocytes (A), THP-1 monocytes (B) and THP-1 MDM (C) GagZipGFP cells were treated with 2 µg/ml of Dox alone or combined with either 500 nM JQ1, 5nM HMBA, 4µM SAHA, 90nM Chaetocin, 500nM Disulfiram, or 1µM Prostratin up to 5 days. The relative expression of GagZipGFP was normalized by GAPDH. Western blot band intensity was quantified using ImageJ software (MacBiophotonics). Curves were obtained by non-linear regression analysis using Prism software (Graphpad) (done jointly with Dr. Elodie Rance). Figure adapted from [150]

peaked at day 3. Further, the lymphocytes reached a higher relative expression than the other cell types. Finally, the potency of the other LRAs varied among the three cell types.

Cell viability following LRA treatment

To determine the toxicity of the LRAs, cells were treated and cell viability was measured as a function of their metabolism of MTT over a 5-day time course following treatment (figure 14). 100% viability was set to the mock treated cells. The viability is also compared to that of Dox alone, which was greater than 90% over the entire time course. The most toxic LRA across the groups were Chaetocin and SAHA, while Prostratin was the least toxic. As with the reactivation levels, the patterns are cell type specific. For example, the lymphocytes were much more sensitive than the macrophages across all LRAs, but generally the relative level of toxicity for each LRA remained similar between each group.

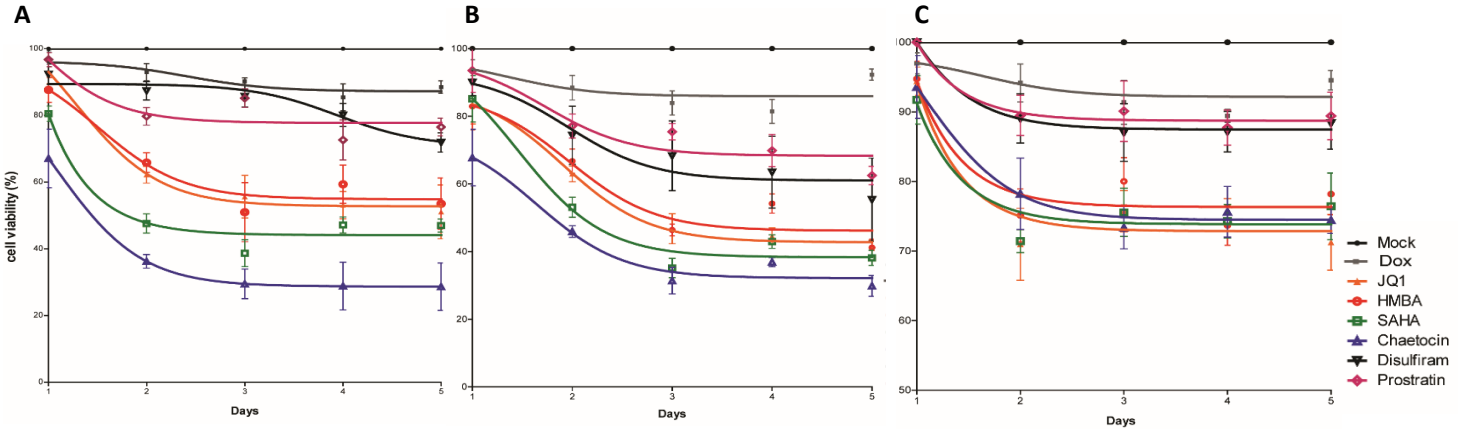


Figure 14: Cell viability pattern after LRA treatments. CEM lymphocytes (A), THP-1 monocytes (B) and THP-1 MDM (C) GagZipGFP cells were treated with 2 µg/ml of Dox alone or combined with either 500 nM JQ1, 5nM HMBA, 4µM SAHA, 90nM Chaetocin, 500nM Disulfiram, or 1µM Prostratin up to 5 days. Cell viability following LRA treatments was measured by the metabolism of MTT, with the viability of the mock set to 100% (done jointly with Dr. Elodie Rance). Figure adapted from [150]

Determining the optimal day of reactivation for each LRA

It is necessary to find a balance between reactivation and toxicity of the LRA in order to use this model to generate the RNA expression pattern in our second aim, so the cells need to be both viable and reactivated. The optimal day of reactivation was defined as greater than 95% reactivation and greater than 50% viability. By merging the results of the Western blot

analysis for HIV-1 reactivation (measured by expression of GagZipGFP) with the cell viability assay, a graph was created for each LRA, with a threshold bar to denote 95% of relative reactivation (figure 15). Each LRA had at least one day that met the criteria outlined above, therefore the optimal day was defined as the earliest time point at which reactivation was above 95%. The optimal day of reactivation for each LRA in each cell line can be seen in table 5.

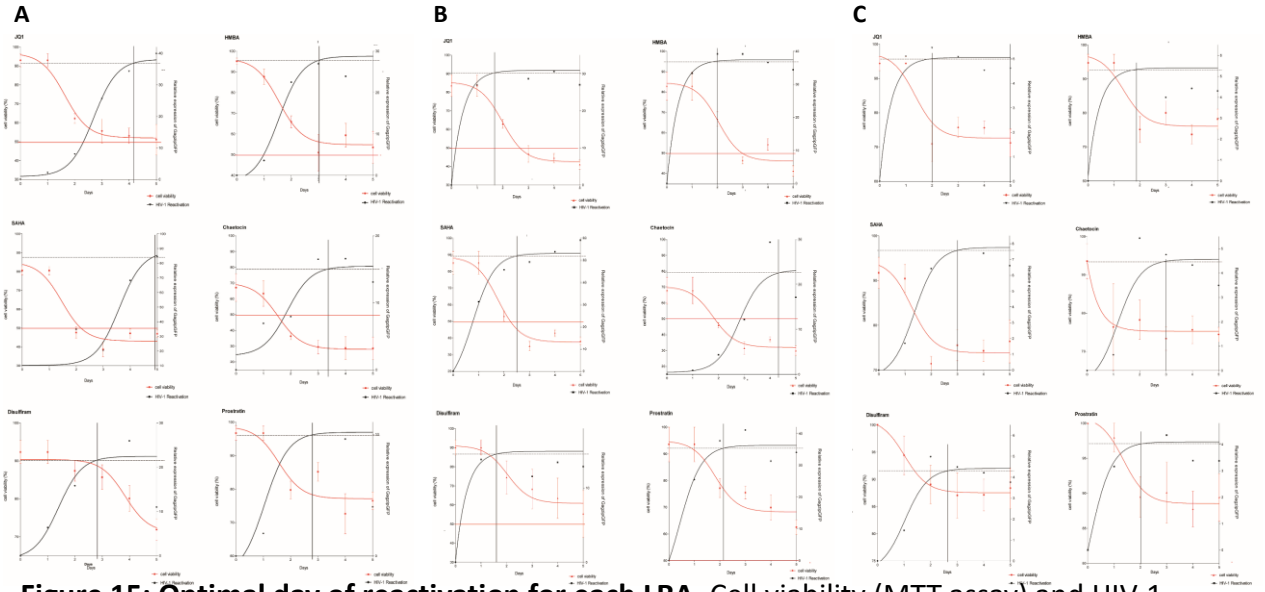


Figure 15: Optimal day of reactivation for each LRA. Cell viability (MTT assay) and HIV-1 reactivation (Western blot analysis of GagZipGFP curves) were merged in order to determine the best day of reactivation for each LRA, which has been defined as the time point that has above 95% reactivation and above 50% cell viability. In purple is the relative reactivation measured by GagZipGFP expression and in green is the cell viability (done jointly with Dr. Elodie Rance). Figure adapted from [150]

Table 5: Optimal day of reactivation for each LRA

	CEM lymphocytes	THP-1 monocytes	MDM macrophages
Prostratin	3	2	2
Disulfiram	3	1-2	2-3
Chaetocin	3	4	3
SAHA	5	2-3	3
JQ1	4	2	2
HMBA	3	2	2

Characterization of HIV-1 reactivation for each LRA, under optimal conditions

Next, we wanted to characterize the HIV-1 reactivation of the optimal day for each LRA by both qualitatively and quantitatively, measuring the number of reactivated cells (GagZipGFP+). Fluorescence imaging was used as a qualitative measure (figure 16), while flow cytometry was used to quantitatively measure the percentage of reactivated cells (GFP+) (figure 17). In support of the Western blot results, SAHA was the best reactivator at 66.5% for both the lymphocytes and monocytes, and 93% for the macrophages. Across all LRA, the macrophages

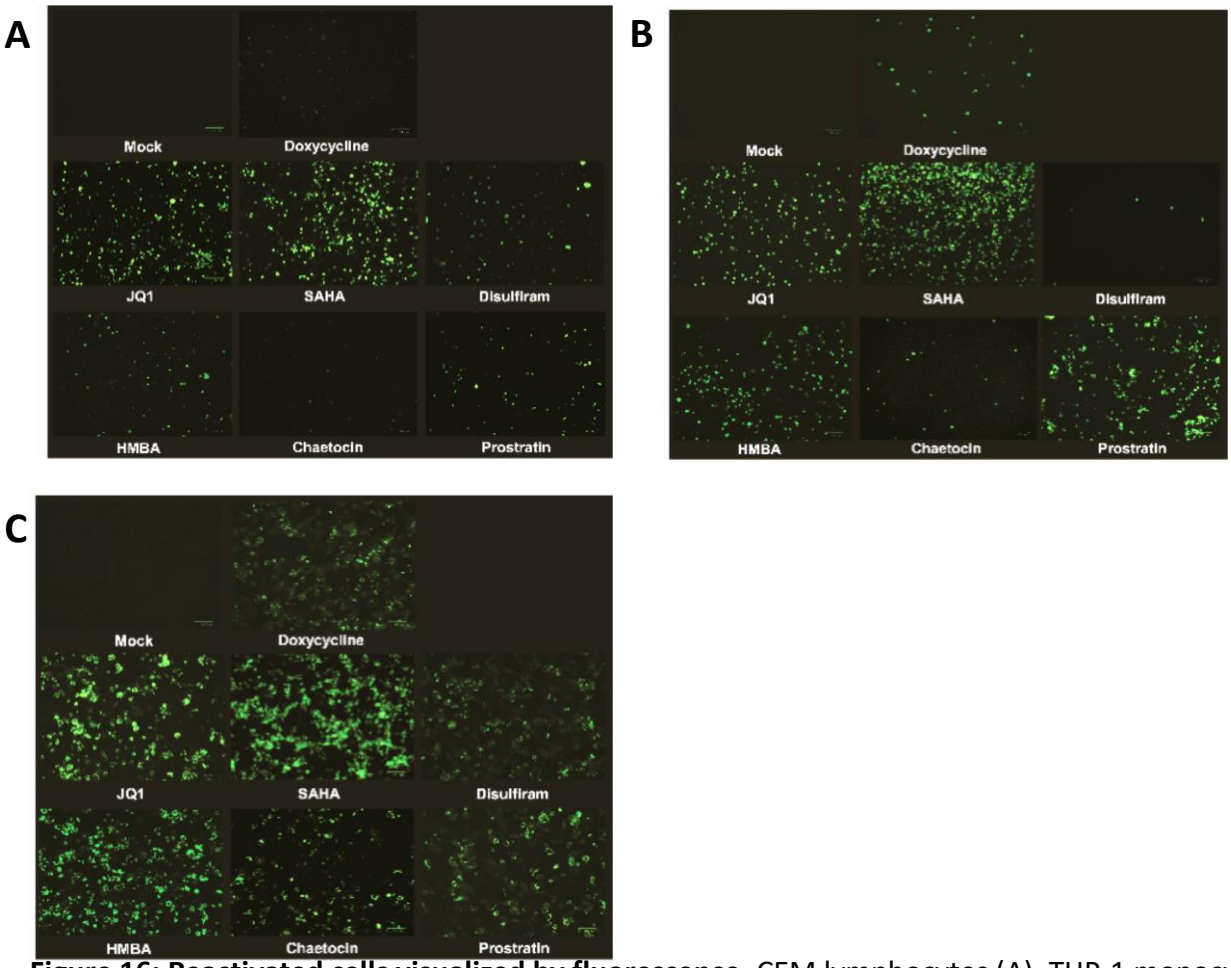


Figure 16: Reactivated cells visualized by fluorescence. CEM lymphocytes (A), THP-1 monocytes (B) and THP-1 MDM (C) GagZipGFP cells were treated with 2 µg/ml of Dox alone or combined with either 500 nM JQ1, 5nM HMBA, 4µM SAHA, 90nM Chaetocin, 500nM Disulfiram, or 1µM Prostratin on the optimal day of reactivation for each LRA (Table 4). GFP+ cells were captured using the ZOE Fluorescent Cell Imager (Bio-Rad) on the optimal day of reactivation for each LRA. Figure adapted from [150]

were more easily reactivated, as seen in both the fluorescence imaging and the flow cytometry data. Finally, when comparing the lymphocytes and monocytes, reactivation levels are similar between the LRAs, except for Prostratin, HMBA, and JQ1, which have greater activity in the monocytes.

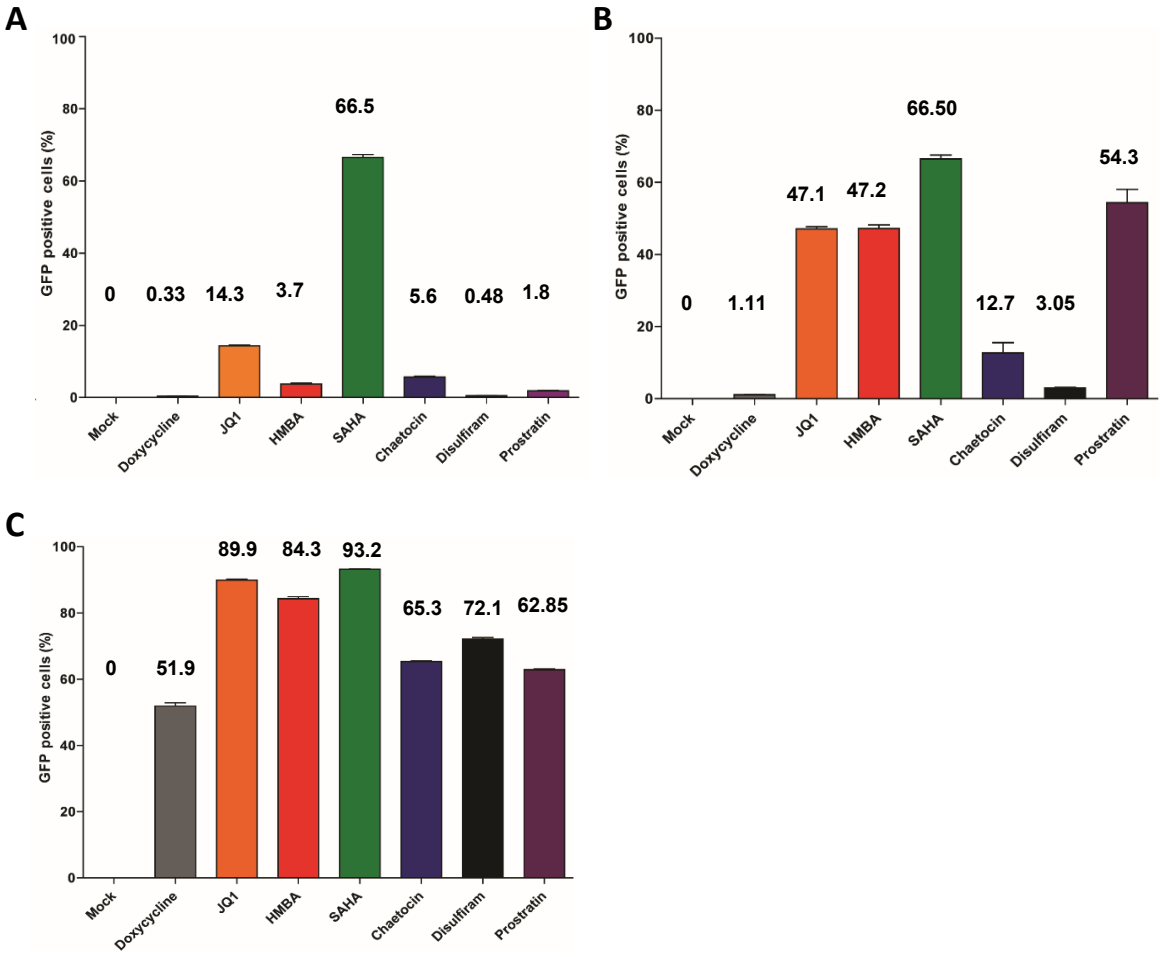


Figure 17: Percentage of reactivated cells for each LRA on their optimal day of reactivation. CEM lymphocytes (A), THP-1 monocytes (B) or THP-1 MDM were treated with 2 µg/ml of Dox alone or combined with either 500 nM JQ1, 5nM HMBA, 4µM SAHA, 90nM Chaetocin, 500nM Disulfiram, or 1µM Prostratin on the best day of reactivation for each LRA. The number of GFP+ cells represents the number of cells that have been reactivated. The percentage of GFP+ cells was measured by LSRFortessa cell analyzer and subsequently analyzed with FlowJo software (done jointly with Dr. Elodie Rance). Figure adapted from [150]

II) Differential expression patterns of miRNA in reactivated versus non-reactivated cells

RNA isolation and sequencing

We wanted to generate a differential expression pattern of miRNA during HIV-1 reactivation using the model we have set up. miRNAs identified could be markers of HIV-1 latency or factors mediating latency in macrophages. To measure the differential expression patterns of miRNA in reactivated versus non-reactivated lymphocytes, cells were treated with different LRAs or LRA combinations. SAHA and Prostratin were chosen because they have a different and potentially complementary mechanism of reactivation. These cells were then sorted by their expression of GFP, a marker for active expression of the HIV-1 GagZipGFP model. The percentage of reactivated cells for each treatment can be seen in figure 18. SAHA was very efficient at reactivating the cells (88% GFP+), while the addition of Prostratin increased this reactivation slightly (by 2%). Prostratin was only slightly more efficient at reactivating the cells than Dox alone. The amount of RNA isolated in each condition is presented in table 6. The desired amount of RNA for each sample is 3µg. Conditions with less than 3µg are highlighted. Samples will be sent to Genome Quebec for RNA-seq as soon as we

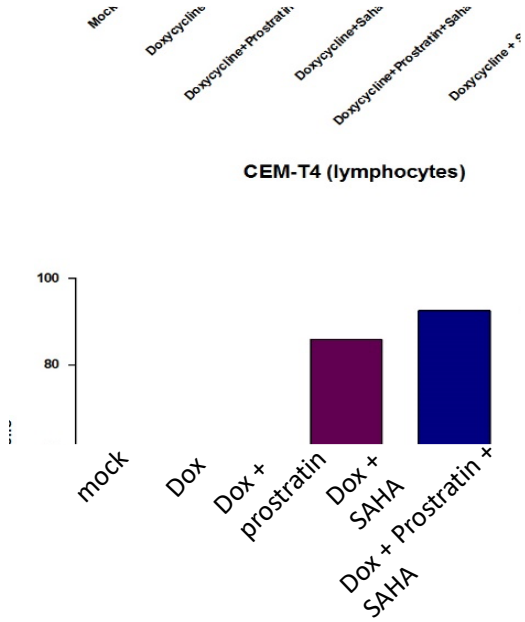


Figure 18: Percentage of reactivated CEM lymphocytes, following treatment with various LRA. Cells were treated with 2µg/ml Dox alone (white) or combined with either 4µM SAHA (blue), 1µM Prostratin (purple), or the two drugs together (black). The number of GFP+ cells represents the number of cells that have been reactivated. The percentage of GFP+ cells was measured by FACSAria Fusion and subsequently analyzed by FlowJo software (done jointly with Dr. Elodie Rance)

gather enough RNA. After this analysis, miRNAs that are differentially regulated in reactivated cells will then be confirmed by qRT-PCR and analyzed for their involvement in HIV-1 latency in lymphocytes, monocytes and macrophages.

Table 6: Amount of RNA collected from lymphocytes for differential RNA expression profile

Treatment	Mock	Doxycycline		Doxycycline+Prostratin		Doxycycline+Saha		Doxycycline+Prostratin+Saha		
Cell populations		GFP -	GFP +	GFP -	GFP +	GFP -	GFP +	GFP -	GFP +	
RNA quantity	3	3		3	3	3	3	3	3	ug
RNA quantity (duplicate)	3	3		3	2	0.3	3	1.5	3	ug
% GFP cells	0		0.3		1.25		81		87	

•Sequenced

•Sequenced soon

III) Sequence and Cytopathicity Analysis Between a Thai and a Brazilian ZIKV isolate

Sequence comparison between ZIKV BRZ and ZIKV THAI shows 13 amino acid polymorphisms

Based on past phylogenetic analyses, both strains of ZIKV are of the Asian lineage. However, ZIKV THAI branches off from the phylogenetic tree before the virus was associated with neurological complications. Thus, we wanted to compare these two isolates at the amino acid level to see if any polymorphisms exist between these two strains[260]. Polymorphisms could help explain why the more recent strains from the outbreaks in the Pacific Islands and Americas appear more pathogenic. As seen in figure 19, there are only 13 amino acid (aa) polymorphisms between the two strains, in the ER anchor of the C, Pr/PrM, NS2A, NS3, NS4A and NS5 proteins. A more precise comparison can be found in table 7.

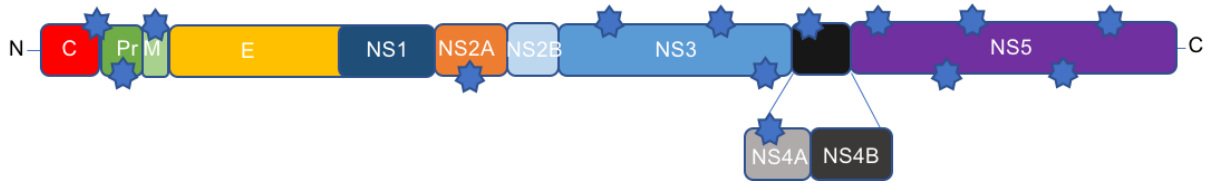


Figure 19: Amino acid polymorphisms between ZIKV BRZ and ZIKV THAI. 13 AA polymorphisms exist between the ZIKV BRZ and ZIKV THAI, 1 in the C, 2 in the PrM, 1 in the NS2A, 3 in the NS3, 1 in the NS4A, and 5 in the NS5 proteins. Figure from [260]

Table 7: Amino acid polymorphisms between ZIKV BRZ and ZIKV THAI

Strain	Amino acid position												
MR-766	A106	A139	S273	A1263	D1622	Y2086	L2123	L2167	Y2594	M2634	V2842	V2894	P3162
ZIKV THAI	T106	S139	S273	A1263	D1622	Y2086	F2123	L2167	Y2594	M2634	I2842	I2894	S3162
ZIKV BRZ	A106	N139	R273	V1263	G1622	H2086	L2123	M2167	H2594	V2634	V2842	V2894	P3162
Protein	ER anchor	pr/prM	prM	NS2A	NS3	NS3	NS4A	NS4A	NS5	NS5	NS5	NS5	NS5

Red: amino acid (aa) that differ from MR766. Blue: ZIKV proteins.

ZIKV BRZ is more cytopathic than ZIKV THAI in all three cell lines studied

When we began working with these two strains of ZIKV, it appeared that ZIKV BRZ was killing cells much more than ZIKV THAI. Therefore, we performed a time course infection to visualize the disruptions to the cell monolayers caused by these two strains in Vero cells. As

seen in figure 20, ZIKV BRZ is creating large holes in the cell monolayer, while ZIKV THAI does not seem to have this effect at the same MOI. This is increasingly evident at 24h post-infection. To quantify the cytotoxic effect of these two ZIKV strains, Vero, HEK-293T and SH-SY5Y Neuroblastoma cells were infected with either virus at increasing MOI (0, 0.01, 0.1, 0.5, 1). At 24h (Figure 21A-C) and 48h (Figure 21D-F), cell metabolism was measured by means of the WST-1 assay. The OD 450nm of the mock was set as 100% viability, and each condition was expressed as a percentage of the mock. At 24h (Figure 21A-C), the Brazilian strain has an augmented cytopathic effect on the Vero cells compared to the Canadian, while the difference between the two strains is negligible for the HEK-293T cells and Neuroblastoma cells. At 48h (Figure 21D-F), we see a large difference between the two strains in all three cell lines tested, most notably the Vero cells.

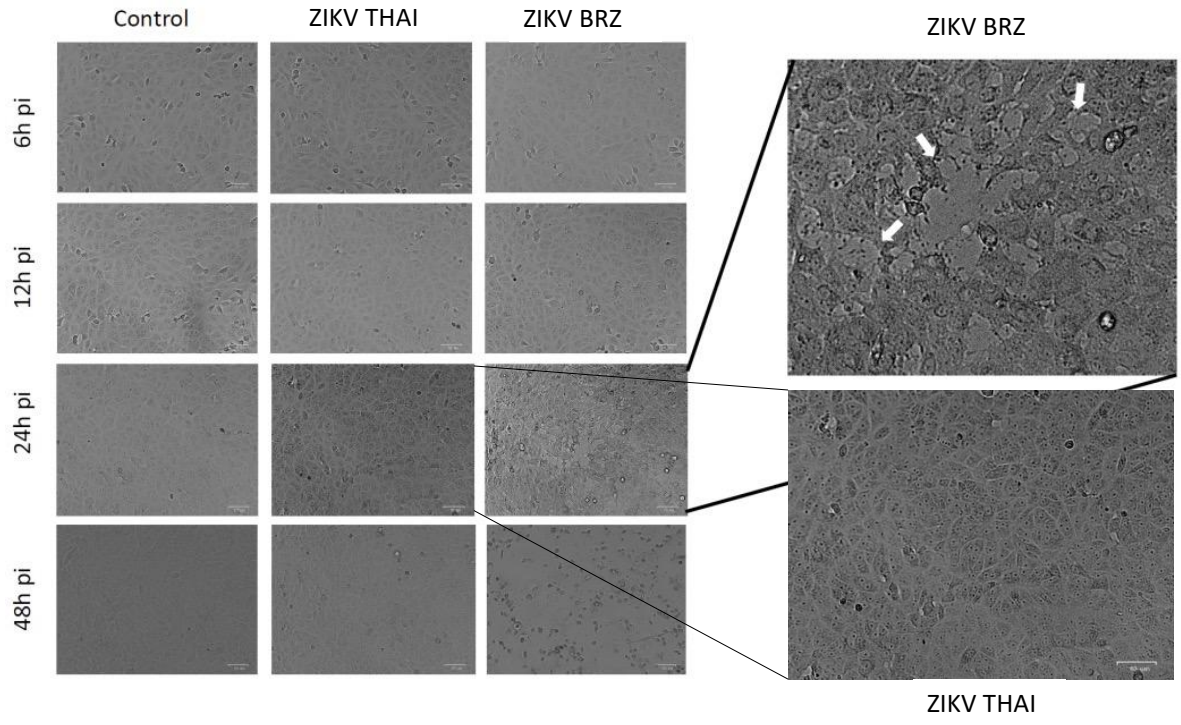


Figure 20: ZIKV BRZ has pronounced cytopathic effect compared to ZIKV THAI. Vero cells were infected at MOI 0.1, and observed 6, 12, 24, and 48h post infection. Cells were fixed with 4% PFA in PBS and examined under the microscope (Done jointly with Sergio Alpuche). Figure from [260]

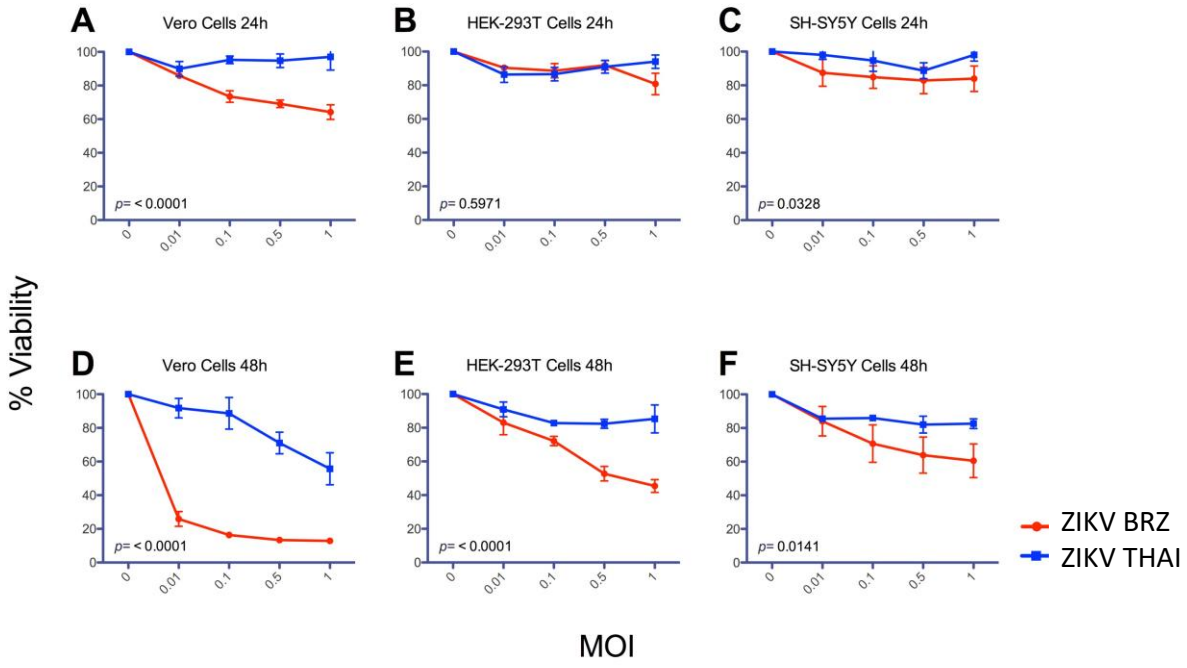


Figure 21: ZIKV BRZ has higher cytopathic effect than the THAI strain. Vero, HEK-293T and SH-SY5Y Neuroblastoma cells were infected with either ZIKV BRZ (red) or ZIKV THAI (blue) at increasing MOI (0, 0.01, 0.1, 0.5, 1). Cell metabolism was measured at 24h (A-C) and 48h (D-F) post infection via WST-1 assay. Figure from [260]

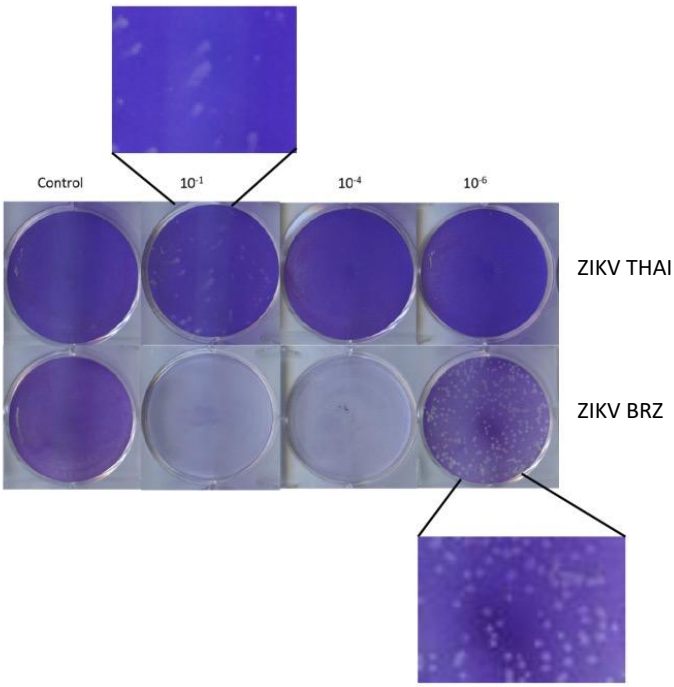
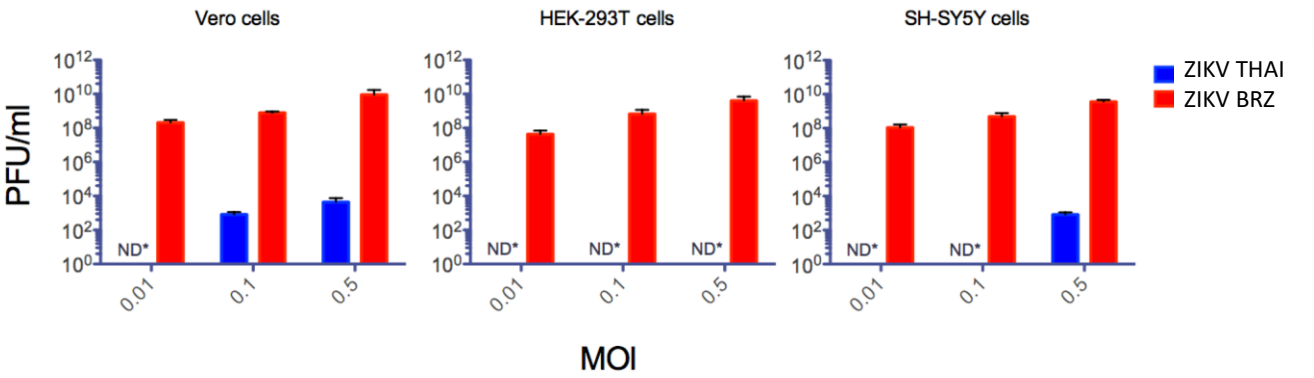


Figure 22: ZIKV BRZ produces higher titers than the THAI strain. Vero cells were infected at MOI 0.5 with either ZIKV BRZ or ZIKV THAI and supernatants were tittered via plaque assay. (Done jointly with Sergio Alpuche)

<u>ZIKV BRZ produces higher viral titers than ZIKV THAI in all three cell lines tested</u>

Next, we wanted to compare the viral titers produced by the two strains. Vero, HEK-293T and SH-SY5Y cells were infected at 0.01, 0.1, and 0.5 MOI, and 24h later supernatants from the infected cells were collected and the titer was determined by plaque assay. An example of this assay can be seen in figure 22, where we see that ZIKV BRZ gives countable plaques at the 10^{-6} dilution. In

contrast ZIKV THAI plaques can be counted at the 10^{-1} dilution. ZIKV BRZ produced much higher viral titers than ZIKV THAI, measured in plaque forming units (PFU), in all three cell lines tested, at all three MOI (figure 23). Interestingly the morphology of the plaques was consistently different between the two strains. ZIKV BRZ formed circular plaques with distinct borders, while ZIKV THAI produced plaques in undefined boundaries and in the shape of comets.



23: ZIKV BRZ produces higher titers than the THAI strain. Vero, HEK-293T and SH-SY5Y Neuroblastoma cells were infected with either ZIKV BRZ or ZIKV THAI at MOI 0.01, 0.1, and 0.5, and supernatants were tittered via plaque assay. (Done jointly with Sergio Alpuche). Figure from [260]

IV) Development of siRNA against ZIKV

siRNA design

To determine the best possible siRNAs to target most ZIKV circulating strains, we first aligned the nucleotide sequence of all ZIKV isolates present in GenBank using Jalview software, and we identified areas of high conservation (greater than 97%). The sequences from these areas were then run through the Dharmacon, Thermofisher, and DesiRM siRNA algorithms, which predict the most potent siRNAs against a given sequence. Furthermore, a literature analysis was performed to identify other putative siRNAs against ZIKV[258,259]. 6 siRNAs were selected for further analysis, 3 in the sequences corresponding to the E protein, 1 in the NS1, 1 in the NS5, and 1 in the 3' UTR, seen in table 3. These 6 siRNAs were synthetized by Dharmacon.

siRNA screen

To quickly and efficiently determine if any of these siRNA could inhibit the virus, we performed a screen using the WST-1 assay, working with ZIKV BRZ. First, we wanted to determine the optimal cell concentration and day post-infection to give a clear result, and select a MOI just before we see maximal cytopathic effects of the virus (figure 24). The

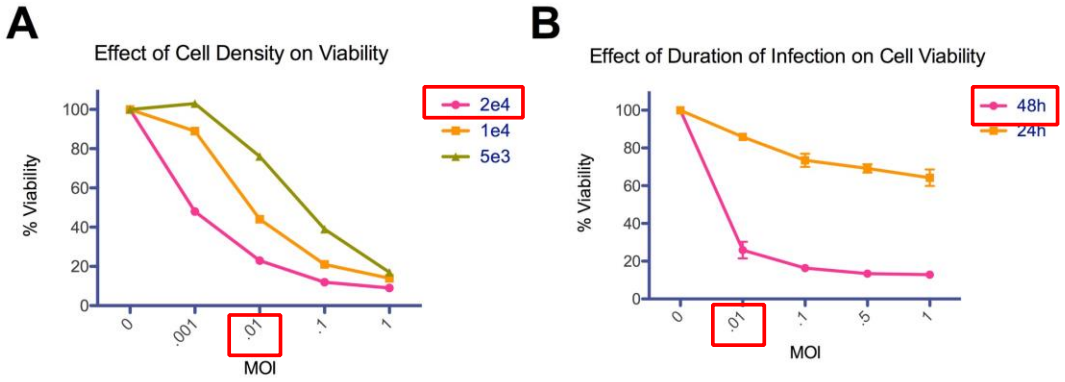


Figure 24: Optimization steps for siRNA screen. In order to optimize the siRNA screen, the effect of cell density (A) and duration of infection (B) were determined. Different densities of Vero cells were infected with ZIKV BRZ at varying MOI for either 24h or 48h. Values before the graph plateaued were selected for use in the screen.

observed decrease in viability was low after 24h but high after 48h. Therefore, tests will be conducted 48h post infection. The cell density selected was $2x10^4$ cells per well, as the lower densities showed less impact on cell viability. The MOI selected was 0.01, as the curve plateaus beyond this point. Cells were transfected with the siRNAs, then 4h later the cells were infected with ZIKV BRZ. Here we are looking for the siRNAs to recover the viability to that of the mock infected cells. As seen in figure 25, the control siRNAs and the transfection reagent alone are able to partially recover cell viability. However, of particular interest are the si-NS1 and si-NS5, both of which are able to almost completely recover cell viability, well above levels of the controls.

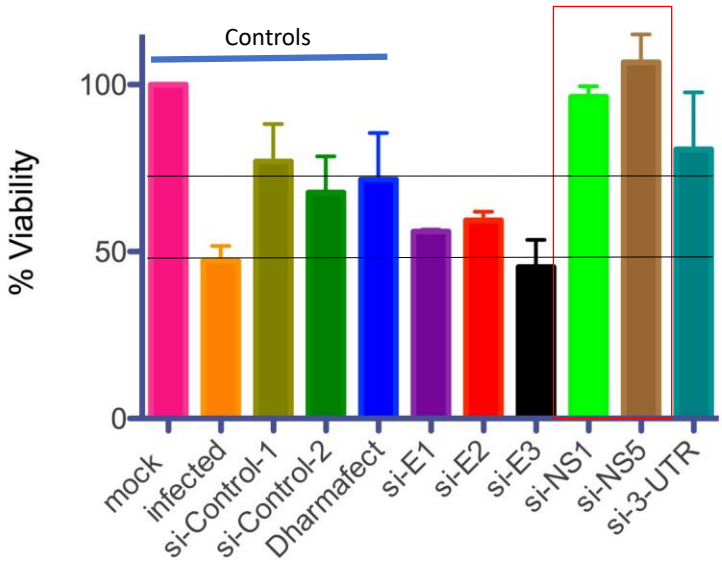


Figure 25: si-NS1 and siNS5 recover cell viability. In the siRNA screen, Vero cells were transfected with siRNAs and 4h later they were infected with ZIKV BRZ at MOI 0.01. Two days post infection, cell viability was estimated via WST-1 assay, and values were normalized to the mock infected cells. First line denotes the viability of the infected cells (48%) while line 2 denotes the viability of the cells treated only with the transfection reagent, Dharmafect (70%). Both si-NS1 and si-NS5 are able to completely recover cell viability.

Effect of siRNA treatment on viral titers

Next, we wanted to test the ability of the two most potent siRNAs, si-NS1 and si-NS5, to inhibit the viral production by measuring their ability to decrease viral titers. Cells were infected at MOI 0.01 and supernatants were collected 24h later. The supernatants were tittered via plaque assay (figure 26). While the siRNAs of interest were able to decrease viral titers, so was the transfection reagent alone. Therefore, we decided to test a number of different

transfection reagents for their ability to inhibit ZIKV via WST-1, seen in figure 27. Lipofectamine had the least impact on cell viability, so we repeated the siRNA screen (figure 28). Again, we see the si-NS1 and si-NS5 are able to recover the cell viability above the controls. However, when we look at the viral titers again, we have the same result as the previous experiment, where the transfection reagent alone is decreasing titers to the same level (figure 29).

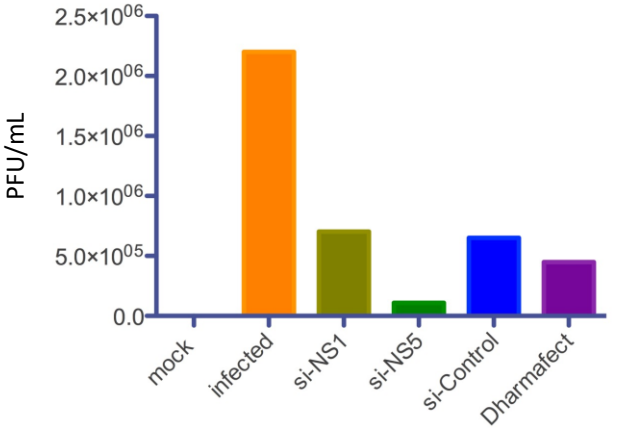
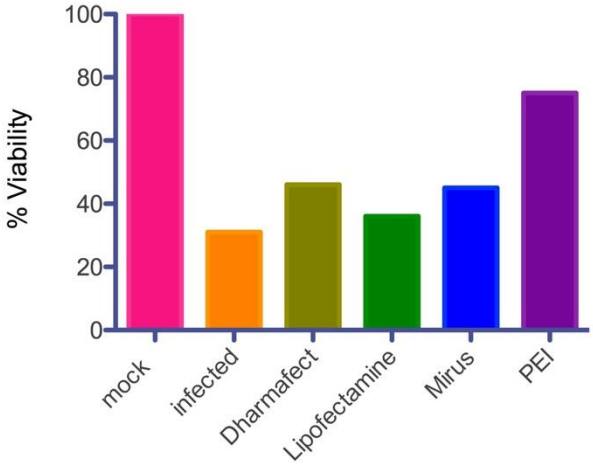


Figure 26: siRNAs decrease viral titers but so do the controls. Vero cells were transfected with siRNAs and 4h later they were infected with ZIKV BRZ at MOI 0.01. 24h later, supernatants were collected and tittered via plaque assay. Si-NS5 has the greatest decrease in viral titers, however the transfection reagent alone (Dharmafect) is also able to decrease titers.

Figure 27: Ability of transfection reagent to recover cell viability after ZIKV infection. Transfection reagents were added to Vero cells and 4h later they were infected with ZIKV BRZ at MOI 0.01. 48h later cell viability was measured via WST-1 assay.



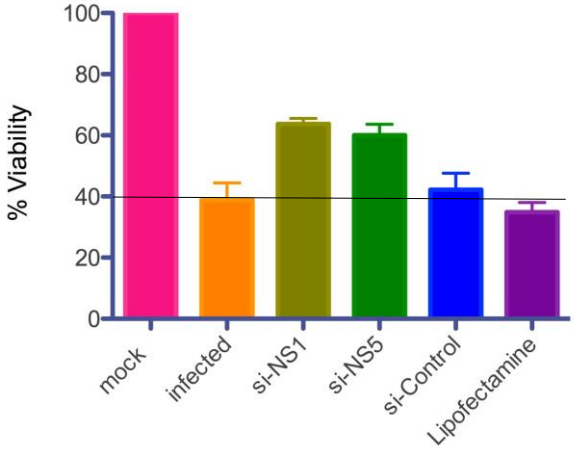


Figure 28: si-NS1 and siNS5 recover cell viability. Vero cells were transfected with siRNAs and 4h later they were infected with ZIKV BRZ at MOI 0.01. Two days post infection, cell viability was estimated via WST-1 assay, and values were normalized to the mock infected cells. The black line denotes the viability of the infected cells (39%) Both si-NS1 and si-NS5 partially recover cell viability with Lipofectamine.

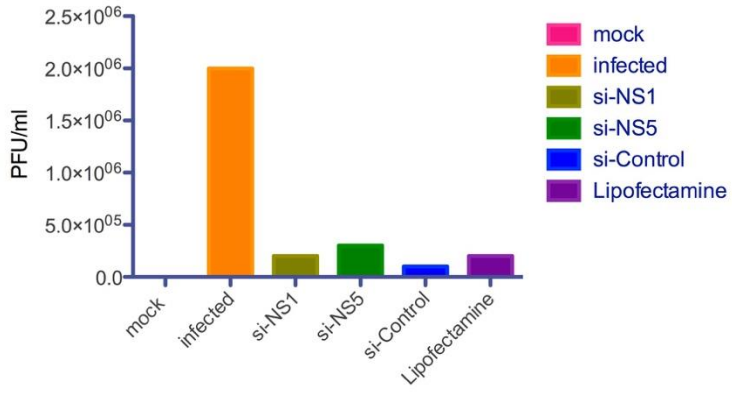


Figure 29: siRNAs decrease viral titers but so do the controls. Vero cells were transfected with siRNAs and 4h later they were infected with ZIKV BRZ at MOI 0.01. 24h later, supernatants were collected and tittered via plaque assay. Si-NS5 has the greatest decrease in viral titers, however the transfection reagent alone (Lipofectamine) is also able to decrease titers.

Measuring viral production after siRNA treatment

We wanted to take a look at virus production via Western blot, however, we were unable to detect ZIKV BRZ with the 4G2 pan-flavivirus antibody, which binds a highly conserved region of the viral E protein, seen in figure 31A. Interestingly, we do detect ZIKV THAI with this antibody, despite the fact that our sequence comparison revealed no AA polymorphisms between the two strains in the E protein. Therefore, we had enlisted the services of antibody manufacturer, MediMabs. We gave them the sequences of both the E and C protein, which they then used to predict the most immunogenic peptides from these proteins (table 4). Six mice were immunized against these peptides. Sera from these mice are then used to detect proteins from both strains of the virus. In figure 31B, we see that serum from mouse #856 is able to detect a protein from ZIKV BRZ at the expected band size of ≈50kDa, but surprisingly not of ZIKV THAI at this size. We do see a band specific to ZIKV THAI, but at a higher molecular weight (≈70kDa). Serum from mouse #859 (figure 30C) also had bands specific to each strain, but again, these bands were at unexpected sizes, ≈34kDa for ZIKV BRZ and the same ≈70kDa band for ZIKV THAI. Furthermore, none of the mouse sera were able to detect the C protein (≈10kDa).

With the serum from mouse #856 we were able to detect ZIKV BRZ, so we infected cells at a MOI of 0.5 and collected cell lysates 24h later. As seen in figure 31, there was no observable difference between the treatment and control conditions.

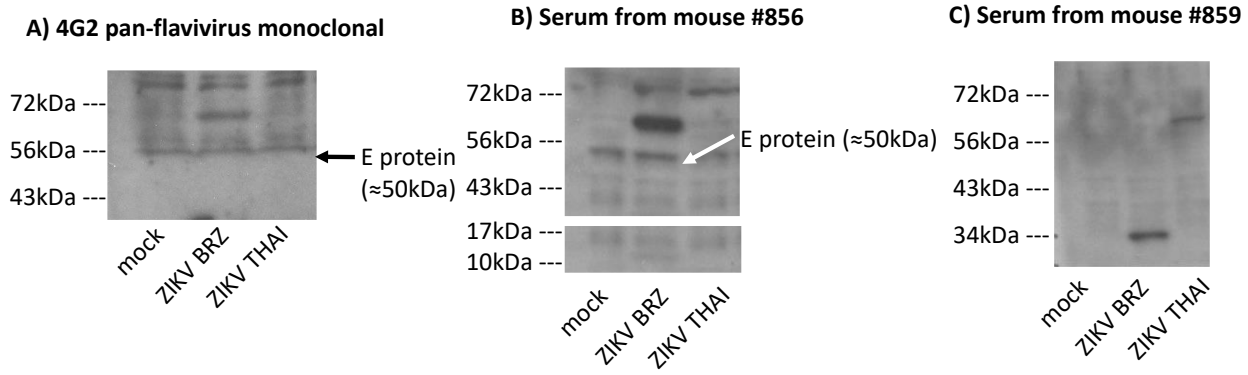


Figure 30: Testing antibodies and mouse serum against ZIKV BRZ and ZIKV THAI protein extract. Vero cells were infected with either ZIKV BRZ at MOI 0.1 or ZIKV THAI at MOI 5. 24h later, protein was collected from cell lysates. The 4G2 pan-flavivirus monoclonal antibody (A), serum from mouse #856 (B) or serum from mouse #859 (C) were tested for their ability to bind proteins from the two strains. The 4G2 binds the E protein of ZIKV THAI, but not ZIKV BRZ. Conversely, serum from mouse #856 recognizes the E protein of ZIKV BRZ, but not ZIKV THAI. Finally, serum from mouse #859 has a band specific to ZIKV BRZ, but not at the size of either the E or C protein.

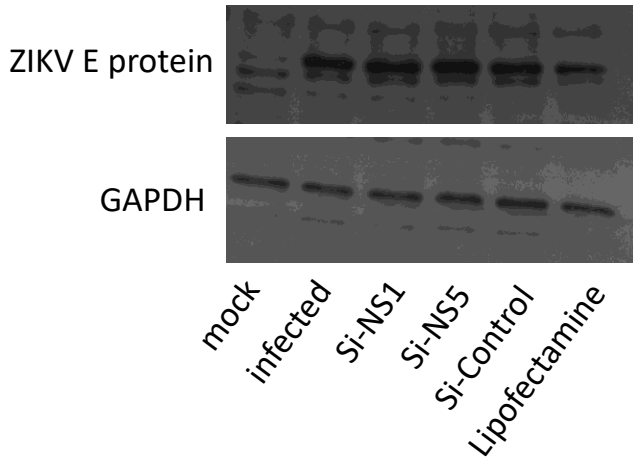


Figure 31: siRNA treatment does not affect ZIKV production. Vero cells were transfected with siRNAs and infected with ZIKV BRZ at MOI 0.5 4h later. After 24h, protein extract was collected and analyzed via Western Blot using serum from mouse #856. GAPDH was used as loading control. There was no observable difference between samples.

Discussion

I) Developing a model to study HIV-1 latency in lymphocytes, monocytes, and macrophages

While cART has dramatically changed the lives of patients living with HIV-1[92,93], the limitations of this treatment underline the importance of developing a cure for this virus[94-96,99]. The ability of HIV-1 to integrate into the host genome and remain latent in cells represents the largest barrier towards a sterilizing cure for HIV-1[117]. Furthermore, the number of possible reservoir cell types makes it difficult to target all of them[261]. In addition, to completely eliminate the virus from the body, all reservoirs must be targeted. Therefore, mechanisms of latency in all types of reservoir cells must be elucidated to achieve a sterilizing cure. Lymphocytes have long been known to harbor latent HIV-1, and while latency in these cells has been studied, our model allows us to study Tat independent reactivation of HIV-1. Monocytes and macrophages are potential reservoirs for HIV-1, but the mechanisms of latency in these cells is poorly understood.

Studying HIV-1 latency is difficult due to the lack of *in vivo* and *in vitro* models. Ideally, latently infected cells would be isolated from patients and studied. However, the limitations of this approach are the low number of latently infected cells, the number of possible latently infected cell types, and the absence of a well-established marker of latently infected cells.

To study the reactivation of HIV-1 reservoir cells, a latency model was set up based on a conditionally replicating virus developed by Das et al. [262] as an approach towards an HIV-1 vaccine. Transcription of the virus occurs only in the presence of Dox. The virus was further engineered by Dr. Alan Cochrane to express a GagZipGFP fusion protein and is mutated in the Protease and RT genes (figure 3). Therefore, the virus is now non-infectious and reactivated cells can easily be selected by expression of GFP. The virus was stably integrated into CEM

lymphocytes as well as monocytic THP-1 cells. This model can thus be used to study cellular changes upon reactivation in CEM lymphocytes, THP-1 monocytes and THP-1 MDM[150] (figure 11).

Various drugs were used to reactivate the virus along with Dox. These drugs have been previously reported in the literature as LRAs[128,129,136]. In order to target chromatin silencing, we used SAHA, an HDAC inhibitor and Chaetocin, a HMT inhibitor. To stimulate transcriptional elongation, we used JQ1, a BET inhibitor and HMBA, a p-TEFb agonist. Finally, in order to recruit host transcription factors, we used Prostratin, a PKC agonist, and Disulfiram, an Akt signaller. The ability of these LRAs to reactivate HIV-1 was studied by Western blot analysis during a 5-day time course following treatment. Each LRA had a different pattern of reactivation in each cell line, but SAHA was the most potent reactivator in all three cell types tested (figure 13). At the same time, we wanted to see how well these drugs were tolerated by the cells and cell viability was measured by the metabolism of MTT. While reactivation levels varied depending on the LRA, cell viability profiles remained consistent across the cell types. SAHA and Chaetocin were the most toxic, whereas Prostratin and Disulfiram were the least toxic. Interestingly, the THP-1 MDM showed the highest tolerance to the LRA. For example, cell viability with Chaetocin reached as low as 25-30% for the CEM lymphocytes and THP-1 monocytes, while the THP-1 MDM were greater than 70% viable (figure 14).

It is important to find a balance between reactivation and toxicity of the LRA because if these drugs are to be used in patients, they must be safe and efficient. Therefore, the results of the reactivation and the results of the toxicity were merged to determine the optimal day of reactivation for each LRA tested (figure 15). This allowed us to determine the optimal day of reactivation for each LRA (table 5).

While the Western blot analysis was able to determine the relative expression of GagZipGFP, it does not allow us to compare the total reactivation across each LRA. By this method alone, it is not possible to determine whether the relative GagZipGFP expression is due to a small population of cells with high expression, or to a large population of cells with low expression. The most important feature of an LRA in the context of the "shock and kill" method towards a sterilizing HIV-1 cure is their ability to reactivate a large population of cells so that they can be targeted by other antiretroviral drugs or a strong immune response. Therefore, we wanted to characterize the reactivation of each LRA on their optimal day of reactivation, based on the Western blot and MTT assays. Fluorescence imaging of GFP and flow cytometry analysis were used, and we determined that SAHA was the best LRA for reactivation (figure 16, 17). While we are using this model to generate a differential expression pattern of mRNA, miRNA and lncRNA, this model could also be used to identify markers for, or factors mediating HIV-1 latency. Furthermore, it can be used to test the effect of other compounds as LRAs.

II) Determining the differential expression patterns of miRNA during HIV-1 reactivation of lymphocytes

With a model to study HIV-1 reactivation in lymphocytes now established, it can be used to study cellular changes that occur upon reactivation. More specifically, it can be used to determine the differential expression patterns of miRNAs during HIV-1 reactivation in these cells. Dox and SAHA (HDACi) were used to reactivate cells. We also wanted to use SAHA in combination with another LRA that acts through another mechanism and showed relatively low toxicity in our

model. Therefore, we elected to use Prostratin (PKC agonist). This would hopefully give another unique miRNA expression pattern.

While the best day of reactivation for SAHA is day 3 and Prostratin is day 2, we decided to use day 3 to study the miRNA expression patterns. The reactivation and toxicity of Prostratin are very similar between day 2 and 3, while there is a significant increase in reactivation from day 2 to 3 with SAHA. 3 days following treatment with the LRA, macrophages were sorted by their expression of GFP (figure 9). The GFP+ population represents those that were reactivated, while the GFP- population represents those that were induced but not reactivated. Therefore, any differences in the miRNA expression between these cells should not be due to effects of the LRA, but are instead either a result of, or factor for HIV-1 reactivation. We have not yet gathered enough RNA from the treated cells to send to Genome Quebec for RNA-seq. We have not been able to isolate enough RNA in the GFP- populations of the SAHA alone and SAHA with Prostratin treatments. These LRA are very effective at reactivating the cells, so there are not many GFP- cells and this work is ongoing.

Upon completing the second set of RNA, miRNA and lncRNA that are differentially expressed in the reactivated and non-reactivated cells must be validated by RT-qPCR. Then, these RNAs could be tested for their role in the maintenance or disruption of HIV latency through either an over expression of the miRNA/lnc RNA or by using a miRNA sponge to knock down their activity[263-265].

III) ZIKV Strain Comparison

Recent outbreaks of ZIKV have highlighted the virus' potential for rapid transmission and ability to cause neuropathologies. However, this was not seen in earlier outbreaks. We obtain a

strain from a Canadian traveller returning from Thailand in 2013 (ZIKV THAI), as well as a strain from the outbreak in Bahia, Brazil in 2015 (ZIKV BRZ). As seen in figure 5, the lineage of ZIKV THAI branches off before the outbreaks in French Polynesia and Brazil where the virus was associated with Guillain-Barré syndrome and microcephaly. Therefore, there could be differences between these two strains that would help explain modifications in the virus's pathogenicity. Indeed, when we began working with these two strains, our first observation was that ZIKV BRZ was forming large holes in the monolayers of our cells, so we did a time point infection, where at 24h and 48h, we see ZIKV BRZ has a pronounced cytopathic effect compared to ZIKV THAI (figure 20). To quantify this cytopathic effect, we used the WST-1 assay, a measure of cell viability similar to the MTT described above. The results presented in figure 21 confirm our initial observation that ZIKV BRZ has an enhanced cytopathic effect compared to ZIKV THAI. This is especially evident in the Vero cells, and even more pronounced at 48h post infection.

Next, we wanted to compare these two strains in their ability to generate viral titers in the plaque assay. In the plaque assay, plaques are formed when the virus lyses the cells. Therefore, the titer is a factor of the virus's ability to replicate and the cytopathic effect of the virus. As we see in figure 21, ZIKV BRZ is more cytopathic. However, at most, this difference is about 3-fold in the Vero cells at 48h. When we look at the plaque assay (figure 22, 23), ZIKV BRZ is able to generate much higher viral titers, on the order of 8 to 10 logs. While some of this difference is likely due to the difference in cytopathicity, there is also likely an enhanced ability for ZIKV BRZ to produce progeny virions to infect other cells, forming more plaques. Another difference observed between the two strains is their plaque morphology. While ZIKV BRZ gives round plaques with distinct borders, ZIKV THAI gives comet shaped plaques with undefined boundaries.

Finally, we wanted to look at the virus's ability to accumulate vRNA in the cells. This work was pursued by S. Alpuche who performed RT-qPCR to evaluate the virus's ability to accumulate vRNA in the cells. ZIKV BRZ accumulated higher vRNA in both Vero and HEK 293T cells but not in the SH-SY5Y [260.]

As seen in figure 19, there are 13 aa polymorphisms between these two strains. Interestingly, previous work has shown that five aa polymorphisms in ZIKV allowed the virus to move from mosquitoes to humans. Furthermore, another group showed that an aa polymorphism in the NS1 protein was able to increase infectivity in mosquitoes[266]. The role of the 13 aa changes between our strains should be studied to determine which region is responsible for the increase cytopathic effect and ability to generate higher viral RNA and viral titers, seen in our experiments (figure 21, 23). In fact, one paper showed that the S139N polymorphism in the PrM protein increased infectivity in human and mouse neural progenitor cells and contributes to fetal microcephaly in mice[184]. These results are consistent with our experiments which show that ZIKV BRZ has an increased cytotoxic effect compared to the ZIKV THAI (figure 21). Furthermore, since the prM is involved in virus maturation and secretion, this could also help explain the increased replicative capacity we see in our experiments (table 7), due to an increase in viral fitness. However, the increased replicative capacity could be explained by further mutations. For example, phylogenetic analyses have shown the while the S139N mutation arose just prior to the French Polynesia outbreak, there was a subsequent mutation in the NS5 polymerase M/T2634V, that was identified in all Latin American viruses but not in French Polynesia isolates[267]. Further research is required on effect of these subsequent mutations on ZIKV pathogenesis.

IV) siRNA against ZIKV

To create an siRNA with the ability to inhibit the replication of multiple ZIKV strains, the sequence of all known strains of ZIKV were aligned to identify areas of high conservation. These sequences were then run through multiple siRNA algorithms to predict to most potent siRNA against the virus. In order to screen these siRNAs for their ability to inhibit the virus, we decided to use the WST-1 assay. We used the ability of the siRNA treatment to recover cell viability as a proxy for their ability to inhibit virus replication, since the 96-well format was much more time and cost-efficient than using Western blot, plaque assay, or RT-qPCR. Only the top siRNAs would be tested via these assays. In figure 25, we see that the siRNAs si-NS1 and si-NS5 are able to recover the viability of the cells. Therefore, we wanted to continue testing these siRNAs. While results from the plaque assay (figure 26) showed that these siRNAs were also able to decrease viral titers, the transfection reagent alone (Dharmafect) also decreased titers. It is possible that the transfection reagent alone is inhibiting the virus's ability to infect or be released from these cells. Therefore, we tested an array of transfection reagents by WST-1, to determine which one(s) would have the least impact on ZIKV infection. In figure 27, we see that Dharmafect, Mirus, and PEI are all able to recover cell viability, while Lipofectamine has the least impact. Therefore, we repeated these experiments with Lipofectamine as our transfection reagent. Again, si-NS1 and si-NS5 recover cell viability (figure 28), however, Lipofectamine also decreases viral titers as seen with Dharmafect (figure 29).

We also wanted to look at the effect of these siRNA on the production of ZIKV proteins. The pan-flavivirus 4G2 monoclonal antibody has been used to study many flaviviruses[268-270]. It binds to a highly conserved fusion loop in domain 2 of the E protein[271]. There are no aa polymorphisms between our two strains in the E protein (figure 19). However, as seen in figure

30A, the 4G2 antibody only recognizes our ZIKV THAI strain. For this work, we need an antibody capable of detecting ZIKV BRZ. Therefore, we had mice immunized against immunogenic peptides derived from the E and C protein sequences common amongst our two strains. Interestingly, the serum from mouse #856 reacted to ZIKV BRZ and not ZIKV THAI (figure 30B). These results would suggest that there is a difference in the E protein between these two strains that is not explained at the aa level. One possible explanation for this would be different post translational modifications, such as alternative glycosylation sites[272]. Also, since we did find aa polymorphisms in the NS3 protease protein, perhaps there is an alternative proteolytic function that results in the formation of different epitopes of the E protein.

Now that we had serum capable of recognizing the ZIKV BRZ strain, we were able to verify the effect of the siRNA treatment on ZIKV production. However, as seen in figure 31, there was no observable difference. However, in this experiment, a higher MOI (0.5) was used, to ensure we would have enough protein. It is possible that the amount of virus was too high for the concentration of siRNA used and should be repeated with either a lower MOI or higher siRNA concentration.

Finally, we wanted to verify the ability of these siRNA to knock-down vRNA levels. However, we have been unsuccessful in amplifying the virus in these conditions and this work is still on-going.

To increase the potency of the siRNAs, we want to optimize their length. Currently we are studying canonical 21nt length siRNAs. However, Dr. Scarborough, a postdoctoral fellow in our lab, has designed an siRNA against HIV and he has shown that longer siRNAs increased their potency[114]. Next, if these siRNAs were ever to be used in for human therapy, a cocktail of siRNAs would be preferable in case the virus were to mutate so combinations of the more

potent siRNAs should be explored. Furthermore, when considering siRNA therapy in humans, it is important to ensure that they are not toxic nor that they induce a dsRNA mediated innate immune response. Toxicity can be assayed via the WST-1 assay in HEK-293T cells, since this is a human cell line that we have already worked with for Zika. To measure immune activation, transfected cells can be blotted for phosphor-PKR and phosphor-TLR3, both markers of immune activation.

Another consideration in using siRNA for human therapy are delivery options. For the siRNAs targeting Ebola virus[242], they used lipid nanoparticles through intravenous injection. Studies for siRNAs in mice against other viruses have also shown to be effective through intranasal administration[239]. Finally, these siRNAs could be used to breed ZIKV resistant mosquitoes. This has been done by a previous group against DENV by expressing the siRNA sequence under the control of a mosquito specific promoter[273]. Therefore, the efficacy of our siRNAs in mosquito cells should be explored.

Conclusion

Project 1 focused on HIV-1. In aim 1 we successfully set up a model to study HIV-1 latency in lymphocytes, monocytes and macrophages. We used this model to test the ability of certain LRA to reactivate latent HIV-1. This model can be used to test other putative LRA to be used in a strategy towards an HIV-1 cure via the "shock and kill" method, described in the introduction. Furthermore, this model can help us understand other mechanisms involved in HIV-1 latency. In aim 2, we used this model to generate a differential expression pattern of mRNA, miRNA, and lncRNA in cells that were reactivated compared to cells that were induced to reactivate, but did not. When enough RNA is gathered for a second round of sequencing, we hope this data will provide insight on other mechanisms related to miRNA and lncRNA that are factors which promote or inhibit HIV-1 latency.

Project 2 focused on ZIKV. In aim 3, we identified key differences between a contemporary strain of ZIKV from the 2015 outbreak in Brazil and a Thai strain of the virus whose lineage predates the viruses' association to neurological complications, namely GBS and microcephaly. Further analyses are required to determine exactly how these mutations may have led to the increased spread and pathogenies of the virus in recent outbreaks. In aim 4, we have developed 2 siRNAs that may have the potential to inhibit replication of the virus, however, further studies and optimization of these siRNA is required. These siRNAs could be used in a much-needed therapy for the virus, or in the breeding of genetically resistant mosquitoes that could end transmission of the virus.

References

1. WHO. HIV Global Statistics. Vol. 2018 (World Heath Organization, 2016).
2. Summary: Estimates of HIV Incidence, Prevalence and Proportion Undiagnosed in Canada. Vol. 2018 (Public Health Agency of Canada, 2015).
3. Barre-Sinoussi, F*., et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**, 868-871 (1983).
4. Gottlieb, M.S*., et al.* Pneumocystis carinii Pneumonia and Mucosal Candidiasis in Previously Healthy Homosexual Men. *New England Journal of Medicine* **305**, 1425-1431 (1981).
5. Hahn, B.H., Shaw, G.M., De Cock, K.M. & Sharp, P.M. AIDS as a zoonosis: scientific and public health implications. *Science* **287**, 607-614 (2000).
6. Marlink, R*., et al.* Reduced rate of disease development after HIV-2 infection as compared to HIV-1. *Science* **265**, 1587-1590 (1994).
7. Rowland-Jones, S.L. & Whittle, H.C. Out of Africa: what can we learn from HIV-2 about protective immunity to HIV-1? *Nature immunology* **8**, 329-331 (2007).
8. Hemelaar, J. The origin and diversity of the HIV-1 pandemic. *Trends in molecular medicine* **18**, 182-192 (2012).
9. Osmanov, S., Pattou, C., Walker, N., Schwardländer, B. & Esparza, J. Estimated global distribution and regional spread of HIV-1 genetic subtypes in the year 2000. *Journal of acquired immune deficiency syndromes (1999)* **29**, 184-190 (2002).
10. Hazenberg, M.D*., et al.* Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS (London, England)* **17**, 1881-1888 (2003).
11. Dragic, T*., et al.* HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**, 667-673 (1996).
12. Holmes, C.B., Losina, E., Walensky, R.P., Yazdanpanah, Y. & Freedberg, K.A. Review of human immunodeficiency virus type 1-related opportunistic infections in sub-Saharan Africa. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **36**, 652-662 (2003).
13. Chang, Y*., et al.* Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *science* **266**, 1865-1869 (1994).
14. Fan, H*., et al.* Epstein–Barr viral load as a marker of lymphoma in AIDS patients. *Journal of medical virology* **75**, 59-69 (2005).
15. Maiman, M*., et al.* Cervical cancer as an AIDS-defining illness. *Obstetrics & Gynecology* **89**, 76-80 (1997).
16. Feng, Y., Broder, C.C., Kennedy, P.E. & Berger, E.A. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872-877 (1996).
17. Deeks, S.G., Overbaugh, J., Phillips, A. & Buchbinder, S. HIV infection. *Nature reviews. Disease primers* **1**, 15035 (2015).
18. Koenig, S*., et al.* Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. *Science* **233**, 1089-1093 (1986).

19. Embretson, J.*, et al.* Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* **362**, 359 (1993).
20. Hu, W.-S. & Hughes, S.H. HIV-1 reverse transcription. *Cold Spring Harbor perspectives in medicine* **2**, a006882 (2012).
21. Mitchell, R.S.*, et al.* Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol* **2**, e234 (2004).
22. Gatignol, A. Transcription of HIV: Tat and cellular chromatin. *Advances in pharmacology (San Diego, Calif.)* **55**, 137-159 (2007).
23. Bannwarth, S. & Gatignol, A. HIV-1 TAR RNA: the target of molecular interactions between the virus and its host. *Current HIV research* **3**, 61-71 (2005).
24. Tazi, J.*, et al.* Alternative splicing: regulation of HIV-1 multiplication as a target for therapeutic action. *The FEBS journal* **277**, 867-876 (2010).
25. Greenberg, M.E.*, et al.* Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation. *The EMBO Journal* **16**, 6964-6976 (1997).
26. Schwartz, O., Maréchal, V., Le Gall, S., Lemonnier, F. & Heard, J.-M. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV–1 Nef protein. *Nature medicine* **2**, 338-342 (1996).
27. Stumptner-Cuvelette, P.*, et al.* HIV-1 Nef impairs MHC class II antigen presentation and surface expression. *Proceedings of the National Academy of Sciences* **98**, 12144-12149 (2001).
28. Neil, S.J., Zang, T. & Bieniasz, P.D. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* **451**, 425 (2008).
29. Archin, N.M., Sung, J.M., Garrido, C., Soriano-Sarabia, N. & Margolis, D.M. Eradicating HIV-1 infection: seeking to clear a persistent pathogen. *Nature reviews. Microbiology* **12**, 750-764 (2014).
30. Siliciano, J.D.*, et al.* Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nat. Med.* **9**, 727-728 (2003).
31. Finzi, D.*, et al.* Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* **278**, 1295-1300 (1997).
32. Chun, T.W.*, et al.* Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* **387**, 183-188 (1997).
33. Finzi, D.*, et al.* Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* **278**, 1295-1300 (1997).
34. Carter, C.C.*, et al.* HIV-1 infects multipotent progenitor cells causing cell death and establishing latent cellular reservoirs. *Nature medicine* **16**, 446-451 (2010).
35. Alexaki, A. & Wigdahl, B. HIV-1 infection of bone marrow hematopoietic progenitor cells and their role in trafficking and viral dissemination. *PLoS pathogens* **4**, e1000215 (2008).
36. Keele, B.F.*, et al.* Characterization of the follicular dendritic cell reservoir of human immunodeficiency virus type 1. *Journal of virology* **82**, 5548-5561 (2008).
37. Kitchen, S.G. & Zack, J.A. CXCR4 expression during lymphopoiesis: implications for human immunodeficiency virus type 1 infection of the thymus. *Journal of virology* **71**, 6928-6934 (1997).

38. Brack-Werner, R. Astrocytes: HIV cellular reservoirs and important participants in neuropathogenesis. *AIDS (London, England)* **13**, 1-22 (1999).
39. Koenig, S.*, et al.* Detection of AIDS virus in macrophages in brain tissue from AIDS patients with encephalopathy. *Science* **233**, 1089-1093 (1986).
40. Lambotte, O.*, et al.* Detection of infectious HIV in circulating monocytes from patients on prolonged highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* **23**, 114-119 (2000).
41. Aquaro, S.*, et al.* Macrophages and HIV infection: therapeutical approaches toward this strategic virus reservoir. *Antiviral research* **55**, 209-225 (2002).
42. Zhu, T.*, et al.* Evidence for human immunodeficiency virus type 1 replication in vivo in CD14+ monocytes and its potential role as a source of virus in patients on highly active antiretroviral therapy. *Journal of virology* **76**, 707-716 (2002).
43. Lusic, M. & Giacca, M. Regulation of HIV-1 latency by chromatin structure and nuclear architecture. *Journal of molecular biology* **427**, 688-694 (2015).
44. Van Lint, C., Bouchat, S. & Marcello, A. HIV-1 transcription and latency: an update. *Retrovirology* **10**, 67 (2013).
45. de Ruijter, A.J., van Gennip, A.H., Caron, H.N., Kemp, S. & van Kuilenburg, A.B. Histone deacetylases (HDACs): characterization of the classical HDAC family. *The Biochemical journal* **370**, 737-749 (2003).
46. Arrowsmith, C.H., Bountra, C., Fish, P.V., Lee, K. & Schapira, M. Epigenetic protein families: a new frontier for drug discovery. *Nature reviews. Drug discovery* **11**, 384-400 (2012).
47. Van Lint, C., Emiliani, S., Ott, M. & Verdin, E. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. *The EMBO journal* **15**, 1112-1120 (1996).
48. Williams, S.A.*, et al.* NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. *Embo j* **25**, 139-149 (2006).
49. Blazkova, J.*, et al.* CpG methylation controls reactivation of HIV from latency. *PLoS pathogens* **5**, e1000554 (2009).
50. Boehm, D.*, et al.* SMYD2-Mediated Histone Methylation Contributes to HIV-1 Latency. *Cell host & microbe* **21**, 569-579.e566 (2017).
51. Marban, C.*, et al.* COUP-TF interacting protein 2 represses the initial phase of HIV-1 gene transcription in human microglial cells. *Nucleic acids research* **33**, 2318-2331 (2005).
52. Marban, C.*, et al.* Recruitment of chromatin-modifying enzymes by CTIP2 promotes HIV-1 transcriptional silencing. *The EMBO journal* **26**, 412-423 (2007).
53. du Chéné, I.*, et al.* Suv39H1 and HP1γ are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency. *The EMBO journal* **26**, 424-435 (2007).
54. Kauder, S.E., Bosque, A., Lindqvist, A., Planelles, V. & Verdin, E. Epigenetic regulation of HIV-1 latency by cytosine methylation. *PLoS pathogens* **5**, e1000495 (2009).
55. Blazkova, J.*, et al.* CpG methylation controls reactivation of HIV from latency. *PLoS pathogens* **5**, e1000554 (2009).
56. Sobhian, B.*, et al.* HIV-1 Tat assembles a multifunctional transcription elongation complex and stably associates with the 7SK snRNP. *Molecular cell* **38**, 439-451 (2010).

57. Griffin, G.E., Leung, K., Folks, T.M., Kunkel, S. & Nabel, G.J. Activation of HIV gene expression during monocyte differentiation by induction of NF-kB. *Nature* **339**, 70 (1989).
58. Wang, X.*, et al.* Cellular microRNA expression correlates with susceptibility of monocytes/macrophages to HIV-1 infection. *Blood* **113**, 671-674 (2009).
59. Huang, J.*, et al.* Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. *Nature medicine* **13**, 1241 (2007).
60. Sung, T.-L. & Rice, A.P. miR-198 inhibits HIV-1 gene expression and replication in monocytes and its mechanism of action appears to involve repression of cyclin T1. *PLoS pathogens* **5**, e1000263 (2009).
61. Ahluwalia, J.K.*, et al.* Human cellular microRNA hsa-miR-29a interferes with viral nef protein expression and HIV-1 replication. *Retrovirology* **5**, 117 (2008).
62. Triboulet, R.*, et al.* Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* **315**, 1579-1582 (2007).
63. Janssen, H.L.*, et al.* a Randomized, Double-blind, Placebo (plb) Controlled Safety And Anti-viral Proof Of Concept Study Of Miravirsen (mir), An Oligonucleotide Targeting Mir-122, In Treatment Naïve Patients With Genotype 1 (gt1) Chronic Hcv Infection: lb-6. *Hepatology* **54**, 1430A (2011).
64. Fischl , M.A.*, et al.* The Efficacy of Azidothymidine (AZT) in the Treatment of Patients with AIDS and AIDS-Related Complex. *New England Journal of Medicine* **317**, 185-191 (1987).
65. Wei, X.*, et al.* Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**, 117 (1995).
66. Preston, B.D., Poiesz, B.J. & Loeb, L.A. Fidelity of HIV-1 reverse transcriptase. *Science* **242**, 1168-1171 (1988).
67. Meyerhans, A.*, et al.* Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. *Cell* **58**, 901-910 (1989).
68. Coffin, J.M. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. *Science* **267**, 483-489 (1995).
69. Erice, A.*, et al.* Primary infection with zidovudine-resistant human immunodeficiency virus type 1. *New England Journal of Medicine* **328**, 1163-1165 (1993).
70. Little, S.J.*, et al.* Antiretroviral-drug resistance among patients recently infected with HIV. *New England Journal of Medicine* **347**, 385-394 (2002).
71. Lederman, M.M.*, et al.* Immunologic responses associated with 12 weeks of combination antiretroviral therapy consisting of zidovudine, lamivudine, and ritonavir: results of AIDS Clinical Trials Group Protocol 315. *The Journal of infectious diseases* **178**, 70-79 (1998).
72. Palella , F.J.J.*, et al.* Declining Morbidity and Mortality among Patients with Advanced Human Immunodeficiency Virus Infection. *New England Journal of Medicine* **338**, 853-860 (1998).
73. Matthews, T.*, et al.* Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. *Nature reviews Drug discovery* **3**, 215-225 (2004).

74. Eron, J.J.*, et al.* Treatment with lamivudine, zidovudine, or both in HIV-positive patients with 200 to 500 CD4+ cells per cubic millimeter. *New England Journal of Medicine* **333**, 1662-1669 (1995).
75. Staszewski , S.*, et al.* Efavirenz plus Zidovudine and Lamivudine, Efavirenz plus Indinavir, and Indinavir plus Zidovudine and Lamivudine in the Treatment of HIV-1 Infection in Adults. *New England Journal of Medicine* **341**, 1865-1873 (1999).
76. Walmsley, S.L.*, et al.* Dolutegravir plus abacavir–lamivudine for the treatment of HIV-1 infection. *New England Journal of Medicine* **369**, 1807-1818 (2013).
77. Clotet, B.*, et al.* Efficacy and safety of darunavir-ritonavir at week 48 in treatment-experienced patients with HIV-1 infection in POWER 1 and 2: a pooled subgroup analysis of data from two randomised trials. *The Lancet* **369**, 1169-1178 (2007).
78. Pilcher, C.D., Miller, W.C., Beatty, Z.A. & Eron, J.J. Detectable HIV-1 RNA at levels below quantifiable limits by Amplicor HIV-1 Monitor is associated with virologic relapse on antiretroviral therapy. *AIDS (London, England)* **13**, 1337-1342 (1999).
79. Hunt, P.W.*, et al.* Continued CD4 cell count increases in HIV-infected adults experiencing 4 years of viral suppression on antiretroviral therapy. *AIDS (London, England)* **17**, 1907-1915 (2003).
80. Bhargava, M., Cajas, J.M., Wainberg, M.A., Klein, M.B. & Pant Pai, N. Do HIV-1 non-B subtypes differentially impact resistance mutations and clinical disease progression in treated populations? Evidence from a systematic review. *Journal of the International AIDS Society* **17**, 18944 (2014).
81. Haggblom, A., Svedhem, V., Singh, K., Sonnerborg, A. & Neogi, U. Virological failure in patients with HIV-1 subtype C receiving antiretroviral therapy: an analysis of a prospective national cohort in Sweden. *The lancet. HIV* **3**, e166-174 (2016).
82. Estes, J.D.*, et al.* Defining total-body AIDS-virus burden with implications for curative strategies. *Nature medicine* (2017).
83. Fletcher, C.V.*, et al.* Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues. *Proceedings of the National Academy of Sciences* **111**, 2307-2312 (2014).
84. Lorenzo-Redondo, R.*, et al.* Persistent HIV-1 replication maintains the tissue reservoir during therapy. *Nature* **530**, 51-56 (2016).
85. Hemelaar, J., Gouws, E., Ghys, P.D. & Osmanov, S. Global trends in molecular epidemiology of HIV-1 during 2000–2007. *AIDS (London, England)* **25**, 679 (2011).
86. Brenner, B.G. Resistance and viral subtypes: how important are the differences and why do they occur? *Current Opinion in HIV and AIDS* **2**, 94-102 (2007).
87. Eshleman, S.H.*, et al.* Nevirapine (NVP) resistance in women with HIV-1 subtype C, compared with subtypes A and D, after the administration of single-dose NVP. *The Journal of infectious diseases* **192**, 30-36 (2005).
88. Eshleman, S.H.*, et al.* Characterization of Nevirapine Resistance Mutations in Women With Subtype A Vs. D HIV-1 6–8 Weeks After Single-Dose Nevirapine (HIVNET 012). *JAIDS Journal of Acquired Immune Deficiency Syndromes* **35**, 126-130 (2004).
89. Sethi, A.K., Celentano, D.D., Gange, S.J., Moore, R.D. & Gallant, J.E. Association between adherence to antiretroviral therapy and human immunodeficiency virus drug resistance. *Clinical Infectious Diseases* **37**, 1112-1118 (2003).

90. Walsh, J.C., Pozniak, A.L., Nelson, M.R., Mandalia, S. & Gazzard, B.G. Virologic rebound on HAART in the context of low treatment adherence is associated with a low prevalence of antiretroviral drug resistance. *Journal of acquired immune deficiency syndromes (1999)* **30**, 278-287 (2002).
91. Leigh Brown, A.J*., et al.* Transmission fitness of drug-resistant human immunodeficiency virus and the prevalence of resistance in the antiretroviral-treated population. *The Journal of infectious diseases* **187**, 683-686 (2003).
92. HIV-Causal-Collaboration. The effect of combined antiretroviral therapy on the overall mortality of HIV-infected individuals. *AIDS (London, England)* **24**, 123 (2010).
93. Narayan, K.V*., et al.* HIV and noncommunicable disease comorbidities in the era of antiretroviral therapy: a vital agenda for research in low-and middle-income country settings. *JAIDS Journal of Acquired Immune Deficiency Syndromes* **67**, S2-S7 (2014).
94. Melkova, Z., Shankaran, P., Madlenakova, M. & Bodor, J. Current views on HIV-1 latency, persistence, and cure. *Folia microbiologica* (2016).
95. von Wyl, V*., et al.* Early Antiretroviral Therapy During Primary HIV-1 Infection Results in a Transient Reduction of the Viral Setpoint upon Treatment Interruption. *PLoS One* **6**(2011).
96. Chen, R.Y*., et al.* Distribution of health care expenditures for HIV-infected patients. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **42**, 1003-1010 (2006).
97. Ammassari, A*., et al.* Self-reported symptoms and medication side effects influence adherence to highly active antiretroviral therapy in persons with HIV infection. *JAIDS Journal of Acquired Immune Deficiency Syndromes* **28**, 445-449 (2001).
98. Mukherjee, J.S., Ivers, L., Leandre, F., Farmer, P. & Behforouz, H. Antiretroviral therapy in resource-poor settings: decreasing barriers to access and promoting adherence. *JAIDS Journal of Acquired Immune Deficiency Syndromes* **43**, S123-S126 (2006).
99. Boden, D*., et al.* HIV-1 drug resistance in newly infected individuals. *Jama* **282**, 1135-1141 (1999).
100. Hütter , G*., et al.* Long-Term Control of HIV by CCR5 Delta32/Delta32 Stem-Cell Transplantation. *New England Journal of Medicine* **360**, 692-698 (2009).
101. Allers, K*., et al.* Evidence for the cure of HIV infection by CCR5Delta32/Delta32 stem cell transplantation. *Blood* **117**, 2791-2799 (2011).
102. Persaud, D*., et al.* Absence of detectable HIV-1 viremia after treatment cessation in an infant. *The New England journal of medicine* **369**, 1828-1835 (2013).
103. Luzuriaga, K*., et al.* Viremic Relapse Following Prolonged Antiretroviral-Free HIV-1 Remission in a Perinatally Infected Child. *The New England journal of medicine* **372**, 786-788 (2015).
104. Sáez-Cirión, A*., et al.* Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. *PLoS pathogens* **9**, e1003211 (2013).
105. Frange, P*., et al.* HIV-1 virological remission lasting more than 12 years after interruption of early antiretroviral therapy in a perinatally infected teenager enrolled in the French ANRS EPF-CO10 paediatric cohort: a case report. *The Lancet HIV* **3**, e49-e54 (2016).

106. Mousseau, G. & Valente, S.T. Didehydro-Cortistatin A: a new player in HIV-therapy? *Expert review of anti-infective therapy* **14**, 145-148 (2016).
107. Kessing, C.F.*, et al.* In vivo suppression of HIV rebound by didehydro-cortistatin A, a "Block-and-Lock" strategy for HIV-1 treatment. *Cell reports* **21**, 600-611 (2017).
108. Qin, X.-F., An, D.S., Chen, I.S. & Baltimore, D. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proceedings of the National Academy of Sciences* **100**, 183-188 (2003).
109. Feng, Y.*, et al.* Inhibition of CCR5-dependent HIV-1 infection by hairpin ribozyme gene therapy against CC-chemokine receptor 5. *Virology* **276**, 271-278 (2000).
110. Tebas, P.*, et al.* Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *New England Journal of Medicine* **370**, 901-910 (2014).
111. Kohn, D.B.*, et al.* A clinical trial of retroviral-mediated transfer of a rev-responsive element decoy gene into CD34(+) cells from the bone marrow of human immunodeficiency virus-1-infected children. *Blood* **94**, 368-371 (1999).
112. DiGiusto, D.L.*, et al.* RNA-based gene therapy for HIV with lentiviral vector-modified CD34(+) cells in patients undergoing transplantation for AIDS-related lymphoma. *Science translational medicine* **2**, 36ra43 (2010).
113. Scarborough, R.J.*, et al.* A Conserved Target Site in HIV-1 Gag RNA is Accessible to Inhibition by Both an HDV Ribozyme and a Short Hairpin RNA. *Molecular therapy. Nucleic acids* **3**, e178 (2014).
114. Scarborough, R.J., Adams, K.L., Daher, A. & Gatignol, A. Effective inhibition of HIV-1 production by short hairpin RNAs and small interfering RNAs targeting a highly conserved site in HIV-1 Gag RNA is optimized by evaluating alternative length formats. *Antimicrobial agents and chemotherapy* **59**, 5297-5305 (2015).
115. Judge, A.D.*, et al.* Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nature biotechnology* **23**, 457 (2005).
116. Mizrahy, S., Hazan-Halevy, I., Dammes, N., Landesman-Milo, D. & Peer, D. Current Progress in Non-viral RNAi-Based Delivery Strategies to Lymphocytes. *Molecular therapy : the journal of the American Society of Gene Therapy* **25**, 1491-1500 (2017).
117. Pierson, T., McArthur, J. & Siliciano, R.F. Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. *Annual review of immunology* **18**, 665-708 (2000).
118. Savarino, A.*, et al.* " Shock and kill" effects of class I-selective histone deacetylase inhibitors in combination with the glutathione synthesis inhibitor buthionine sulfoximine in cell line models for HIV-1 quiescence. *Retrovirology* **6**, 1 (2009).
119. Campbell, J.H., Hearps, A.C., Martin, G.E., Williams, K.C. & Crowe, S.M. The importance of monocytes and macrophages in HIV pathogenesis, treatment, and cure. *AIDS (London, England)* **28**, 2175-2187 (2014).
120. Lassen, K., Han, Y., Zhou, Y., Siliciano, J. & Siliciano, R.F. The multifactorial nature of HIV-1 latency. *Trends in molecular medicine* **10**, 525-531 (2004).
121. Xu, W., Parmigiani, R. & Marks, P. Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene* **26**, 5541-5552 (2007).
122. Marks, P.A. & Breslow, R. Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. *Nature biotechnology* **25**, 84-90 (2007).

123. Atadja, P. Development of the pan-DAC inhibitor panobinostat (LBH589): successes and challenges. *Cancer letters* **280**, 233-241 (2009).
124. Piekarz, R.L*., et al.* Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous T-cell lymphoma. *Journal of clinical oncology* **27**, 5410 (2009).
125. VanderMolen, K.M., McCulloch, W., Pearce, C.J. & Oberlies, N.H. Romidepsin (Istodax, NSC 630176, FR901228, FK228, depsipeptide): a natural product recently approved for cutaneous T-cell lymphoma. *The Journal of antibiotics* **64**, 525 (2011).
126. Duvic, M. & Vu, J. Vorinostat: a new oral histone deacetylase inhibitor approved for cutaneous T-cell lymphoma. *Expert opinion on investigational drugs* **16**, 1111-1120 (2007).
127. Laubach, J.P., Moreau, P., San-Miguel, J.F. & Richardson, P.G. Panobinostat for the treatment of multiple myeloma. *Clinical cancer research*, clincanres. 0530.2015 (2015).
128. Gray, L.R*., et al.* Toxicity and in vitro activity of HIV-1 latency-reversing agents in primary CNS cells. *Journal of neurovirology* **22**, 455-463 (2016).
129. Archin, N.M*., et al.* Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. *Nature* **487**, 482-485 (2012).
130. Bernhard, W*., et al.* The Suv39H1 methyltransferase inhibitor chaetocin causes induction of integrated HIV-1 without producing a T cell response. *FEBS letters* **585**, 3549-3554 (2011).
131. Peterlin, B.M. & Price, D.H. Controlling the elongation phase of transcription with P-TEFb. *Molecular cell* **23**, 297-305 (2006).
132. Bisgrove, D.A., Mahmoudi, T., Henklein, P. & Verdin, E. Conserved P-TEFb-interacting domain of BRD4 inhibits HIV transcription. *Proceedings of the National Academy of Sciences* **104**, 13690-13695 (2007).
133. Contreras, X., Barboric, M., Lenasi, T. & Peterlin, B.M. HMBA releases P-TEFb from HEXIM1 and 7SK snRNA via PI3K/Akt and activates HIV transcription. *PLoS pathogens* **3**, e146 (2007).
134. Doyon, G., Zerbato, J., Mellors, J.W. & Sluis-Cremer, N. Disulfiram reactivates latent HIV-1 expression through depletion of the phosphatase and tensin homolog. *AIDS (London, England)* **27**, F7-F11 (2013).
135. Ghosh, S. & Baltimore, D. Activation In Vitro of NF-(kappa) B by Phosphorylation of Its Inhibitor I (kappa) B. *Nature* **344**, 678 (1990).
136. Williams, S.A*., et al.* Prostratin antagonizes HIV latency by activating NF-kappaB. *Journal of Biological Chemistry* (2004).
137. Mehla, R*., et al.* Bryostatin modulates latent HIV-1 infection via PKC and AMPK signaling but inhibits acute infection in a receptor independent manner. *PloS one* **5**, e11160 (2010).
138. Jiang, G*., et al.* Reactivation of HIV latency by a newly modified Ingenol derivative via protein kinase Cδ–NF-κB signaling. *AIDS (London, England)* **28**, 1555 (2014).
139. Bednarik, D.P., Cook, J. & Pitha, P. Inactivation of the HIV LTR by DNA CpG methylation: evidence for a role in latency. *The EMBO journal* **9**, 1157 (1990).

140. Sung, T.-L. & Rice, A.P. miR-198 inhibits HIV-1 gene expression and replication in monocytes and its mechanism of action appears to involve repression of cyclin T1. *PLoS Pathog* **5**, e1000263 (2009).
141. Adoro, S.*, et al.* IL-21 induces antiviral microRNA-29 in CD4 T cells to limit HIV-1 infection. *Nature communications* **6**(2015).
142. Swaminathan, S., Murray, D.D. & Kelleher, A.D. The role of microRNAs in HIV-1 pathogenesis and therapy. *AIDS (London, England)* **26**, 1325-1334 (2012).
143. Yang, H.-C.*, et al.* Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. *The Journal of clinical investigation* **119**, 3473-3486 (2009).
144. Pace, M.J.*, et al.* Directly infected resting CD4+ T cells can produce HIV Gag without spreading infection in a model of HIV latency. *PLoS pathogens* **8**, e1002818 (2012).
145. Saleh, S.*, et al.* Expression and reactivation of HIV in a chemokine induced model of HIV latency in primary resting CD4+ T cells. *Retrovirology* **8**, 80 (2011).
146. Jordan, A., Bisgrove, D. & Verdin, E. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. *The EMBO journal* **22**, 1868-1877 (2003).
147. Clouse, K.A.*, et al.* Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. *The Journal of Immunology* **142**, 431-438 (1989).
148. Folks, T.M., Justement, J., Kinter, A., Dinarello, C.A. & Fauci, A.S. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science* **238**, 800-802 (1987).
149. Das, A.T., Verhoef, K. & Berkhout, B. A conditionally replicating virus as a novel approach toward an HIV vaccine. in *Methods in enzymology*, Vol. 388 359-379 (Elsevier, 2004).
150. Rance E, C., A, McCullogh C, Niu M, Rao S, Bell B, Mouland AJ, Cochrane AW, Gatignol A. A GFP HIV-1 Tat-independent latency model in monocytes, monocytes-derived macrophages and lymphocytic cell lines.
151. Dick, G., Kitchen, S. & Haddow, A. Zika virus (I). Isolations and serological specificity. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **46**, 509-520 (1952).
152. Dick, G.W. Epidemiological notes on some viruses isolated in Uganda; Yellow fever, Rift Valley fever, Bwamba fever, West Nile, Mengo, Semliki forest, Bunyamwera, Ntaya, Uganda S and Zika viruses. *Trans R Soc Trop Med Hyg* **47**, 13-48 (1953).
153. Macnamara, F. Zika virus: a report on three cases of human infection during an epidemic of jaundice in Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **48**, 139-145 (1954).
154. Weinbren, M. & Williams, M. Zika virus: further isolations in the Zika area, and some studies on the strains isolated. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **52**, 263-268 (1958).
155. Haddow, A., Williams, M., Woodall, J., Simpson, D. & Goma, L. Twelve isolations of Zika virus from Aedes (Stegomyia) africanus (Theobald) taken in and above a Uganda forest. *Bulletin of the World Health Organization* **31**, 57 (1964).
156. Marchette, N., Garcia, R. & Rudnick, A. Isolation of Zika virus from Aedes aegypti mosquitoes in Malaysia. *The American journal of tropical medicine and hygiene* **18**, 411-415 (1969).

157. Duffy, M.R.*, et al.* Zika Virus Outbreak on Yap Island, Federated States of Micronesia. *New England Journal of Medicine* **360**, 2536-2543 (2009).
158. Musso, D.*, et al.* Detection of Zika virus in saliva. *Journal of Clinical Virology* **68**, 53-55 (2015).
159. Cao-Lormeau, V.M.*, et al.* Zika virus, French polynesia, South pacific, 2013. *Emerging infectious diseases* **20**, 1085-1086 (2014).
160. Aubry, M.*, et al.* Serosurvey of dengue, Zika and other mosquito-borne viruses in French Polynesia. in *AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE*, Vol. 93 231-232 (AMER SOC TROP MED & HYGIENE 8000 WESTPARK DR, STE 130, MCLEAN, VA 22101 USA, 2015).
161. Musso, D., Nilles, E. & Cao-Lormeau, V.M. Rapid spread of emerging Zika virus in the Pacific area. *Clinical Microbiology and Infection* **20**(2014).
162. Zanluca, C.*, et al.* First report of autochthonous transmission of Zika virus in Brazil. *Memórias do Instituto Oswaldo Cruz* **110**, 569-572 (2015).
163. Hennessey, M., Fischer, M. & Staples, J.E. Zika virus spreads to new areas—region of the Americas, May 2015–January 2016. *American Journal of Transplantation* **16**, 1031-1034 (2016).
164. Zika suspected and confirmed cases reported by countries and territories in the Americas Cumulative cases, 2015-2017. (Pan American Health Organization / World Health Organization).
165. Gubler, D.J. The global emergence/resurgence of arboviral diseases as public health problems. *Archives of medical research* **33**, 330-342 (2002).
166. Kenney, J.L. & Brault, A.C. The role of environmental, virological and vector interactions in dictating biological transmission of arthropod-borne viruses by mosquitoes. *Advances in virus research* **89**, 39-83 (2014).
167. Rajah, M.M., Pardy, R.D., Condotta, S.A., Richer, M.J. & Sagan, S.M. Zika virus: emergence, phylogenetics, challenges, and opportunities. *ACS infectious diseases* **2**, 763-772 (2016).
168. Lanciotti, R.S.*, et al.* Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerging infectious diseases* **14**, 1232 (2008).
169. Lanciotti, R.S., Lambert, A.J., Holodniy, M., Saavedra, S. & Signor, L.d.C.C. Phylogeny of Zika virus in western hemisphere, 2015. *Emerging infectious diseases* **22**, 933 (2016).
170. Grard, G.*, et al.* Zika virus in Gabon (Central Africa)–2007: a new threat from Aedes albopictus? *PLoS neglected tropical diseases* **8**, e2681 (2014).
171. Campos, G.S., Bandeira, A.C. & Sardi, S.I. Zika virus outbreak, bahia, brazil. *Emerging infectious diseases* **21**, 1885 (2015).
172. Musso, D., Nilles, E.J. & Cao-Lormeau, V.M. Rapid spread of emerging Zika virus in the Pacific area. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **20**, O595-596 (2014).
173. Hughes, R.A. & Cornblath, D.R. Guillain-barre syndrome. *The Lancet* **366**, 1653-1666 (2005).
174. Oehler, E.*, et al.* Zika virus infection complicated by Guillain-Barré syndrome – case report, French Polynesia, December 2013. *Eurosurveillance* **19**, 20720 (2014).

175. Musso, D., Nilles, E. & Cao-Lormeau, V.M. Rapid spread of emerging Z ika virus in the P acific area. *Clinical Microbiology and Infection* **20**, O595-O596 (2014).
176. Zika virus epidemic in the Americas: potential association with microcephaly and Guillain-Barré syndrome. (European Center for Disease Prevention and Control, 2016).
177. Epidemiological update. Neurological syndrome, congenital anomalies, and Zika virus infection. (Pan American Health Organization., 2016).
178. Faria, N.R.*, et al.* Zika virus in the Americas: Early epidemiological and genetic findings. *Science* (2016).
179. Calvet, G.*, et al.* Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. *The Lancet Infectious Diseases* **16**, 653-660 (2016).
180. Cordeiro, M.T., Pena, L.J., Brito, C.A., Gil, L.H. & Marques, E.T. Positive IgM for Zika virus in the cerebrospinal fluid of 30 neonates with microcephaly in Brazil. *The Lancet* **387**, 1811-1812 (2016).
181. Rapid risk assessment: Microcephaly in Brazil potentially linked to the Zika virus epidemic. (European Centre for Disease Prevention and Control, 2015).
182. Kuhn, R.J.*, et al.* Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* **108**, 717-725 (2002).
183. Wang, S., He, R. & Anderson, R. PrM- and cell-binding domains of the dengue virus E protein. *Journal of virology* **73**, 2547-2551 (1999).
184. Yuan, L.*, et al.* A single mutation in the prM protein of Zika virus contributes to fetal microcephaly. *Science* **358**, 933-936 (2017).
185. Chung, K.M.*, et al.* West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 19111-19116 (2006).
186. Crook, K.R., Miller-Kittrell, M., Morrison, C.R. & Scholle, F. Modulation of innate immune signaling by the secreted form of the West Nile virus NS1 glycoprotein. *Virology* **458-459**, 172-182 (2014).
187. Youn, S., Ambrose, R.L., Mackenzie, J.M. & Diamond, M.S. Non-structural protein-1 is required for West Nile virus replication complex formation and viral RNA synthesis. *Virology journal* **10**, 339 (2013).
188. Xie, X., Zou, J., Puttikhunt, C., Yuan, Z. & Shi, P.Y. Two distinct sets of NS2A molecules are responsible for dengue virus RNA synthesis and virion assembly. *Journal of virology* **89**, 1298-1313 (2015).
189. Yoon, K.J.*, et al.* Zika-Virus-Encoded NS2A Disrupts Mammalian Cortical Neurogenesis by Degrading Adherens Junction Proteins. *Cell stem cell* **21**, 349-358.e346 (2017).
190. Yusof, R., Clum, S., Wetzel, M., Murthy, H.K. & Padmanabhan, R. Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids in vitro. *Journal of Biological Chemistry* **275**, 9963-9969 (2000).
191. Cahour, A., Falgout, B. & Lai, C. Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junctions is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. *Journal of virology* **66**, 1535-1542 (1992).

192. Yamshchikov, V.F. & Compans, R.W. Processing of the intracellular form of the west Nile virus capsid protein by the viral NS2B-NS3 protease: an in vitro study. *Journal of virology* **68**, 5765-5771 (1994).
193. Amberg, S.M., Nestorowicz, A., McCourt, D.W. & Rice, C.M. NS2B-3 proteinase-mediated processing in the yellow fever virus structural region: in vitro and in vivo studies. *Journal of virology* **68**, 3794-3802 (1994).
194. Gorbalenya, A.E., Koonin, E.V., Donchenko, A.P. & Blinov, V.M. Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic acids research* **17**, 4713-4730 (1989).
195. Matusan, A.E., Pryor, M.J., Davidson, A.D. & Wright, P.J. Mutagenesis of the Dengue virus type 2 NS3 protein within and outside helicase motifs: effects on enzyme activity and virus replication. *Journal of virology* **75**, 9633-9643 (2001).
196. Munoz-Jordan, J.L*., et al.* Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. *Journal of virology* **79**, 8004-8013 (2005).
197. Issur, M*., et al.* The flavivirus NS5 protein is a true RNA guanylyltransferase that catalyzes a two-step reaction to form the RNA cap structure. *RNA (New York, N.Y.)* **15**, 2340-2350 (2009).
198. Laurent-Rolle, M*., et al.* The NS5 protein of the virulent West Nile virus NY99 strain is a potent antagonist of type I interferon-mediated JAK-STAT signaling. *Journal of virology* **84**, 3503-3515 (2010).
199. Calvez, E*., et al.* Genetic diversity and phylogeny of Aedes aegypti, the main arbovirus vector in the Pacific. *PLoS neglected tropical diseases* **10**, e0004374 (2016).
200. Boorman, J. & Porterfield, J. A simple technique for infection of mosquitoes with viruses transmission of Zika virus. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **50**, 238-242 (1956).
201. Wong, P.-S.J., Li, M.-z.I., Chong, C.-S., Ng, L.-C. & Tan, C.-H. Aedes (Stegomyia) albopictus (Skuse): a potential vector of Zika virus in Singapore. *PLoS neglected tropical diseases* **7**, e2348 (2013).
202. Basu, R. & Tumban, E. Zika Virus on a Spreading Spree: what we now know that was unknown in the 1950's. *Virology journal* **13**, 165 (2016).
203. Musso, D*., et al.* Potential sexual transmission of Zika virus. *Emerging infectious diseases* **21**, 359 (2015).
204. Motta, I.J*., et al.* Evidence for transmission of Zika virus by platelet transfusion. *New England Journal of Medicine* **375**, 1101-1103 (2016).
205. Hamel, R*., et al.* Biology of Zika virus infection in human skin cells. *Journal of virology* **89**, 8880-8896 (2015).
206. Suthar, M.S., Diamond, M.S. & Gale Jr, M. West Nile virus infection and immunity. *Nature Reviews Microbiology* **11**, 115 (2013).
207. Heinz, F.X. & Allison, S.L. Flavivirus structure and membrane fusion. *Advances in virus research* **59**, 63-99 (2003).
208. Lindenbach, B.D. & Rice, C.M. Unravelling hepatitis C virus replication from genome to function. *Nature* **436**, 933 (2005).

209. Screaton, G., Mongkolsapaya, J., Yacoub, S. & Roberts, C. New insights into the immunopathology and control of dengue virus infection. *Nature Reviews Immunology* **15**, 745 (2015).
210. Barrett, A.D. Zika vaccine candidates progress through nonclinical development and enter clinical trials. *NPJ vaccines* **1**, 16023 (2016).
211. Abbink, P.*, et al.* Protective efficacy of multiple vaccine platforms against Zika virus challenge in rhesus monkeys. *Science*, aah6157 (2016).
212. Dowd, K.A.*, et al.* Rapid development of a DNA vaccine for Zika virus. *Science*, aai9137 (2016).
213. Harris, A.F.*, et al.* Field performance of engineered male mosquitoes. *Nature biotechnology* **29**, 1034 (2011).
214. Carvalho, D.O.*, et al.* Suppression of a field population of Aedes aegypti in Brazil by sustained release of transgenic male mosquitoes. *PLoS neglected tropical diseases* **9**, e0003864 (2015).
215. Hammond, A.*, et al.* A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. *Nature biotechnology* **34**, 78 (2016).
216. Pugh, J. Driven to extinction? The ethics of eradicating mosquitoes with gene-drive technologies. *Journal of medical ethics*, medethics-2016-103462 (2016).
217. Wightman, B., Ha, I. & Ruvkun, G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell* **75**, 855-862 (1993).
218. Fire, A.*, et al.* Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806 (1998).
219. Zhu, L.*, et al.* Understanding the core of RNA interference: The dynamic aspects of Argonaute-mediated processes. *Progress in biophysics and molecular biology* (2016).
220. Sayed, D. & Abdellatif, M. MicroRNAs in development and disease. *Physiological reviews* **91**, 827-887 (2011).
221. Yi, R., Qin, Y., Macara, I.G. & Cullen, B.R. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & development* **17**, 3011-3016 (2003).
222. Daniels, S.M. & Gatignol, A. The multiple functions of TRBP, at the hub of cell responses to viruses, stress, and cancer. *Microbiology and molecular biology reviews : MMBR* **76**, 652-666 (2012).
223. Hammond, S.M. Dicing and slicing: the core machinery of the RNA interference pathway. *FEBS letters* **579**, 5822-5829 (2005).
224. Sevignani, C., Calin, G.A., Siracusa, L.D. & Croce, C.M. Mammalian microRNAs: a small world for fine-tuning gene expression. *Mammalian Genome* **17**, 189-202 (2006).
225. Lewis, B.P., Shih, I.-h., Jones-Rhoades, M.W., Bartel, D.P. & Burge, C.B. Prediction of mammalian microRNA targets. *Cell* **115**, 787-798 (2003).
226. Pillai, R.S.*, et al.* Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* **309**, 1573-1576 (2005).
227. Giraldez, A.J.*, et al.* Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *science* **312**, 75-79 (2006).
228. Fabian, M.R., Sonenberg, N. & Filipowicz, W. Regulation of mRNA translation and stability by microRNAs. *Annual review of biochemistry* **79**, 351-379 (2010).

229. Friedman, R.C., Farh, K.K.-H., Burge, C.B. & Bartel, D.P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome research* **19**, 92-105 (2009).
230. Daniels, S.M. & Gatignol, A. The multiple functions of TRBP, at the hub of cell responses to viruses, stress, and cancer. *Microbiology and Molecular Biology Reviews* **76**, 652-666 (2012).
231. Carthew, R.W. & Sontheimer, E.J. Origins and mechanisms of miRNAs and siRNAs. *Cell* **136**, 642-655 (2009).
232. Zhao, M.*, et al.* Lipofectamine RNAiMAX: an efficient siRNA transfection reagent in human embryonic stem cells. *Molecular biotechnology* **40**, 19-26 (2008).
233. Whitehead, K.A., Langer, R. & Anderson, D.G. Knocking down barriers: advances in siRNA delivery. *Nature reviews Drug discovery* **8**, 129 (2009).
234. Zamore, P.D., Tuschl, T., Sharp, P.A. & Bartel, D.P. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25-33 (2000).
235. Reynoso, R.*, et al.* MicroRNAs differentially present in the plasma of HIV elite controllers reduce HIV infection in vitro. *Scientific reports* **4**(2014).
236. Neff, C.P.*, et al.* An aptamer-siRNA chimera suppresses HIV-1 viral loads and protects from helper CD4(+) T cell decline in humanized mice. *Science translational medicine* **3**, 66ra66 (2011).
237. Palliser, D.*, et al.* An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature* **439**, 89-94 (2006).
238. Li, B.-j.*, et al.* Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nature medicine* **11**, 944 (2005).
239. Ge, Q.*, et al.* Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 8676-8681 (2004).
240. Zhang, W.*, et al.* Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. *Nature medicine* **11**, 56 (2005).
241. DeVincenzo, J.*, et al.* A randomized, double-blind, placebo-controlled study of an RNAi-based therapy directed against respiratory syncytial virus. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 8800-8805 (2010).
242. Thi, E.P.*, et al.* Lipid nanoparticle siRNA treatment of Ebola-virus-Makona-infected nonhuman primates. *Nature* **521**, 362 (2015).
243. Zhang, W.*, et al.* Attenuation of dengue virus infection by adeno-associated virus-mediated siRNA delivery. *Genetic vaccines and therapy* **2**, 8 (2004).
244. Anantpadma, M. & Vrati, S. siRNA-mediated suppression of Japanese encephalitis virus replication in cultured cells and mice. *Journal of antimicrobial chemotherapy* **67**, 444-451 (2011).
245. Kumar, P., Lee, S.K., Shankar, P. & Manjunath, N. A single siRNA suppresses fatal encephalitis induced by two different flaviviruses. *PLoS medicine* **3**, e96 (2006).
246. Anderson, J. & Akkina, R. HIV-1 resistance conferred by siRNA cosuppression of CXCR4 and CCR5 coreceptors by a bispecific lentiviral vector. *AIDS research and therapy* **2**, 1 (2005).

247. DiGiusto, D.L.*, et al.* Development of Hematopoietic Stem Cell Based Gene Therapy for HIV-1 Infection: Considerations for Proof of Concept Studies and Translation to Standard Medical Practice. *Viruses* **5**, 2898-2919 (2013).
248. Al-Hashimi, H.M. Structural biology: Aerial view of the HIV genome. *Nature* **460**, 696 (2009).
249. Scarborough, R.J.*, et al.* A conserved target site in HIV-1 Gag RNA is accessible to inhibition by both an HDV ribozyme and a short hairpin RNA. *Molecular Therapy-Nucleic Acids* **3**(2014).
250. Kim, D.H. & Rossi, J.J. Strategies for silencing human disease using RNA interference. *Nature Reviews Genetics* **8**, 173 (2007).
251. Robbins, M., Judge, A. & MacLachlan, I. siRNA and innate immunity. *Oligonucleotides* **19**, 89-102 (2009).
252. Keene, K.M.*, et al.* RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; Togaviridae) infection of Anopheles gambiae. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 17240-17245 (2004).
253. Kemp, C. & Imler, J.-L. Antiviral immunity in drosophila. *Current opinion in immunology* **21**, 3-9 (2009).
254. Franz, A.W.*, et al.* Fitness impact and stability of a transgene conferring resistance to dengue-2 virus following introgression into a genetically diverse Aedes aegypti strain. *PLoS Negl Trop Dis* **8**, e2833 (2014).
255. Slater, T., Sawyer, B. & Sträuli, U. Studies on succinate-tetrazolium reductase systems: III. Points of coupling of four different tetrazolium salts III. Points of coupling of four different tetrazolium salts. *Biochimica et biophysica acta* **77**, 383-393 (1963).
256. Fonseca, K.*, et al.* First case of Zika virus infection in a returning Canadian traveler. *Am J Trop Med Hyg* **91**, 1035-1038 (2014).
257. Costa, V.V.*, et al.* N-Methyl-d-Aspartate (NMDA) Receptor Blockade Prevents Neuronal Death Induced by Zika Virus Infection. *mBio* **8**(2017).
258. Hashem, M. & Shuvo, M.A. A Computational Approach to Design Potential Antiviral RNA for 3'UTR Post Transcriptional Gene Silencing of Different Strains of Zika Virus. *Journal of Young Pharmacists* **9**(2017).
259. Shawan, M.*, et al.* Design and prediction of potential RNAi (siRNA) molecules for 3'UTR PTGS of different strains of zika virus: a computational approach. *Nat. Sci* **13**, 37-50 (2015).
260. Alpuche-Lazcano, S.P.*, et al.* Higher Cytopathic Effects of a Zika Virus Brazilian Isolate from Bahia Compared to a Canadian-Imported Thai Strain. *Viruses* **10**, 53 (2018).
261. Chun, T.-W., Moir, S. & Fauci, A.S. HIV reservoirs as obstacles and opportunities for an HIV cure. *Nature immunology* **16**, 584 (2015).
262. Das, A.T., Verhoef, K. & Berkhout, B. A conditionally replicating virus as a novel approach toward an HIV vaccine. *Methods in enzymology* **388**, 359-379 (2004).
263. Ebert, M.S. & Sharp, P.A. MicroRNA sponges: progress and possibilities. *RNA (New York, N.Y.)* (2010).
264. Tong, A. & Nemunaitis, J. Modulation of miRNA activity in human cancer: a new paradigm for cancer gene therapy? *Cancer gene therapy* **15**, 341 (2008).

265. Gandhi, N.S., Tekade, R.K. & Chougule, M.B. Nanocarrier mediated delivery of siRNA/miRNA in combination with chemotherapeutic agents for cancer therapy: current progress and advances. *Journal of Controlled Release* **194**, 238-256 (2014).
266. Liu, Y.*, et al.* Evolutionary enhancement of Zika virus infectivity in Aedes aegypti mosquitoes. *Nature* **545**, 482-486 (2017).
267. Pettersson, J.H.-O.*, et al.* How did Zika virus emerge in the Pacific Islands and Latin America? *mBio* **7**, e01239-01216 (2016).
268. Aubry, M., Richard, V., Green, J., Broult, J. & Musso, D. Inactivation of Zika virus in plasma with amotosalen and ultraviolet A illumination. *Transfusion* **56**, 33-40 (2016).
269. Henchal, E., Gentry, M., McCown, J. & Brandt, W. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. *The American journal of tropical medicine and hygiene* **31**, 830-836 (1982).
270. Haridas, V.*, et al.* Bispidine-amino acid conjugates act as a novel scaffold for the design of antivirals that block Japanese encephalitis virus replication. *PLoS neglected tropical diseases* **7**, e2005 (2013).
271. Crill, W.D. & Chang, G.-J.J. Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. *Journal of virology* **78**, 13975-13986 (2004).
272. Sagar, M., Wu, X., Lee, S. & Overbaugh, J. Human immunodeficiency virus type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over the course of infection, and these modifications affect antibody neutralization sensitivity. *Journal of virology* **80**, 9586-9598 (2006).
273. Franz, A.W.*, et al.* Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified Aedes aegypti. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 4198-4203 (2006).