Investigating the combinatorial use of RNA interference and CRISPR-Cas9 for long-term control of HIV-1 infection

by

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AAV: adeno-associated virus
Ago2: argonaute RISC catalytic component 2
AIDS: acquired immunodeficiency syndrome
AML: acute myeloid leukemia
ANOVA: analysis of variance
APOBEC3G: apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G
ARV: AIDS-associated retrovirus
ATI: analytical treatment interruption
AZT: azidothymidine
CA: capsid
cART: combination antiretroviral therapy
Cas: CRISPR associated
CCR5: C-C chemokine receptor type 5
CD4: cluster of differentiation 4
CDC: Centre for Disease Control
CRF: circulating recombinant forms
CRISPR: clustered regularly interspaced palindromic repeats
CROI: Conference on Retroviruses and Opportunistic Infections
crRNA: CRISPR RNA
CTD: C-terminal domain
CXCR4: C-X-C chemokine receptor type 4
DMEM: Dulbecco's modified eagle medium
dpi: days post infection
DSB: double-stranded break
dsDNA: double-stranded DNA
dsRNA: double-stranded RNA
Env: envelope
GFP: green fluorescence protein
gRNA: guide RNA
GVHD: graft versus host disease
h: hours
HDR: homology directed repair
HIV: human immunodeficiency virus
HLA: human leukocyte antigen
HSC: hematopoietic stem cell
HSCT: hematopoietic stem cell transplant
IAS: International AIDS Society
IC_{50}: half maximal inhibitory concentration
IN: integrase
Indel: insertion or deletion mutation

kb: kilobases
LAV: lymphadenopathy-associated virus
LEDGF: lens epithelium-derived growth factor
LTR: long-terminal repeat
MA: matrix
mCh: mCherry
min: minutes
miRNA: micro RNA
MLV: murine leukemia virus
MMEJ: microhomology-mediated end joining
MSM: men who have sex with men
NC: nucleocapsid
NHEJ: non-homologous end joining
NNRTI: non-nucleoside/nucleotide reverse transcriptase inhibitor
NRTI: nucleoside/nucleotide reverse transcriptase inhibitor
nt: nucleotide
PAM: protospacer adjacent motif
PARN: poly-A specific ribonuclease
PARP1: poly-ADP ribose polymerase 1
PBMC: peripheral blood mononuclear cells
PBS: phosphate buffer saline
PIC: pre-integration complex
PLWH: people living with HIV
Pol: polymerase
PR: protease
RCL: replication competent lentiviruses
Rev: RNA splicing regulator
RNAi: RNA interference
RPMI: Roswell Park Memorial Institute
RT: reverse transcriptase
RTC: reverse transcription complex
s: seconds
SEM: standard error of the mean
sgRNA: single guide RNA
shRNA: short hairpin RNA
siRNA: short interfering RNA
SIV: simian immunodeficiency virus
TALEN: transcription activator-like effector nuclease
TAR: transactivation response element
Tat: transactivator of transcription
tracrRNA: trans-activating RNA
TRBP: TAR RNA binding protein

TRIM5alpha-HRH: human-rhesus macaque tripartite motif chimeric protein
tRNA: transfer RNA
UNAIDS: The Joint United Nations Programme on HIV/AIDS
UTR: untranslated region
V3 loop: 3rd variable loop
VSV-G: vesicular stomatitis virus glycoprotein G
WHO: World Health Organization
WT: wild type
ZFN: zinc finger nuclease
ZFP: zinc finger protein

Abstract

To enter its target cell, the human immunodeficiency virus (HIV-1) must bind to both the host CD4 receptor and either the CCR5 or CXCR4 coreceptor. The virus' ability to integrate into the host genome and the long-lasting life span of their preferential target cells, $CD4^+$ T lymphocytes, allow HIV-1 to become permanently established in tissues as latent reservoirs that are so far impossible to completely eliminate. To cure HIV-1 infection, attention has turned to gene therapies to equip cells with antiviral molecules. Short hairpin (sh)RNAs are synthetic molecules that exploit the RNA interference pathway to target an RNA through complementary sequence recognition. They may be designed to target viral RNA and are promising candidates for HIV-1 gene therapy. In recent years, allogeneic hematopoietic stem cell transplants with naturally CCR5-deficient donor cells have gained traction as another possible method for achieving long-term remission in patients who are also diagnosed with a hematological malignancy. The success of this strategy has so far been demonstrated in five patients; two of these individuals who have been in remission for five and thirteen years are considered cured. With gene editing technologies, it is possible to modify a patient's cells *ex vivo* to recreate the naturally occurring *ccr5* mutation with the objective of increasing the frequency of transplant-based cures. However, these cells remain susceptible to CXCR4-tropic viruses. It may be possible to generate pan-resistant cells by further modifying them with antiviral shRNAs that target conserved regions in the viral genome. To determine whether a dual-pronged approach could be used to generate non-cytotoxic HIV-1 resistant T cells, we identified 23 shRNAs that most effectively target highly conserved regions in the HIV-1 genome. From this panel, we selected three shRNAs that significantly delayed viral replication to test them under three different human type 3 RNA polymerase III promoters, H1, 7SK, and U6. We found that shRNAs expressed from the 7SK and U6 promoters had greater inhibitory activity against HIV-1 production and replication but some of these are cytotoxic. However, the degree of cytotoxicity depended on the shRNA sequence. To test whether antiviral shRNA expression and CCR5 gene editing on T cells can collectively inhibit replication of CXCR4-tropic (X4) and CCR5-tropic (R5) HIV-1, we used CRISPR/Cas9 to knock out *ccr5* in the PM-1 cell line. We identified two monoclonal colonies with insertion or deletion mutations that generate an early stop codon due to a frameshift mutation. Pending validation of the absence of CCR5 surface expression, we may have generated an appropriate cell line to test whether implementing a stepwise blockade

that limits entry of R5 viruses then blocks replication of X4 viruses is a viable approach to make autologous stem cell transplants a reliable strategy for a HIV-1 cure.

Résumé

Pour pénétrer dans sa cellule cible, le virus de l'immunodéficience humaine (VIH-1) doit se lier à la fois au récepteur CD4 de l'hôte et au corécepteur CCR5 ou CXCR4. Grâce à l'identité rétrovirale du virus et à la longue durée de vie des cellules T $CD4^+$, le virus s'établit de façon permanente dans les tissus sous forme de réservoirs latents qu'il est jusqu'à présent impossible d'éliminer complètement. Pour guérir de l'infection par le VIH-1, l'attention s'est portée sur les thérapies géniques afin de doter les cellules de molécules antivirales. Les ARN courts en épingle à cheveux (shARN) sont des molécules synthétiques qui exploitent la voie de l'interférence ARN pour cibler un ARN par reconnaissance de la séquence complémentaire. Ils peuvent être conçus pour cibler l'ARN viral et sont des candidats prometteurs pour la thérapie génique du VIH-1. Ces dernières années, les allogreffes de cellules souches hématopoïétiques avec des cellules de donneurs naturellement déficients en CCR5 ont gagné en popularité comme autre méthode possible pour obtenir une rémission à long terme chez les patients chez qui on a également diagnostiqué une hémopathie maligne. Le succès de cette stratégie a été démontré jusqu'à présent chez cinq patients; deux de ces personnes, en rémission depuis cinq et treize ans, sont considérées comme guéries. Grâce aux technologies d'édition de gènes, il est possible de modifier ex vivo les cellules d'un patient afin de recréer la mutation ccr5 présente dans la nature, l'objectif étant d'augmenter la fréquence des guérisons par transplantation. Cependant, ces cellules restent sensibles aux virus CXCR4-tropiques. Il pourrait être possible de générer des cellules pan-résistantes en les modifiant davantage avec des shARN antiviraux qui ciblent des régions conservées du génome viral. Pour déterminer si une approche à deux volets pouvait être utilisée pour générer des cellules T non cytotoxiques résistantes au VIH-1, nous avons identifié 23 shARN qui ciblent le plus efficacement des régions très conservées du génome du VIH-1. À partir de cet ensemble, nous avons sélectionné trois shARN qui retardaient significativement la réplication virale pour les tester sous trois différents promoteurs humains de l'ARN polymérase III de type 3, H1, 7SK et U6. Nous avons constaté que les shARN exprimés à partir des promoteurs 7SK et U6 avaient une plus grande activité inhibitrice contre la production et la réplication du VIH-1, mais que certains d'entre eux étaient cytotoxiques. Cependant, le degré de cytotoxicité dépendait de la séquence du shARN. Pour vérifier si la combinaison de l'expression de shARN antiviraux et l'édition du gène CCR5 sur les cellules T peuvent inhiber la réplication du VIH-1 CXCR4-tropique (X4) et CCR5-tropique (R5), nous avons utilisé CRISPR/Cas9 pour éliminer ccr5 dans la lignée cellulaire PM-1. Nous

avons identifié deux colonies monoclonales avec des mutations d'insertion ou de délétion qui génèrent un codon stop précoce dû à une mutation causant un décalage de phase de lecture. En attendant la validation de l'absence d'expression de surface de CCR5, nous avons peut-être généré une lignée cellulaire appropriée pour tester si la mise en œuvre d'un blocage par étapes qui limite l'entrée des virus R5 puis bloque la réplication des virus X4 est une approche viable pour faire des greffes de cellules souches autologues une stratégie fiable pour une guérison de l'infection par le VIH-1.

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I embarked on my MSc journey in the Fall of 2020, when the world was still reeling from the shock of a global pandemic and only just gathering itself to steel against future blows from the coronavirus. It is against this backdrop of medical courage and scientific leadership that three years later, I feel unendingly grateful to Dr. Anne Gatignol for giving me the opportunity to study possible solutions for a similarly devastating pandemic. She gave me the freedom to explore this project and provided direction that advanced my work. Without her encouragement and mentorship, I would not have had the opportunities to attend conferences or contribute to papers, experiences that helped me develop scientific communication skills that I will carry beyond my MSc. I would also like to thank Dr. Robert Scarborough for his guidance, support, and patience as I navigated through difficult experiments and the writing of this thesis. His guidance and willingness to answer my questions significantly improved my experience in the lab and the readability of this thesis. Aïcha Daher was the reason I kept my sanity in the lab. Her approachability and endless technical knowledge helped me overcome challenges I faced in the lab, both large and small. I would also like to thank the newly minted Dr. Ryan Goguen for sharing his knowledge and last years of his PhD with me. His helpfulness and mantra "it is what it is" helped me pull through roadblocks with a smile.

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I think most would agree that grad school can be an isolating experience, and the temporal circumstances of 2020 made this especially true for me. I would like to extend my gratitude to my friends for laughing and commiserating with me as I rode through the ups and downs of a unique graduate experience. Though she cannot read, I make sure to let my cat know every day how much I appreciate her adorable, usually welcome distractions. Finally, I would like to thank my family for their unfaltering support throughout my entire post-secondary education. My brother has been the anchor that moored me to a safe harbour when I felt adrift at sea (and I was adrift, *a lot*). My parents expensed their own dreams for me to pursue mine, including this MSc, and I know for a fact I can never repay this sacrifice. I can only promise to forever try.

With gratitude,

Michelle

Preface and Author Contributions

This thesis was written by the candidate under the supervision of Drs. Anne Gatignol and Robert J. Scarborough in accordance with the guidelines set out by the McGill University Graduate and Postdoctoral Studies. This thesis is written in the traditional format, beginning with a comprehensive literature review that establishes the project rationale, hypothesis, and research aims. This is followed by the materials and methods, results, discussion, and conclusion, and ends with a bibliography.

Sections 2.1.2, 2.1.3, and 2.3 of the Introduction were published in a review: **Michelle J. Chen**, Anne Gatignol, Robert J. Scarborough. "The Discovery and Development of RNA-Based Therapies for Treatment of HIV-1 Infection". Expert Opinion on Drug Discovery, 2023. 18: 163-179. Section 2.1.2 corresponds to Section 3.3 of the review and sections 2.1.3 and 2.3 correspond to section 3.5 of the review, both of which were written by the candidate and edited by Drs. Anne Gatignol and Robert J. Scarborough.

Section 2.2 of the Introduction and Figures 4-6 will be published in a review: Ryan P. Goguen, **Michelle J. Chen**, Owen R.S. Dunkley, Anne Gatignol, Robert J. Scarborough. "Gene Therapy to Cure HIV Infection". Virologie (accepted for publication). Section 2.2 corresponds to the section "Gene editing to generate HIV-resistant cells" in the review, which was written by the candidate and edited by all authors. Figures 4-6 were created by the candidate.

Aim 1 was conceived by Drs. Anne Gatignol and Robert Scarborough. Aim 2 was conceived by Drs. Anne Gatignol, Robert J. Scarborough, Stéphane Isnard, and Jean-Pierre Routy. The candidate performed all experiments and prepared all figures in this thesis with some assistance from RJS, except where stated. Data shown in Figure 7B and Figure 8 were from experiments performed by Camille Malard and graphed by the candidate. The experiment shown in Figures 13C and D was set up by Dr. Ryan Goguen; data from Day 8 onwards were collected by the candidate.

Chapter 1: Introduction & literature review

1.1 The type 1 human immunodeficiency virus (HIV-1)

1.1.1 Discovery

The first inklings of a burgeoning epidemic in North America appeared in two terse reports published by the Centre for Disease Control in the summer of 1981. Doctors in California and New York were raising alarm to an unusual increase in the number of patients presenting with rare and deadly diseases[1,2]. These included Kaposi's sarcoma, an uncommon neoplasm that predominantly impacts elderly males, and opportunistic fungal infections that are characteristic of profound immunosuppression. Indeed, the patients were also found to have severely reduced white blood cell counts[3]. Over the next year, scientists termed a growing collection of severe secondary infections as acquired immunodeficiency syndrome (AIDS) and characterized lymphopenia and lymphadenopathy as syndromes preceding AIDS. The sudden occurrence of rare, life-threatening medical conditions in younger patients and preferentially in men who have sex with men (MSM) was highly unusual and prompted a feverish search for its cause. Epidemiological data and observations of depleted white blood cell counts provided clues that the etiological agent targeted T cells. Serendipitously, the first human retrovirus, a human T lymphotropic virus (HTLV), had only been discovered a few years earlier and propelled a search for a similar virus in AIDS patients, one we now know as type 1 human immunodeficiency virus (HIV-1)[4].

The discovery of HIV-1 rode on the coattails of advances in retrovirology made in previous decades. Initial interest in retroviruses was sparked in the early 1900s when ultra-filterable agents were found to cause leukemia in non-primate animals[5-8]. However, the search for oncogenic human retroviruses was fruitless until the late 1970s. The discovery was made shortly after Howard Temin and David Baltimore independently and simultaneously proved the existence of reverse transcriptase (RT)[9,10], the activity of which became a sensitive assay for detecting new retroviruses. Additionally, the 1976 discovery of interleukin 2 (IL-2) was essential for establishing a culture system that supported longitudinal growth of uncontaminated primary T cells[11]. Previously, contamination of cell culture systems with animal retroviruses nullified claims of human retrovirus discovery. Together with RT activity, the ability to culture T cells provided a method to propagate and detect human retroviruses. These tools led to the discovery of the first two human retroviruses: HTLV-I in 1979[12] and HTLV-II in 1981[13]. The HTLVs were isolated from patients with T cell

leukemias or lymphomas and were confirmed to be distinct from known animal retroviruses in a barrage of molecular experiments[14,15]. Importantly, their tropism for T cells inspired Luc Montagnier and Robert C. Gallo's groups to look for a retrovirus in the HTLV family as the cause of AIDS.

In 1983, both Montagnier and Gallo published evidence that a virus similar to HTLV-I could be isolated from patients with AIDS or displaying pre-AIDS symptoms[16,17]. Montagnier's group detected RT activity from supernatants of cultured biopsied lymph node cells from a patient with pre-AIDS symptoms. They showed that the isolates displayed preferentially high RT activity in the presence of an RNA template over a DNA template which confirmed the virus' retroviral identity. Its magnesium-dependent activity also classified it as a primate retrovirus since non-primate retroviral RTs are independent of magnesium. Importantly, Montagnier's immunoprecipitation and electron microscopy experiments showed that the virus was similar to but distinct from HTLV-I and -II, and they termed it lymphadenopathy-associated virus (LAV), though the link between LAV and AIDS remained tenuous at this point. Gallo's group also described a retrovirus in an AIDS patient and developed a cell line that allowed the virus to be propagated and studied[18]. They named it HTLV-III, a misnomer that stemmed from their immunological experiments characterizing its antigens which led them to erroneously conclude that the virus belonged to the HTLV family[19]. Nonetheless, in 1984 they strengthened the link between the new virus and AIDS by showing that the virus could be isolated from a large proportion of patients with pre-AIDS or AIDS but not from patients without the disease, and that the majority of serum samples from these populations contained antibodies against HTLV-III antigens[20,21]. The association between a new retrovirus and AIDS was further bolstered by Levy et al. who detected viruses, which they termed AIDS-associated retroviruses (ARV) and which cross-reacted with Montagnier's LAV isolate, from 22 out of 45 patients in San Francisco[22]. The causal role between AIDS and a new retrovirus distinct from HTLVs was established as researchers detected them from patients with AIDS or pre-AIDS symptoms with accelerating frequency[23-27]. The new human retrovirus that had been referred to as LAV, ARV, and HTLV-III was officially renamed to Human Immunodeficiency Virus in 1986 by the International Committee on the Taxonomy of Viruses[4].

As HIV-1 was being characterized, an anxious search for a treatment was simultaneously being carried out in laboratories. Drug screens identified the nucleoside analogue azidothymidine

(AZT) as a promising candidate for HIV-1 inhibition and early clinical trials, though rushed, showed that it had some positive effects on clinical progression and survival[28]. In 1987, AZT was approved by the Food and Drug Administration as the first drug to treat HIV-1, but its hopeful success was short-lived. Patients had to remain on AZT otherwise virus would rebound[29], but severe side effects and escape mutants from AZT monotherapy presented a significant barrier to continual administration[30-32]. By the time other drugs targeting different parts of the HIV-1 replication cycle were developed, researchers were battling the virus' rapid ability to resist monotherapy with multi-drug combinations. Three-drug regimens known as combination antiretroviral therapy (cART)[33] were widely implemented in the 1990s and have since saved – and continue to save – millions of lives.

1.1.2 Epidemiology

HIV-1 is transmitted through body fluids including blood, semen, vaginal secretions, and breast milk. Vertical transmission from mother to child can also occur but is drastically reduced with the use of antiretroviral drugs[34]. Infection begins with an acute stage characterized by high levels of viral RNA (vRNA) ($10^6 – 10^7$ copies/ml at peak) that develop within two weeks of initial exposure[35]. The acute stage generally lasts three to four weeks but can persist for months, during which patients may be asymptomatic or present flu-like symptoms[36]. At this stage, the levels of vRNA and HIV-1 proteins in the blood surge ahead of HIV-1 specific antibody titres and are used as early clinical markers of infection. vRNA levels remain elevated until infection progresses into the chronic stage where the immune system begins to control replication but fails to completely eliminate infection. After several years, cluster of differentiation (CD)4^+ T cell counts which normally measure between 500-1500 cells/mm^3 fall to less than 200 cells/mm^3, severely compromising the immune system's ability to manage opportunistic infections. Without cART, infection progresses into AIDS within a decade. Clinical manifestations of AIDS include protozoal, bacterial, and viral opportunistic infections, as well as aggressive cancers such as Kaposi's sarcoma, Non-Hodgkin's lymphoma, and Hodgkin's disease[37].

Since the start of the HIV-1 pandemic, the virus has claimed over 40 million lives worldwide[38]. At the peak of the pandemic in the early 2000s, two million individuals died each year due to AIDS-related illnesses[38]. The widespread reach of the virus mobilized a concerted global endeavour to eradicate AIDS, and through synergistic efforts between scientists,

policymakers, and community leaders, the world has made massive strides in realizing this vision. The World Health Organization's (WHO) "3 by 5" initiative that aimed to treat three million people in low- and middle-income countries by 2005[39] was achieved in 2007, and the Joint United Nations Programme on HIV/AIDS' (UNAIDS) "15 by 15" target succeeded in providing cART access to 15 million people by 2015[40]. Most recently, UNAIDS adopted the "95-95-95" goal to have 95% of all people living with HIV (PLWH) know their status, have 95% of all PLWH have access to cART, and achieve viral suppression in 95% of all PLWH by 2030[41,42]. Clearly defined goals like these mobilized governments to implement policies that expedited treatment rollout. Additionally, unflagging community leadership by HIV/AIDS advocates has accelerated treatment rollout and scale-up. Combined efforts by scientific, global, and community leaders have reduced the virus' impact and brought about safer and more effective treatments that transformed HIV-1 infections from a fatal diagnosis to a manageable chronic illness. Today, less than 700,000 individuals around the world die from AIDS-related illness each year which represents a 68% reduction from the peak of the pandemic[38].

Despite this progress, HIV-1 still weighs significantly on the global disease burden. Today, more than 38 million people are living with HIV-1 and over one million individuals are newly infected each year[38]. The vast majority of individuals living with HIV-1 reside in eastern and southern Africa, where most new infections also take place; the second highest incidence of new infections are in Asia and the Pacific Islands[38]. Among the general population, HIV-1 incidence has stabilized or decreased, but it remains disproportionately prevalent among key populations[43]. Globally, MSM, transgender people, sex workers, intravenous drug users, and people in prisons and other enclosed settings are most at risk for infection and can influence the dynamics of HIV-1 epidemics[44]. To effectively address the current HIV-1 epidemiological landscape, the WHO has recommended policies that focus on addressing the needs of these key populations[44].

1.1.3 Classification & evolution

HIV belongs to the *Retroviridae* family and is further categorized into the *Orthoviridae* subfamily and the Lentivirus genus. There are two known species: HIV-1 is responsible for the global reach of the virus while HIV-2 is mostly confined to West Africa. Both types can result in AIDS, but HIV-2 is less transmissible and is less likely to progress to the secondary disease[45]. HIV-1 is divided into groups M (major), O (outlier), N (non-major, non-outlier), and P. Group M

is responsible for the majority of HIV-1 infections and is further classified into subtypes, also known as clades. Subtype B is the most common in North America and Europe while subtype C is most common in Southern Africa and India; subtype C accounts for half of HIV-1 subtype diversity worldwide[46] (Figure 1). Genetic diversity within subtypes range between 15-20%[47] and individuals can be multiply infected with different subtypes leading to transmissible recombinant forms. Recombinants that are found in at least three non-epidemiologically linked individuals are considered to be "circulating" recombinant forms (CRF); these make up the majority of infections in Central and Western Africa and Southeast Asia[46,48] (Figure 1).

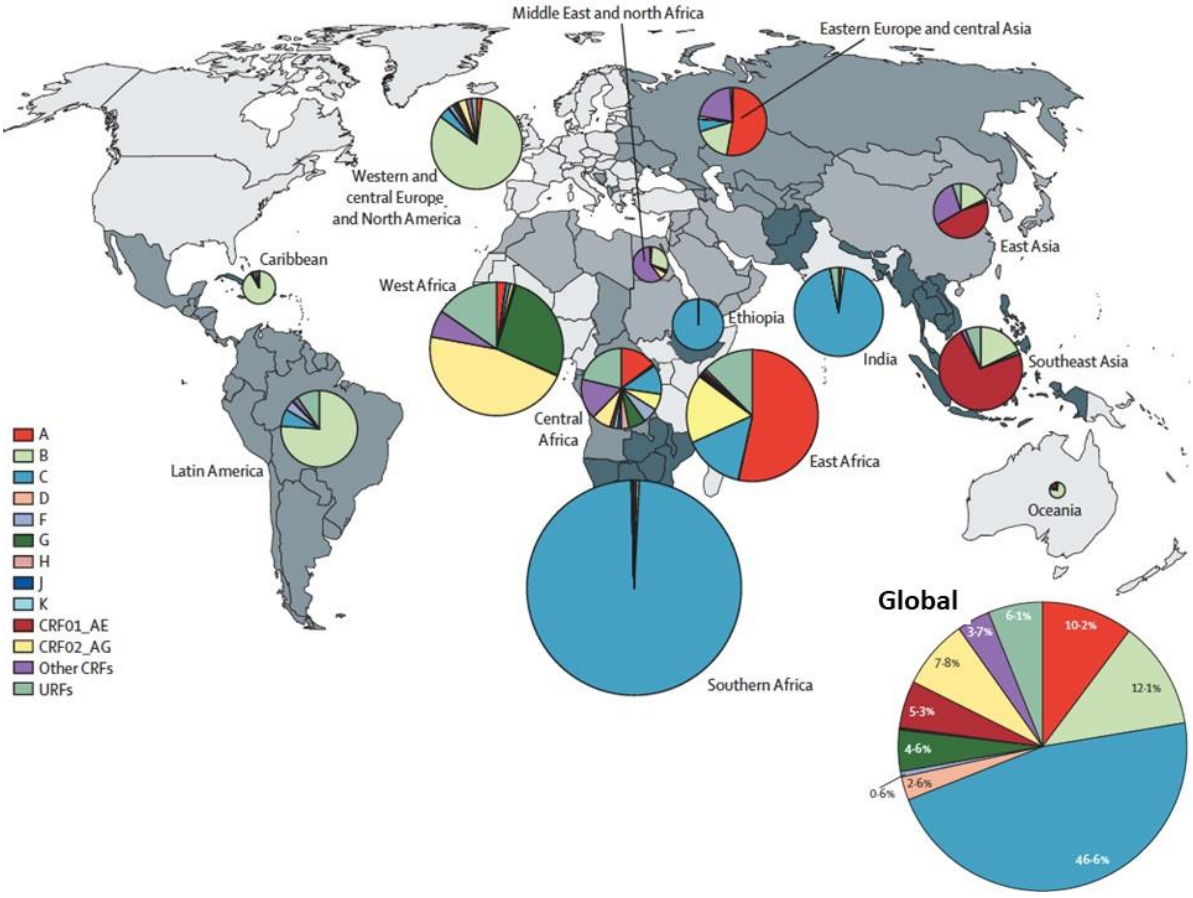

Figure 1. Global geographic distribution of HIV-1 subtype diversity. Reprinted from The Lancet, Vol. 19 Hemelaar *et al*., "Global and regional molecular epidemiology of HIV-1, 1990-2015: a systematic review, global survey, and trend analysis", 143-155, 2019, with permission from Elsevier.

HIV-1 may be additionally classified by its host coreceptor usage for entry into the cell. Coreceptor tropism is determined by the third variable (V3) loop of the glycoprotein (gp)120

subunit of the viral Envelope (Env) receptor[49]. Interaction between gp120 and host CD4 induces a conformational change in Env that allows the V3 loop to bind either CCR5 or CXCR4[50] (Figure 2). Isolates that exclusively use C-C chemokine receptor type 5 (CCR5) are termed "R5", isolates that exclusively use C-X-C chemokine receptor type 4 (CXCR4) are termed "R4", and isolates that can use both are termed "R5/X4"[51]. The majority of clinical isolates use CCR5 to mediate entry and R5 viruses dominate early stages of infection. In some patients, coreceptor switching to CXCR4 occurs with disease progression[52].

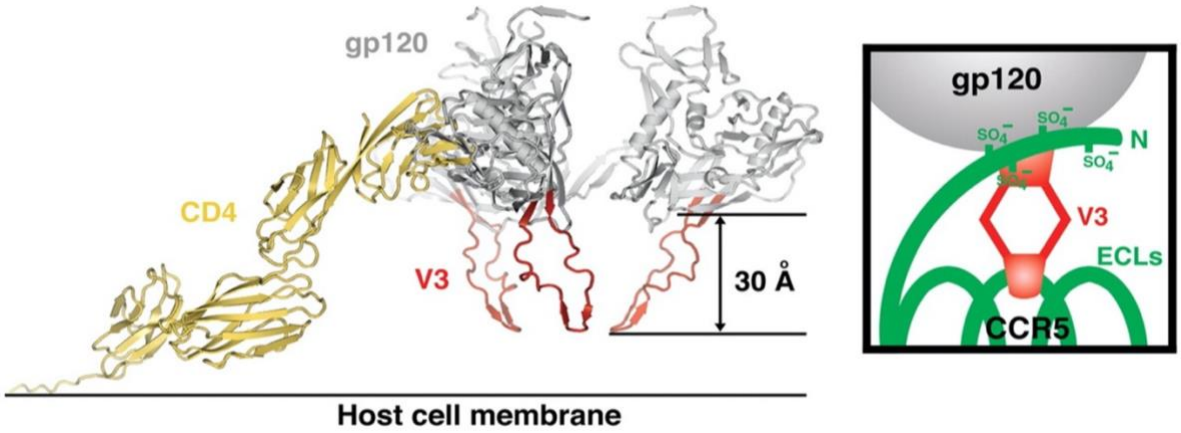

Figure 2. Structural model of a gp120 trimer binding to CD4 and CCR5. Left: CD4 bound to one unit of a gp120 trimer. Binding induces a conformational change that orients the Env complex toward the cellular membrane where the V3 loop may interact with CCR5 or CXCR4. Right: Interaction between the V3 loop and CCR5. From Huang *et al.*, 2005 (Science). Reprinted with permission from AAAS.

The HIV species evolved from simian immunodeficiency viruses (SIVs) found in non-human primates in West-Central Africa. Close contact between humans and non-human primates by way of bushmeat hunting allowed for independent zoonotic transmissions from non-human primates to humans which gave rise to the four HIV-1 groups, M, N, O, and P. Mutations and selection events further added to the diversity of HIV lineages found today. Phylogenetic studies have shown that HIV-1 group M originated from SIV_{cpz} found in the chimpanzee species *Pan troglodytes troglodytes* and was likely spread with human activity along the Congo River[48]. The first documented case of an HIV-1 infection was found in Kinshasa and is speculated to be the origin of the global HIV-1 pandemic[53]. In contrast, HIV-2 originated from SIV_{smm} found in sooty mangabey monkeys[54].

1.1.4 The viral particle & genomic architecture

The nucleic acid core of a HIV-1 particle consists of two ~9kb positive-sense, single-stranded RNAs (+ssRNA) flanked by a 5' cap and a 3' polyadenylated tail. The dimer is stabilized by nucleocapsid (NC) proteins[55] and bound to a host transfer (t)RNA^{Lys3} that acts as a primer for reverse transcription[56]. Surrounding the genome-NC-tRNA are three protective layers. The lattice immediately encapsidating the genome is composed of capsid protein oligomerized into a conical net of pentamers and hexamers that shields the reverse transcription complex (RTC) and facilitates nuclear targeting[57]. This capsid core is further enclosed by a matrix shell that discontinuously lines the inner leaflet of the viral membrane, the final layer derived from host plasma membrane and decorated with viral Env. In addition to packaging genomic information, the mature virion also contains protease, reverse transcriptase, integrase, and three accessory proteins: viral infectivity factor (Vif)[58], viral protein R (Vpr)[59,60], and negative factor (Nef)[61] (Figure 3A).

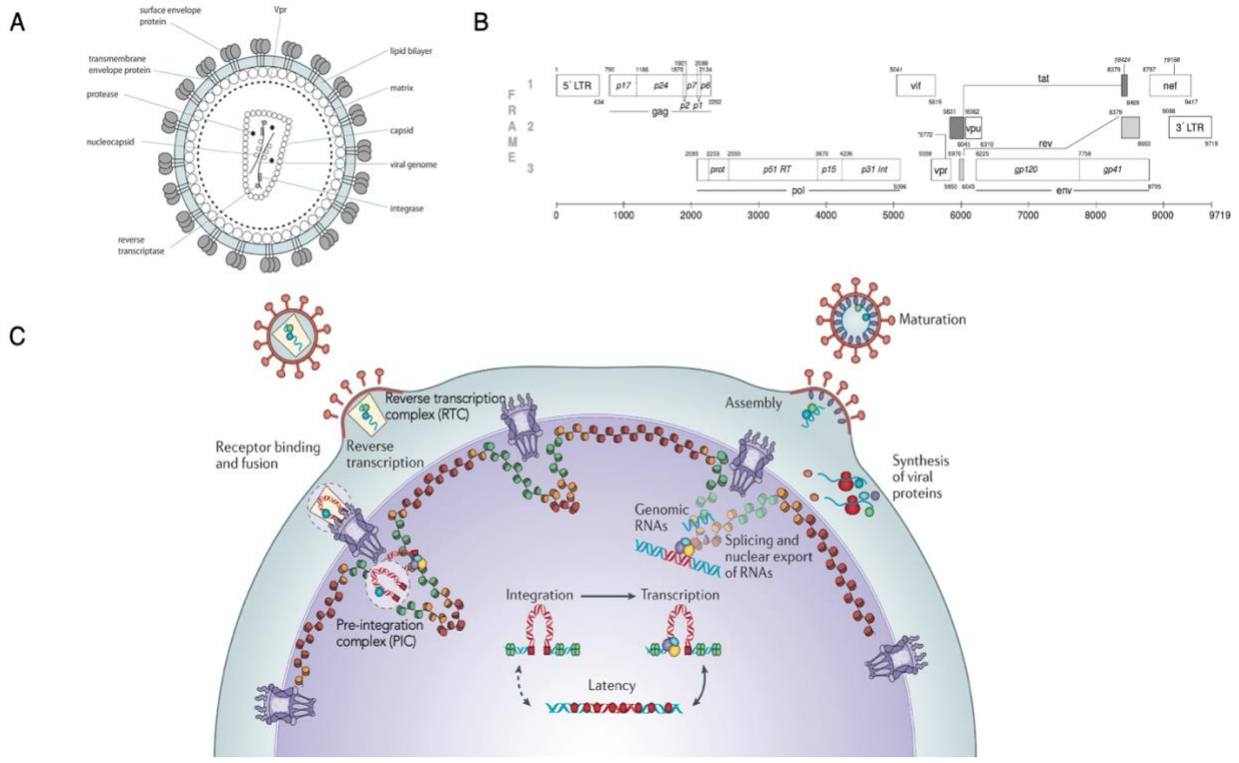

Figure 3. The HIV-1 virion, genome map, and replication cycle. A) Architecture of a mature HIV-1 virion. Image obtained from the London School of of Hygiene and Tropical Medicine[62]. B) The HIV-1 RNA genome and its coded proteins. Figure reproduced with permission from the HIV Sequence Compendium 2021. C) Major steps of the replication cycle. Figure adapted from Lusic & Siliciano, 2017 (Nature Reviews Microbiology) and reproduced with permission from Springer Nature.

The genomic RNA encodes 15 viral proteins that mediate various steps in the HIV-1 replication cycle, promote infectivity and replication, and reduce host antiviral defenses[63] (Figure 3B). The *gag* gene codes for a polyprotein encoding p6, the spacer proteins SP1 and SP2, and the structural proteins matrix (MA/p17), capsid (CA/p24), and nucleocapsid (NC/p7), which are later assembled to form the core. The *pol* gene encodes a polyprotein that gives rise to protease (PR/p12), reverse transcriptase (RT/[p51/p66]), and integrase (IN/p32). These essential enzymes govern viral polyprotein processing, synthesis of a DNA intermediate, and integration into the host genome, respectively. The *env* gene translates into a polyprotein that gives rise to surface gp120 and transmembrane gp41, which together form the Env protein. Polyproteins are assembled into the immature viral particle where they must be cleaved by protease to develop into an infectious virion.

The transactivator protein (Tat) and RNA splicing-regulator (Rev) play important roles in regulating viral gene expression[64] and their transcripts are among the first to be translated. Tat binds to the trans-activating response element (TAR), a RNA hairpin present at the 5' end of viral transcripts, and increases processivity of RNA polymerase (pol) II by enhancing phosphorylation at its C-terminal domain (CTD)[65]. Rev allows for cytoplasmic accumulation of partially-spliced ~4 kb RNAs and unspliced ~9 kb RNAs[66]. Rev shuttles between the nucleus and cytoplasm, exporting incompletely spliced and unspliced viral RNAs by binding to the Rev response element (RRE) present on partially spliced and full-length transcripts.

HIV-1 also encodes accessory proteins that each play multiple roles in obstructing host cell antiviral defenses, promoting HIV-1 replication, and assisting in viral assembly. Vif has a well-defined role in targeting the host antiviral enzyme APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G). Virions deficient in Vif have greatly reduced infectivity[67,68] because APOBEC3G is incorporated into the virion and introduces extensive hypermutations in viral cDNA[69]. Vif blocks this activity by impairing APOBEC3G translation and targeting the protein for proteasomal degradation. Vif was later shown to also activate the tumor suppressor gene p53 to induce G2 cell cycle arrest which facilitates viral replication[70]. Vpr is implicated in viral trafficking, transcription, and permissivity. It associates with importin-α which helps transport the pre-integration complex (PIC) into the nucleus, and functions as a coactivator at the long terminal repeat (LTR) where it associates with Tat to cooperatively enhance viral production[71]. Vpr is important for infection of non-dividing cells and thus implicated in establishing reservoirS3. Viral protein U (Vpu) downregulates cell surface CD4 expression and

enhances release of immature viral particles from infected cells by interfering with the host restriction factor tetherin[72]. Finally, Nef supports viral replication by interfering with immunosurveillance[73]. It hijacks endocytic trafficking pathways, stripping the plasma membrane of CD4 and major histocompatibility complex I to target them for lysosomal degradation or subcellular localization, respectively[74,75]. Absence of these molecules prevents superinfection and antibody-dependent cellular cytotoxicity by which effector immune cells identify and kill infected cells through antibody-Fc receptor interactions. Nef also perturbs signalling pathways that decrease T cell motility and produce an intracellular environment conducive for replication[76].

1.1.5 The replication cycle

HIV-1 primarily targets $CD4^+$ T cells but can also infect other cell types harbouring the required entry receptors, including other T cell subsets, macrophages[77], and astrocytes[78]. Early phase events of the replication cycle begin with cell binding (Figure 3C). Virions traverse across the cell surface by way of non-specific interactions with proteoglycans, integrins, and receptors until they are in close proximity with CD4. Binding between CD4 and Env gp120 is essential for entry into the cell. A second critical interaction between gp120 and CCR5 or CXCR4, selection of which depends on the V3 loop of gp120, induces hinge-like conformational changes in the Env complex. The conformational changes expose the gp41 fusion peptide which anchors the viral and cellular membranes together and supply the Env complex with the potential energy required to fuse the membranes together, releasing the viral capsid core into the cell[79,80]. Immediately beneath the cellular membrane lies a dense layer of actin; disassembly of the matrix shell upon fusion releases Nef which aids in depolymerizing the microfilament network[81].

Inside the cytoplasm, the core is known as a reverse transcription complex (RTC) and eventually becomes a pre-integration complex (PIC) that is imported into the nucleus. Reverse transcription and CA uncoating must occur sometime between core entry and proviral integration, but the exact location and kinetics of these steps are still under study. It was previously thought that the CA layer disintegrates upon entry which triggers reverse transcription, but paradoxically, other studies show CA stability is required for efficient reverse transcription and that CA itself engages nuclear import machinery to translocate the PIC across the nuclear membrane[82]. Recent evidence suggests that uncoating and reverse transcription finalizes in the nucleus[83], which aligns with other studies showing that CA remains associated with the PIC in the nucleus[84,85].

Inside the nucleus, viral cDNA is preferentially integrated into transcriptionally active sites of the host genome[86]. This process is mediated by integrase, which cleaves two nucleotides from each 3' end of the viral cDNA. The exposed 3' hydroxyl groups then undergo nucleophilic attack on two phosphodiester bonds located five nucleotides apart on the target DNA, ultimately incorporating itself as a provirus[87]. Cellular DNA repair enzymes then resolve unpaired nucleotides to complete integration.

Viral transcription by host factors marks the beginning of viral expression. RNA pol II assembles on the 5' LTR with other transcription factors to initiate production of new viral mRNAs. Initial transcripts are completely spliced into ~2 kb mRNAs that are exported to the cytoplasm and encode Tat, Rev, and Nef; Tat and Rev proteins enter the nucleus to regulate viral mRNA expression and processing. Tat complexes with host transcription and elongation factors that form the super elongation complex (SEC)[88], including positive transcription elongation factor b (P-TEFb) which is composed of Cyclin T1 and cyclin-dependent kinase 9 (CDK9)[89]. Tat binds TAR RNA present at the 5' end of all viral transcripts and recruits proteins from the SEC, which brings CDK9 in close proximity to the CTD of RNA pol II. Phosphorylation of CTD by CDK9 increases transcription rate and promotes transcription elongation[90], producing a large amount of full-length vRNA. As a consequence, the partially-spliced ~4.5 kb mRNAs that encode Env, Vif, Vpu, or Vpr, and unspliced ~9 kb mRNAs that encode Gag, Gag-Pol, or constitute the genomic vRNA, accumulate in the nucleus. Nuclear export of these mRNAs by Rev marks the beginning of the late phase of the replication cycle. Rev binds to the RRE present on Env mRNA[91] and complexes with chromosome maintenance factor 1 (CRM1) and RanGTP. The resulting ribonucleoprotein complex is exported through the nuclear pore using exportin machinery.

Gag is translated in high levels as a 55 kDa polyprotein and acts as the main conductor of viral assembly. Its MA domain contains a basic region that has a high affinity to the negatively charged phospholipid heads on the inner leaflet of the membrane. At this location, electrostatic interactions unveil the MA N-terminal myristate that inserts into the membrane, stably docking Gag beneath the cell surface[92]. Gag also causes lipid rafts to coalesce at the assembly site. The NC domain recruits dimers of unspliced vRNA into nascent virions by binding the Y-element, a packaging signal on the 5' untranslated region (UTR) of vRNA[93]. Gag-Pol polyproteins containing PR, RT, and IN are produced at 5% of Gag production due to ribosomal frameshifting during translation[94] and incorporated into assembling virions. CA causes Gag to multimerize into a lattice

around vRNA. The Gag lattice also plays a role in irreversibly incorporating Env complexes into the membrane; the long cytoplasmic tail of Env glycoproteins, emerging from the secretory pathway as heterodimeric trimers of gp120 and gp41, are likely trapped by the lattice[95]. Finally, the p6 domain, via its N-terminal PTAP peptide motif, recruits the endosomal sorting complex required for transport (ESCRT) which catalyzes membrane fission to complete budding[95].

Maturation occurs after viral particles bud from the cell membrane. In a process necessary for viral infectivity, protease, packaged within the particles, cleaves the Gag and Gag-Pol polyproteins into individual mature proteins, initiating an internal restructuring that is characteristic of virion maturation. Release of individual CA proteins causes them to reassemble into the conical capsid core surrounding viral genomic RNA and individual proteins packaged within the particle[96]. Mutations decreasing the efficiency of PR activity detrimentally impacts viral infectivity leading to non-infectious particles[97].

1.2 Controlling HIV-1 infection

1.2.1 Current anti-HIV-1 drugs & regimens

HIV-1 drugs work on the principle of preventing the formation of new infectious virions by inhibiting specific and necessary steps in the viral replication cycle. Over 30 molecules have been developed to inhibit entry and fusion, reverse transcription, integration, and proteolytic cleavage (Table 1). Monotherapy is not used because the high mutagenicity of HIV-1 allows it to rapidly overcome antiviral effects. However, a combination of these drugs can suppress viral replication in the long-term.

Table 1. Examples of currently FDA-approved antiretroviral drugs[98]

Nucleoside/nucleotide reverse transcriptase inhibitors (NRTI)	Non-nucleoside/nucleotide reverse transcriptase inhibitors (NNRTI)	Protease inhibitors (PI)	Entry and fusion inhibitors	Integrase strand transfer inhibitors (INSTI)
Abacavir	Delavirdine	Atazanavir	Enfuvirtide	Bictegravir
Emtricitabine	Doravirine	Darunavir	Maraviroc	Cabotegravir
Lamivudine	Efavirenz	Fosamprenavir		Elivitegravir
Tenofovir	Etravirine	Indinavir		Dolutegravir
Zidovudine	Nevirapine	Lopinavir		Raltegravir
	Rilpivirene	Nelfinavir		
		Ritonavir		
		Saquinavir		
		Tipranavir		

The first drug used to treat HIV-1 infection was a reverse transcription inhibitor, a class of antiretrovirals that include nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and non-nucleoside/nucleotide reverse transcriptase inhibitors (NNRTI). NRTIs lack the 3'-hydroxyl group necessary for DNA synthesis, effectively preventing creation of the HIV-1 DNA intermediate. Azidothymidine (AZT/zidovudine) paved the way for the discovery of NRTIs as the first class of antiretrovirals developed to treat HIV-1 infection. AZT was initially discovered and investigated in 1964 as an anticancer drug but was repurposed as the first approved antiretroviral drug in 1987 when a clinical trial showed that it reduced mortality and the incidence of opportunistic infections in AIDS patients[28]. Clinical trials for other nucleoside analogues followed, including didanosine, zalcitabine, stavudine, alovudine, and lamivudine. Some of these have been dropped from clinical trials or discontinued due to toxicity. A list of currently used drugs is shown in Table 1.

NNRTIs act as non-competitive inhibitors of HIV-1 reverse transcriptase. The first-generation NNRTIs (efavirenz, delavirdine, and nevirapine) bind to the p66 subunit of RT in close proximity to the DNA polymerase active site. Binding distorts the architecture of the active site, reducing its catalytic efficiency[99]. Because these NNRTIs target the same binding pocket, they have an especially low genetic barrier to resistance. A single mutation against one drug can generate class-resistance to the others thus precluding their use[100]. While they are generally well-tolerated, severe rashes occur in a small portion of patients and additionally, they can cause neurological side effects, and be teratogenic or hepatotoxic[101,102]. Additionally, unlike other inhibitors, most are ineffective against HIV-2 because of different residues within the binding site[103]. Chemical advancements in drug development have yielded second-generation NNRTIs (etravirine, doravirene, riplivirene) that have more flexible chemical structures, allowing them to adapt to conformational changes brought about by mutations and maintain important interactions necessary for inhibition[104].

With NRTIs, protease inhibitors (PI) were the second chemical class included in early cART regimens. They can either bind directly to HIV-1 protease to inhibit its activity, or they augment the activity of other inhibitors by restricting metabolic cytochromes from metabolizing the inhibitors[105]. Inhibitors that bind protease directly mimic the polyprotein cleavage site to interact with relatively conserved residues in the HIV-1 protease active site. This structural mimicry sequesters protease away from viral polyproteins and prevents the formation of new

infectious virions. Those that inhibit metabolic cytochromes, such as ritonavir, are used in combination with another protease inhibitor to boost its bioavailability.

HIV-1 entry may be considered as three distinct steps: binding of CD4, binding of coreceptor, and fusion, each of which presents a unique drug target. Attachment inhibitors were developed to block the first two steps by disrupting interactions with CD4, CCR5, and CXCR4. However, targeting cellular receptors, particularly CD4 and CXCR4, is undesirable because of their essential roles in T cell function. The exception is CCR5. Its antagonist maraviroc is the only attachment inhibitor approved by the FDA and Health Canada. Clinical trials showed that it was well-tolerated, able to reduce viral load, and showed similar efficacy to an established NNRTI, efavirenz[106]. However, maraviroc is ineffective against CXCR4-tropic isolates and resistance can develop quickly against it as HIV-1 can mutate to use maraviroc-bound CCR5[107]. Fusion inhibitors bind to the gp41 subunit of HIV-1 Env and prevent the formation of the fusion peptide and subsequent fusion of viral and host cell membranes.. Enfuvirtide was the first approved chemical in this class.

There are five approved integrase strand transfer inhibitors (INSTI): the first-generation drugs are raltegravir and elvitegravir and the second-generation drugs are dolutegravir, bictegravir, and cabotegravir. Strand transfer refers to the second integration step where the viral DNA, previously cleaved at the 3' ends, is inserted into host DNA. INSTIs are active in intasomes, the nucleoprotein complex containing viral DNA, host and viral proteins that assembles before the integration step. They destabilize the integrase-DNA complex by interacting with Mg^{2+} cofactors and displacing viral DNA from integrase[108,109]. They have a high genetic barrier to resistance, though resistant mutants against all available INSTIs have been reported. Cabotegravir is the most recently approved INSTI and is notable for its long-acting effects given its elimination half-life of 5.6-11.5 weeks following intramuscular injection[110].

Three-drug regimens known as highly active antiretroviral therapy were introduced in the mid-1990s. Now named combination antiretroviral therapy (cART), it remains the gold standard for treating HIV-1 infections. For a majority of patients, cART prevents progression to AIDS and effectively reduces the viral load in blood below the limits of clinical detection (<50 copies HIV-1 RNA/ml)[111] so that the virus is no longer transmitted[112-114]. Improvements to the drug profile, as described above, have made cART even more effective and tolerable; the decline in number of new HIV-1 infections since the early 2000s is a testament to its effectiveness. The exact

combination used depends on the treatment history of the patient but commonly consists of two NRTIs and an inhibitor from another class. Treatment failure can arise from a multitude of reasons – poor adherence, poor absorption, drug resistance, dosing issues, drug-drug interactions – and treatment regimens are adjusted by switching out specific drugs to retain virologic suppression.

Two-drug regimens are under study. Treatment options that use fewer chemicals may avoid the toxicities associated with lifetime exposure to a cocktail of drugs, but they run the risk of virologic failure and drug-resistant strain emergence[115]. However, clinical trials showing the non-inferiority of multiple two-drug formulations compared to three-drug cocktails are giving credence to the possibility of implementing two-drug regimens[115]. For example, two phase III clinical trials showed that monthly injections of long acting cabotegravir and rilpivirene were noninferior to a standard three-drug oral therapy[116,117]. Today, US, European, and international guidelines include the use of two-drug regimens for both treatment-naïve and virologically suppressed individuals, but with significant caveats[115,118].

Monotherapy should not be used because of rapid viral evolution to resist single-drug regimens, but challenges with adherence and cART failure among HIV-positive children in resource-limited settings have led to strategies using lamivudine monotherapy in youth. However, the risk of developing worse outcomes is high as shown by prospective studies in Asia and South Africa which found that pediatric individuals on lamivudine monotherapy experienced significant CD4 decline[119,120].

1.2.2 Prevention

The inhibitors used to treat HIV-1 infection are also used in drug formulations for preventing infections. Non-infected individuals who are at a higher risk for contracting the virus can protect themselves against infection by taking pre-exposure prophylaxis (PrEP)[121-123]. Three formulations are currently approved by the FDA, including two once-daily oral medications (emtricitabine/tenofovir disoproxil fumarate[123] and emtricitabine/tenofovir alafenamide[124]) and one long-acting injectable medication (cabotegravir extended-release injectable suspension). The oral pills are generally well-tolerated and widely available but require daily adherence to be maximally effective. Extended-release formulations that require less frequent doses would expand treatment options for individuals for which daily medications present a barrier. Cabotegravir, an INSTI discovered in 2013, was investigated as a strong candidate because of its slow metabolism,

low aqueous solubility, and high melting point, properties that allow it to be formulated as an injectable drug[125]. In two stage 3 randomized, double-blind, controlled noninferiority or superiority trials, bimonthly injections of cabotegravir were shown to be as effective as emtricitabine/tenofovir disoproxil fumarate[126,127]. These results were consistent in cisgender men, transgender women, and cisgender women. In December 2021, the FDA approved cabotegravir as the first long-acting injectable prevention medication for individuals 12 years of age and over. However, its prohibitive cost at $3700 per dose, a price tag 6100% higher than generic emtricitabine/tenofovir oral formulations[128], prevents its use for individuals in under-resourced communities where long-acting prevention strategies could make the most impact. Given the high efficacy of HIV-1 preventative drugs, increasing governmental investment in PrEP programs can contribute to reducing transmission.

Other science-based methods that mitigate the risk of infection include condom use, clean needle exchange programs, circumcision, and screening[129,130]. Studies have also shown that comprehensive sexual education can impact behaviour in a way that reduces transmission[37,131].

1.3 Towards a HIV-1 cure

1.3.1 Drawbacks to cART

cART has revolutionized the treatment of HIV-1 infection, transforming a fatal diagnosis into a manageable chronic infection[132]. Despite their efficacy and general tolerability, lifetime dependence on antiretroviral drugs is associated with a higher risk for premature development of age-related diseases such as cardiovascular diseases, diabetes, and frailty[133,134] which puts increasing pressure on public health systems as the demographics of PLWH shift to an older population[135]. Furthermore, virologic failure can occur with poor adherence and for individuals with low (< 200 cells/mm^3) CD4$^+$ T cell counts[136]. Finally, cART is unable to eradicate HIV-1 reservoirs, and persistent but low-level replication of the virus in patients on cART[137] triggers chronic inflammatory responses that lead to comorbid diseases. Therefore, the search for a cure that had started around 15 years ago is necessary and becoming increasingly urgent[138]. Equipped with a greater understanding of HIV-1 biology, insight into cellular reservoirs, and lessons from individuals who have been cured, researchers are slowly piecing together the components that may one day come together into a cure for all PLWH.

1.3.2 Latent reservoirs as a barrier to cures

Since the start of the HIV-1 pandemic, methods that completely eliminate the virus from the body have proved to be difficult to achieve. The main barrier is the latent HIV-1 reservoir in resting $CD4^+$ memory T cells. In the latent state, proviral DNA remains stably integrated in the genome but is not actively expressed, thus viral proteins are not targeted by cART drugs and infected cells avoid immune recognition[139]. This mechanism of viral persistence is not intrinsic to the replication cycle but is rather a consequence of the fundamental immunological dynamics of the $CD4^+$ T cells the virus preferentially infects. Two mechanisms of latency have been suggested. The first, known as pre-integration latency, involves the infection of naïve resting $CD4^+$ T cells. These cells support HIV-1 reverse transcription but integration of viral complementary (c)DNA fails to occur[140]. If the cells were activated while viral DNA was still intact, then integration and production of infectious virions could take place. Thus, activating a reservoir of recently infected naïve T cells can be a mechanism of latency but for patients on cART, this reservoir is likely limited as any new virions would be targeted by the drugs. Post-integration latency likely constitutes the mechanism for the majority of latently infected $CD4^+$ T cells[141]. In this scenario, viral cDNA is integrated into host DNA. These cells are transcriptionally active and most susceptible to productive infection. Most infected cells die because of cytopathic effects of the virus and the naturally short lifespan of effector cells, but some persist as memory T cells, which have slowed metabolic rates and importantly, are transcriptionally inactive until they encounter an activator which leads to their production[139]. In this latent state, the provirus is unable to be targeted by antiretroviral drugs. Induction of transcription factors that leads to cellular reactivation promotes transcription of the provirus, leading to a fresh round of viral replication. The presence of latent HIV proviruses necessitates lifetime dependence on cART drugs to control viral production from these reservoirs[142,143].

Cure strategies based on immune activation combined with cART have been attempted with the reasoning that the cytotoxicity of a massive quantity of virions purged by cytokines that induce viral replication such as IL-2, IL-6, and TNFα[144,145] would kill reservoir cells while antiretrovirals would prevent new infections[146]. While this "shock and kill" strategy did reduce the pool of latently infected $CD4^+$ T cells in circulation, patients who were subsequently taken off cART showed rapid viral rebound indicating that activation at the cellular level is insufficient for wholly purging latent reservoirs[147,148]. Other activation methods at the chromatin level have also

been investigated for reservoir activation. Histone deacetylases (HDACs) condense chromatin and reduce transcription, thus HDAC inhibitors should increase transcription of proviruses. Nevertheless, experiments with these inhibitors have largely been unfruitful[149,150]. Overall, all shock and kill strategies so far have failed to meaningfully delay viral rebound[151].

1.3.3 Functional cures

The pessimism surrounding the feasibility of a cure lifted in 2010 when Timothy Ray Brown, previously known as the Berlin patient, was announced as the first person in the world to be cured of HIV-1. Slated to receive an allogeneic hematopoietic stem cell transplant (HSCT) to treat an unrelated acute myeloid leukemia (AML), Brown's doctors matched him with a donor whose hematopoietic stem cells (HSCs) contained a homozygous 32 bp deletion in the *ccr5* gene (*ccr5* Δ32/Δ32)[152]. Since the late nineties, researchers had noted that individuals carrying a homozygous or heterozygous deletion in *ccr5* were more resistant to HIV compared to individuals with the wild type (WT) gene[153-156]. Cells with this genetic mutation lack CCR5 expression on their surface and are thus resistant to R5 viruses. Undergoing an irradiating treatment that removes all bone marrow cells followed by a transplant with resistant cells had the potential to reduce the viral reservoir and prevent new infections, a combination that would obviate the need for a daily cART regimen. This turned out to be the outcome. Despite being off cART for 13 years, Brown remained in remission from HIV-1 infection and was considered cured. He died in 2021 as a result of a resurgence in his leukemia.

Brown's case is an example of a functional cure, a long-term viral control strategy without the administration of antiretroviral drugs, and it is not a singular one. Since the landmark paper describing Brown's remission, four other patients have displayed an ongoing absence of viral rebound after cART interruption following an allogeneic HSCT with *ccr5* Δ32/Δ32 donor cells (Table 2). Adam Castillejo, previously known as "the London patient", received analytic treatment interruption (ATI) 16 months post-transplant and according to the most recently published update in 2020, had not experienced rebound for 2.5 years since ATI[157]. The Düsseldorf patient received a transplant in February 2013 to treat recurring AML and stopped cART in November 2018. In the most recent update published in Nature Medicine in February 2023, he had been in remission for 4 years[158] Another case of long-term remission without cART comes from the IMPAACT study in the USA and is the first example of a female patient who is potentially cured of HIV. This

patient received a transplant with a mix of donor cells containing haploidentical peripheral stem cells and cord blood stem cells harbouring *ccr5* Δ32/Δ32[159]. At the most recent update presented at CROI in February 2022, the patient had been in remission for 14 months. The most recent example of a potential cure, the "City of Hope patient", was presented in August 2022 at the International AIDS Society (IAS) Conference on HIV Science. The patient, who received an allogeneic transplant with *ccr5* Δ32/Δ32 HSCs to treat acute myelogenous leukemia, is the oldest individual to achieve remission from both the cancer and HIV-1. He underwent ATI in March 2021 and had been in remission for over 16 months at the time of announcement[160]. Collectively, these cases give hope to the feasibility of a functional cure and so far, HSCTs with *ccr5* Δ32/Δ32 are the only method that have demonstrably achieved a cure result.

Table 2. Stem cell transplants in patients diagnosed with HIV-1 and a blood cancer

Patient	Hematological diagnosis	Donor type/graft source	Donor CCR5 genotype	Time of ATI	Length of remission since ATI	Ref
Timothy Ray Brown (Berlin Patient)	Acute myeloid leukemia	HLA-matched HSCs (transplanted twice)	Δ32/Δ32	1 day pre-transplant	13 years	[152]
Adam Castillejo (London patient)	Hodgkin's lymphoma	HLA-matched HSCs	Δ32/Δ32	16 months post-transplant	30 months in last published update May 2020; currently 5.5 years	[157,161]
Düsseldorf patient	Acute myeloid leukemia	HLA-matched HSCs	Δ32/Δ32	5 years post-transplant	4 years (last update Feb 2023)	[158]
City of Hope patient	Acute myelogenous leukemia	HLA-matched HSCs	Δ32/Δ32	2 years post-transplant	16 months (last update Aug 2022 at IAS)	[160]
USA patient	Acute myeloid leukemia	Cord blood stem cells + haploidentical HSCs from relative	Δ32/Δ32	37 months post-transplant	14 months (last update Feb 2022 at CROI)	[159], NCT02140944
Essen patient	Non-Hodgkin's lymphoma	HLA-matched HSCs	Δ32/Δ32	7 days pre-transplant	3 weeks before rebound	[162]
Boston patients (patient 1; patient 2)	Hodgkin's lymphoma; myelodysplastic syndrome	Single HLC-C mismatched (7/8 match) HSCs from unrelated donor; HLA-matched HSCs from sibling	WT; WT		84 days before rebound; 225 days before rebound	[163,164]

Though none of these patients have shown viral rebound, trace detections of HIV-1 DNA have been observed in some samples of $CD4^+$ T cells and tissues from each patients[165,166]. Due to limitations in the sensitivity of detection methods, whether these signals are false positives or indicative of a reduced but persistent viral reservoir is impossible to know without taking large tissue biopsies. IciStem is an international effort to compile all known cases of viral suppression after allogeneic HSCTs (icistem.org, accessed on 15 November 2022)[167]. Two patients registered in this study died a few months after transplant and their tissues were analyzed with digital droplet PCR to investigate the existence of reservoirs. Proviral DNA was undetectable in peripheral blood mononuclear cells (PBMCs), but non-negligible copies of HIV-1 DNA were found in the lungs, spleen, and liver[168]. Without functional tests such as viral outgrowth assays, whether they are replication-competent cannot be ascertained, but these outcomes show that tissue reservoirs are present after allogeneic HSCT and may be a source for viral rebound.

There are major caveats to using stem cell transplants to cure HIV-1 infection. First, given the cost and resources required for the transplant and its aftercare, the procedure is not scalable to the millions of people living with the virus, particularly for those living in under-resourced communities that shoulder most of the disease burden. Second, HSCTs have risks and side effects; the most common cause of death after allogeneic HSCT, apart from relapse of the original disease, is graft versus host disease (GVHD) but patients are vulnerable to infections and respiratory and cardiovascular diseases as well[169]. Currently, use of HSCT to treat HIV-1 is reserved as a last-resort for individuals who are also diagnosed with a blood cancer. Finally, *ccr5* Δ32/Δ32 cells remain penetrable to X4 and X4/R5 viruses. R5 viruses dominate early stages of infection but they are known to switch coreceptor use to CXCR4 in later stages[170-172] and in cART-treated patients[173]. Transplants with *ccr5* Δ32/Δ32 cells may subject X4 or X4/R5 viruses to selection pressure, promoting their emergence. An example of this case was demonstrated with the Essen patient who underwent ATI before transplantation with *ccr5* Δ32/Δ32 cells. Prior to the transplant, genotypic analyses of the gp120 V3 loop, the amino acid architecture that determines coreceptor use, revealed viral preference for CCR5, but the patient experienced a viral rebound in CXCR4-tropic viruses 20 days post-transplantation[162]. Interestingly, in Brown's case, X4 viruses were also detected at low levels in plasma but they did not lead to a rebound[152]. Given the heterogenous nature of HIV-1 infection between individuals, it is possible that replication-competent X4 viruses existed in a larger proportion in the Essen patient and were able to reseed infection in the reconstituted immune

system. Ultimately, this case cautions against universal application of transplants as a cure, especially for individuals with advanced infection or treatment-experienced individuals.

1.4 RNA-based gene therapies for a functional cure

1.4.1 RNA interference (RNAi)

The discovery that exogenous dsRNA can interfere with mRNA expression was discovered in *C. elegans* in 1998[174]. dsRNA is processed through the RNA interference (RNAi) pathway to generate a double-stranded small-interfering RNA (siRNA) with one strand complementary to a sequence on the original dsRNA. First, the dsRNA is cleaved into a short, double-stranded siRNA by the RNase Dicer. The Dicer-siRNA complex associates with other cellular proteins to form the RNA-induced silencing complex (RISC), where one strand of the siRNA is removed by an Argonaute RNase (Ago2). The remaining guide strand leads the complex to the complementary sequence in the target mRNA which is cleaved by Ago2. In plants and invertebrates, RNAi serves as a defense mechanism against viruses. In mammalian cells, this pathway may be harnessed to target specific foreign RNA pathogens.

1.4.2 Harnessing shRNAs for gene therapy

While there have been major advances in the development of siRNAs as drugs, their short half-lives and difficult delivery limit their success as a drug, particularly for HIV-1. An alternative is to harness the antiviral properties of RNAi instead with shRNAs to precisely target HIV-1 transcripts or host mRNAs for degradation, ultimately preventing production of new virions in a gene therapy setting. shRNAs consist of a ~19 bp stem that form the sense and antisense strands, a ~9 nucleotide (nt) hairpin loop, and a 2 nt 3' UU overhang[175]. Early studies showed that they are more accurately and efficiently expressed from RNA pol III promoters, results which were later corroborated by comparisons studies[175-177]. They can be transiently introduced into mammalian cells with vectors either by cell transfection with DNA plasmids or with viral vectors that do not persist in cells, such as adeno-associated viruses. They can also be permanently expressed in cells through transduction with retroviral vectors. Upon transcription, these small single-stranded RNA molecules spontaneously rearrange into dsRNA hairpins that are processed into active ~19 bp siRNAs through Dicer cleavage of the loop structure. Dicer in complex with the siRNA and the TAR RNA binding protein (TRBP) then associates with Ago2 to form the RISC. One strand is

cleaved by Ago2, leaving an active guide that is complementary to the target which is subsequently degraded by Ago2. In addition to this canonical shRNA format, up to three targeting moieties can be designed into a single extended shRNA. The extended stem is cleaved into separate siRNAs by Dicer before being further processed in the RISC. Furthermore, shRNAs with shorter stems can directly complex with Ago2 (Ago-shRNAs) to avoid cleavage by Dicer[178]. With polyA-specific ribonuclease (PARN), Ago2 promotes degradation of the strand on the 3' end of the shRNA, resulting in a single-stranded guide RNA (gRNA). Absence of a second strand eliminates concerns over off-target activity by the passenger strand therefore reducing its cellular toxicity, but also reduces RNAi activity[178].

1.4.3 Delivery

To be used in a curative manner, the DNA sequences for antiviral shRNAs need to be permanently inserted into the host cell genome to prevent their dilution in subsequent generations of cells. Insertion can be achieved with viral vectors based on retroviruses that integrate their payload into the host genome without disturbing essential functions. The replication cycle of retroviruses is unique in its generation of a dsDNA intermediate that is then integrated into the host genome. All retroviruses code for at least three genes, *gag*, *pol*, and *env*, that are necessary for generating new virions. Their viral genomes also contain LTRs at the 5' and 3' ends that contain transcriptional elements required for replication and reverse transcription. Retroviral systems are a collection of vectors that only generate replication-competent virus when expressed concurrently in a cell. The *gag, pol,* and *env* genes are supplied on separate vectors, as are the LTRs and the therapeutic gene of interest. The vector carrying *Env* is often pseudotyped with the coding sequence for glycoprotein G from vesicular stomatitis virus (VSV-G) to increase the recombinant virus's tropism across a greater number of cell types [179]. Early clinical gene therapy trials in HSCs used vectors based on murine leukemia viruses (MLVs) to correct immune disorders[180,181]. While one study successfully demonstrated the safety and durability of gene therapy[181], the other saw five patients develop leukemia as a result of insertional oncogenesis[180,182]. Additional studies with gamma-retroviruses such as MLVs showed that they preferentially insert into transcriptionally active sites and can activate oncogenes[183]. This redirected focus to a different retrovirus genus, the lentiviruses, which are less likely to insert in the vicinity of oncogenes and led to the development of self-inactivating vectors[184]. Lentiviral systems are typically derived from HIV-1 and consist of

different plasmids that carry the transgene and LTRs, as well as genes encoding Env and packaging proteins. These systems have been extensively improved over the years. The most current iteration are the self-inactivating, third generation lentiviral vectors that lack HIV-1 accessory virulence factors, separate *gag* and *pol* from *env* and *rev*, and contain deletions in the 3' LTR to disrupt its promoter/enhancer function[185]. Such modifications are meant to reduce insertional oncogenesis and prevent formation of replication competent lentiviruses (RCL), virions that could newly infect cells. Third generation systems have been used in gene therapy trials to treat HIV-1 infection[186], genetic diseases[187-189], and lymphomas[190-193] by modifying T cells and HSCs. None of these studies observed adverse events related to the lentiviral vector. A recent study combed through 460 transduced cell products across 26 clinical trials for RCL and found none[194]. The first HIV-1 gene therapy trials in autologous $CD4^+$ T cells showed persistence of modified cells for up to 5 years in some patients, integration enrichment in non-oncogenic regions, and absence of RCL[195,196].

1.5 Direct gene editing for a functional cure

1.5.1 Zinc finger nucleases (ZFNs)

The first programmable editing systems developed were the zinc finger nucleases (ZFNs). These enzymatic complexes are engineered from the DNA-binding sites of zinc finger proteins (ZFPs) and the nuclease domain of the bacterial Fok1 restriction enzyme[197]. The DNA-binding sites of ZFPs consist of repeated amino acid motifs that each fold into conserved beta-hairpin and alpha helix modules in the presence of zinc, forming Cys2-His2 "fingers"[198] (Figure 4). A tandem series of fingers, each separated by a linker region, contact a segment of DNA through noncovalent interactions between residues on the alpha helix and DNA nucleotides. Canonically, each finger docks onto a nucleotide triplet but some may contact four base pairs[199]. Each finger may be modified by changing alpha helical residues to bind a nucleotide of choice, resulting in an engineered ZFP that targets a desired sequence. The endonuclease domain of Fok1 cleaves non-specifically when it is dimerized around DNA. When coupled with the DNA-binding zinc fingers, enzymatic activity is limited to a specific region defined by the ZFP, thus yielding a molecular tool for cleaving any desired sequence. Upon expression in the cell, ZFNs generate double-stranded breaks (DSBs) at the target sequence which are subsequently rejoined by cellular DNA repair mechanisms including non-homologous end joining (NHEJ) and homology directed repair (HDR).

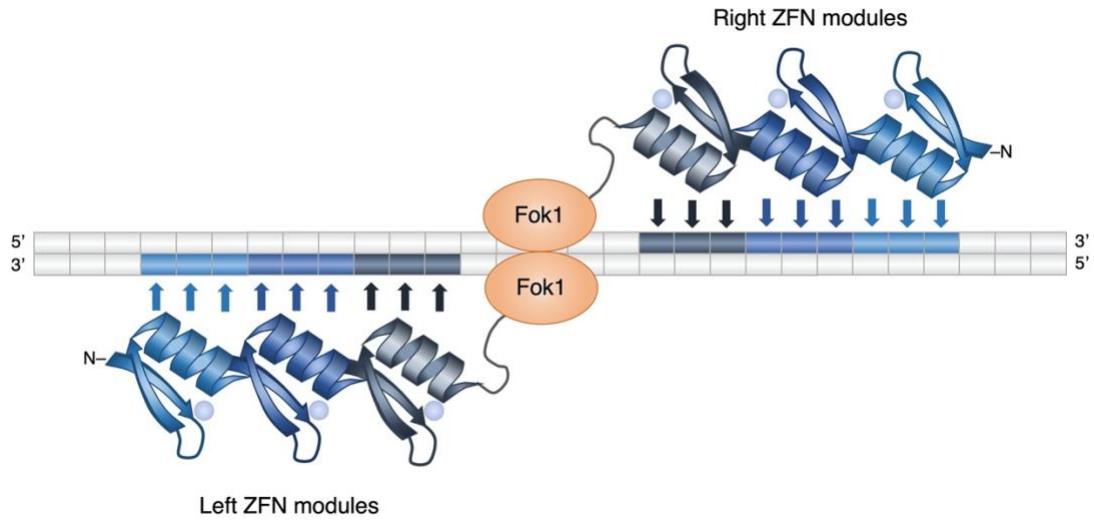

Figure 4. Schematic of a zinc finger nuclease. ZFNs consist of alpha-helix/beta-hairpin modules (three per ZFN are illustrated here) fused at its C-terminal with the Fok1 endonuclease. The tertiary structure of each zinc-finger module is stabilized through interactions with a zinc ion, represented by a blue circle. Each module corresponds to a triplicate of nucleotides which taken together, comprise the target sequence flanking the cleavage site. ZFNs function as homodimers to generate a DSB.

1.5.2 Transcription activator-like effector nucleases (TALENs)

Another class of designer nucleases are the transcription activator-like effector nucleases (TALENs). Like ZFNs, TALENs are enzymatic chimeras engineered to cleave the genome through protein-DNA interactions. The DNA-binding domain is derived from transcription activator-like effectors (TALE), proteins naturally produced by *Xanthomonas* bacteria to induce infection susceptibility in plant cells[200]. DNA recognition is achieved through interactions between the genomic sequence and an array of TALE modules, each containing 33-34 repeating residues; a pair of variable residues located at positions 12 and 13 determines the nucleotide specificity of each module[201,202] (Figure 5). TALEs customized at the two variable residues can be combined with the Fok1 endonuclease to generate TALENs that cleave a target sequence. TALENs host a series of advantages over ZFNs. They are easier to synthesize and may be designed against a wider range of targets than ZFNs because the specificity of a TALE module for a nucleotide residue is one-to-one, whereas each zinc finger binds to a nucleotide triplet and may overlap[203]. Neighboring zinc fingers influence the orientation of residues on the binding interface, thus potentially reducing the specificity of ZFNs compared to TALENs[204]. Imprecise binding of zinc fingers to DNA

coupled with non-specific Fok1 endonuclease activity can cause a high rate of genome-wide off-target effects, resulting in cytotoxicity[203]. On the other hand, the large size of TALENs[205] makes cellular delivery more difficult compared to ZFNs[206].

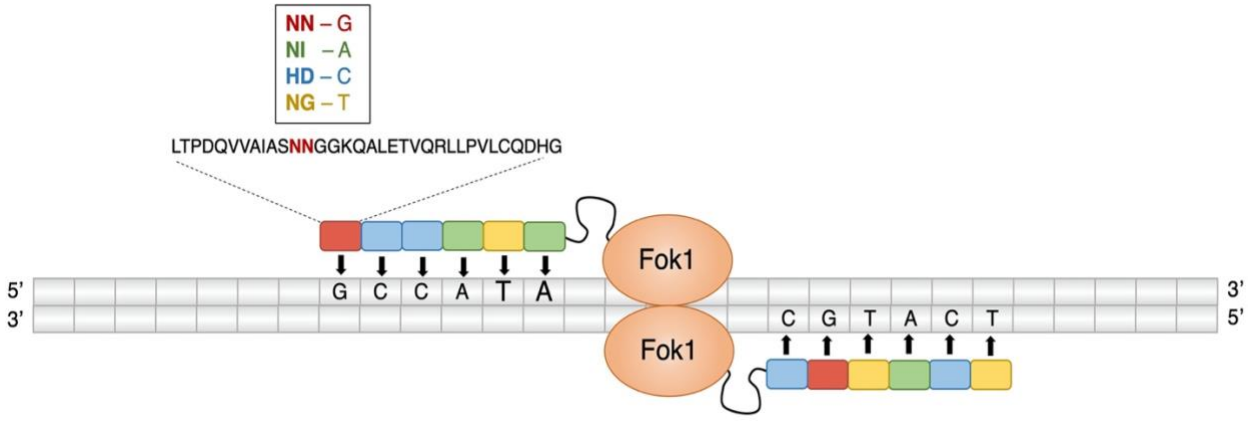

Figure 5. Schematic of a transcription activator-like effector nuclease. TALENs are composed of a Fok1 endonuclease fused with 13-28 modular TALE proteins that each correspond to a single nucleotide (6 modules per TALEN are illustrated as an example). All modules are identical in their ~34 amino acid makeup except for two variable residues present at positions 12 and 13. These variable residues determine the nucleotide specificity of the module. Variable residues "NN" have high specificity to guanine; variable residues "NI" have high specificity to adenine; variable residues "HD" have high specificity to cytosine; variable residues "NG" have high specificity to thymine. Upon binding to the target sequences flanking the cleavage site, TALENs function has homodimers to generate DSBs.

1.5.3 Clustered regularly interspaced palindromic repeats (CRISPR)

The most recent additions to designer nucleases are the CRISPR (clustered regularly interspaced palindromic repeats) associated (Cas) nucleases. Although they arrived later to the scene as a functional genome-editing tool, their comparative ease of use catapulted this technology to the forefront of genome engineering. There are two CRISPR-Cas systems (Class 1 and 2) subdivided into several types, and CRISPR-Cas9, a member of the Class 2 type II family, is the best-characterized and most widely used. CRISPR-Cas9 was derived from an RNA-guided defense mechanism in *Streptococcus pyogenes* that protects the bacterium against foreign DNA, most often of a viral origin. Canonically, fragmented sections of invading DNA are incorporated into a CRISPR locus consisting of multiple foreign-derived sequences interspaced by short repeats[207]. Post-transcriptional processing of the RNA expressed from this locus yields CRISPR RNAs (crRNAs) complementary to invading DNA. Type II systems require that crRNAs mature by

duplexing with a trans-activating RNA (tracrRNA), a small RNA encoded upstream of the CRISPR locus[208]. Upon colocalization with Cas9, the duplexed RNAs guide the nuclease to target and destroy complementary invading DNA. Molecular engineering has simplified the RNA requirements and produced an easily manipulated CRISPR-Cas gene editing system that involves two components: a single guide RNA (sgRNA) formed by hybridizing the crRNA and tracrRNA, and the Cas endonuclease that generates a DSB[209] (Figure 6). sgRNA target selection is limited by the requirement of a protospacer adjacent motif (PAM) next to the target site, which is determined by the chosen variant of Cas.

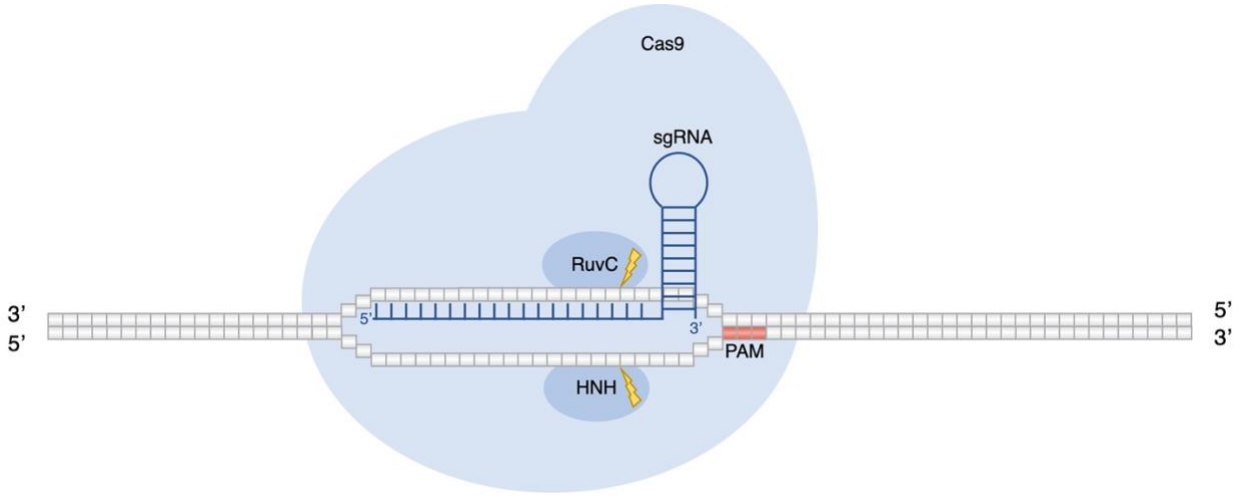

Figure 6. Schematic of Cas9-sgRNA ribonucleoprotein targeting DNA. CRISPR/Cas9 has been engineered to require only a sgRNA to guide the Cas9 enzyme to a target site adjacent to a PAM sequence, a set of 3 nucleotides necessary for enzymatic activity. The hairpin structure at the 3' end of the sgRNA binds Cas9, while the single-stranded 5' sequence mediates binding to the complementary DNA region. Recognition of the target sequence and the PAM triggers DNA melting and sgRNA invasion to form an RNA-DNA heteroduplex. The catalytic RuvC and HNH domains of Cas9 mediate a double-stranded break.

1.6 Targets for HIV-1 gene therapy

1.6.1 Viral targets

shRNAs have massive potential as anti-HIV-1 agents because of their ease of design and specificity of action. Some of the early siRNAs sequences were then adapted to be delivered with lentiviral vectors and to determine if their promising activity was maintained during long-term expression in lymphocytes or in HSCs. Early attempts targeted either HIV-1 sequences or the

coreceptors[210-213]. To ensure global activity against all HIV-1 strains, they must target conserved regions of the HIV-1 genome. In the first genome-wide screen to identify conserved target sites, the authors aligned 20 nt sequences in the HIV-1 subtype B LAI isolate to 170 complete HIV-1 sequences in the Los Alamos HIV-1 database and looked for complete sequence homology in at least 75% of the 170 genomes[214]. 86 shRNAs were designed targeting 19 regions, and 21 demonstrated strong inhibitory effects against HIV-1 replication. The four most effective shRNAs were cloned into a lentiviral vector and tested *in vivo* in a mouse model; one was immunotoxic but the combined expression of the remaining three was shown to safely inhibit HIV-1 replication[215]. A second genome-wide screen evaluated sequence homology in 19 nt windows across HIV-1 full length and partial fragments in the Los Alamos database[216]. Target sites were selected based on perfect matches and highest conservation estimates, resulting in the design of 96 shRNAs. Approximately two thirds were highly active against HIV-1 strain NL4-3 production. A third set of 26 shRNAs was designed by probing HIV-1 RNA architecture and identifying accessible target regions[217]. The majority of these shRNAs reduced HIV-1 production in cell assays, and eight were found to completely inhibit or significantly delay replication in a human T lymphocytic cell line. Safety evaluations showed that five out of these eight shRNAs did not have effects on cell growth. Using shRNAs identified from previous screens the efficacy of four top-performing candidates in mouse models reconstituted with transduced human HSCs was evaluated and three of them did not affect normal hematopoiesis[218]. Using a ribozyme screen to identify a highly accessible antisense target site in HIV-1 RNA, an shRNA was identified targeting a conserved region in the *gag* coding sequence of HIV-1 RNA that significantly reduced viral production in diverse HIV-1 strains from different clades[219]. Like cART, effective shRNAs will need to be combined with each other or with other antiviral molecules to ensure lasting protection against new infections because HIV-1 will quickly develop escape mutations against single shRNA regimens[220,221].

1.6.2 Cellular targets

In addition to targeting viral genes, shRNAs may be developed against host factors that mediate viral infection such as CCR5. Given its critical role as an HIV-1 entry receptor, disrupting the interaction between HIV-1 Env and CCR5 would prevent infection. The efficacy of targeting this protein has been demonstrated with the FDA-approved CCR5 antagonist maraviroc, which is used to treat HIV-1 infection particularly in patients who failed to respond to or are intolerant to

cART regimens[106]. One study showed that a CCR5 shRNA combined with C46, a membrane-bound fusion inhibitor, significantly suppressed CCR5-tropic viral infection in cell lines and PBMCs[222]. This combination has been transduced into $CD4^+$ T cells and $CD34^+$ HSCs in a clinical trial (NCT01734850). However, the preliminary results published on the clinical trials website show that it did not reduce viral load by 48 weeks, likely because the shRNA and C46 were not stably expressed as neither was detectable by qPCR at the end of the study period. A separate phase I/II trial currently underway aims to transduce human HSCs with a CCR5 shRNA in combination with a human-rhesus macaque tripartite motif chimeric protein (TRIM5α-HRH) and a TAR decoy (NCT02797470). Another host factor that has been shown to be a cofactor of HIV-1 replication is lens epithelium-derived growth factor (LEDGF)/p75[223]. Interactions between LEDGF/p75 and HIV-1 integrase is important for supporting infection as demonstrated in cell knockout experiments[223]. Because LEDGF/p75 also mediates critical host genome-protein interactions, prudence is required to disrupt only its interactions with HIV-1, which has been achievable with specific missense mutations[223].

Other cellular factors that support HIV-1 replication may be identified using genome-wide siRNA libraries assaying for genetic targets that, when knocked down, result in reduced viral production. Three such siRNA screens have been performed, each identifying between 224 and 295 factors not previously associated with HIV-1 replication[224-226]. While these screens may not be comprehensive because they failed to identify known cofactors such as LEDGF/p75, they did independently flag overlapping mediator proteins that play a role in the interactions between Tat and the transcription machinery. Indeed, the knock-down of some mediator proteins induce an inhibition of HIV-1 production, but sustained knocking-down of these factors may affect long-term cellular functions and should be considered cautiously[227]. A meta-analysis of cofactors involved in HIV-1 replication implicate pathways related to transport across the nuclear pore, GTP binding, and protein complex assembly as therapeutic targets[228]. While targeting cellular factors to prevent HIV-1 replication is an interesting method that could lead to a more constant target than the viral genome, besides CCR5, there is currently no other gene that can be considered for a long-term inhibition without affecting cellular functions.

1.7 Hypothesis and Objectives

1.7.1 Rationale

The success of the only reported cures of HIV-1 are based on the absence of cell-surface CCR5 coreceptors on HSCs and their progeny. Additionally, mutations in *ccr5* have no known negative effects. Thus, recapitulating the mutant *ccr5* genotype in patient cells may prevent entry of R5 viruses. Inhibition would not be complete because of the presence of X4 and R5/X4 viruses. This thesis aims to determine the feasibility of generating cells that are resistant to R5 and X4 viruses by using CRISPR-Cas9 to generate a homozygous mutation in the *ccr5* gene, then further modifying these cells with an effective and non-toxic shRNA to inhibit production of CXCR4-tropic isolates.

1.7.2 Aim 1: Determine the efficacy and toxicity of promoter-shRNA cassettes

shRNAs are typically expressed from one of three human type 3 RNA pol III promoters, 7SK, U6, or H1. Each of these promoters has a different transcriptional efficiency that affect the potency and toxicity of its downstream gene. Additionally, each shRNA may have sequence-specific potencies and toxicities which may be modulated by the promoter from which it is expressed. Our lab had previously showed that sh1498, a shRNA we identified as being highly conserved and effective at inhibiting HIV-1 production in HEK293T cells[219], and sh5983, a shRNA targeting the *tat/rev* region that is undergoing clinical trials[186], are more potent when expressed from the 7SK and U6 promoters[176]. We had also compiled a panel of shRNAs that target regions in the HIV-1 genome with high sequence conservation and identified three, L1, P2, and S3, that significantly delayed viral replication in a T cell line when expressed from the H1 promoter. Because we found that promoter selection can modulate shRNA potency, it is worthwhile to investigate whether these three shRNAs can be optimized with a different promoter. Thus, the first aim is to determine an optimal promoter-shRNA cassette by comparing the efficacy and toxicity of L1, P2, and S3 expressed under each of the three promoters.

1.7.3 Aim 2: Generate R5 and X4 resistant cells with ccr5 *gene editing and a shRNA*

Individuals with a heterozygous Δ32/WT or homozygous Δ32/Δ32 mutation in the *ccr5* gene are, respectively, partially or completely resistant to infection with R5 viruses. This deletion results in a truncated protein that is not expressed at the cell surface, thus preventing viral entry.

CRISPR/Cas9 can be used to induce DSB in *ccr5* that, when resolved by cellular repair machinery, may result in indels that cause frameshift mutations and an early stop codon that truncate translation of the protein. Because the collection of indels produced by cellular repair machinery are heterogeneous, a pair of gRNAs may be used to better control the anticipated deletion. Two different pairs of guide RNAs (gRNA) with minimal off-target activity that bind sequences in the protein coding region of the *ccr5* gene have been shown to restrict viral replication in primary human HSCs, primary human $CD4^+$ T cells, or T cell lines [229-231]. However, modifying cells with these deletions will not restrict CXCR4-tropic strains. Thus, the second aim is to generate a monoclonal T cell line with a homozygous deletion in *ccr5* then determine whether these cells, when further transduced with an antiviral shRNA, can restrict entry of R5 viruses and replication of X4 viruses.

Chapter 2: Materials and Methods

2.1 Cell culture maintenance

HEK293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (HyClone). SUP-T1 (ATCC) and PM-1 (NIH AIDS Reagent Program) cells were grown in Roswell Park Memorial Institute (RPMI) medium (HyClone). Both media were supplemented with 1% penicillin-streptomycin (Life Technologies) and 10% heat-inactivated, filtered fetal bovine serum (FBS) (HyClone). Media supplemented in this manner are termed "complete". All cell lines were maintained in a 37°C incubator supplemented with 5% CO_2.

2.2 Sequence conservation of shRNA target sites

20 nt shRNA target site sequences were input into the QuickAlign tool on the Los Alamos National Laboratory website (https://www.hiv.lanl.gov/content/sequence/QUICK_ALIGNv2/QuickAlign.html). Sequences were entered using the conventional option and "Web alignment" was selected to align them against all complete sequences in the database. Percent conservation was calculated for all sequences in the database as well as for subtype B or C sequences only.

2.3 Construction of promoter-shRNA constructs

shRNAs were cloned into psiRNA4-h7SKGFP::Zeo and psiRNA-hH1GFP::Zeo (Invivogen) under the 7SK and H1 promoters, respectively. A third vector expressing U6 was generated by replacing H1 from psiRNA-hH1GFPzeo with U6 from the pSIREN-shuttle vector (Clontech Laboratories, Mountain View, CA, USA). Complementary DNA oligonucleotides of each shRNA were ordered from ThermoFisher and annealed in a 40 µl reaction (1.25 µM of each oligonucleotide, 75 mM NaCl, ddH_2O to 40 µl). The reaction was incubated at 80°C for 5 min, after which the temperature was allowed to decrease to 37°C, at which point the reaction was removed. 100 ng of the resulting dsDNA oligos were ligated with 100 ng of BpiI-digested psiRNA vectors with T4 ligase following the manufacturer's protocol (ThermoFisher). Ligations were transformed into high efficiency chemically competent DHα bacterial cells (Invitrogen). Single transformed colonies were selected by zeocin-resistance and loss of X-galactose blue staining from the β-galactose gene in the empty vector that is replaced by the shRNA insert. Selected colonies were incubated at 37°C, 220 rpm for 12 h in LB broth (Miller) (Sigma-Aldrich) supplemented with

zeocin. Vectors were purified with a Miniprep kit (Qiagen) and confirmed with Sanger sequencing (Genome Quebec) before large-scale purification with a Maxiprep kit (Qiagen).

2.4 Cotransfection assay

The efficacy of promoter-shRNA cassettes was determined by cotransfecting HEK293T cells with 100 ng of the HIV-1 molecular clone pNL4-3 and 100, 10, 1, 0.1, or 0.01 ng of each shRNA vector. 150 μl cells were seeded at a concentration of 250,000 cells/ml in 96 well plates. After 24 h, the cells were cotransfected with a mixture of shRNA vectors and pNL4-3. Briefly, *Trans*IT LT1 transfection reagent (Mirus Bio) was mixed with DNA in OptiMEM (ThermoFisher) at a ratio of 3 μl reagent to 1 μg DNA. The mixture was incubated at room temperature for 20 min to allow formation of *Trans*IT-LT1:DNA complexes before it was evenly pipetted over the cells. Supernatants were harvested 48 h after cotransfection and assessed for RT activity.

2.5 Lentivirus production

Promoter-shRNA cassettes were cloned from the psiRNA vectors into the lentiviral transfer vector HIV7-EGFP with PCR using the forward primer 5'-TATGCGGCCGCAGGGATTTTGGT CATGTTCTTAATCGATACTA-3' and reverse primer 5'-GTAACGCCTGCAGGTTAATTAA GTCTAGAAGCTTTTCCAA-3'. Purified PCR products were digested with NotI and XbaI. 400 ng of the digested products were ligated with 100 ng of NotI- and XbaI-digested HIV7-EGFP using T4 ligase according to the manufacturer's protocol. Plasmids were purified with a Miniprep kit and confirmed with Sanger sequencing (Genome Quebec) before large-scale purification with a Maxiprep kit. To generate lentiviruses carrying the cassettes, 20 ml of HEK293T cells were seeded at 275,000 cells/ml in T75 flasks. After 24 h, cells were transfected with 9 μg HIV7-EGFP, 3.4 μg envelope plasmid CMV-G (gift from Dr. J. Rossi, City of Hope, Duarte, California), and 50 μg Pax2 packaging plasmid (Addgene #12260). Plasmids were incubated for 15 min with polyethylenimine (PEI) and Optimem (ThermoFisher). The plasmid mixture was then pipetted evenly over the plated cells and incubated at 37^{o}C for 48 h. Supernatants were filtered once through a 45 mm filter to remove debris before lentiviruses in the supernatant were concentrated with the Lenti-X concentrator (Takara Bio) and harvested according to the manufacturer's protocol.

Lentiviruses were collected in 700 µl RPMI, separated into 100 µl aliquots, and stored at -80°C until use.

2.6 Lentivirus titration

Lentiviruses were titrated by measuring % GFP expression in SUP-T1 cells on a dilution curve. 8 µg/ml of polybrene (Sigma-Aldrich) was added to SUP-T1s at a concentration of 175,000 cells/ml, then 200 µl of these cells were seeded in a 96 well plate. Lentiviruses were serially diluted and added to the cells. In the first dilution, 50 µl original lentivirus stock was diluted in 150 µl RPMI. Nine two-fold dilutions were then made. GFP expression was measured with flow cytometry on a LSR Fortessa machine (BD Biosciences). Untransduced SUP-T1 cells were used as a negative control to set GFP^+ gates.

2.7 Infection and competitive growth assays

To determine how well shRNA constructs inhibited viral replication over the long-term, transduced cells were infected with HIV-1 strain NL4-3 and their RT activity in cell culture supernatants was followed over time. SUP-T1 cells were transduced with lentiviruses at a multiplicity of infection (MOI) of 1 using 8 µg/ml of polybrene. GFP^+ cells were obtained with fluorescence-activated cell sorting (FACS) on a FACSAria Fusion cell sorter (BD BioSciences). 100 µl sorted cells were plated at a concentration of 200,000 cells/ml in 96 well U-bottom plates. After 24 h, cells were infected in triplicate with 100 µl of high dose (10 µl stock NL4-3 in 5 ml RPMI) or low dose (5 µl stock NL4-3 in 5 ml RPMI) of HIV-1 NL4-3. Sorted, untransduced cells and cells transduced with lentiviruses carrying an empty cassette (absent of promoter-shRNA) were also infected to serve as positive controls for infection. Supernatants were harvested 3 times a week and assessed for RT activity. The harvested volume was replaced with fresh RPMI.

To probe for toxicity of the shRNA constructs, 100 µl of sorted, transduced GFP^+ cells were plated in triplicates with 100 µl of sorted, untransduced GFP^- cells in 96 well U-bottom plates. Both were plated at a concentration of 200,000 cells/ml. 200 µl of sorted, untransduced GFP^- cells were plated as negative controls for GFP expression. GFP^+ samples included cells transduced with lentiviruses carrying an empty cassette which served as negative controls for toxicity. Percent GFP expression was measured with flow cytometry 24 h later (day 1) after which it was assessed weekly

for 4 weeks, then assessed biweekly thereafter. Declining GFP expression indicates death or a growth defect of cells transduced with the shRNA construct.

2.8 RT assay

Viral production and replication were assessed with RT assays. 2.5 µl or 5 µl of supernatant harvested from cotransfection and infection assays were incubated for 5 min with a non-radioactive cocktail (1% NP-40, 1.04 mM EDTA, 5mM $MgCl_2$, 60 mM TrisCl, 75 mM KCl) to disrupt viral membranes and release RT. This mixture was subsequently incubated for 2 h at 37°C with 12.5 or 25 µl of a radioactive cocktail (10 µg/ml polyA (Roche), 0.33 µg/ml oligo(dT) (Life Technologies), 65 mM DTT, 0.05 miC/ml a^{32}P-TTP (3000 Ci/mmol, Perkin Elmer), 1.04 mM EDTA, 5mM $MgCl_2$, 60 mM TrisCl, 75 mM KCl). 9 × 12 grids were drawn onto positively-charged nylon membranes (Hybond-N$^+$, Amersham Biosciences), upon which 5 µl of the reaction mixture were then pipetted on every other gridded square. Once dried, unincorporated [^{32}P] dTTP were washed from the membranes with five 5 min washes in 2× saline-sodium citrate (SSC) buffer, followed by two 1 min washes with 95% ethanol. After drying for 24 h, the amount of RT in each sample which is proportional to the radioactivity readout was measured in counts per minute (CPM) with a microplate scintillation counter (MicroBeta TriLux, PerkinElmer).

2.9 Generation of CRISPR-Cas9 plasmids

Two pairs of published gRNAs[229,230] targeting different regions in the *ccr5* coding region were selected to produce a CCR5-ablated PM-1 cell line. The four gRNA sequences are listed in Table 3 and the expected results of the paired gRNA activity are presented in Table 4. ssDNA oligos were ordered from ThermoFisher (Table 3) and annealed according to the protocol described above. dsDNA fragments were then ligated into BpiI-digested mCherry vectors (pU6-mCherry) containing Cas9 (Addgene #64324) or Esp31-digested GFP vectors (pLKO5-GFP) (Addgene #57822). Vectors were amplified with a Miniprep kit and sequenced to ensure proper cloning before amplification with a Maxiprep kit. The sequencing primers used for pU6-mCherry and pLKO5-GFP were 5'-GAGGGCCTATTTCCCATGATT-3' and 5'-TTTGCTGTACTTTCTATAGTG-3' respectively.

Table 3. gRNA sequences

gRNA	gRNA Sequence (5' → 3')	ssDNA Oligo Sequence (5' → 3')	Vector into which dsDNA oligo was cloned
gRNA1	CAGAAUUGAUACUGACUGUA	**CACCG**CAGAATTGATACTGACTGTA	pU6-mCherry (Addgene #64324)
		AAACTACAGTCAGTATCAATTCTG**C**	
gRNA2	AGAUGACUAUCUUUAAUGUC	**CACCG**AGATGACTATCTTTAATGT**C**	pLKO5-GFP (Addgene #57822)
		AAACGACATTAAAGATAGTCATCT**C**	
gRNA3	GACUAUGCUGCCGCCCAGU	**CACCG**GACTATGCTGCCGCCCAGT	pU6-mCherry (Addgene #64324)
		AAACACTGGGCGGCAGCATAGTCC	
gRNA4	GCAGAAGGGGACAGUAAGA	**CACCG**GCAGAAGGGGACAGTAAGA	pLKO5-GFP (Addgene #57822)
		AAACTCTTACTGTCCCCTTCTGCC	

Table 4. Expected results from gRNA pairs

gRNA Pair	Name	Expected Deletion	Reference
gRNA1 + gRNA2	Δ32-gRNA	32-bp	Qi *et al.*, 2018[229]
gRNA3 + gRNA4	Δ35-gRNA	35-bp	Xu, *et al.*, 2017[230]

2.10 Generation of monoclonal cells with a pair of gRNAs

gRNA1 and gRNA3 were individually cloned into pU6-mCherry while gRNA2 and gRNA4 were individually cloned into pLKO5-GFP. gRNA1 is paired with gRNA2 to generate a 32 bp deletion, and gRNA3 is paired with gRNA4 to generate a 35 bp deletion. 7.5 mg of each pair of vectors were electroporated into 1×10^7 cells in 300 ml of RPMI without FBS or antibiotics using the Gene Pulser II (BioRad). The capacitance knob was set to high and a single pulse with time constant of ~21 ms with voltage of 0.250 kV and capacitance of 0.950 mF × 1000 was delivered to the cell-plasmid mixture in a 4 mm cuvette (BioRad). Cellular debris and bubbles were removed with a pipette, and 1 ml of RPMI (absent of FBS and antibiotics) was added to the cells before they were transferred into 6 well plates with 2 ml complete RPMI. After 48 h, cells were filtered with 70 μm filters and resuspended in 500 μl RPMI with 2% FBS in preparation for sorting. Non-electroporated PM-1 cells were also prepared as a negative control for gating. Single cells double positive for mCherry and GFP signals (mCh^+/GFP^+) were isolated with FACS using the BD FACSAria Fusion cell sorter. Cells were plated in 100 μl conditioned media. Conditioned

media was prepared by mixing fresh complete RPMI with 0.2 μm-filtered supernatant of PM-1 cells in the log stage of growth in a 1:1 ratio. 200 μl of complete RPMI was added to the cells 4 days after sorting. Plates were monitored for growth of monoclonal cell clusters which were expanded in 2 ml complete RPMI in 6-well plates.

2.11 Generation of monoclonal cells with a single gRNA

gRNA1 and gRNA3 were individually cloned into pU6-mCherry and separately transfected into PM-1 cells to generate indels or point mutations that might ablate CCR5 surface expression. 7.5 μg of gRNA:pU6-mCherry were added to 1×10^7 cells in 300 μl of RPMI without FBS or antibiotics. Cells were either electroporated with the Gene Pulser II or the Neon Transfection System (ThermoFisher). Using the Gene Pulser, an exponential decay system, the capacitance knob was set to high and a single pulse (time constant ~ 21 ms; voltage = 0.250 kV; capacitance = 0.950 μF × 1000) was delivered to the cell-plasmid mixture in a 4 mm cuvette (BioRad). Upon removal of cellular debris and bubbles, the cells were resuspended with 1 ml of RPMI (absent of FBS and antibiotics) and transferred to a 6 well plate with 2 ml complete RPMI. Using the Neon Transfection System, cells were washed with PBS and resuspended at a concentration of 2×10^7 cells/ml according to manufacturer's protocol. The following parameters were input into the system: pulse voltage = 1325 V, pulse width = 10 ms, pulse number = 3. Electroporated samples were immediately put into 2 ml RPMI with 10% FBS and no antibiotics. All electroporated samples were incubated at 37°C. After 48 h, cells were filtered with 70 μm filters and resuspended in 500 μl RPMI with 2% FBS in preparation for sorting. Non-electroporated PM-1 cells were also prepared as a negative control for gating. Single, mCh^+ cells were sorted with FACS into 96 well plates with 100 μl conditioned media. 200 μl of complete RPMI was added to the cells four days after sorting. Plates were monitored for growth of monoclonal cell clusters. These were expanded in 2 ml RPMI in 6 well plates.

2.12 Selection and validation of edited monoclonal cells

DNA from monoclonal cells were assessed for genomic editing with PCR and Sanger sequencing. Briefly, 1 ml of cells were centrifuged and resuspended in 10 – 40 μl TE buffer. Cells were incubated at 95°C then on ice for 5 min each. Cell debris were pelleted by centrifuging at

13000 rpm for 5 min before supernatant containing DNA was collected. For PCR, 12.5 µl of Q5 High-Fidelity 2× PCR MasterMix (New England BioLabs) was mixed with 1.25 µl each of forward and reverse primers diluted to 10 mM (Table), 5 µl of DNA, and water for a total reaction volume of 25 µl. PCR was performed with a thermocycler (Biometra T1 thermocycler, Jena, Germany) under the following conditions: preheated lid to 100°C, initial denaturation at 98°C for 3 min, followed by 35 rounds of denaturation (98°C for 30 s), annealing (67°C for 30 s for CRISPR-Δ32 primers and 55°C for 30 s for CRISPR-Δ35 primers), and extension (72°C for 20 s), and ended with a final extension at 72°C for 2 min. PCR products were ran on a 1.5% low electroendosmosis (EEO) agarose gel (Fisher Bioreagents) prepared with 1× Tris-acetate-EDTA (TAE) buffer for 45 – 60 min at 120 V.

PCR products were purified with the QIAquick PCR purification kit according to the manufacturer's instructions before they were sent to Genome Quebec for Sanger sequencing (primers listed in Table 6). Sequencing results were manually aligned using A Plasmid Editor (ApE) software (jorgensen.biology.utah.edu/wayned/ape/). Translation products were also predicted with ApE.

Table 5. PCR primers for analyzing DNA from monoclonal cells

Primer	**Sequence (5' → 3')**	**PCR Product Size (bp)**
CRISPR-Δ32 F	GTGACAAGTGTGATCACTTGGGTGGTGGC	339
CRISPR-Δ32 R	GGTGTTCAGGAGAAGGACAATGTTGTAGGGAGC	
CRISPR-Δ35 F	ATGTGAAGCAAATCGCAGCC	416
CRISPR-Δ35 R	TGGGAGAGACGCAAACACAG	

Table 6. Sequencing primers for analyzing PCR products from monoclonal cells

Primer	**Sequence (5' → 3')**
CRISPR-Δ32 F	GTGACAAGTGTGATCACTTGGGTGGTGGC
CRISPR-Δ35 F	ATGTGAAGCAAATCGCAGCC

2.13 Statistical analyses

Multi-group analyses were done with one-way ANOVA with a significance threshold set at $p = 0.05$. Tukey honestly significant difference (HSD) post-hoc tests were conducted on significant ANOVA results, with a significance threshold set at $p = 0.05$. Two-tailed student t-tests assuming equal variance with a significance threshold set at $p = 0.05$ were performed on pairs of independent samples.

To generate the IC_{50} curves in Figure 10, RT activity from each construct within a single experiment was normalized against its corresponding empty vector (psiRNA-7SK, psiRNA-U6, or psiRNA-H1), averaged, then plotted against the construct dose. Data was fitted with a power curve, and the IC_{50} value was found using the equation $y = mx^{-b}$ by setting y to 0.5 where y is the relative RT activity, x is the dose, and m and b are constants. IC_{50} values were calculated from three independent experiments, averaged, then compared using one-way ANOVA with a significance threshold set at $p = 0.05$.

Chapter 3: Results

3.1 Aim 1: Determining the efficacy and toxicity of promoter-shRNA cassettes

3.1.1 Identifying potent shRNA candidates against HIV-1 molecular clone NL4-3

Others have demonstrated that single or combinations of shRNAs designed to target conserved sequences within the HIV-1 genome exhibit potent inhibition against viral replication[214,216,217]. Our lab previously collated these results to identify a battery of 16 shRNAs whose targets had the highest sequence conservation, demonstrated high levels of inhibition *in vitro*, or were included in pre-clinical trials (Figure 7A). We included sh1498 which was identified by our lab[232], and sh5983 which has advanced to clinical trials[186], as positive controls for inhibition in our assays. These target the *gag* and *tat/rev* coding regions, respectively. We additionally included G5 as a positive control for T cell toxicity. sh582, sh688, sh4749, and sh7797 were newly designed for investigation as they target highly conserved regions in the 5' LTR, *psi*, *pol*, and *env* coding regions, respectively. These 23 shRNAs were cloned into an expression vector under the H1 promoter then co-expressed at different quantities with the HIV-1 molecular clone pNL4-3 in HEK293T cells to compare their ability to inhibit viral production (Figure 7B). Ten candidates with the highest antiviral activity from this assay (sh1498, sh5983, G5, L1, LTR516, P10, P28, P2, T3, S3) were cloned into lentiviral vectors and expressed in SUP-T1, a human T cell line, to test for their ability to inhibit viral replication over the long-term (Figure 8A, B). In cells transduced with an empty control and a nonsense shRNA (shNS), relative RT activity had risen to near peak levels at 8 days post-infection (dpi). Notably, cells transduced with L1, P2, and S3 had suppressed RT levels until 17 dpi and peaked at 20 dpi, representing a delay in viral replication by 10 days compared to the empty control. When tested for toxicity in a competitive growth assay, all cocultures displayed constant levels of GFP, indicating that the shRNAs were not causing a growth disadvantage (Figure 8C). L1, P2, and S3 were thus selected for further investigation.

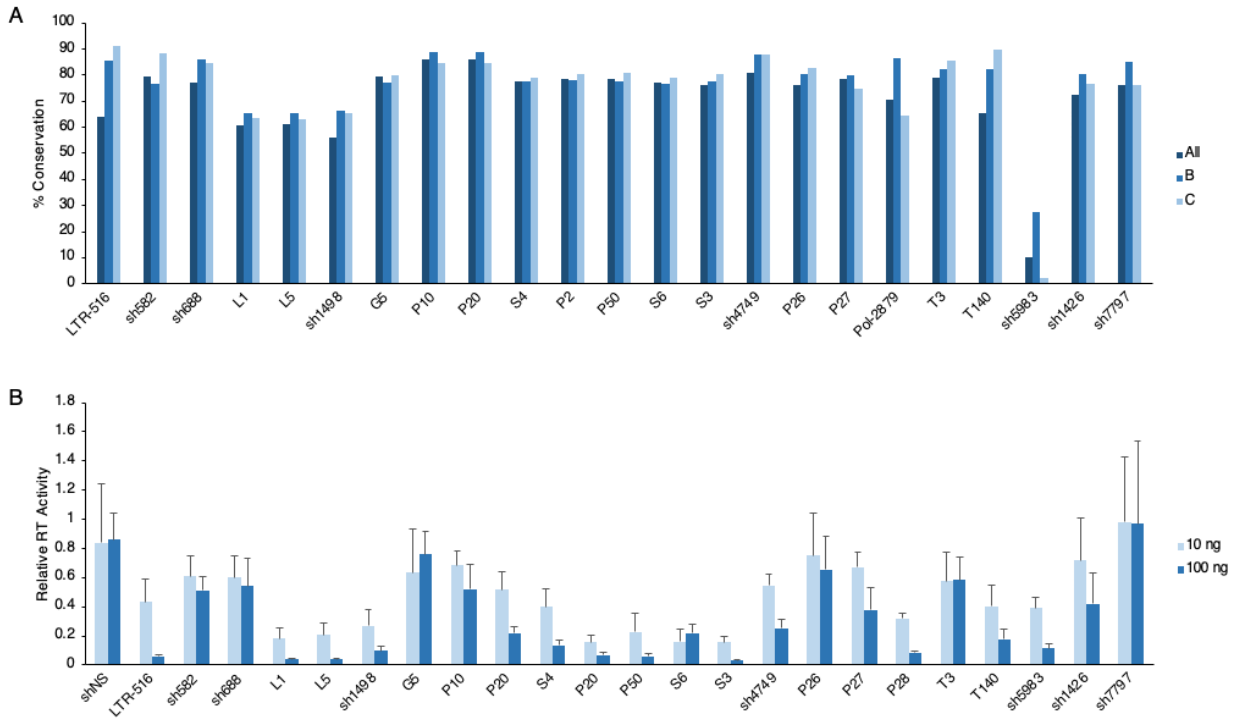

Figure 7. Screening the efficacy of the top 23 shRNA candidates. A) Sequence conservation of target sites of the selected 23 shRNA candidates. Target site conservation percentages were calculated among all HIV-1 sequences, all clade B HIV-1 sequences, and all clade C sequences in the Los Alamos National Laboratory HIV Sequence Database. B) HEK293T cells were cotransfected with 200 ng of the HIV-1 molecular clone pNL4-3 and 10 or 100 ng of each shRNA. Supernatants were harvested after 48 h and assessed for RT activity measured in counts per minute (CPM). Each CPM readout was normalized to the RT readout from cells cotransfected with an empty expression vector. Figure represents 2-4 independent experiments (n = 1-4). Error bars show SEM. Data from Figure 7B were collected by Camille Malard.

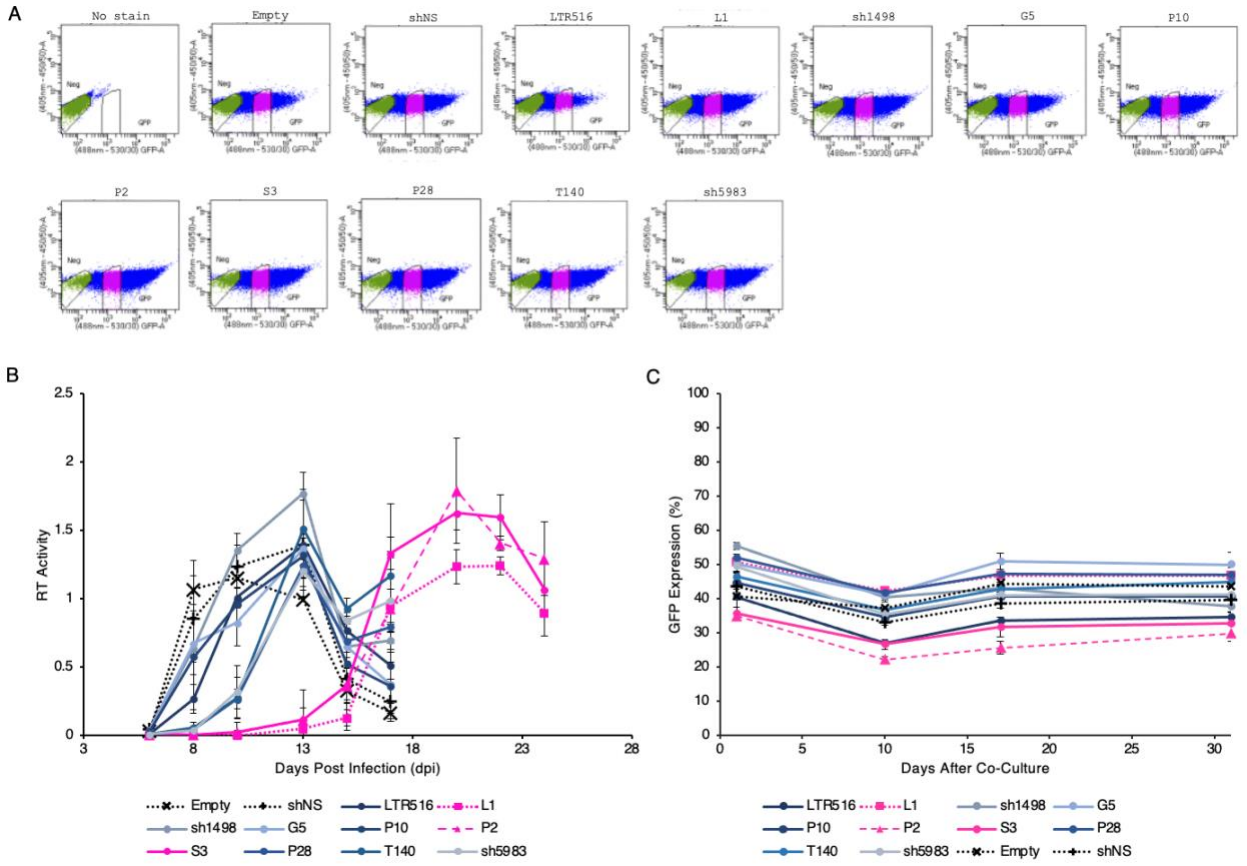

Figure 8. L1, P2, and S3 delay viral replication and do not exhibit cytotoxicity. A) Gating strategy for isolating GFP$^+$ cells. SUP-T1 cells were transduced with lentiviral vectors carrying shRNA and GFP gene cassettes. Cells highlighted in purple were used in infection and competitive growth assays shown in B and C. B) HIV-1 replication kinetics. SUP-T1 cells were transduced with lentiviral vectors carrying a shRNA. Transduced cells were then infected with the HIV-1 strain NL4-3. Supernatants were collected at multiple time points and assessed for RT activity, measured in CPM. Each CPM readout was normalized to a standard NL4-3 RT activity. C) Competitive growth experiment. Sorted, transduced cells (GFP$^+$) were plated in a 1:1 ratio with sorted, untransduced cells (GFP$^-$). % GFP expression was measured at multiple timepoints. All data from Figure 8 were collected by Camille Malard.

3.1.2 Comparing the potency of promoter-shRNA cassettes against HIV-1 production

Given the differential transcriptional efficiencies of the human type 3 RNA pol III promoters 7SK, U6, and H1[176], we hypothesized that the efficacy of L1, P2, and S3 may change depending on the promoter from which they are expressed from. We cloned each shRNA into psiRNA expression vectors under the control of 7SK or U6 (Figure 9) then compared the efficacy of each of these cassettes to the H1-shRNA combinations.

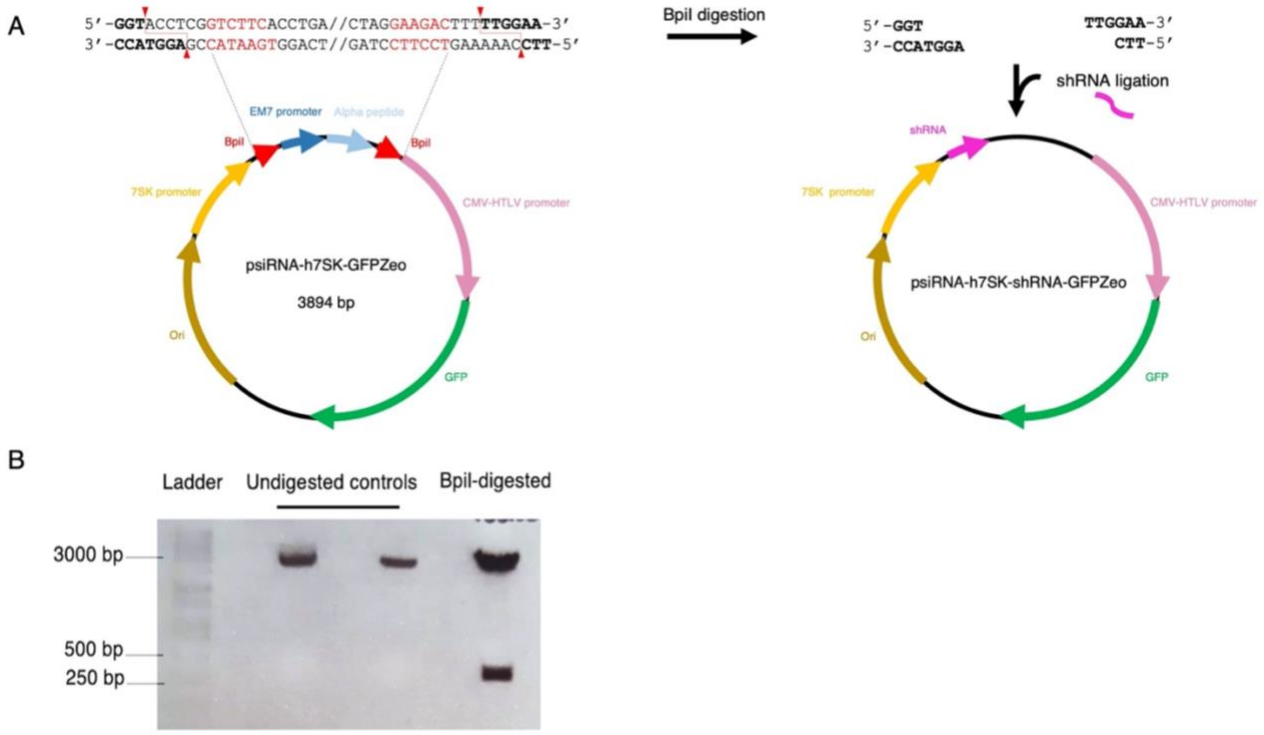

Figure 9. Workflow for cloning shRNAs into psiRNA expression vectors. A) Expression vectors containing the 7SK, U6, or H1 promoter were used. A vector containing 7SK is illustrated here as an example. The vector was digested with BpiI. ssDNA shRNA oligos were designed with overhangs complementary to BpiI restriction overhangs, annealed, then ligated into the expression vector. Expression vectors were verified with Sanger sequencing. B) The digested vector was separated from the 360-bp lacZ α-peptide cassette site (light blue) between BpiI restriction sites (red) using gel electrophoresis.

We cotransfected HEK293T cells with 0.01 – 100 ng of each cassette and with 100 ng of the HIV-1 molecular clone pNL4-3; supernatants were assessed for RT activity after 48 h (Figure 10A). Empty expression vectors encoding the promoter without a shRNA were also cotransfected with virus to provide a baseline readout of vector activity against HIV-1 production. Empty vectors showed no significant inhibitory activity against viral production, and there was no difference in viral production between empty vectors containing 7SK, U6, or H1 promoters as determined by one-way ANOVA ($F(2,15) = 3.193$, $p = 0.0699$) (Figure 10B). Each promoter-shRNA cassette dose-dependently inhibited viral production. We plotted the dose of each shRNA against its resulting relative RT activity and fitted the data with a power curve trendline (Figure 10C, E, G). All but two had R^2 values > 0.93; 7SK-L1 had a R^2 value of 0.8643 and H1-S3 had a R^2 value of

0.8202. We calculated the half maximal inhibitory concentration (IC_{50}) for each cassette based on the equation of these trendlines. All shRNAs produced from U6 had a lower IC_{50} than when expressed from H1 (Figure 10D F, H), though the results are not significant. 7SK-P2 and 7SK-S3 also had lower IC_{50}s compared to H1-P2 and H1-S3, as does 7SK-L1 compared to H1-L1 but additional experimental repeats should be performed for 7SK-L1. Overall, these cotransfection experiments suggest that shRNAs expressed from the U6 promoter mediate greater inhibition against HIV-1 production compared to H1, and shRNAs promoted from 7SK are generally more potent compared to those promoted from H1.

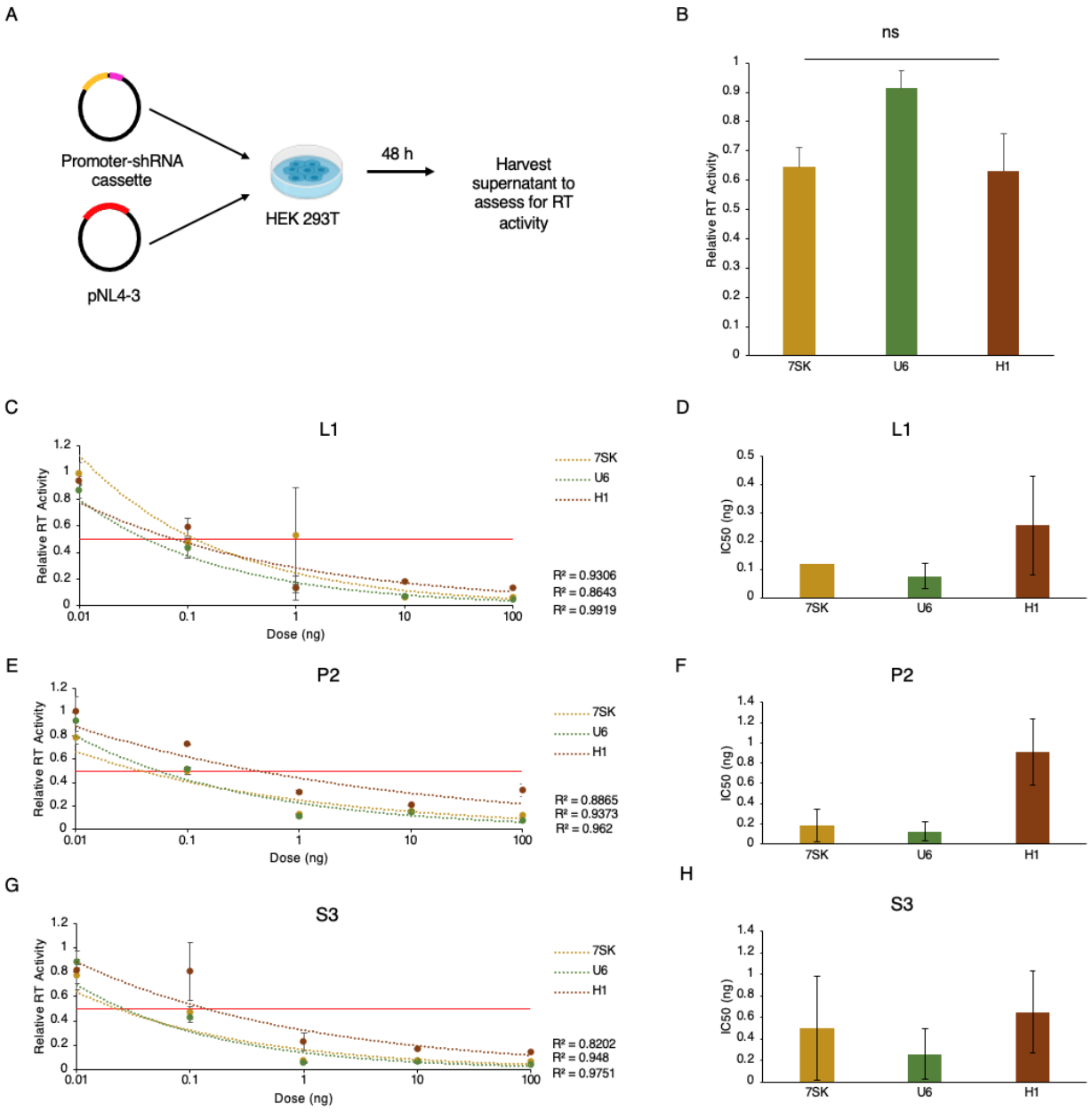

Figure 10. shRNAs expressed from H1 show less potency against viral production compared to shRNAs expressed from 7SK or U6. A) Cotransfection workflow. 0.01, 0.1, 1, 10 and 100 ng of expression vectors containing either 7SK, U6, or H1 without shRNA (empty vector), or promoter-shRNA cassettes were cotransfected with 100 ng pNL4-3 in HEK293T cells. Supernatants were harvested 48 h after cotransfection and viral production was assessed with a RT assay. B) Empty vectors do not affect viral production at 100 ng ($F(2,15) = 3.193$, $p = 0.0699$). RT activity of each data point was standardized to the average RT readout of pNL4-3 transfection alone and the mean was plotted with $n = 6$ across two independent experiments. ns = not significant. C, E, G) RT activity of shRNAs at each dose was standardized to the average RT readout of its corresponding empty vector (7SK-psiRNA, U6-psiRNA, or H1-psiRNA) and the mean was

plotted. Each graph is one representative experiment. Dotted lines represent the line of best fit. The intersection of the solid red line and the line of best fit represent the IC_{50}. Each data point represents the average standardized RT activity from one independent experiment with n = 2. Error bars show SEM. D, F, H) IC_{50} values were calculated from the equation generated from each line of best fit, then averaged across 3 independent experiments. Error bars shows SEM. Only 1 experimental replicate was available for 7SK-L1 in D, therefore no error bar is shown.

3.1.3 Long-term inhibition of NL4-3 replication using promoter-shRNA cassettes

To assess the longitudinal efficacy of each cassette, we transduced the SUP-T1 T cell line with lentiviruses carrying each cassette at a MOI of 1. Cells were sorted to obtain similar levels of cassette insertion as determined by their GFP expression (Figure 11).

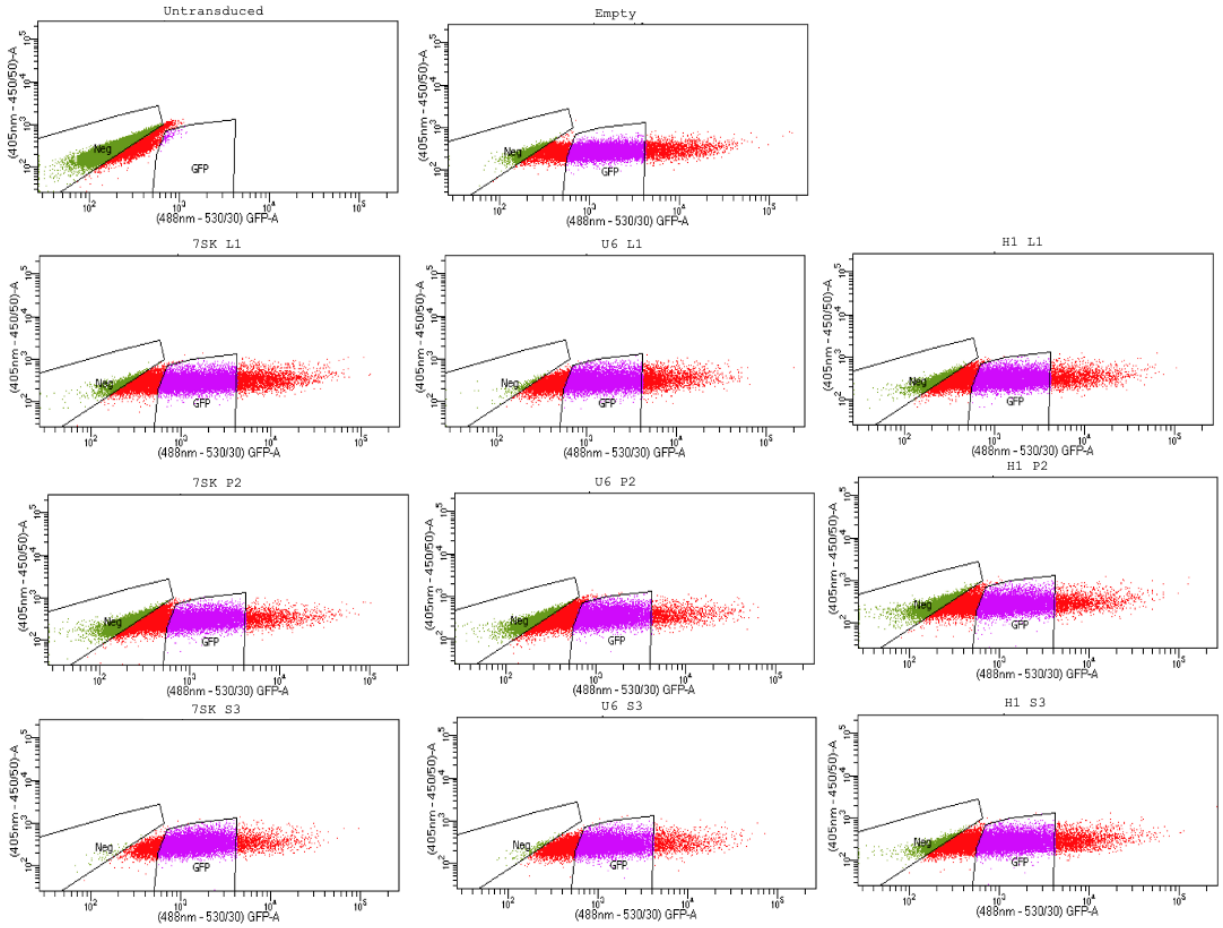

Figure 11. Gating strategy for sorting GFP$^+$ cells. Gates were set based on the negative control (untransduced; top left). Cells highlighted in purple were sorted out for use in the infection and competitive growth assays.

Following sorting, cells were infected with either a high or low inoculum of HIV-1 NL4-3. We measured RT activity in harvested supernatants to assess viral kinetics in transduced cells over the long-term. In both high- and low-inoculum conditions, RT activity in untransduced cells and cells transduced with the empty vector rose rapidly after 8 dpi and exhibited a peak in infection 15 dpi, indicating that the vector alone does not affect HIV-1 replication (Figure 12A, B). In the high inoculum condition, all cells transduced with H1-promoted shRNAs showed similar viral kinetics over 8-15 dpi (Figure 12A). At 8 dpi, there was no difference in relative RT activity between cells transduced with any promoter-shRNA cassette and the empty vector control (Figure 12C). There was significantly less RT activity in H1-shRNA-transduced cells compared to empty vector-transduced cells at 12 dpi indicating replication suppression, but the difference is no longer significant by 15 dpi. On the contrary, during this time frame, all cells transduced with 7SK- and U6-promoted shRNAs exhibited notably lower RT activity compared to empty vector-transduced cells, and RT activity remained low for at least 21 dpi. These results indicate that 7SK- and U6-promoted shRNAs are better at delaying HIV-1 replication compared to shRNAs expressed from the H1 promoter. In the low inoculum condition, all cells transduced with 7SK-and U6-promoted shRNAs also exhibited suppressed RT activity compared to cells transduced with H1-promoted shRNAs, but the viral kinetics between H1-promoted shRNAs were more variable compared to those seen in the high-dose condition (Figure 12B). Over 8-15 dpi, cells transduced with H1-L1 display similar RT activity compared to empty and untransduced cells, but cells transduced with H1-P2 or H1-S3 show significantly reduced activity ($p < 0.05$) (Figure 12D). These results suggest that P2 and S3 may be more effective inhibitors of HIV-1 replication compared to L1, but this effect is only visible at low quantities of virus. Data from low- and high-inoculum conditions demonstrate that inhibition of a high quantity of virus by P2 and S3 is abrogated when promoted from H1 but is durable when promoted from 7SK or U6. Collectively, these assays suggest that 7SK- and U6-promoted shRNAs better delay viral replication compared to H1-promoted shRNAs.

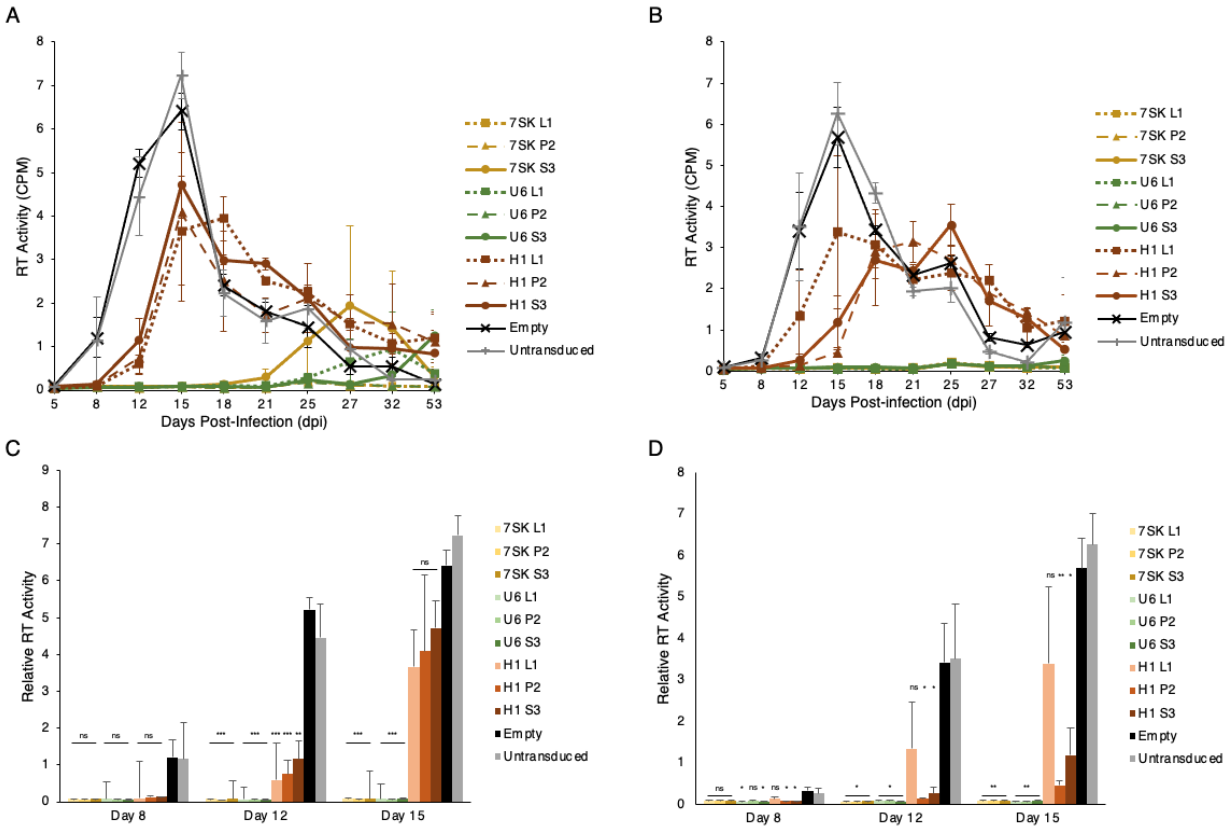

Figure 12. 7SK- and U6-promoted shRNAs better delay viral replication compared to H1-promoted shRNAs. A) Infection with a high inoculum of NL4-3. Data is the result of one experimental replicate with each data point representing the mean RT activity of 3 repeats normalized to the activity of NL4-3. B) Infection with a low inoculum of virus. C & D) Comparison of RT activity in cells infected with a high (C) and low (D) inoculum of NL4-3 over 8-15 dpi. The relative RT activity of each H1-promoted shRNA was compared to the relative RT activity of the empty vector on days 8, 12, and 15. Comparisons were made with two-tailed t-tests assuming equal variance. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *ns* = not significant. Error bars show SEM.

3.1.4 Cytotoxicity of promoter-shRNA cassettes

shRNAs can have cytotoxic effects from off-target binding, activating innate immunity, or saturating RNAi machinery[233-235]. We tested our promoter-shRNA cassettes for cytotoxicity by transducing them into SUP-T1 cells then co-culturing transduced cells with untransduced cells in a 1:1 ratio. We then measured GFP expression over time. Initial GFP expression for most cocultures fell between 40% and 50%, but 7SK-S3, U6-S3, and U6-L1 had an initial expression between 20 and 30% (Figure 13A). By 36 days after coculturing, all H1-promoted shRNAs retained similar GFP expression levels (Figure 13B), results which agree with the competitive

growth results shown in Figure 8B. GFP expression notably decreased in cells transduced with 7SK and U6-promoted shRNAs, except for P2 which did not show a significant change in expression. These results suggest that L1 and S3 mediate cellular growth defects when expressed from 7SK or U6. Unexpectedly, cocultures with the empty vector control showed a significant increase in GFP expression. This may be the result of co-culturing transduced GFP^+ cells with unhealthy GFP^- untransduced cells, leading to an increased proportion of GFP^+ cells over time. This may obscure the toxicity of the H1-shRNAs, 7SK-P2, and U6-P2. Indeed, a second experimental replicate showed that GFP levels in cells transduced with the empty vector remained steady at ~50% after four weeks of coculture (Figure 13C, D). Like the first replicate, the change in GFP expression levels in H1-promoted shRNAs were non-significant, but paired t-tests of GFP expression at day 1 and day 29 showed that all shRNAs expressed from the 7SK and U6 promoters had decreased significantly ($p < 0.01$) (Figure 13D). However, the change in GFP expression in cells transduced with 7SK-P2 and U6-P2 were not as large as those seen in cells transduced with other 7SK and U6 shRNA cassettes. These results indicate that shRNAs promoted from 7SK and U6 are associated with cellular growth defects, but not to the same degree.

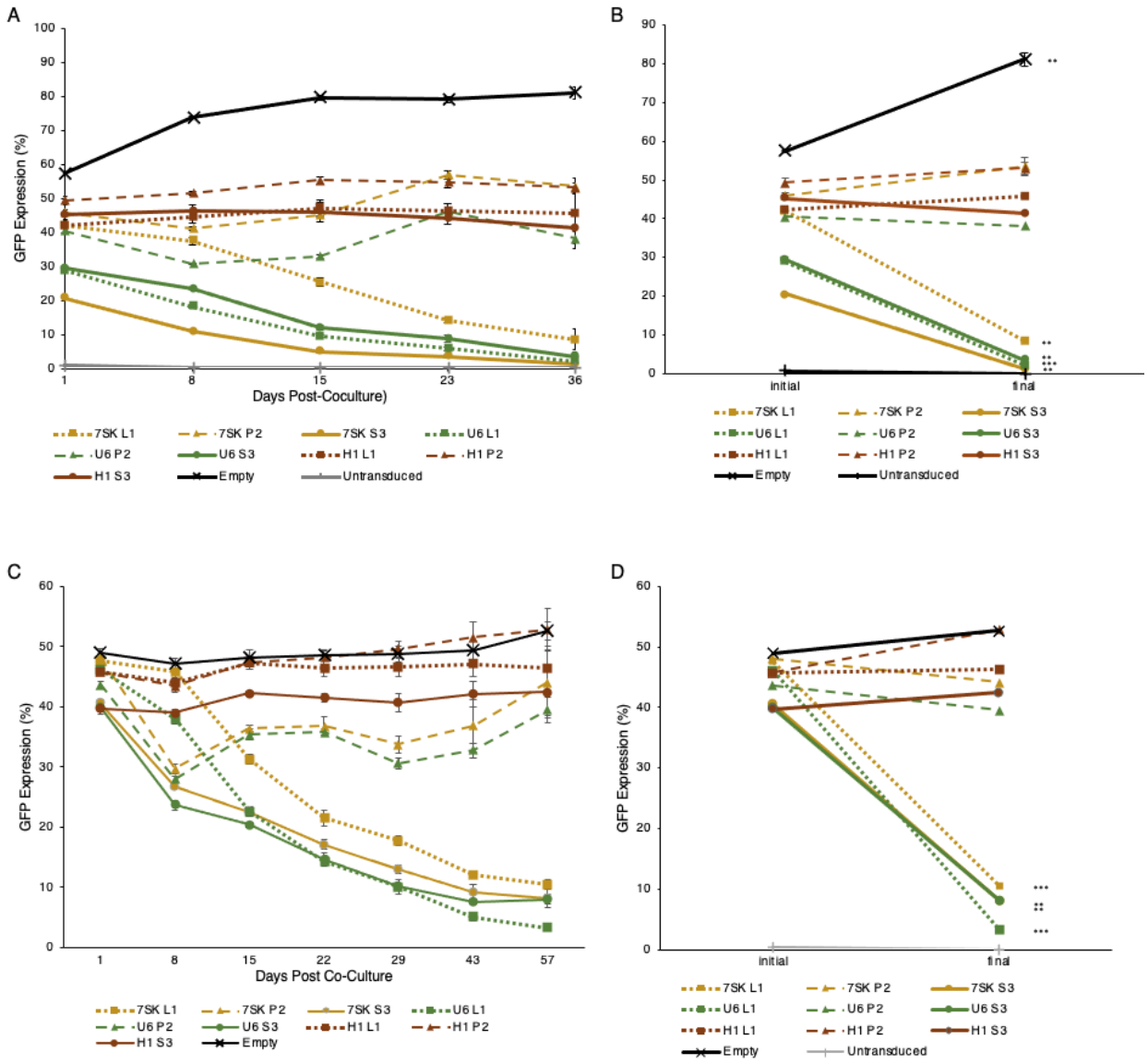

Figure 13. Some shRNAs expressed from 7SK and U6 show signs of cytotoxicity. A & C) Two experimental replicates are shown. Transduced, GFP^+ cells were sorted by FACS and plated in a 1:1 ratio with untransduced, GFP^- cells, then % GFP expression of each culture was measured after 24 h with flow cytometry. Reduction in GFP^+ cells is evident in cultures expressing L1 and S3 from both 7SK and U6. Each data point represents the mean of 3 repeats in 1 experiment. B & D) Initial GFP expression (day 1) of cocultured cells were compared to the final GFP expression (B: day 36; D: day 29) with a paired two-tailed t-test. *$p < 0.05$; **$p<0.01$; ***$p<0.001$. Error bars show SEM. The experimental replicate in C & D was set up by Dr. Ryan Goguen; data from day 8 onwards were collected by the candidate.

3.2 Combining *ccr5* gene editing with a shRNA to generate R5 and X4 resistant cells

3.2.1 Growing monoclonal PM-1 cell lines transfected with a pair of gRNAs

HSCT with *ccr5* Δ32/Δ32 donor cells have resulted in long-term remission from HIV-1, a phenomenon observed in five different individuals (see Table 2). However, it is rare to find human leukocyte antigen (HLA)-matched donors with the protective mutation and viral rebound is possible due to X4 emergence. Thus, we are interested to know whether a patient's own HSCs may be genetically modified to recapitulate the naturally occurring homozygous mutation in *ccr5*, then further modified with antiviral promoter-shRNA cassettes to inhibit X4 replication. To generate CCR5-deficient cells, we selected two pairs of published guide RNAs that were shown to mediate deletion of a 32 bp (CRISPR-Δ32 gRNAs) or 35 bp (CRISPR-Δ35 gRNAs) segment within the protein-coding region of the *ccr5* gene[229,230]. Their targets are illustrated in Figure 14A. The first pair guides a 32 bp deletion that exactly replicates the 32 bp deletion preventing CCR5 surface expression found naturally in some humans, while the second pair guides a 35 bp deletion that also abrogates CCR5 surface expression. Δ35/Δ35 HSCs generated with the second pair are currently undergoing clinical trials (NCT03164135)[231]. We attempted to replicate these precise deletions in PM-1 cells, a T cell line that expresses both CCR5 and CXCR4 coreceptors. We cloned each gRNA pair into a double vector system in which one vector encodes the Cas9 protein, a mCherry protein, and a gRNA scaffold, while the other encodes GFP and a gRNA scaffold (Figure 14B). To reduce off-target effects mediated by constitutive expression of CRISPR machinery[236], we transiently transfected the vectors into cells with standard electroporation and isolated mCh^+/GFP^+ single cells by FACS (Figure 15A). Out of 240 cells, only two that had been transfected with CRISPR-Δ32 gRNAs were expanded. We analyzed their DNA with PCR, using DNA from non-electroporated PM-1 cells as a positive control for the WT gene and DNA from non-electroporated HEK293T cells as a positive control for a heterozygous mutation as they were shown by another group to contain a 32 bp deletion in one allele[237]. PCR products from the two colonies showed a single band with the expected 339-bp size, indicating that the desired 32 bp deletion had not been created (Figure 15B).

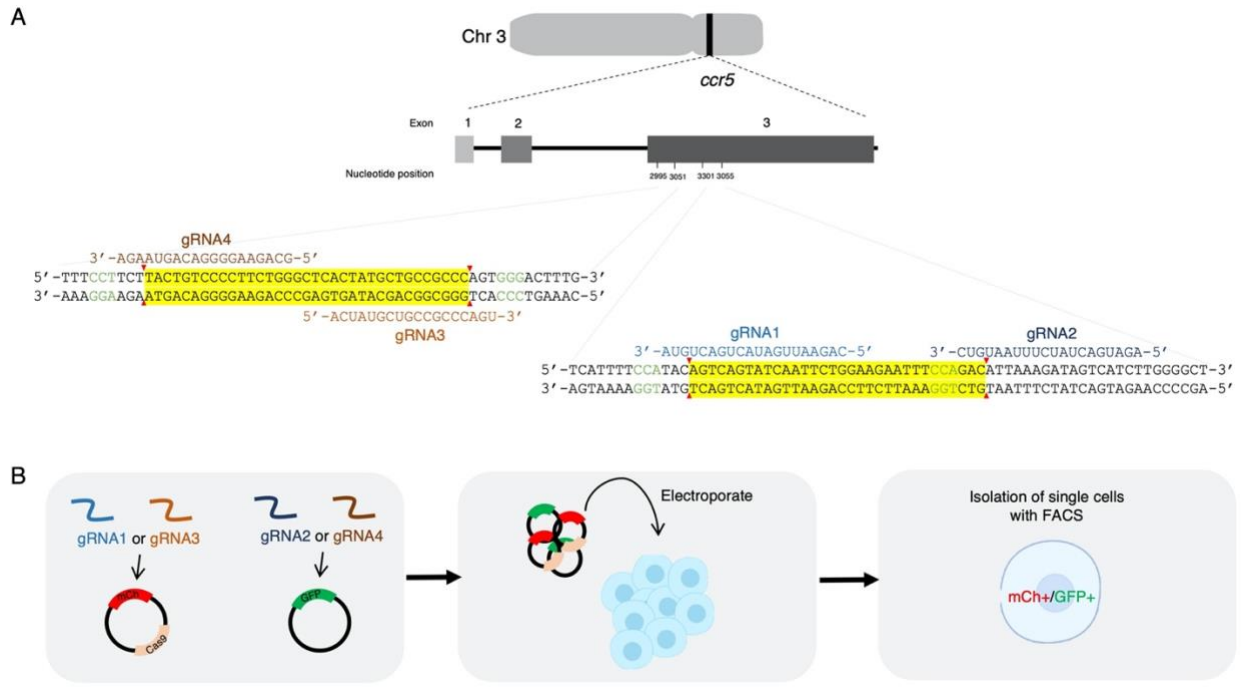

Figure 14. Generating monoclonal cells with a 32 bp or 35 bp deletion in the *ccr5* gene. A) Schematic of gRNA pair binding sites. Yellow highlighted regions indicate the expected deletion. Green nucleotides denote the PAM while red arrows denote the DSB site. B) Workflow for obtaining single cells transfected with CRISPR/Cas9 machinery.

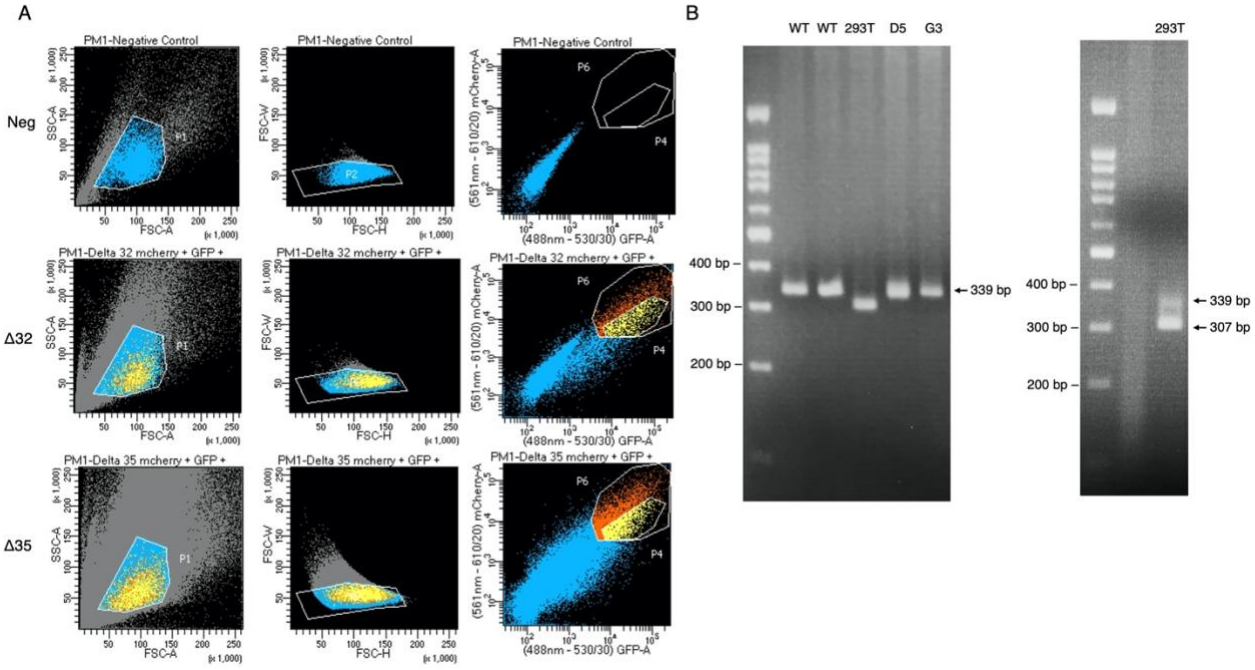

Figure 15. Two monoclonal cells obtained from PM-1 transfected with CRISPR-Δ32 gRNAs are WT. A) Gates for single, mCh^+/GFP^+ were established based on non-electroporated cells

(negative control). Blue (P1) indicates lymphocytes gated from the total population indicated in grey. mCh^+/GFP^+ cells are indicated in yellow (P4) and orange (P6). P4 is a more stringent gate compared to P6. B) D5 and G3 were the only two mCh^+/GFP^+ single cells that expanded into colonies. DNA from these cells were subjected to PCR with primers that generate a 339-bp band in WT PM-1 cells, and the products were ran on a 1.5% agarose gel. HEK293T cells which contain a 32-bp deletion in one *ccr5* allele were used as positive controls for a heterozygous mutation. The WT band is not present in the left image for unknown reasons. PCR of DNA from HEK293T cells using the same primers was performed and the products were again separated with gel electrophoresis; an image of the gel is presented on the right.

3.2.2 Growing monoclonal PM-1 cell lines transfected with a single gRNA

We were unable to generate a significant number of monoclonal cells for analysis using standard electroporation which may have been due to poor cell recovery. We thus proceeded to compare this method with the Neon Transfection System which employs a unique electroporation chamber that generates a more even electric field and reduces the change in pH, properties that should collectively increase cell viability. For this test, we simplified the transfection protocol by transfecting only the pU6-mCherry vector into which we cloned either gRNA1 or gRNA3 (pU6-mCherry:gRNA). First, we electroporated pU6-mCherry:gRNA into PM-1 cells by applying an exponential decay pulse (voltage = 0.25 kV, capacitance = 950 μF, time constant = 21 ms) to 1×10^7 PM-1 cells mixed with the vector. To obtain a monoclonal cell line, we used FACS to isolate single, mCh^+ cells into 96-well plates containing conditioned media. The gating strategy is shown in Figure 16A. Cells were incubated at 37°C and assessed for growth. Of 240 single cells, none developed into viable clusters. Next, using the Neon Transfection System which applies a square-wave pulse at intervals of 1 ms, we transfected the vector into 1×10^6 PM-1 cells, following the manufacturer's recommendations. Single mCh^+ cells were isolated with FACS (Figure 16B) and grown in conditioned media in 96-well plates. Four to five visible clusters of cells per plate began forming at approximately 2 weeks. When the cell cluster diameter was ~1 mm, we transferred them into 6-well plates with 2 ml of RPMI for further expansion.

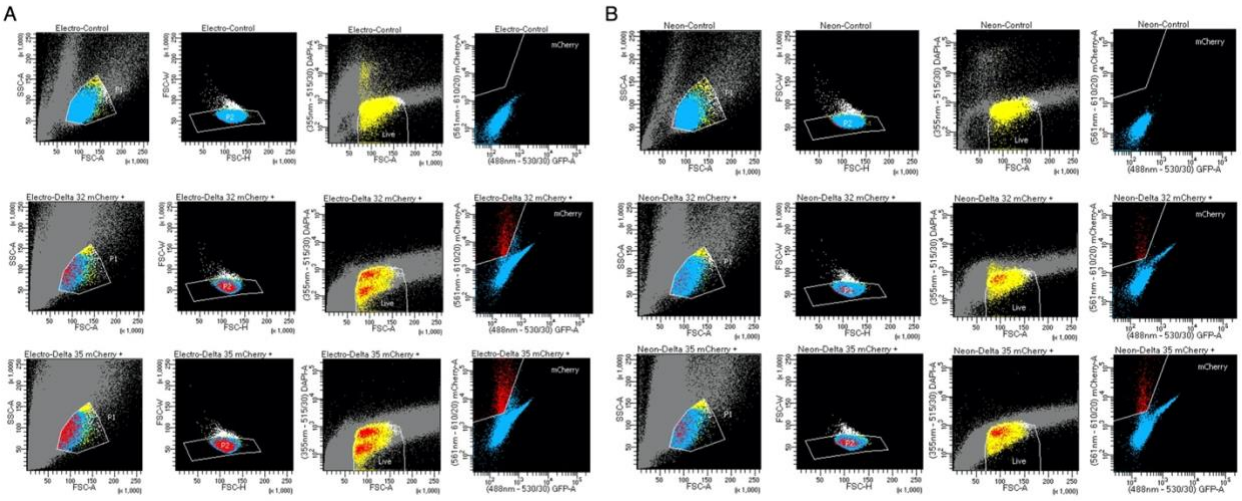

Figure 16. Gating strategy for isolating single mCh$^+$ cells transfected with CRISPR/Cas9:gRNA. PM-1 cells were transfected with expression vectors containing Cas9, gRNA1 or gRNA3, and a mCh fluorescent protein using A) standard electroporation or B) the Neon transfection system. White populations (P1) indicate lymphocytes gated from the total population indicated in grey; yellow populations indicate single cells; blue populations indicate live cells; red populations indicate mCh$^+$ cells. Top: Non-electroporated cells were used as negative controls to set the mCh$^+$ gate. Middle: Cells electroporated with gRNA1. Bottom: Cells electroporated with gRNA3.

We performed PCR analysis on DNA from cells transfected with pU6-mCherry:gRNA3 using the forward and reverse primers CRISPR-Δ35 (Table 5) which yields a 416 bp product. Amplicons of most cells appeared at the expected band size. Notably, clone 9 showed two bands at ~416 bp and ~360 bp, indicating a heterozygous genotype (Figure 17A). We sequenced the PCR products by Sanger sequencing and manually aligned sequences around the DSB site with the WT sequence and found unreadable nucleotides (N) within 5 nt upstream and downstream the DSB site in six samples (Figure 17B). Chromatogram analysis showed that the predicted nucleotide at these sites matches the WT sequence. Additionally, we found a G to T mismatch mutation 2 nt downstream the DSB site in clone 7. Notably, we were unable to align the sequence from clone 9 around the DSB but upon closer examination of the sequence, we found that it harboured a 59 bp deletion that spanned the PAM, DSB, and gRNA binding site (Figure 17C). We used ApE software to predict the amino acid (AA) sequence that results from translation of the *ccr5* gene with the 59 bp deletion and found that two thirds of the protein is predicted to be truncated (Figure 17D).

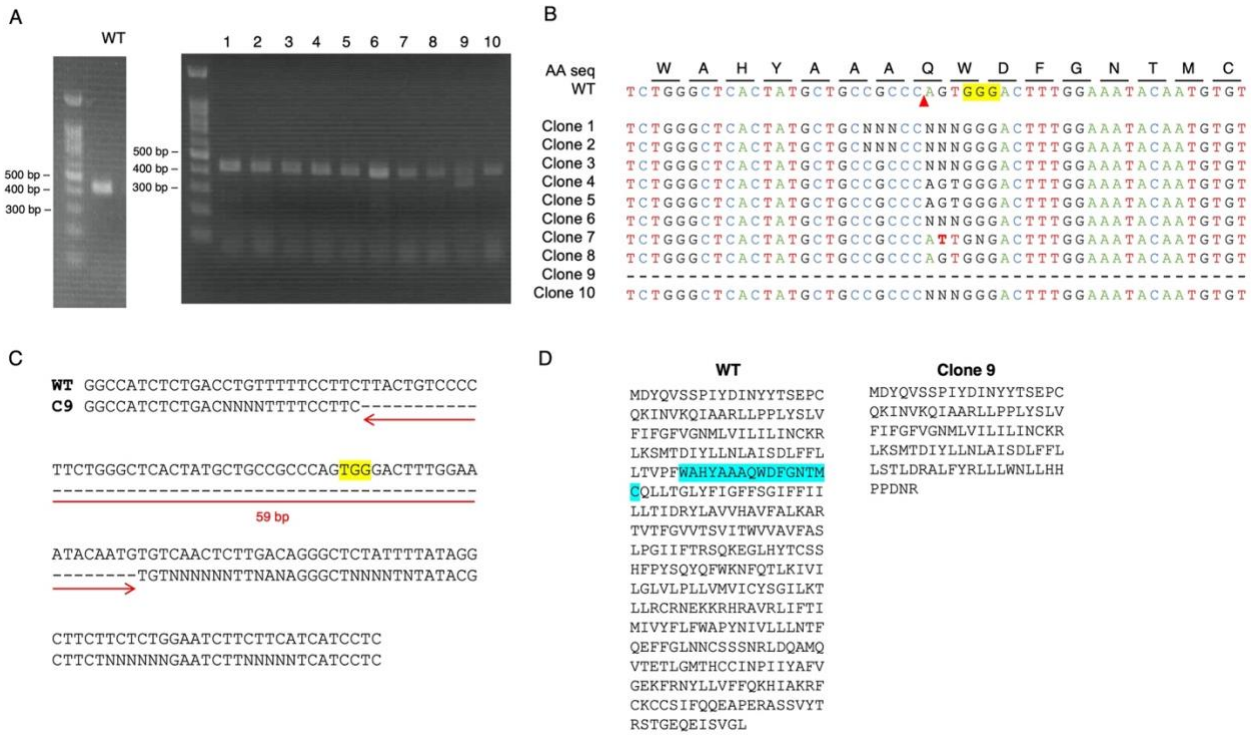

Figure 17. PCR and sequencing analysis of monoclonal cells transfected with gRNA3. A) DNA harvested from monoclonal cells 1-10 was amplified with PCR primers flanking the DSB site (expected product size of 416 bp). The products were resolved on a 1.5% agarose gel. A representative PCR of WT PM-1 cells is also shown. B) Purified PCR products were submitted to Genome Quebec for Sanger sequencing. 25 nt sequences upstream and downstream the DSB site, indicated by the red arrow, were aligned with the WT sequence in ApE. The open reading frame coding for the WT amino acid (AA) sequence is illustrated above the WT nucleic acid sequence. The highlighted three-letter sequence indicates the PAM. Bolded letters indicate a mismatch from the WT sequence. N indicates a low confidence read during Sanger sequencing. C) A 59 bp deletion was found in Clone 9. D) The 59 bp deletion was removed from the WT *ccr5* sequence in ApE and the software was used to predict the AA sequence of the resulting protein. The blue highlighted sequence corresponds to the WT AA sequence shown in B.

Upon PCR analysis, all DNA from cells transfected with pU6-mCherry:gRNA1 showed the expected band size of ~339 bp (Figure 18A). However, sequencing showed that clone 8 harbours a single nucleotide insertion located 4 bp upstream the DSB site which disrupts the reading frame (Figure 18B). This results in an early stop codon that truncates the last 26 AA of the protein (Figure 18C). There is a notable drop in chromatogram quality at position 85 (Figure 18D) where the insertion takes place. This is indicative of a heterozygous DNA sample, suggesting that the indel may have occurred in one allele only. Single allele sequencing should be performed to confirm the genotype.

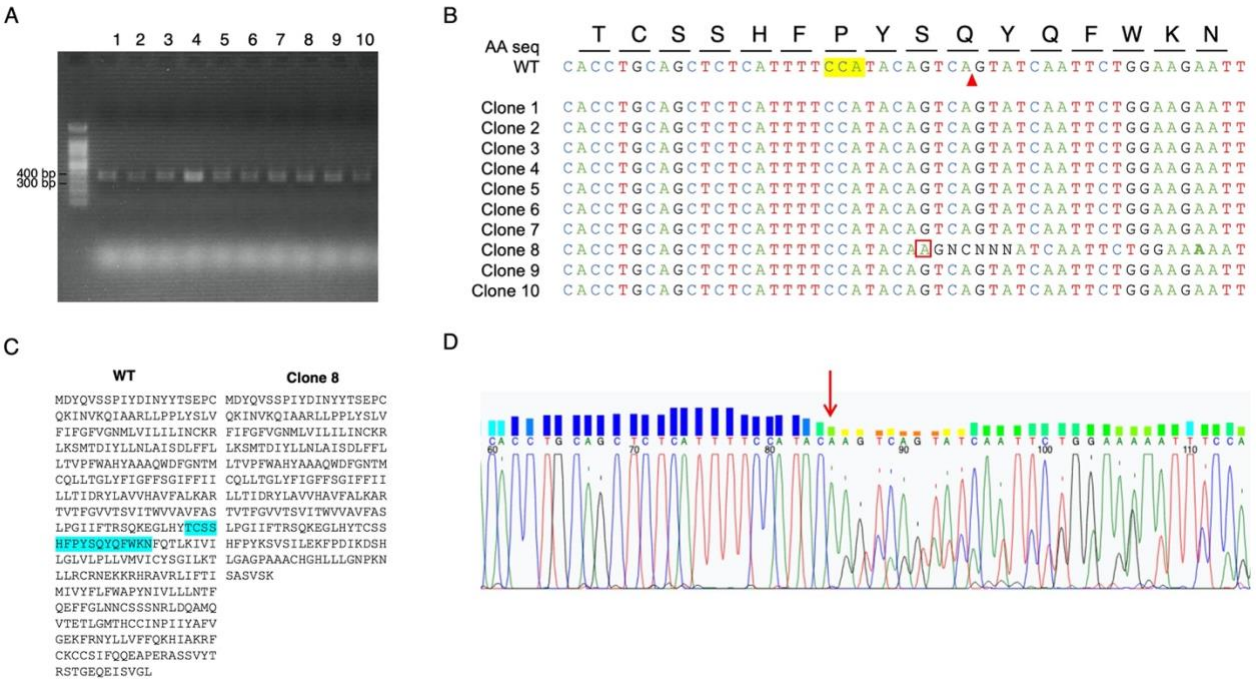

Figure 18. PCR and sequencing analysis of monoclonal cells transfected with gRNA1. A) DNA harvested from monoclonal cells 1-10 was amplified with primers flanking the DSB site (expected product size of 339 bp). B) Clone 8 harbours a single nucleotide insertion, indicated by the red box. The highlighted three-letter sequence indicates the PAM. The red arrow indicates the DSB site. C) ApE software was used to predict the protein product that results from the single nucleotide insertion found in clone 8. D) Chromatograph of the DNA sequence 25 nt upstream and downstream of the DSB of clone 8. The red arrow indicates the position of the single nucleotide insertion.

Chapter 4: Discussion

4.1 Selection of potent promoter-shRNA cassettes

Multiple large-scale studies have determined that shRNAs show antiviral activity against HIV-1 production[214,216,217]. However, HIV-1 is highly mutable because of its error-prone RT and inhibition by a single shRNA is temporary[220]. Like cART, any RNAi-based therapy will require a combination of shRNAs, but individual shRNAs should be carefully selected for their ability to durably inhibit replication. The cited studies had used shRNAs targeting different regions of the HIV-1 genome and expressed from different promoters, making it difficult to determine the best promoter-shRNA cassette that may be pursued for anti-HIV-1 gene therapy. Our lab previously attempted to systematically assess shRNA efficacy by cloning 23 shRNAs under the control of the H1 promoter and investigating their ability to inhibit viral production, replication, and induce cytotoxicity in SUP-T1 cells (Figures 7-8). Out of this panel, we found that L1, P2, and S3, were most effective at delaying viral replication and did not cause defects in cell growth when tested in a competitive growth culture with untransduced cells.

In addition to the shRNA target sequence, another factor that can affect shRNA potency and/or toxicity is the choice of promoter. In the literature, there is conflicting evidence for how the three type 3 RNA pol III promoters impact shRNA potency: three studies found that U6-promoted shRNAs are more active than H1-promoted shRNAs[238-240]; one study found contradictory results with H1-promoted shRNAs being more potent compared to 7SK- and U6-promoted shRNAs[241]; a fifth study found no difference in shRNA potency between the three promoters[242]. Using sh1498, a shRNA targeting a conserved sequence in *gag*[219], and sh5983, a shRNA that targets the tat/rev coding region[186], our lab previously showed that in HEK293T cells, the 7SK and U6 promoter drive expression of more potent shRNAs which correlated with an increased expression of the shRNA[176]. Given that L1, P2, and S3 outperformed sh1498 and sh5983 in delaying viral replication when expressed from the H1 promoter, it is worthwhile to further evaluate the efficacy of these three shRNAs by comparing their activity when expressed from 7SK and U6. Our cotransfection experiments showed that H1-promoted shRNAs generally exhibit less inhibitory activity compared to those expressed from U6 and 7SK. In cell cultures infected with a low inoculum of HIV-1, we found that the inhibitory activity between the three H1-promoted shRNAs could be better teased out: L1 was the least effective at delaying HIV-1 replication compared to P2 and S3 (Figure 12D). This suggests that the shRNA sequence itself offers an additional level of modulation – the

suppressive activity of the cassette therefore depends on both the promoter and the shRNA sequence. Overall, our cotransfection experiments strengthen the conclusion that 7SK and U6 shRNA cassettes result in more potent shRNAs.

Longitudinal infection assays in SUP-T1 cells provided corroborative data that 7SK and U6-promoted shRNAs are more effective at delaying viral replication. In the high-dose condition, cells transduced with H1-shRNAs showed an increase in RT activity at 12 dpi, while cells transduced with U6-S3, the least effective cassette from 7SK or U6, only began showing an increase in RT activity at 25 dpi. The H1-shRNAs showed some ability to delay replication near the beginning of infection (12 dpi, Figure 12C), but the delay was short-lived as RT levels were comparable to the empty control by 15 dpi. However, the overall RT activity at replication peak is reduced compared to infected untransduced cells, indicating that the H1-promoted shRNAs still display some antiviral activity. Given that all cells transduced with 7SK- and U6-promoted shRNAs are suppressed in this time frame, our infection experiments also lend credence to the conclusion that these promoters, by some mechanism, produce more potent shRNAs compared to H1. This apparent potency could be due to an increased production of shRNAs from the 7SK and U6 promoters, as was previously demonstrated in our lab using Northern blots[176]. Another possible mechanism involves the identity of the promoter's transcriptional start site, which is defined by a certain nucleotide distance from the TATA box sequence. The 7SK and U6 promoters mediate transcription at or within 2 nt of the +1 start site, but the H1 start site is more variable and thus produces transcripts with greater variation in the 5' end which can impact downstream shRNA processing and thus its efficiency[243].

It is worthwhile to note that each data point in Figures 12A and 12B is the average of three replicates. While assessing the replicates individually for each sample, we noticed that, in a minority of samples, the viral kinetics differed between replicates. For some, viral breakthrough appeared at different times, but for most anomalies infection simply did not take hold in one of the three replicates. Figure 12 includes these anomalous replicates. The distinct temporal signatures of viral peaks were consistent: the samples that peaked earlier did so in all replicates as did the samples that peaked later; the anomalies are mentioned here to acknowledge that they obscure the precise amplitude and width of viral peaks. Overall, these long-term data suggest that while H1-promoted shRNAs exhibit some antiviral activity, it is not potent enough to delay replication breakthrough. Our past experiments comparing the most effective shRNAs (Figure 7B) show that

shRNA potency depends on the shRNA sequence itself, but the data here reveal that assessing shRNAs independent of promoter activity gives incomplete information of its overall performance. Together with results from the cotransfection experiment, our infection assays show that H1-promoted shRNAs are less effective as they do not significantly delay the peak of viral replication, and that 7SK and U6 can be used to express shRNAs that durably inhibit HIV-1 replication. However, overexpression of transcripts from these promoters can be detrimental to cell health and thus each promoter-cassette should be tested for cytotoxic effects.

4.2 Assessing the safety of optimized promoter-shRNA cassettes

shRNA expression needs to be carefully optimized as overexpression can lead to cell death. Having determined that 7SK and U6 reduce the IC_{50} of our three shRNAs, we wondered whether the potency was to the detriment of cell health. We evaluated the toxicity of each promoter-shRNA cassette with a competitive growth assay. Co-culturing transduced cells (GFP^+) with untransduced cells (GFP^-) in a 1:1 ratio should yield a GFP expression of 50%. If the shRNAs were cytotoxic or caused defects that slowed cellular proliferation, the proportion of GFP fluorescence in cocultures would reduce over time. We observed this decreasing pattern in cocultures with 7SK-L1, 7SK-S3, U6-L1, and U6-S3. The same shRNAs sustained a steady GFP expression under transcriptional control of H1. However, our lab had previously tested the cytotoxic effects of sh1498 and sh5983 expressed from each promoter and found that none of the cassettes impacted HEK293T cell proliferation though all cassettes were transfected at high amounts (1.5 and 2 μg)[176]. Juxtaposition of these results suggest that toxicity may be sequence-specific, but we have not yet probed the mechanism for toxicity.

Our lab's past work with sh1498 and sh5983 showed that their potency correlated with increased expression of the guide strand – if the same is true for the three shRNAs tested here, the reason for cell death might be explained by the dysregulation from overexpression of shRNAs. This concept has been demonstrated *in vivo* in mice, where overexpression of shRNAs from adeno-associated virus (AAV)8 vectors in hepatocytes resulted in severe liver injury and death[235]. Essential regulatory microRNAs (miRNAs) that are processed by the RNAi machinery are outcompeted by an overabundance of shRNAs; this dysregulation of miRNAs contributes to cell death. This mechanism could be tested by profiling miRNA expression with RNA sequencing in cells transduced with the cassettes. Another mechanism of cell death includes off-target binding

of transcripts through partial sequence complementarity, resulting in perturbations of essential gene functions; this may be tested through genome-scale expression profiling with microarrays. A final insight into the cytotoxicity mechanism comes from studies into tumour suppressive RNAs. The authors found that a higher GC content in the shRNA seed sequence, defined by positions two through seven at the 5' end of the guide strand, correlated with greater cytotoxicity through off-targeting of the 3' UTR of a network of survival genes in cancer cell lines[244]. In a separate screen, they found that human and mouse cancer cells transfected with siRNAs containing G-rich seeds displayed the lowest viability, and that two major families of tumour suppressive miRNAs contained G-rich seeds that likely contributes to their cancer cell killing ability[245]. From these findings, we can hypothesize that the GC content in S3 and L1 contribute to declining GFP^+ cell viability observed in our competitive growth assays, but only when expressed at high levels from the 7SK and U6 promoters as they do not show the same degree of cytotoxicity when expressed from the H1 promoter.

Curiously, cells transduced with an empty vector that served as a negative control for toxicity displayed a significant increase in GFP expression over time ($p = 0.004$ by two-tailed t-test). Selection for GFP^+ cells may indicate that the untransduced GFP^- cells were unhealthy at the seeding stage. Over time, the unhealthy cells would slowly die off and leave a greater proportion of GFP^+ cells. This aligns with what is seen with the empty vector control, but we would also expect for the other samples to have increased GFP+ proportions. It could be possible that we do not see these results because the cytotoxic effects of the shRNAs compensate for the increased proportion of GFP^+ cells. This would be problematic as the cytotoxic effects of seemingly safe shRNAs would be masked. Another replicate should be conducted to verify these results. However, from current data, we can confidently conclude that L1 and S3 are cytotoxic when expressed from 7SK and U6, and that the same shRNA can have dramatically different cytotoxic effects depending on the promoter from which is expressed from. Tentatively, competitive growth and infection experiments collectively point to 7SK-P2 and U6-P2 as the best candidates to pursue for combination therapy.

4.3 Generation of a CCR5-knockout cell line

All individuals considered cured of HIV received stem cell transplants from donors with a natural homozygous mutation in the *ccr5* gene that results in loss of cell-surface CCR5 expression. Given that this phenotype has no known detrimental effects, that the CCR5 coreceptor serves as

one of the first major points of contact required for infection, and that viral coreceptor switching can lead to emergence of CXCR4-tropic viruses, we began to investigate whether a combination of cellular modifications with the shRNAs described above and *ccr5*-gene editing could inhibit replication of R5 and X4 viruses. We first attempted to create a monoclonal CCR5-knockout cell line before proceeding with further modifications. We chose to set up this modification in PM-1 cells, a T lymphocytic cell line that expresses both CCR5 and CXCR4. Clinical trials using CRISPR-Cas9 modified HSPCs are currently underway, and preliminary reports show no gene-editing related adversities[231]. Given these results and its ease of use, we also chose to use CRISPR-Cas9 to create homozygous deletions in the *ccr5* gene. A double-stranded break by Cas9 is repaired through NHEJ which introduces indels at the repair site. These can cause frameshift mutations that result in truncated proteins. Truncated versions of CCR5 are not expressed at the cell surface, but because indels are randomly generated, we wanted to employ a CRISPR-Cas9 system that could generate a predictable deletion that reliably results in truncated CCR5. Two groups had published two different pairs of gRNAs that generate a 32 or 35 bp deletion in the *ccr5* gene that results in early stop codons. Thus, we attempted to transfect PM-1 cells with these gRNA pairs by electroporating cells with a mix of two vectors, each cloned with one of the two gRNAs. One of the vectors, pU6-mCherry, also coded for the Cas9 protein as well as the mCherry fluorescent protein. The other vector coded for GFP.

During our first attempt at transfecting cells with CRISPR machinery, we used a standard electroporation method with an exponential decay pulse to transfect Δ32-gRNA (Table 4). Though electroporation causes massive cell death, we attempted to allow the cells to recover by incubating them for 48 h before using FACS to isolate single, mCh^+/GFP^+ cells. Despite culturing single cells in conditioned media containing growth factors secreted by healthy WT cells, out of multiple rounds of electroporation and sorting with 240 single cells sorted per round, we were only successful in amplifying two samples in total. These were WT by PCR analysis (Figure 15B). Electroporation creates a cellular stress response[246] and it is possible that the 48 h between transfection and sorting, another stressful event, was not enough for recovery. Because mCh and GFP expression deteriorates over time, the window we could provide between electroporation and cell sorting was limited.

Hypothesizing that the electroporation method used was detrimental to cell health, we compared it to a second method using the Neon Transfection System where the electric current is

delivered in square wave pulses and a more uniform electric field is generated by a thinner electrode. Another group had achieved favourable transfection results on other T cell lines with this system[247]. We also opted to transfect a single gRNA (gRNA1 and gRNA 3, Table 3). Single cells were again cultured in conditioned media after FACS. With the Neon Transfection System, we found five to six monoclonal cell colonies per 96-well plate containing 60 sorted cells while standard electroporation resulted in at most one colony per plate. These results indicate that the Neon Transfection System induces less cellular stress, allowing cells to recover more quickly. We expanded the cells by transferring them from 96-well plates to 6-well plates but not all colonies grew after transfer. While the Neon Transfection System improved cell viability, the low percentage of amplification and the variable health of the colonies that did form indicate that electroporation parameters need to be carefully optimized. Transfection methods that further reduce cellular stress may increase cell viability and thus increase the probability of identifying the desired homozygous mutation.

Of the 10 clones obtained from gRNA3, six (clones 1, 2, 3, 6, 7, 10) had at least one unreadable nucleotide within five nucleotides upstream and downstream the DSB site. Trace analysis of the chromatograms of clones 1, 2, 3, 6, 7, and 10 showed overlapping peaks in the unreadable regions. The nucleotides predicted by the software matches the WT sequence, but the presence of overlapping traces suggest that there were competing nucleotides at those positions, indicating that one of the alleles had been altered. Single allele sequencing should be performed to determine the genotype of the mutations. Regardless of whether the unreadable nucleotides are substitution mutations, unless they result in an early stop codon, they are less likely to cause a major protein truncation compared to a frameshift mutation or a large deletion. PCR analysis of clone 9 indicated that one of the alleles had a ~25 bp deletion, but sequencing more precisely revealed that the deletion spanned a 59 bp region that included the gRNA binding site and PAM. This is unusually large for NHEJ as indels are usually less than 10 bp[248]. An alternative NHEJ mechanism, microhomology-mediated end-joining (MMEJ), has been reported in human cells[249] and involves a more extensive resection of the exposed ends of the DSB to generate microhomologous arms that are annealed before gaps are filled in and ligated[250]. Selection of MMEJ over NHEJ is influenced by the cell cycle and competition between the DNA repair proteins Ku and poly ADP-ribose polymerase 1 (PARP1). PARP1 is activated by DSB and plays a role in mediating MMEJ, which is most active during the S phase of the cell cycle[251]. Cell conditions that

favour MMEJ could be a possible explanation for the large deletion observed. Compared to the wild type 352 AA CCR5 protein, the predicted protein product is truncated at the 105th AA and unlikely to be expressed at the cell surface. Flow cytometry experiments should be performed to assess this hypothesis.

Of the 10 clones obtained from gRNA1, all appeared to have WT bands. Sequencing data revealed a single nucleotide insertion in clone 8 four nucleotides upstream the DSB site, demonstrating that small indels can be easily missed with PCR and gel electrophoresis. Electrophoresis could be better optimized to detect indels, as another study reported that resolution as low as 3 bp is possible with gel electrophoresis using 4-6% low electroendosmosis agarose[252]. Like clone 9, the predicted protein product is also truncated and may indicate not be expressed at the cell surface. Chromatogram noise increased significantly at the site of the indel, indicating a heterozygous mix of alleles. Its genotype may be confirmed with single allele sequencing.

Overall, results from single gRNA-editing points to the variability in repair products that result from Cas9-mediated DSB. Given that the Neon Transfection System results in greater cell viability post-FACS, future steps involve transfecting gRNA pairs into PM-1 cells with this system to generate more reliable deletions. We will select monoclonal cells homozygous for this deletion and confirm downregulation of surface CCR5 expression with flow cytometry. We expect that these cells will be resistant to R5 viruses and will determine whether they can also be resistant to X4 viruses upon transduction with 7SK-P2 or U6-P2.

Conclusions

Antiviral molecules developed for use in HIV-1 gene therapy require stringent testing and optimization to ensure that they can durably inhibit viral replication and cause no harm to cells. All anti-HIV shRNA screens in the literature tested shRNAs from a single promoter and were performed in different cell lines. This thesis collated the best candidates from these screens and systematically tested the highest performing ones from three different human RNA Pol III Type 3 promoters. We found that promoter choice significantly impacts shRNA efficacy with 7SK and U6 outperforming H1when it comes to inhibiting viral production and delaying viral replication, which confirms past findings by our lab. We also showed that shRNA toxicity is dependent on the sequence, as under a single promoter, not all shRNAs were cytotoxic to the same degree. Multiple shRNAs, either in combination with each other or with other classes of antiviral molecules, is required to prevent escape mutations. Our results indicated that 7SK-P2 and U6-P2 are the best candidates to pursue for combination testing but optimization is needed to minimize their toxicity. We showed that PM-1 cell survival post-sorting is compromised when they are electroporated with exponential decay pulses but improves when the Neon Transfection System is employed. Using the CRISPR/Cas9 system with a single gRNA, we identified two monoclonal cell colonies that potentially contain truncating mutations. These will be tested for their genotype and CCR5 surface expression but given the heterogenous nature of CRISPR/Cas9-mediated indels, we are more likely to obtain consistent deletion products when we use a pair of gRNAs. The results of this thesis guide toward a better understanding of developing a monoclonal CCR5-KO cell line that can be combined with safe and potent anti-HIV-1 shRNAs. These results may inform how future patient HSCs should be modified for autologous transplants to protect against both CCR5- and CXCR4-tropic viruses.

Bibliography

1 CDC. Pneumocystis Pneumonia --- Los Angeles. 250-252 (Centers for Disease Control (CDC), 1981).

2 CDC. Kaposi's Sarcoma and Pneumocystis Pneumonia Among Homosexual Men — New York City and California. 305-308 (Centers for Disease Control (CDC), 1981).

3 Gottlieb, M. S., Schroff, R., Schanker, H. M., Weisman, J. D., Fan, P. T., Wolf, R. A. & Saxon, A. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med* **305**, 1425-1431, doi:10.1056/NEJM198112103052401 (1981).

4 Coffin, J., Haase, A., Levy, J. A., Montagnier, L., Oroszlan, S., Teich, N., Temin, H., Toyoshima, K., Varmus, H. & Vogt, P. Human immunodeficiency viruses. *Science* **232**, 697, doi:10.1126/science.3008335 (1986).

5 Rous, P. A Sarcoma of the Fowl Transmissible by an Agent Separable from the Tumor Cells. *J Exp Med* **13**, 397-411, doi:10.1084/jem.13.4.397 (1911).

6 Bittner, J. J. Some Possible Effects of Nursing on the Mammary Gland Tumor Incidence in Mice. *Science* **84**, 162, doi:10.1126/science.84.2172.162 (1936).

7 Gross, L. Development and serial cellfree passage of a highly potent strain of mouse leukemia virus. *Proc Soc Exp Biol Med* **94**, 767-771, doi:10.3181/00379727-94-23080 (1957).

8 Jarrett, W. F., Martin, W. B., Crighton, G. W., Dalton, R. G. & Stewart, M. F. Transmission Experiments with Leukemia (Lymphosarcoma). *Nature* **202**, 566-567, doi:10.1038/202566a0 (1964).

9 Baltimore, D. RNA-dependent DNA polymerase in virions of RNA tumour viruses. *Nature* **226**, 1209-1211, doi:10.1038/2261209a0 (1970).

10 Temin, H. M. & Mizutani, S. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature* **226**, 1211-1213, doi:10.1038/2261211a0 (1970).

11 Morgan, D. A., Ruscetti, F. W. & Gallo, R. Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science* **193**, 1007-1008, doi:10.1126/science.181845 (1976).

12 Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D. & Gallo, R. C. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci U S A* **77**, 7415-7419, doi:10.1073/pnas.77.12.7415 (1980).

13 Kalyanaraman, V. S., Sarngadharan, M. G., Robert-Guroff, M., Miyoshi, I., Golde, D. & Gallo, R. C. A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia. *Science* **218**, 571-573, doi:10.1126/science.6981847 (1982).

14 Poiesz, B. J., Ruscetti, F. W., Reitz, M. S., Kalyanaraman, V. S. & Gallo, R. C. Isolation of a new type C retrovirus (HTLV) in primary uncultured cells of a patient with Sezary T-cell leukaemia. *Nature* **294**, 268-271, doi:10.1038/294268a0 (1981).

15 Kalyanaraman, V. S., Sarngadharan, M. G., Poiesz, B., Ruscetti, F. W. & Gallo, R. C. Immunological properties of a type C retrovirus isolated from cultured human T-lymphoma cells and comparison to other mammalian retroviruses. *J Virol* **38**, 906-915, doi:10.1128/JVI.38.3.906-915.1981 (1981).

16 Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. & Montagnier, L. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**, 868-871, doi:10.1126/science.6189183 (1983).

17 Gallo, R. C., Sarin, P. S., Gelmann, E. P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V. S., Mann, D., Sidhu, G. D., Stahl, R. E., Zolla-Pazner, S., Leibowitch, J. & Popovic, M. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* **220**, 865-867, doi:10.1126/science.6601823 (1983).

18 Popovic, M., Sarngadharan, M. G., Read, E. & Gallo, R. C. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**, 497-500, doi:10.1126/science.6200935 (1984).

19 Schupbach, J., Popovic, M., Gilden, R. V., Gonda, M. A., Sarngadharan, M. G. & Gallo, R. C. Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS. *Science* **224**, 503-505, doi:10.1126/science.6200937 (1984).

20 Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., Palker, T. J., Redfield, R., Oleske, J. & Safai, B. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**, 500-503, doi:10.1126/science.6200936 (1984).

21 Sarngadharan, M. G., Popovic, M., Bruch, L., Schupbach, J. & Gallo, R. C. Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. *Science* **224**, 506-508, doi:10.1126/science.6324345 (1984).

22 Levy, J. A., Hoffman, A. D., Kramer, S. M., Landis, J. A., Shimabukuro, J. M. & Oshiro, L. S. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* **225**, 840-842, doi:10.1126/science.6206563 (1984).

23 Brun-Vezinet, F., Rouzioux, C., Barre-Sinoussi, F., Klatzmann, D., Saimot, A. G., Rozenbaum, W., Christol, D., Gluckmann, J. C., Montagnier, L. & Chermann, J. C. Detection of IgG antibodies to lymphadenopathy-associated virus in patients with AIDS or lymphadenopathy syndrome. *Lancet* **1**, 1253-1256, doi:10.1016/s0140-6736(84)92444-9 (1984).

24 Schupbach, J., Haller, O., Vogt, M., Luthy, R., Joller, H., Oelz, O., Popovic, M., Sarngadharan, M. G. & Gallo, R. C. Antibodies to HTLV-III in Swiss patients with AIDS and pre-AIDS and in groups at risk for AIDS. *N Engl J Med* **312**, 265-270, doi:10.1056/NEJM198501313120502 (1985).

25 Ellrodt, A., Barre-Sinoussi, F., Le Bras, P., Nugeyre, M. T., Palazzo, L., Rey, F., Brun-Vezinet, F., Rouzioux, C., Segond, P. & Caquet, R. Isolation of human T-lymphotropic retrovirus (LAV) from Zairian married couple, one with AIDS, one with prodromes. *Lancet* **1**, 1383-1385, doi:10.1016/s0140-6736(84)91877-4 (1984).

26 Vilmer, E., Barre-Sinoussi, F., Rouzioux, C., Gazengel, C., Brun, F. V., Dauguet, C., Fischer, A., Manigne, P., Chermann, J. C. & Griscelli, C. Isolation of new lymphotropic retrovirus from two siblings with haemophilia B, one with AIDS. *Lancet* **1**, 753-757, doi:10.1016/s0140-6736(84)91275-3 (1984).

27 Zagury, D., Bernard, J., Leibowitch, J., Safai, B., Groopman, J. E., Feldman, M., Sarngadharan, M. G. & Gallo, R. C. HTLV-III in cells cultured from semen of two patients with AIDS. *Science* **226**, 449-451, doi:10.1126/science.6208607 (1984).

28 Fischl, M. A., Richman, D. D., Grieco, M. H., Gottlieb, M. S., Volberding, P. A., Laskin, O. L., Leedom, J. M., Groopman, J. E., Mildvan, D. & Schooley, R. T. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. A double-blind, placebo-controlled trial. *N Engl J Med* **317**, 185-191, doi:10.1056/NEJM198707233170401 (1987).

29 Spear, J. B., Benson, C. A., Pottage, J. C., Jr., Paul, D. A., Landay, A. L. & Kessler, H. A. Rapid rebound of serum human immunodeficiency virus antigen after discontinuing zidovudine therapy. *J Infect Dis* **158**, 1132-1133, doi:10.1093/infdis/158.5.1132 (1988).

30 Richman, D. D. Emergence of mutant HIV reverse transcriptase conferring resistance to AZT. *J Enzyme Inhib* **6**, 55-64, doi:10.3109/14756369209041356 (1992).

31 Larder, B. A., Darby, G. & Richman, D. D. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. *Science* **243**, 1731-1734, doi:10.1126/science.2467383 (1989).

32 Larder, B. A. & Kemp, S. D. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* **246**, 1155-1158, doi:10.1126/science.2479983 (1989).

33 Lederman, M. M., Connick, E., Landay, A., Kuritzkes, D. R., Spritzler, J., St Clair, M., Kotzin, B. L., Fox, L., Chiozzi, M. H., Leonard, J. M., Rousseau, F., Wade, M., Roe, J. D., Martinez, A. & Kessler, H. Immunologic responses associated with 12 weeks of combination antiretroviral therapy consisting of zidovudine, lamivudine, and ritonavir: results of AIDS Clinical Trials Group Protocol 315. *J Infect Dis* **178**, 70-79, doi:10.1086/515591 (1998).

34 Zijenah, L. S., Bandason, T., Bara, W., Chipiti, M. M. & Katzenstein, D. A. Impact of Option B(+) Combination Antiretroviral Therapy on Mother-to-Child Transmission of HIV-1, Maternal and Infant Virologic Responses to Combination Antiretroviral Therapy, and Maternal and Infant Mortality Rates: A 24-Month Prospective Follow-Up Study at a Primary Health Care Clinic, in Harare, Zimbabwe. *AIDS Patient Care STDS* **36**, 145-152, doi:10.1089/apc.2021.0217 (2022).

35 Robb, M. L., Eller, L. A., Kibuuka, H., Rono, K., Maganga, L., Nitayaphan, S., Kroon, E., Sawe, F. K., Sinei, S., Sriplienchan, S., Jagodzinski, L. L., Malia, J., Manak, M., de Souza, M. S., Tovanabutra, S., Sanders-Buell, E., Rolland, M., Dorsey-Spitz, J., Eller, M. A., Milazzo, M., Li, Q., Lewandowski, A., Wu, H., Swann, E., O'Connell, R. J., Peel, S., Dawson, P., Kim, J. H., Michael, N. L. & Team, R. V. S. Prospective Study of Acute HIV-1 Infection in Adults in East Africa and Thailand. *N Engl J Med* **374**, 2120-2130, doi:10.1056/NEJMoa1508952 (2016).

36 Miller, W. C., Rosenberg, N. E., Rutstein, S. E. & Powers, K. A. Role of acute and early HIV infection in the sexual transmission of HIV. *Curr Opin HIV AIDS* **5**, 277-282, doi:10.1097/COH.0b013e32833a0d3a (2010).

37 Leslie-Harwit, M. & Meheus, A. Sexually transmitted disease in young people: the importance of health education. *Sex Transm Dis* **16**, 15-20, doi:10.1097/00007435-198901000-00004 (1989).

38 UNAIDS. *Global HIV & AIDS statistics — Fact sheet*, 2021).

39 Predicting the failure of 3 by 5. *Lancet* **365**, 1597 (2005).

40 Deborah L. Birx, M. C., Mark Dybul, Michel Sidibé. “15 By 15” A Global Target Achieved. (UNAIDS, Geneva, Switzerland, 2015).

41 Frescura, L., Godfrey-Faussett, P., Feizzadeh, A. A., El-Sadr, W., Syarif, O., Ghys, P. D., on & behalf of the testing treatment target Working, G. Achieving the 95 95 95 targets for all: A pathway to ending AIDS. *PLoS One* **17**, e0272405, doi:10.1371/journal.pone.0272405 (2022).
42 UNAIDS. Understanding Fast-Track: Accelerating Action to end the AIDS Epidemic by 2030. (UNAIDS, Geneva, Switzerland).
43 Garnett, G. P. Reductions in HIV incidence are likely to increase the importance of key population programmes for HIV control in sub-Saharan Africa. *J Int AIDS Soc* **24**, e25727, doi:10.1002/jia2.25727 (2021).
44 Organization, W. H. Consolidated guidelines on HIV prevention, diagnosis, treatment and care for key populations – 2016 update. (World Health Organization, 2016).
45 Nyamweya, S., Hegedus, A., Jaye, A., Rowland-Jones, S., Flanagan, K. L. & Macallan, D. C. Comparing HIV-1 and HIV-2 infection: Lessons for viral immunopathogenesis. *Rev Med Virol* **23**, 221-240, doi:10.1002/rmv.1739 (2013).
46 Hemelaar, J., Elangovan, R., Yun, J., Dickson-Tetteh, L., Fleminger, I., Kirtley, S., Williams, B., Gouws-Williams, E., Ghys, P. D. & Characterisation, W.-U. N. f. H. I. Global and regional molecular epidemiology of HIV-1, 1990-2015: a systematic review, global survey, and trend analysis. *Lancet Infect Dis* **19**, 143-155, doi:10.1016/S1473-3099(18)30647-9 (2019).
47 Hemelaar, J., Gouws, E., Ghys, P. D. & Osmanov, S. Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *AIDS* **20**, W13-23, doi:10.1097/01.aids.0000247564.73009.bc (2006).
48 Taylor, B. S., Sobieszczyk, M. E., McCutchan, F. E. & Hammer, S. M. The challenge of HIV-1 subtype diversity. *N Engl J Med* **358**, 1590-1602, doi:10.1056/NEJMra0706737 (2008).
49 Hwang, S. S., Boyle, T. J., Lyerly, H. K. & Cullen, B. R. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science* **253**, 71-74, doi:10.1126/science.1905842 (1991).
50 Wyatt, R. & Sodroski, J. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280**, 1884-1888, doi:10.1126/science.280.5371.1884 (1998).
51 Berger, E. A., Doms, R. W., Fenyo, E. M., Korber, B. T., Littman, D. R., Moore, J. P., Sattentau, Q. J., Schuitemaker, H., Sodroski, J. & Weiss, R. A. A new classification for HIV-1. *Nature* **391**, 240, doi:10.1038/34571 (1998).
52 Connor, R. I., Sheridan, K. E., Ceradini, D., Choe, S. & Landau, N. R. Change in coreceptor use correlates with disease progression in HIV-1--infected individuals. *J Exp Med* **185**, 621-628, doi:10.1084/jem.185.4.621 (1997).
53 Hemelaar, J. The origin and diversity of the HIV-1 pandemic. *Trends Mol Med* **18**, 182-192, doi:10.1016/j.molmed.2011.12.001 (2012).
54 Hirsch, V. M., Olmsted, R. A., Murphey-Corb, M., Purcell, R. H. & Johnson, P. R. An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339**, 389-392, doi:10.1038/339389a0 (1989).
55 Levin, J. G., Mitra, M., Mascarenhas, A. & Musier-Forsyth, K. Role of HIV-1 nucleocapsid protein in HIV-1 reverse transcription. *RNA Biol* **7**, 754-774, doi:10.4161/rna.7.6.14115 (2010).
56 Yu, Q. & Morrow, C. D. Essential regions of the tRNA primer required for HIV-1 infectivity. *Nucleic Acids Res* **28**, 4783-4789, doi:10.1093/nar/28.23.4783 (2000).

57 Rossi, E., Meuser, M. E., Cunanan, C. J. & Cocklin, S. Structure, Function, and Interactions of the HIV-1 Capsid Protein. *Life (Basel)* **11**, doi:10.3390/life11020100 (2021).
58 Camaur, D. & Trono, D. Characterization of human immunodeficiency virus type 1 Vif particle incorporation. *J Virol* **70**, 6106-6111, doi:10.1128/JVI.70.9.6106-6111.1996 (1996).
59 Paxton, W., Connor, R. I. & Landau, N. R. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. *J Virol* **67**, 7229-7237, doi:10.1128/JVI.67.12.7229-7237.1993 (1993).
60 Muller, B., Tessmer, U., Schubert, U. & Krausslich, H. G. Human immunodeficiency virus type 1 Vpr protein is incorporated into the virion in significantly smaller amounts than gag and is phosphorylated in infected cells. *J Virol* **74**, 9727-9731, doi:10.1128/jvi.74.20.9727-9731.2000 (2000).
61 Welker, R., Harris, M., Cardel, B. & Krausslich, H. G. Virion incorporation of human immunodeficiency virus type 1 Nef is mediated by a bipartite membrane-targeting signal: analysis of its role in enhancement of viral infectivity. *J Virol* **72**, 8833-8840, doi:10.1128/JVI.72.11.8833-8840.1998 (1998).
62 (London School of Hygiene and Tropical Medicine).
63 Watts, J. M., Dang, K. K., Gorelick, R. J., Leonard, C. W., Bess, J. W., Jr., Swanstrom, R., Burch, C. L. & Weeks, K. M. Architecture and secondary structure of an entire HIV-1 RNA genome. *Nature* **460**, 711-716, doi:10.1038/nature08237 (2009).
64 Rosen, C. A. Tat and Rev: positive modulators of human immunodeficiency virus gene expression. *Gene Expr* **1**, 85-90 (1991).
65 Frankel, A. D. & Young, J. A. HIV-1: fifteen proteins and an RNA. *Annu Rev Biochem* **67**, 1-25, doi:10.1146/annurev.biochem.67.1.1 (1998).
66 Pollard, V. W. & Malim, M. H. The HIV-1 Rev protein. *Annu Rev Microbiol* **52**, 491-532, doi:10.1146/annurev.micro.52.1.491 (1998).
67 Fisher, A. G., Ensoli, B., Ivanoff, L., Chamberlain, M., Petteway, S., Ratner, L., Gallo, R. C. & Wong-Staal, F. The sor gene of HIV-1 is required for efficient virus transmission in vitro. *Science* **237**, 888-893, doi:10.1126/science.3497453 (1987).
68 Strebel, K., Daugherty, D., Clouse, K., Cohen, D., Folks, T. & Martin, M. A. The HIV 'A' (sor) gene product is essential for virus infectivity. *Nature* **328**, 728-730, doi:10.1038/328728a0 (1987).
69 Stopak, K., de Noronha, C., Yonemoto, W. & Greene, W. C. HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. *Mol Cell* **12**, 591-601, doi:10.1016/s1097-2765(03)00353-8 (2003).
70 Izumi, T., Io, K., Matsui, M., Shirakawa, K., Shinohara, M., Nagai, Y., Kawahara, M., Kobayashi, M., Kondoh, H., Misawa, N., Koyanagi, Y., Uchiyama, T. & Takaori-Kondo, A. HIV-1 viral infectivity factor interacts with TP53 to induce G2 cell cycle arrest and positively regulate viral replication. *Proc Natl Acad Sci U S A* **107**, 20798-20803, doi:10.1073/pnas.1008076107 (2010).
71 Kogan, M. & Rappaport, J. HIV-1 accessory protein Vpr: relevance in the pathogenesis of HIV and potential for therapeutic intervention. *Retrovirology* **8**, 25, doi:10.1186/1742-4690-8-25 (2011).
72 Dube, M., Bego, M. G., Paquay, C. & Cohen, E. A. Modulation of HIV-1-host interaction: role of the Vpu accessory protein. *Retrovirology* **7**, 114, doi:10.1186/1742-4690-7-114 (2010).

73 Staudt, R. P., Alvarado, J. J., Emert-Sedlak, L. A., Shi, H., Shu, S. T., Wales, T. E., Engen, J. R. & Smithgall, T. E. Structure, function, and inhibitor targeting of HIV-1 Nef-effector kinase complexes. *J Biol Chem* **295**, 15158-15171, doi:10.1074/jbc.REV120.012317 (2020).
74 Dirk, B. S., Pawlak, E. N., Johnson, A. L., Van Nynatten, L. R., Jacob, R. A., Heit, B. & Dikeakos, J. D. HIV-1 Nef sequesters MHC-I intracellularly by targeting early stages of endocytosis and recycling. *Sci Rep* **6**, 37021, doi:10.1038/srep37021 (2016).
75 Kwon, Y., Kaake, R. M., Echeverria, I., Suarez, M., Karimian Shamsabadi, M., Stoneham, C., Ramirez, P. W., Kress, J., Singh, R., Sali, A., Krogan, N., Guatelli, J. & Jia, X. Structural basis of CD4 downregulation by HIV-1 Nef. *Nat Struct Mol Biol* **27**, 822-828, doi:10.1038/s41594-020-0463-z (2020).
76 Basmaciogullari, S. & Pizzato, M. The activity of Nef on HIV-1 infectivity. *Front Microbiol* **5**, 232, doi:10.3389/fmicb.2014.00232 (2014).
77 Carter, C. A. & Ehrlich, L. S. Cell biology of HIV-1 infection of macrophages. *Annu Rev Microbiol* **62**, 425-443, doi:10.1146/annurev.micro.62.081307.162758 (2008).
78 Lutgen, V., Narasipura, S. D., Barbian, H. J., Richards, M., Wallace, J., Razmpour, R., Buzhdygan, T., Ramirez, S. H., Prevedel, L., Eugenin, E. A. & Al-Harthi, L. HIV infects astrocytes in vivo and egresses from the brain to the periphery. *PLoS Pathog* **16**, e1008381, doi:10.1371/journal.ppat.1008381 (2020).
79 Wilen, C. B., Tilton, J. C. & Doms, R. W. HIV: cell binding and entry. *Cold Spring Harb Perspect Med* **2**, doi:10.1101/cshperspect.a006866 (2012).
80 Pancera, M., Majeed, S., Ban, Y. E., Chen, L., Huang, C. C., Kong, L., Kwon, Y. D., Stuckey, J., Zhou, T., Robinson, J. E., Schief, W. R., Sodroski, J., Wyatt, R. & Kwong, P. D. Structure of HIV-1 gp120 with gp41-interactive region reveals layered envelope architecture and basis of conformational mobility. *Proc Natl Acad Sci U S A* **107**, 1166-1171, doi:10.1073/pnas.0911004107 (2010).
81 Iordanskiy, S. & Bukrinsky, M. Reverse transcription complex: the key player of the early phase of HIV replication. *Future Virol* **2**, 49-64, doi:10.2217/17460794.2.1.49 (2007).
82 Schaller, T., Ocwieja, K. E., Rasaiyaah, J., Price, A. J., Brady, T. L., Roth, S. L., Hue, S., Fletcher, A. J., Lee, K., KewalRamani, V. N., Noursadeghi, M., Jenner, R. G., James, L. C., Bushman, F. D. & Towers, G. J. HIV-1 capsid-cyclophilin interactions determine nuclear import pathway, integration targeting and replication efficiency. *PLoS Pathog* **7**, e1002439, doi:10.1371/journal.ppat.1002439 (2011).
83 Dharan, A., Bachmann, N., Talley, S., Zwikelmaier, V. & Campbell, E. M. Nuclear pore blockade reveals that HIV-1 completes reverse transcription and uncoating in the nucleus. *Nat Microbiol* **5**, 1088-1095, doi:10.1038/s41564-020-0735-8 (2020).
84 Matreyek, K. A. & Engelman, A. The requirement for nucleoporin NUP153 during human immunodeficiency virus type 1 infection is determined by the viral capsid. *J Virol* **85**, 7818-7827, doi:10.1128/JVI.00325-11 (2011).
85 Peng, K., Muranyi, W., Glass, B., Laketa, V., Yant, S. R., Tsai, L., Cihlar, T., Muller, B. & Krausslich, H. G. Quantitative microscopy of functional HIV post-entry complexes reveals association of replication with the viral capsid. *Elife* **3**, e04114, doi:10.7554/eLife.04114 (2014).
86 Schroder, A. R., Shinn, P., Chen, H., Berry, C., Ecker, J. R. & Bushman, F. HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* **110**, 521-529, doi:10.1016/s0092-8674(02)00864-4 (2002).

87 Craigie, R. & Bushman, F. D. HIV DNA integration. *Cold Spring Harb Perspect Med* **2**, a006890, doi:10.1101/cshperspect.a006890 (2012).
88 Chou, S., Upton, H., Bao, K., Schulze-Gahmen, U., Samelson, A. J., He, N., Nowak, A., Lu, H., Krogan, N. J., Zhou, Q. & Alber, T. HIV-1 Tat recruits transcription elongation factors dispersed along a flexible AFF4 scaffold. *Proc Natl Acad Sci U S A* **110**, E123-131, doi:10.1073/pnas.1216971110 (2013).
89 Chen, D., Fong, Y. & Zhou, Q. Specific interaction of Tat with the human but not rodent P-TEFb complex mediates the species-specific Tat activation of HIV-1 transcription. *Proc Natl Acad Sci U S A* **96**, 2728-2733, doi:10.1073/pnas.96.6.2728 (1999).
90 Gatignol, A. Transcription of HIV: Tat and cellular chromatin. *Adv Pharmacol* **55**, 137-159, doi:10.1016/S1054-3589(07)55004-0 (2007).
91 Fernandes, J., Jayaraman, B. & Frankel, A. The HIV-1 Rev response element: an RNA scaffold that directs the cooperative assembly of a homo-oligomeric ribonucleoprotein complex. *RNA Biol* **9**, 6-11, doi:10.4161/rna.9.1.18178 (2012).
92 Ono, A. HIV-1 Assembly at the Plasma Membrane: Gag Trafficking and Localization. *Future Virol* **4**, 241-257, doi:10.2217/fvl.09.4 (2009).
93 Berglund, J. A., Charpentier, B. & Rosbash, M. A high affinity binding site for the HIV-1 nucleocapsid protein. *Nucleic Acids Res* **25**, 1042-1049, doi:10.1093/nar/25.5.1042 (1997).
94 Brierley, I. Ribosomal frameshifting viral RNAs. *J Gen Virol* **76 (Pt 8)**, 1885-1892, doi:10.1099/0022-1317-76-8-1885 (1995).
95 Freed, E. O. HIV-1 assembly, release and maturation. *Nat Rev Microbiol* **13**, 484-496, doi:10.1038/nrmicro3490 (2015).
96 Briggs, J. A., Riches, J. D., Glass, B., Bartonova, V., Zanetti, G. & Krausslich, H. G. Structure and assembly of immature HIV. *Proc Natl Acad Sci U S A* **106**, 11090-11095, doi:10.1073/pnas.0903535106 (2009).
97 Sundquist, W. I. & Krausslich, H. G. HIV-1 assembly, budding, and maturation. *Cold Spring Harb Perspect Med* **2**, a006924, doi:10.1101/cshperspect.a006924 (2012).
98 FDA. *HIV and AIDS: Medicines to Help You*, <https://www.fda.gov/consumers/free-publications-women/hiv-and-aids-medicines-help-you> (2020).
99 Xia, Q., Radzio, J., Anderson, K. S. & Sluis-Cremer, N. Probing nonnucleoside inhibitor-induced active-site distortion in HIV-1 reverse transcriptase by transient kinetic analyses. *Protein Sci* **16**, 1728-1737, doi:10.1110/ps.072829007 (2007).
100 Wainberg, M. A. HIV resistance to nevirapine and other non-nucleoside reverse transcriptase inhibitors. *J Acquir Immune Defic Syndr* **34 Suppl 1**, S2-7, doi:10.1097/00126334-200309011-00002 (2003).
101 Benedicto, A. M., Fuster-Martinez, I., Tosca, J., Esplugues, J. V., Blas-Garcia, A. & Apostolova, N. NNRTI and Liver Damage: Evidence of Their Association and the Mechanisms Involved. *Cells* **10**, doi:10.3390/cells10071687 (2021).
102 Minuto, J. J. & Haubrich, R. Etravirine: a second-generation NNRTI for treatment-experienced adults with resistant HIV-1 infection. *Futur HIV Ther* **2**, 525-537, doi:10.2217/17469600.2.6.525 (2008).
103 Sluis-Cremer, N. & Tachedjian, G. Mechanisms of inhibition of HIV replication by non-nucleoside reverse transcriptase inhibitors. *Virus Res* **134**, 147-156, doi:10.1016/j.virusres.2008.01.002 (2008).

104 Udier-Blagovic, M., Tirado-Rives, J. & Jorgensen, W. L. Validation of a model for the complex of HIV-1 reverse transcriptase with nonnucleoside inhibitor TMC125. *J Am Chem Soc* **125**, 6016-6017, doi:10.1021/ja034308c (2003).
105 Kempf, D. J., Marsh, K. C., Kumar, G., Rodrigues, A. D., Denissen, J. F., McDonald, E., Kukulka, M. J., Hsu, A., Granneman, G. R., Baroldi, P. A., Sun, E., Pizzuti, D., Plattner, J. J., Norbeck, D. W. & Leonard, J. M. Pharmacokinetic enhancement of inhibitors of the human immunodeficiency virus protease by coadministration with ritonavir. *Antimicrob Agents Chemother* **41**, 654-660, doi:10.1128/AAC.41.3.654 (1997).
106 Woollard, S. M. & Kanmogne, G. D. Maraviroc: a review of its use in HIV infection and beyond. *Drug Des Devel Ther* **9**, 5447-5468, doi:10.2147/DDDT.S90580 (2015).
107 Westby, M., Smith-Burchnell, C., Mori, J., Lewis, M., Mosley, M., Stockdale, M., Dorr, P., Ciaramella, G. & Perros, M. Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry. *J Virol* **81**, 2359-2371, doi:10.1128/JVI.02006-06 (2007).
108 Miri, L., Bouvier, G., Kettani, A., Mikou, A., Wakrim, L., Nilges, M. & Malliavin, T. E. Stabilization of the integrase-DNA complex by Mg2+ ions and prediction of key residues for binding HIV-1 integrase inhibitors. *Proteins* **82**, 466-478, doi:10.1002/prot.24412 (2014).
109 Machado, L. A. & Guimaraes, A. C. R. Evidence for Disruption of Mg(2+) Pair as a Resistance Mechanism Against HIV-1 Integrase Strand Transfer Inhibitors. *Front Mol Biosci* **7**, 170, doi:10.3389/fmolb.2020.00170 (2020).
110 Hodge, D., Back, D. J., Gibbons, S., Khoo, S. H. & Marzolini, C. Pharmacokinetics and Drug-Drug Interactions of Long-Acting Intramuscular Cabotegravir and Rilpivirine. *Clin Pharmacokinet* **60**, 835-853, doi:10.1007/s40262-021-01005-1 (2021).
111 Shen, L. & Siliciano, R. F. Viral reservoirs, residual viremia, and the potential of highly active antiretroviral therapy to eradicate HIV infection. *J Allergy Clin Immunol* **122**, 22-28, doi:10.1016/j.jaci.2008.05.033 (2008).
112 Cohen, M. S., Chen, Y. Q., McCauley, M., Gamble, T., Hosseinipour, M. C., Kumarasamy, N., Hakim, J. G., Kumwenda, J., Grinsztejn, B., Pilotto, J. H., Godbole, S. V., Mehendale, S., Chariyalertsak, S., Santos, B. R., Mayer, K. H., Hoffman, I. F., Eshleman, S. H., Piwowar-Manning, E., Wang, L., Makhema, J., Mills, L. A., de Bruyn, G., Sanne, I., Eron, J., Gallant, J., Havlir, D., Swindells, S., Ribaudo, H., Elharrar, V., Burns, D., Taha, T. E., Nielsen-Saines, K., Celentano, D., Essex, M., Fleming, T. R. & Team, H. S. Prevention of HIV-1 infection with early antiretroviral therapy. *N Engl J Med* **365**, 493-505, doi:10.1056/NEJMoa1105243 (2011).
113 Rodger, A. J., Cambiano, V., Bruun, T., Vernazza, P., Collins, S., Degen, O., Corbelli, G. M., Estrada, V., Geretti, A. M., Beloukas, A., Raben, D., Coll, P., Antinori, A., Nwokolo, N., Rieger, A., Prins, J. M., Blaxhult, A., Weber, R., Van Eeden, A., Brockmeyer, N. H., Clarke, A., Del Romero Guerrero, J., Raffi, F., Bogner, J. R., Wandeler, G., Gerstoft, J., Gutierrez, F., Brinkman, K., Kitchen, M., Ostergaard, L., Leon, A., Ristola, M., Jessen, H., Stellbrink, H. J., Phillips, A. N., Lundgren, J. & Group, P. S. Risk of HIV transmission through condomless sex in serodifferent gay couples with the HIV-positive partner taking suppressive antiretroviral therapy (PARTNER): final results of a multicentre, prospective, observational study. *Lancet* **393**, 2428-2438, doi:10.1016/S0140-6736(19)30418-0 (2019).

114 Bavinton, B. R., Pinto, A. N., Phanuphak, N., Grinsztejn, B., Prestage, G. P., Zablotska-Manos, I. B., Jin, F., Fairley, C. K., Moore, R., Roth, N., Bloch, M., Pell, C., McNulty, A. M., Baker, D., Hoy, J., Tee, B. K., Templeton, D. J., Cooper, D. A., Emery, S., Kelleher, A., Grulich, A. E. & Opposites Attract Study, G. Viral suppression and HIV transmission in serodiscordant male couples: an international, prospective, observational, cohort study. *Lancet HIV* **5**, e438-e447, doi:10.1016/S2352-3018(18)30132-2 (2018).

115 Gibas, K. M., Kelly, S. G., Arribas, J. R., Cahn, P., Orkin, C., Daar, E. S., Sax, P. E. & Taiwo, B. O. Two-drug regimens for HIV treatment. *Lancet HIV*, doi:10.1016/S2352-3018(22)00249-1 (2022).

116 Orkin, C., Arasteh, K., Gorgolas Hernandez-Mora, M., Pokrovsky, V., Overton, E. T., Girard, P. M., Oka, S., Walmsley, S., Bettacchi, C., Brinson, C., Philibert, P., Lombaard, J., St Clair, M., Crauwels, H., Ford, S. L., Patel, P., Chounta, V., D'Amico, R., Vanveggel, S., Dorey, D., Cutrell, A., Griffith, S., Margolis, D. A., Williams, P. E., Parys, W., Smith, K. Y. & Spreen, W. R. Long-Acting Cabotegravir and Rilpivirine after Oral Induction for HIV-1 Infection. *N Engl J Med* **382**, 1124-1135, doi:10.1056/NEJMoa1909512 (2020).

117 Swindells, S., Andrade-Villanueva, J. F., Richmond, G. J., Rizzardini, G., Baumgarten, A., Masia, M., Latiff, G., Pokrovsky, V., Bredeek, F., Smith, G., Cahn, P., Kim, Y. S., Ford, S. L., Talarico, C. L., Patel, P., Chounta, V., Crauwels, H., Parys, W., Vanveggel, S., Mrus, J., Huang, J., Harrington, C. M., Hudson, K. J., Margolis, D. A., Smith, K. Y., Williams, P. E. & Spreen, W. R. Long-Acting Cabotegravir and Rilpivirine for Maintenance of HIV-1 Suppression. *N Engl J Med* **382**, 1112-1123, doi:10.1056/NEJMoa1904398 (2020).

118 Saag, M. S., Gandhi, R. T., Hoy, J. F., Landovitz, R. J., Thompson, M. A., Sax, P. E., Smith, D. M., Benson, C. A., Buchbinder, S. P., Del Rio, C., Eron, J. J., Jr., Fatkenheuer, G., Gunthard, H. F., Molina, J. M., Jacobsen, D. M. & Volberding, P. A. Antiretroviral Drugs for Treatment and Prevention of HIV Infection in Adults: 2020 Recommendations of the International Antiviral Society-USA Panel. *JAMA* **324**, 1651-1669, doi:10.1001/jama.2020.17025 (2020).

119 Bartlett, A. W., Lumbiganon, P., Kurniati, N., Sudjaritruk, T., Mohamed, T. J., Hansudewechakul, R., Ly, P. S., Truong, K. H., Puthanakit, T., Nguyen, L. V., Chokephaibulkit, K., Do, V. C., Kumarasamy, N., Nik Yusoff, N. K., Fong, M. S., Watu, D. K., Nallusamy, R., Sohn, A. H., Law, M. G. & Asia-Pacific, T. A. P. H. O. D. o. I. Use and Outcomes of Antiretroviral Monotherapy and Treatment Interruption in Adolescents With Perinatal HIV Infection in Asia. *J Adolesc Health* **65**, 651-659, doi:10.1016/j.jadohealth.2019.05.025 (2019).

120 Patten, G., Bernheimer, J., Fairlie, L., Rabie, H., Sawry, S., Technau, K., Eley, B., Davies, M. A. & for Ie, D. E. A. S. A. Lamivudine monotherapy as a holding regimen for HIV-positive children. *PLoS One* **13**, e0205455, doi:10.1371/journal.pone.0205455 (2018).

121 Donnell, D., Baeten, J. M., Bumpus, N. N., Brantley, J., Bangsberg, D. R., Haberer, J. E., Mujugira, A., Mugo, N., Ndase, P., Hendrix, C. & Celum, C. HIV protective efficacy and correlates of tenofovir blood concentrations in a clinical trial of PrEP for HIV prevention. *J Acquir Immune Defic Syndr* **66**, 340-348, doi:10.1097/QAI.0000000000000172 (2014).

122 Baeten, J. M., Donnell, D., Ndase, P., Mugo, N. R., Campbell, J. D., Wangisi, J., Tappero, J. W., Bukusi, E. A., Cohen, C. R., Katabira, E., Ronald, A., Tumwesigye, E., Were, E., Fife, K. H., Kiarie, J., Farquhar, C., John-Stewart, G., Kakia, A., Odoyo, J., Mucunguzi, A., Nakku-Joloba, E., Twesigye, R., Ngure, K., Apaka, C., Tamooh, H., Gabona, F., Mujugira, A., Panteleeff, D., Thomas, K. K., Kidoguchi, L., Krows, M., Revall, J.,

Morrison, S., Haugen, H., Emmanuel-Ogier, M., Ondrejcek, L., Coombs, R. W., Frenkel, L., Hendrix, C., Bumpus, N. N., Bangsberg, D., Haberer, J. E., Stevens, W. S., Lingappa, J. R., Celum, C. & Partners Pr, E. P. S. T. Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N Engl J Med* **367**, 399-410, doi:10.1056/NEJMoa1108524 (2012).

123 Grant, R. M., Lama, J. R., Anderson, P. L., McMahan, V., Liu, A. Y., Vargas, L., Goicochea, P., Casapia, M., Guanira-Carranza, J. V., Ramirez-Cardich, M. E., Montoya-Herrera, O., Fernandez, T., Veloso, V. G., Buchbinder, S. P., Chariyalertsak, S., Schechter, M., Bekker, L. G., Mayer, K. H., Kallas, E. G., Amico, K. R., Mulligan, K., Bushman, L. R., Hance, R. J., Ganoza, C., Defechereux, P., Postle, B., Wang, F., McConnell, J. J., Zheng, J. H., Lee, J., Rooney, J. F., Jaffe, H. S., Martinez, A. I., Burns, D. N., Glidden, D. V. & iPrEx Study, T. Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. *N Engl J Med* **363**, 2587-2599, doi:10.1056/NEJMoa1011205 (2010).

124 Mayer, K. H., Molina, J. M., Thompson, M. A., Anderson, P. L., Mounzer, K. C., De Wet, J. J., DeJesus, E., Jessen, H., Grant, R. M., Ruane, P. J., Wong, P., Ebrahimi, R., Zhong, L., Mathias, A., Callebaut, C., Collins, S. E., Das, M., McCallister, S., Brainard, D. M., Brinson, C., Clarke, A., Coll, P., Post, F. A. & Hare, C. B. Emtricitabine and tenofovir alafenamide vs emtricitabine and tenofovir disoproxil fumarate for HIV pre-exposure prophylaxis (DISCOVER): primary results from a randomised, double-blind, multicentre, active-controlled, phase 3, non-inferiority trial. *Lancet* **396**, 239-254, doi:10.1016/S0140-6736(20)31065-5 (2020).

125 Cattaneo, D. & Gervasoni, C. Pharmacokinetics and Pharmacodynamics of Cabotegravir, a Long-Acting HIV Integrase Strand Transfer Inhibitor. *Eur J Drug Metab Pharmacokinet* **44**, 319-327, doi:10.1007/s13318-018-0526-2 (2019).

126 Delany-Moretlwe, S., Hughes, J. P., Bock, P., Ouma, S. G., Hunidzarira, P., Kalonji, D., Kayange, N., Makhema, J., Mandima, P., Mathew, C., Spooner, E., Mpendo, J., Mukwekwerere, P., Mgodi, N., Ntege, P. N., Nair, G., Nakabiito, C., Nuwagaba-Biribonwoha, H., Panchia, R., Singh, N., Siziba, B., Farrior, J., Rose, S., Anderson, P. L., Eshleman, S. H., Marzinke, M. A., Hendrix, C. W., Beigel-Orme, S., Hosek, S., Tolley, E., Sista, N., Adeyeye, A., Rooney, J. F., Rinehart, A., Spreen, W. R., Smith, K., Hanscom, B., Cohen, M. S., Hosseinipour, M. C. & group, H. s. Cabotegravir for the prevention of HIV-1 in women: results from HPTN 084, a phase 3, randomised clinical trial. *Lancet* **399**, 1779-1789, doi:10.1016/S0140-6736(22)00538-4 (2022).

127 Landovitz, R. J., Donnell, D., Clement, M. E., Hanscom, B., Cottle, L., Coelho, L., Cabello, R., Chariyalertsak, S., Dunne, E. F., Frank, I., Gallardo-Cartagena, J. A., Gaur, A. H., Gonzales, P., Tran, H. V., Hinojosa, J. C., Kallas, E. G., Kelley, C. F., Losso, M. H., Madruga, J. V., Middelkoop, K., Phanuphak, N., Santos, B., Sued, O., Valencia Huamani, J., Overton, E. T., Swaminathan, S., Del Rio, C., Gulick, R. M., Richardson, P., Sullivan, P., Piwowar-Manning, E., Marzinke, M., Hendrix, C., Li, M., Wang, Z., Marrazzo, J., Daar, E., Asmelash, A., Brown, T. T., Anderson, P., Eshleman, S. H., Bryan, M., Blanchette, C., Lucas, J., Psaros, C., Safren, S., Sugarman, J., Scott, H., Eron, J. J., Fields, S. D., Sista, N. D., Gomez-Feliciano, K., Jennings, A., Kofron, R. M., Holtz, T. H., Shin, K., Rooney, J. F., Smith, K. Y., Spreen, W., Margolis, D., Rinehart, A., Adeyeye, A., Cohen, M. S., McCauley, M., Grinsztejn, B. & Team, H. S. Cabotegravir for HIV Prevention in Cisgender Men and Transgender Women. *N Engl J Med* **385**, 595-608, doi:10.1056/NEJMoa2101016 (2021).

128 Prather, C. & Jeon, C. Cabotegravir: The first long-acting injectable for HIV pre-exposure prophylaxis. *Am J Health Syst Pharm* **79**, 1898-1905, doi:10.1093/ajhp/zxac201 (2022).
129 Pinkerton, S. D. & Abramson, P. R. Effectiveness of condoms in preventing HIV transmission. *Soc Sci Med* **44**, 1303-1312, doi:10.1016/s0277-9536(96)00258-4 (1997).
130 Aspinall, E. J., Nambiar, D., Goldberg, D. J., Hickman, M., Weir, A., Van Velzen, E., Palmateer, N., Doyle, J. S., Hellard, M. E. & Hutchinson, S. J. Are needle and syringe programmes associated with a reduction in HIV transmission among people who inject drugs: a systematic review and meta-analysis. *Int J Epidemiol* **43**, 235-248, doi:10.1093/ije/dyt243 (2014).
131 Fonner, V. A., Armstrong, K. S., Kennedy, C. E., O'Reilly, K. R. & Sweat, M. D. School based sex education and HIV prevention in low- and middle-income countries: a systematic review and meta-analysis. *PLoS One* **9**, e89692, doi:10.1371/journal.pone.0089692 (2014).
132 Deeks, S. G., Lewin, S. R. & Havlir, D. V. The end of AIDS: HIV infection as a chronic disease. *Lancet* **382**, 1525-1533, doi:10.1016/S0140-6736(13)61809-7 (2013).
133 Torres, R. A. & Lewis, W. Aging and HIV/AIDS: pathogenetic role of therapeutic side effects. *Lab Invest* **94**, 120-128, doi:10.1038/labinvest.2013.142 (2014).
134 Streeck, H., Maestri, A., Habermann, D., Crowell, T. A., Esber, A. L., Son, G., Eller, L. A., Eller, M. A., Parikh, A. P., Horn, P. A., Maganga, L., Bahemana, E., Adamu, Y., Kiweewa, F., Maswai, J., Owuoth, J., Robb, M. L., Michael, N. L., Polyak, C. S., Hoffmann, D., Ake, J. A. & Group, A. S. Dissecting drivers of immune activation in chronic HIV-1 infection. *EBioMedicine* **83**, 104182, doi:10.1016/j.ebiom.2022.104182 (2022).
135 Costagliola, D. Demographics of HIV and aging. *Curr Opin HIV AIDS* **9**, 294-301, doi:10.1097/COH.0000000000000076 (2014).
136 Lailulo, Y., Kitenge, M., Jaffer, S., Aluko, O. & Nyasulu, P. S. Factors associated with antiretroviral treatment failure among people living with HIV on antiretroviral therapy in resource-poor settings: a systematic review and metaanalysis. *Syst Rev* **9**, 292, doi:10.1186/s13643-020-01524-1 (2020).
137 Martinez-Picado, J. & Deeks, S. G. Persistent HIV-1 replication during antiretroviral therapy. *Curr Opin HIV AIDS* **11**, 417-423, doi:10.1097/COH.0000000000000287 (2016).
138 Dunkley, O. R. S., Scarborough, R. J. & Gatignol, A. 40th year anniversary of HIV discovery: Evolving paradigms for cure strategies. *Virologie (Montrouge)* **26**, 1-3, doi:10.1684/vir.2022.0938 (2022).
139 Siliciano, R. F. & Greene, W. C. HIV latency. *Cold Spring Harb Perspect Med* **1**, a007096, doi:10.1101/cshperspect.a007096 (2011).
140 Bonczkowski, P., De Scheerder, M. A., Malatinkova, E., Borch, A., Melkova, Z., Koenig, R., De Spiegelaere, W. & Vandekerckhove, L. Protein expression from unintegrated HIV-1 DNA introduces bias in primary in vitro post-integration latency models. *Sci Rep* **6**, 38329, doi:10.1038/srep38329 (2016).
141 Ruelas, D. S. & Greene, W. C. An integrated overview of HIV-1 latency. *Cell* **155**, 519-529, doi:10.1016/j.cell.2013.09.044 (2013).
142 Finzi, D., Blankson, J., Siliciano, J. D., Margolick, J. B., Chadwick, K., Pierson, T., Smith, K., Lisziewicz, J., Lori, F., Flexner, C., Quinn, T. C., Chaisson, R. E., Rosenberg, E., Walker, B., Gange, S., Gallant, J. & Siliciano, R. F. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med* **5**, 512-517, doi:10.1038/8394 (1999).

143 Strain, M. C., Gunthard, H. F., Havlir, D. V., Ignacio, C. C., Smith, D. M., Leigh-Brown, A. J., Macaranas, T. R., Lam, R. Y., Daly, O. A., Fischer, M., Opravil, M., Levine, H., Bacheler, L., Spina, C. A., Richman, D. D. & Wong, J. K. Heterogeneous clearance rates of long-lived lymphocytes infected with HIV: intrinsic stability predicts lifelong persistence. *Proc Natl Acad Sci U S A* **100**, 4819-4824, doi:10.1073/pnas.0736332100 (2003).

144 Chun, T. W., Engel, D., Mizell, S. B., Ehler, L. A. & Fauci, A. S. Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. *J Exp Med* **188**, 83-91, doi:10.1084/jem.188.1.83 (1998).

145 Lafeuillade, A., Poggi, C., Chadapaud, S., Hittinger, G., Chouraqui, M., Pisapia, M. & Delbeke, E. Pilot study of a combination of highly active antiretroviral therapy and cytokines to induce HIV-1 remission. *J Acquir Immune Defic Syndr* **26**, 44-55, doi:10.1097/00126334-200101010-00006 (2001).

146 Deeks, S. G. HIV: Shock and kill. *Nature* **487**, 439-440, doi:10.1038/487439a (2012).

147 Hokello, J., Sharma, A. L., Dimri, M. & Tyagi, M. Insights into the HIV Latency and the Role of Cytokines. *Pathogens* **8**, doi:10.3390/pathogens8030137 (2019).

148 Gruell, H., Gunst, J. D., Cohen, Y. Z., Pahus, M. H., Malin, J. J., Platten, M., Millard, K. G., Tolstrup, M., Jones, R. B., Conce Alberto, W. D., Lorenzi, J. C. C., Oliveira, T. Y., Kummerle, T., Suarez, I., Unson-O'Brien, C., Nogueira, L., Olesen, R., Ostergaard, L., Nielsen, H., Lehmann, C., Nussenzweig, M. C., Fatkenheuer, G., Klein, F., Caskey, M. & Sogaard, O. S. Effect of 3BNC117 and romidepsin on the HIV-1 reservoir in people taking suppressive antiretroviral therapy (ROADMAP): a randomised, open-label, phase 2A trial. *Lancet Microbe* **3**, e203-e214, doi:10.1016/S2666-5247(21)00239-1 (2022).

149 Archin, N. M., Cheema, M., Parker, D., Wiegand, A., Bosch, R. J., Coffin, J. M., Eron, J., Cohen, M. & Margolis, D. M. Antiretroviral intensification and valproic acid lack sustained effect on residual HIV-1 viremia or resting CD4+ cell infection. *PLoS One* **5**, e9390, doi:10.1371/journal.pone.0009390 (2010).

150 Siliciano, J. D., Lai, J., Callender, M., Pitt, E., Zhang, H., Margolick, J. B., Gallant, J. E., Cofrancesco, J., Jr., Moore, R. D., Gange, S. J. & Siliciano, R. F. Stability of the latent reservoir for HIV-1 in patients receiving valproic acid. *J Infect Dis* **195**, 833-836, doi:10.1086/511823 (2007).

151 Thomas, J., Ruggiero, A., Paxton, W. A. & Pollakis, G. Measuring the Success of HIV-1 Cure Strategies. *Front Cell Infect Microbiol* **10**, 134, doi:10.3389/fcimb.2020.00134 (2020).

152 Hutter, G., Nowak, D., Mossner, M., Ganepola, S., Mussig, A., Allers, K., Schneider, T., Hofmann, J., Kucherer, C., Blau, O., Blau, I. W., Hofmann, W. K. & Thiel, E. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med* **360**, 692-698, doi:10.1056/NEJMoa0802905 (2009).

153 Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A. & Landau, N. R. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**, 367-377, doi:10.1016/s0092-8674(00)80110-5 (1996).

154 Paxton, W. A., Kang, S. & Koup, R. A. The HIV type 1 coreceptor CCR5 and its role in viral transmission and disease progression. *AIDS Res Hum Retroviruses* **14 Suppl 1**, S89-92 (1998).

155 Paxton, W. A., Liu, R., Kang, S., Wu, L., Gingeras, T. R., Landau, N. R., Mackay, C. R. & Koup, R. A. Reduced HIV-1 infectability of CD4+ lymphocytes from exposed-uninfected individuals: association with low expression of CCR5 and high production of beta-chemokines. *Virology* **244**, 66-73, doi:10.1006/viro.1998.9082 (1998).

156 Pasi, K. J., Sabin, C. A., Jenkins, P. V., Devereux, H. L., Ononye, C. & Lee, C. A. The effects of the 32-bp CCR-5 deletion on HIV transmission and HIV disease progression in individuals with haemophilia. *Br J Haematol* **111**, 136-142, doi:10.1046/j.1365-2141.2000.02325.x (2000).

157 Gupta, R. K., Peppa, D., Hill, A. L., Galvez, C., Salgado, M., Pace, M., McCoy, L. E., Griffith, S. A., Thornhill, J., Alrubayyi, A., Huyveneers, L. E. P., Nastouli, E., Grant, P., Edwards, S. G., Innes, A. J., Frater, J., Nijhuis, M., Wensing, A. M. J., Martinez-Picado, J. & Olavarria, E. Evidence for HIV-1 cure after CCR5Delta32/Delta32 allogeneic haemopoietic stem-cell transplantation 30 months post analytical treatment interruption: a case report. *Lancet HIV* **7**, e340-e347, doi:10.1016/S2352-3018(20)30069-2 (2020).

158 Jensen, B. O., Knops, E., Cords, L., Lubke, N., Salgado, M., Busman-Sahay, K., Estes, J. D., Huyveneers, L. E. P., Perdomo-Celis, F., Wittner, M., Galvez, C., Mummert, C., Passaes, C., Eberhard, J. M., Munk, C., Hauber, I., Hauber, J., Heger, E., De Clercq, J., Vandekerckhove, L., Bergmann, S., Dunay, G. A., Klein, F., Haussinger, D., Fischer, J. C., Nachtkamp, K., Timm, J., Kaiser, R., Harrer, T., Luedde, T., Nijhuis, M., Saez-Cirion, A., Schulze Zur Wiesch, J., Wensing, A. M. J., Martinez-Picado, J. & Kobbe, G. In-depth virological and immunological characterization of HIV-1 cure after CCR5Delta32/Delta32 allogeneic hematopoietic stem cell transplantation. *Nat Med* **29**, 583-587, doi:10.1038/s41591-023-02213-x (2023).

159 Hsu J, V. B. K., Glesby M, Coletti A, Pahwa S, Warshaw M, Golner A, Bone F, Tobin N, Riches M, Mellors J, Browning R, Persaud D, Bryson Y. in *CROI*.

160 J. Dickter, S. W., A. Cardoso, S. Li, K. Gendzekhadze, Y. Feng, S. Dadwal, R. Taplitz, J. Ross, A. Aribi, R. Stan, T. Kidambi, L. Lai, S. Chang, A. Chaillon, M. Al Malki, J. Alvarnas, S. Forman, J. Zaia & in *AIDS 2022* https://programme.aids2022.org/Abstract/Abstract/?abstractid=12508 (Montreal, Canada, 2022).

161 Gupta, R. K., Abdul-Jawad, S., McCoy, L. E., Mok, H. P., Peppa, D., Salgado, M., Martinez-Picado, J., Nijhuis, M., Wensing, A. M. J., Lee, H., Grant, P., Nastouli, E., Lambert, J., Pace, M., Salasc, F., Monit, C., Innes, A. J., Muir, L., Waters, L., Frater, J., Lever, A. M. L., Edwards, S. G., Gabriel, I. H. & Olavarria, E. HIV-1 remission following CCR5Delta32/Delta32 haematopoietic stem-cell transplantation. *Nature* **568**, 244-248, doi:10.1038/s41586-019-1027-4 (2019).

162 Kordelas, L., Verheyen, J., Beelen, D. W., Horn, P. A., Heinold, A., Kaiser, R., Trenschel, R., Schadendorf, D., Dittmer, U., Esser, S. & Essen, H. I. V. A. G. Shift of HIV tropism in stem-cell transplantation with CCR5 Delta32 mutation. *N Engl J Med* **371**, 880-882, doi:10.1056/NEJMc1405805 (2014).

163 Henrich, T. J., Hu, Z., Li, J. Z., Sciaranghella, G., Busch, M. P., Keating, S. M., Gallien, S., Lin, N. H., Giguel, F. F., Lavoie, L., Ho, V. T., Armand, P., Soiffer, R. J., Sagar, M., Lacasce, A. S. & Kuritzkes, D. R. Long-term reduction in peripheral blood HIV type 1 reservoirs following reduced-intensity conditioning allogeneic stem cell transplantation. *J Infect Dis* **207**, 1694-1702, doi:10.1093/infdis/jit086 (2013).

164 Henrich, T. J., Hanhauser, E., Marty, F. M., Sirignano, M. N., Keating, S., Lee, T. H., Robles, Y. P., Davis, B. T., Li, J. Z., Heisey, A., Hill, A. L., Busch, M. P., Armand, P., Soiffer, R. J., Altfeld, M. & Kuritzkes, D. R. Antiretroviral-free HIV-1 remission and viral rebound after allogeneic stem cell transplantation: report of 2 cases. *Ann Intern Med* **161**, 319-327, doi:10.7326/M14-1027 (2014).

165 Yukl, S. A., Boritz, E., Busch, M., Bentsen, C., Chun, T. W., Douek, D., Eisele, E., Haase, A., Ho, Y. C., Hutter, G., Justement, J. S., Keating, S., Lee, T. H., Li, P., Murray, D., Palmer, S., Pilcher, C., Pillai, S., Price, R. W., Rothenberger, M., Schacker, T., Siliciano, J., Siliciano, R., Sinclair, E., Strain, M., Wong, J., Richman, D. & Deeks, S. G. Challenges in detecting HIV persistence during potentially curative interventions: a study of the Berlin patient. *PLoS Pathog* **9**, e1003347, doi:10.1371/journal.ppat.1003347 (2013).

166 Jensen, B. E., Haussinger, D., Knops, E., Wensing, A. M. J., Martinez-Picado, J., Nijhuis, M., Salgado, M., Estes, J., Lubke, N., Kaiser, R., Harrer, T., Fischer, J., Eberhard, J. M., Schulze zur Wiesch, J., Haas, R. & Kobbe, G. in *CROI.*

167 Scarborough, R. J., Goguen, R. P. & Gatignol, A. A second patient cured of HIV infection: hopes and limitations. *Virologie (Montrouge)* **23**, 1-4, doi:10.1684/vir.2019.0778 (2019).

168 Huyveneers, L. E. P., Bruns, A., Stam, A., Ellerbroek, P., de Jong, D., Nagy, N. A., Gumbs, S. B. H., Tesselaar, K., Bosman, K., Salgado, M., Hutter, G., Brosens, L. A. A., Kwon, M., Diez Martin, J., van der Meer, J. T. M., de Kort, T. M., Saez-Cirion, A., Schulze Zur Wiesch, J., Boelens, J. J., Martinez-Picado, J., Kuball, J. H. E., Wensing, A. M. J., Nijhuis, M. & IciStem, C. Autopsy Study Defines Composition and Dynamics of the HIV-1 Reservoir after Allogeneic Hematopoietic Stem Cell Transplantation with CCR5Delta32/Delta32 Donor Cells. *Viruses* **14**, doi:10.3390/v14092069 (2022).

169 Trajkovska, I., Georgievski, B., Cevreska, L., Gacovski, A., Hasan, T. & Nedeska-Minova, N. Early and Late Complications in Patients with Allogeneic Transplantation of Hematopoietic Stem Cell - Case Report. *Open Access Maced J Med Sci* **5**, 340-343, doi:10.3889/oamjms.2017.038 (2017).

170 Verhofstede, C., Nijhuis, M. & Vandekerckhove, L. Correlation of coreceptor usage and disease progression. *Curr Opin HIV AIDS* **7**, 432-439, doi:10.1097/COH.0b013e328356f6f2 (2012).

171 Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A. & Ho, D. D. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science* **261**, 1179-1181, doi:10.1126/science.8356453 (1993).

172 Delobel, P., Sandres-Saune, K., Cazabat, M., Pasquier, C., Marchou, B., Massip, P. & Izopet, J. R5 to X4 switch of the predominant HIV-1 population in cellular reservoirs during effective highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* **38**, 382-392, doi:10.1097/01.qai.0000152835.17747.47 (2005).

173 Castagna, A., Monno, L., Carta, S., Galli, L., Carrara, S., Fedele, V., Punzi, G., Fanti, I., Caramello, P., Lepri, A. C., De Luca, A., Ceccherini-Silberstein, F., Monforte, A. D. & Group, I. F. S. Switch of predicted HIV-1 tropism in treated subjects and its association with disease progression. *Medicine (Baltimore)* **95**, e5222, doi:10.1097/MD.0000000000005222 (2016).

174 Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-811, doi:10.1038/35888 (1998).

175 Brummelkamp, T. R., Bernards, R. & Agami, R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550-553, doi:10.1126/science.1068999 (2002).
176 Goguen, R. P., Del Corpo, O., Malard, C. M. G., Daher, A., Alpuche-Lazcano, S. P., Chen, M. J., Scarborough, R. J. & Gatignol, A. Efficacy, accumulation, and transcriptional profile of anti-HIV shRNAs expressed from human U6, 7SK, and H1 promoters. *Mol Ther Nucleic Acids* **23**, 1020-1034, doi:10.1016/j.omtn.2020.12.022 (2021).
177 Paul, C. P., Good, P. D., Winer, I. & Engelke, D. R. Effective expression of small interfering RNA in human cells. *Nat Biotechnol* **20**, 505-508, doi:10.1038/nbt0502-505 (2002).
178 Liu, Y. P., Karg, M., Herrera-Carrillo, E. & Berkhout, B. Towards Antiviral shRNAs Based on the AgoshRNA Design. *PLoS One* **10**, e0128618, doi:10.1371/journal.pone.0128618 (2015).
179 DePolo, N. J., Reed, J. D., Sheridan, P. L., Townsend, K., Sauter, S. L., Jolly, D. J. & Dubensky, T. W., Jr. VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum. *Mol Ther* **2**, 218-222, doi:10.1006/mthe.2000.0116 (2000).
180 Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J. L., Bousso, P., Deist, F. L. & Fischer, A. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**, 669-672, doi:10.1126/science.288.5466.669 (2000).
181 Aiuti, A., Slavin, S., Aker, M., Ficara, F., Deola, S., Mortellaro, A., Morecki, S., Andolfi, G., Tabucchi, A., Carlucci, F., Marinello, E., Cattaneo, F., Vai, S., Servida, P., Miniero, R., Roncarolo, M. G. & Bordignon, C. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* **296**, 2410-2413, doi:10.1126/science.1070104 (2002).
182 Anguela, X. M. & High, K. A. Entering the Modern Era of Gene Therapy. *Annu Rev Med* **70**, 273-288, doi:10.1146/annurev-med-012017-043332 (2019).
183 Hacein-Bey-Abina, S., Garrigue, A., Wang, G. P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., Beldjord, K., Asnafi, V., MacIntyre, E., Dal Cortivo, L., Radford, I., Brousse, N., Sigaux, F., Moshous, D., Hauer, J., Borkhardt, A., Belohradsky, B. H., Wintergerst, U., Velez, M. C., Leiva, L., Sorensen, R., Wulffraat, N., Blanche, S., Bushman, F. D., Fischer, A. & Cavazzana-Calvo, M. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest* **118**, 3132-3142, doi:10.1172/JCI35700 (2008).
184 Zhou, S., Fatima, S., Ma, Z., Wang, Y. D., Lu, T., Janke, L. J., Du, Y. & Sorrentino, B. P. Evaluating the Safety of Retroviral Vectors Based on Insertional Oncogene Activation and Blocked Differentiation in Cultured Thymocytes. *Mol Ther* **24**, 1090-1099, doi:10.1038/mt.2016.55 (2016).
185 Milone, M. C. & O'Doherty, U. Clinical use of lentiviral vectors. *Leukemia* **32**, 1529-1541, doi:10.1038/s41375-018-0106-0 (2018).
186 DiGiusto, D. L., Krishnan, A., Li, L., Li, H., Li, S., Rao, A., Mi, S., Yam, P., Stinson, S., Kalos, M., Alvarnas, J., Lacey, S. F., Yee, J. K., Li, M., Couture, L., Hsu, D., Forman, S. J., Rossi, J. J. & Zaia, J. A. RNA-based gene therapy for HIV with lentiviral vector-modified CD34(+) cells in patients undergoing transplantation for AIDS-related lymphoma. *Sci Transl Med* **2**, 36ra43, doi:10.1126/scitranslmed.3000931 (2010).

187 Aiuti, A., Biasco, L., Scaramuzza, S., Ferrua, F., Cicalese, M. P., Baricordi, C., Dionisio, F., Calabria, A., Giannelli, S., Castiello, M. C., Bosticardo, M., Evangelio, C., Assanelli, A., Casiraghi, M., Di Nunzio, S., Callegaro, L., Benati, C., Rizzardi, P., Pellin, D., Di Serio, C., Schmidt, M., Von Kalle, C., Gardner, J., Mehta, N., Neduva, V., Dow, D. J., Galy, A., Miniero, R., Finocchi, A., Metin, A., Banerjee, P. P., Orange, J. S., Galimberti, S., Valsecchi, M. G., Biffi, A., Montini, E., Villa, A., Ciceri, F., Roncarolo, M. G. & Naldini, L. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science* **341**, 1233151, doi:10.1126/science.1233151 (2013).

188 Cartier, N., Hacein-Bey-Abina, S., Bartholomae, C. C., Veres, G., Schmidt, M., Kutschera, I., Vidaud, M., Abel, U., Dal-Cortivo, L., Caccavelli, L., Mahlaoui, N., Kiermer, V., Mittelstaedt, D., Bellesme, C., Lahlou, N., Lefrere, F., Blanche, S., Audit, M., Payen, E., Leboulch, P., l'Homme, B., Bougneres, P., Von Kalle, C., Fischer, A., Cavazzana-Calvo, M. & Aubourg, P. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* **326**, 818-823, doi:10.1126/science.1171242 (2009).

189 Cavazzana-Calvo, M., Payen, E., Negre, O., Wang, G., Hehir, K., Fusil, F., Down, J., Denaro, M., Brady, T., Westerman, K., Cavallesco, R., Gillet-Legrand, B., Caccavelli, L., Sgarra, R., Maouche-Chretien, L., Bernaudin, F., Girot, R., Dorazio, R., Mulder, G. J., Polack, A., Bank, A., Soulier, J., Larghero, J., Kabbara, N., Dalle, B., Gourmel, B., Socie, G., Chretien, S., Cartier, N., Aubourg, P., Fischer, A., Cornetta, K., Galacteros, F., Beuzard, Y., Gluckman, E., Bushman, F., Hacein-Bey-Abina, S. & Leboulch, P. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. *Nature* **467**, 318-322, doi:10.1038/nature09328 (2010).

190 Fowler, N. H., Dickinson, M., Dreyling, M., Martinez-Lopez, J., Kolstad, A., Butler, J., Ghosh, M., Popplewell, L., Chavez, J. C., Bachy, E., Kato, K., Harigae, H., Kersten, M. J., Andreadis, C., Riedell, P. A., Ho, P. J., Perez-Simon, J. A., Chen, A. I., Nastoupil, L. J., von Tresckow, B., Ferreri, A. J. M., Teshima, T., Patten, P. E. M., McGuirk, J. P., Petzer, A. L., Offner, F., Viardot, A., Zinzani, P. L., Malladi, R., Zia, A., Awasthi, R., Masood, A., Anak, O., Schuster, S. J. & Thieblemont, C. Tisagenlecleucel in adult relapsed or refractory follicular lymphoma: the phase 2 ELARA trial. *Nat Med* **28**, 325-332, doi:10.1038/s41591-021-01622-0 (2022).

191 Salles, G., Schuster, S. J., Dreyling, M., Fischer, L., Kuruvilla, J., Patten, P. E. M., von Tresckow, B., Smith, S. M., Jimenez-Ubieto, A., Davis, K. L., Anjos, C., Chu, J., Zhang, J., Lobetti Bodoni, C., Thieblemont, C., Fowler, N. H., Dickinson, M., Martinez-Lopez, J., Wang, Y. & Link, B. K. Efficacy comparison of tisagenlecleucel vs usual care in patients with relapsed or refractory follicular lymphoma. *Blood Adv* **6**, 5835-5843, doi:10.1182/bloodadvances.2022008150 (2022).

192 Locke, F. L., Ghobadi, A., Jacobson, C. A., Miklos, D. B., Lekakis, L. J., Oluwole, O. O., Lin, Y., Braunschweig, I., Hill, B. T., Timmerman, J. M., Deol, A., Reagan, P. M., Stiff, P., Flinn, I. W., Farooq, U., Goy, A., McSweeney, P. A., Munoz, J., Siddiqi, T., Chavez, J. C., Herrera, A. F., Bartlett, N. L., Wiezorek, J. S., Navale, L., Xue, A., Jiang, Y., Bot, A., Rossi, J. M., Kim, J. J., Go, W. Y. & Neelapu, S. S. Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. *Lancet Oncol* **20**, 31-42, doi:10.1016/S1470-2045(18)30864-7 (2019).

193 Neelapu, S. S., Locke, F. L., Bartlett, N. L., Lekakis, L. J., Miklos, D. B., Jacobson, C. A., Braunschweig, I., Oluwole, O. O., Siddiqi, T., Lin, Y., Timmerman, J. M., Stiff, P. J.,

Friedberg, J. W., Flinn, I. W., Goy, A., Hill, B. T., Smith, M. R., Deol, A., Farooq, U., McSweeney, P., Munoz, J., Avivi, I., Castro, J. E., Westin, J. R., Chavez, J. C., Ghobadi, A., Komanduri, K. V., Levy, R., Jacobsen, E. D., Witzig, T. E., Reagan, P., Bot, A., Rossi, J., Navale, L., Jiang, Y., Aycock, J., Elias, M., Chang, D., Wiezorek, J. & Go, W. Y. Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. *N Engl J Med* **377**, 2531-2544, doi:10.1056/NEJMoa1707447 (2017).

194 Cornetta, K., Duffy, L., Turtle, C. J., Jensen, M., Forman, S., Binder-Scholl, G., Fry, T., Chew, A., Maloney, D. G. & June, C. H. Absence of Replication-Competent Lentivirus in the Clinic: Analysis of Infused T Cell Products. *Mol Ther* **26**, 280-288, doi:10.1016/j.ymthe.2017.09.008 (2018).

195 Tebas, P., Stein, D., Binder-Scholl, G., Mukherjee, R., Brady, T., Rebello, T., Humeau, L., Kalos, M., Papasavvas, E., Montaner, L. J., Schullery, D., Shaheen, F., Brennan, A. L., Zheng, Z., Cotte, J., Slepushkin, V., Veloso, E., Mackley, A., Hwang, W. T., Aberra, F., Zhan, J., Boyer, J., Collman, R. G., Bushman, F. D., Levine, B. L. & June, C. H. Antiviral effects of autologous CD4 T cells genetically modified with a conditionally replicating lentiviral vector expressing long antisense to HIV. *Blood* **121**, 1524-1533, doi:10.1182/blood-2012-07-447250 (2013).

196 Levine, B. L., Humeau, L. M., Boyer, J., MacGregor, R. R., Rebello, T., Lu, X., Binder, G. K., Slepushkin, V., Lemiale, F., Mascola, J. R., Bushman, F. D., Dropulic, B. & June, C. H. Gene transfer in humans using a conditionally replicating lentiviral vector. *Proc Natl Acad Sci U S A* **103**, 17372-17377, doi:10.1073/pnas.0608138103 (2006).

197 Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S. & Gregory, P. D. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* **11**, 636-646, doi:10.1038/nrg2842 (2010).

198 Krishna, S. S., Majumdar, I. & Grishin, N. V. Structural classification of zinc fingers: survey and summary. *Nucleic Acids Res* **31**, 532-550, doi:10.1093/nar/gkg161 (2003).

199 Wolfe, S. A., Nekludova, L. & Pabo, C. O. DNA recognition by Cys2His2 zinc finger proteins. *Annu Rev Biophys Biomol Struct* **29**, 183-212, doi:10.1146/annurev.biophys.29.1.183 (2000).

200 Doyle, E. L., Stoddard, B. L., Voytas, D. F. & Bogdanove, A. J. TAL effectors: highly adaptable phytobacterial virulence factors and readily engineered DNA-targeting proteins. *Trends Cell Biol* **23**, 390-398, doi:10.1016/j.tcb.2013.04.003 (2013).

201 Moore, R., Chandrahas, A. & Bleris, L. Transcription activator-like effectors: a toolkit for synthetic biology. *ACS Synth Biol* **3**, 708-716, doi:10.1021/sb400137b (2014).

202 Joung, J. K. & Sander, J. D. TALENs: a widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol* **14**, 49-55, doi:10.1038/nrm3486 (2013).

203 Cathomen, T. & Keith Joung, J. Zinc-finger Nucleases: The Next Generation Emerges. *Mol Ther* **16**, 1200-1207, doi:10.1038/mt.2008.114 (2008).

204 Garton, M., Najafabadi, H. S., Schmitges, F. W., Radovani, E., Hughes, T. R. & Kim, P. M. A structural approach reveals how neighbouring C2H2 zinc fingers influence DNA binding specificity. *Nucleic Acids Res* **43**, 9147-9157, doi:10.1093/nar/gkv919 (2015).

205 Bogdanove, A. J. & Voytas, D. F. TAL effectors: customizable proteins for DNA targeting. *Science* **333**, 1843-1846, doi:10.1126/science.1204094 (2011).

206 Martinez-Lage, M., Torres-Ruiz, R. & Rodriguez-Perales, S. CRISPR/Cas9 Technology: Applications and Human Disease Modeling. *Prog Mol Biol Transl Sci* **152**, 23-48, doi:10.1016/bs.pmbts.2017.09.002 (2017).

207 Doudna, J. A. & Charpentier, E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* **346**, 1258096, doi:10.1126/science.1258096 (2014).
208 Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., Eckert, M. R., Vogel, J. & Charpentier, E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* **471**, 602-607, doi:10.1038/nature09886 (2011).
209 Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A. & Charpentier, E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821, doi:10.1126/science.1225829 (2012).
210 Banerjea, A., Li, M. J., Bauer, G., Remling, L., Lee, N. S., Rossi, J. & Akkina, R. Inhibition of HIV-1 by lentiviral vector-transduced siRNAs in T lymphocytes differentiated in SCID-hu mice and CD34+ progenitor cell-derived macrophages. *Mol Ther* **8**, 62-71, doi:10.1016/s1525-0016(03)00140-0 (2003).
211 Lee, M. T., Coburn, G. A., McClure, M. O. & Cullen, B. R. Inhibition of human immunodeficiency virus type 1 replication in primary macrophages by using Tat- or CCR5-specific small interfering RNAs expressed from a lentivirus vector. *J Virol* **77**, 11964-11972, doi:10.1128/jvi.77.22.11964-11972.2003 (2003).
212 Li, M. J., Bauer, G., Michienzi, A., Yee, J. K., Lee, N. S., Kim, J., Li, S., Castanotto, D., Zaia, J. & Rossi, J. J. Inhibition of HIV-1 infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs. *Mol Ther* **8**, 196-206, doi:10.1016/s1525-0016(03)00165-5 (2003).
213 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. *Proc Natl Acad Sci U S A* **100**, 183-188, doi:10.1073/pnas.232688199 (2003).
214 ter Brake, O., Konstantinova, P., Ceylan, M. & Berkhout, B. Silencing of HIV-1 with RNA interference: a multiple shRNA approach. *Mol Ther* **14**, 883-892, doi:10.1016/j.ymthe.2006.07.007 (2006).
215 Centlivre, M., Legrand, N., Klamer, S., Liu, Y. P., Jasmijn von Eije, K., Bohne, M., Rijnstra, E. S., Weijer, K., Blom, B., Voermans, C., Spits, H. & Berkhout, B. Preclinical in vivo evaluation of the safety of a multi-shRNA-based gene therapy against HIV-1. *Mol Ther Nucleic Acids* **2**, e120, doi:10.1038/mtna.2013.48 (2013).
216 McIntyre, G. J., Groneman, J. L., Yu, Y. H., Jaramillo, A., Shen, S. & Applegate, T. L. 96 shRNAs designed for maximal coverage of HIV-1 variants. *Retrovirology* **6**, 55, doi:10.1186/1742-4690-6-55 (2009).
217 Low, J. T., Knoepfel, S. A., Watts, J. M., ter Brake, O., Berkhout, B. & Weeks, K. M. SHAPE-directed discovery of potent shRNA inhibitors of HIV-1. *Mol Ther* **20**, 820-828, doi:10.1038/mt.2011.299 (2012).
218 Knoepfel, S. A., Centlivre, M., Liu, Y. P., Boutimah, F. & Berkhout, B. Selection of RNAi-based inhibitors for anti-HIV gene therapy. *World J Virol* **1**, 79-90, doi:10.5501/wjv.v1.i3.79 (2012).
219 Scarborough, R. J., Levesque, M. V., Boudrias-Dalle, E., Chute, I. C., Daniels, S. M., Ouellette, R. J., Perreault, J. P. & Gatignol, A. A Conserved Target Site in HIV-1 Gag RNA is Accessible to Inhibition by Both an HDV Ribozyme and a Short Hairpin RNA. *Mol Ther Nucleic Acids* **3**, e178, doi:10.1038/mtna.2014.31 (2014).
220 Das, A. T., Brummelkamp, T. R., Westerhout, E. M., Vink, M., Madiredjo, M., Bernards, R. & Berkhout, B. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J Virol* **78**, 2601-2605, doi:10.1128/jvi.78.5.2601-2605.2004 (2004).

221 Sabariegos, R., Gimenez-Barcons, M., Tapia, N., Clotet, B. & Martinez, M. A. Sequence homology required by human immunodeficiency virus type 1 to escape from short interfering RNAs. *J Virol* **80**, 571-577, doi:10.1128/JVI.80.2.571-577.2006 (2006).
222 Ledger, S., Howe, A., Turville, S., Aggarwal, A., Savkovic, B., Ong, A., Wolstein, O., Boyd, M., Millington, M., Gorry, P. R., Murray, J. M. & Symonds, G. Analysis and dissociation of anti-HIV effects of shRNA to CCR5 and the fusion inhibitor C46. *J Gene Med* **20**, e3006, doi:10.1002/jgm.3006 (2018).
223 Shun, M. C., Raghavendra, N. K., Vandegraaff, N., Daigle, J. E., Hughes, S., Kellam, P., Cherepanov, P. & Engelman, A. LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration. *Genes Dev* **21**, 1767-1778, doi:10.1101/gad.1565107 (2007).
224 Brass, A. L., Dykxhoorn, D. M., Benita, Y., Yan, N., Engelman, A., Xavier, R. J., Lieberman, J. & Elledge, S. J. Identification of host proteins required for HIV infection through a functional genomic screen. *Science* **319**, 921-926, doi:10.1126/science.1152725 (2008).
225 Konig, R., Zhou, Y., Elleder, D., Diamond, T. L., Bonamy, G. M., Irelan, J. T., Chiang, C. Y., Tu, B. P., De Jesus, P. D., Lilley, C. E., Seidel, S., Opaluch, A. M., Caldwell, J. S., Weitzman, M. D., Kuhen, K. L., Bandyopadhyay, S., Ideker, T., Orth, A. P., Miraglia, L. J., Bushman, F. D., Young, J. A. & Chanda, S. K. Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. *Cell* **135**, 49-60, doi:10.1016/j.cell.2008.07.032 (2008).
226 Zhou, H., Xu, M., Huang, Q., Gates, A. T., Zhang, X. D., Castle, J. C., Stec, E., Ferrer, M., Strulovici, B., Hazuda, D. J. & Espeseth, A. S. Genome-scale RNAi screen for host factors required for HIV replication. *Cell Host Microbe* **4**, 495-504, doi:10.1016/j.chom.2008.10.004 (2008).
227 Ruiz, A., Pauls, E., Badia, R., Riveira-Muñoz, E., Clotet, B., Ballana, E. & Esté, J. A. Characterization of the influence of mediator complex in HIV-1 transcription. *J Biol Chem* **289**, 27665-27676, doi:10.1074/jbc.M114.570341 (2014).
228 Bushman, F. D., Malani, N., Fernandes, J., D'Orso, I., Cagney, G., Diamond, T. L., Zhou, H., Hazuda, D. J., Espeseth, A. S., Konig, R., Bandyopadhyay, S., Ideker, T., Goff, S. P., Krogan, N. J., Frankel, A. D., Young, J. A. & Chanda, S. K. Host cell factors in HIV replication: meta-analysis of genome-wide studies. *PLoS Pathog* **5**, e1000437, doi:10.1371/journal.ppat.1000437 (2009).
229 Qi, C., Li, D., Jiang, X., Jia, X., Lu, L., Wang, Y., Sun, J., Shao, Y. & Wei, M. Inducing CCR5Delta32/Delta32 Homozygotes in the Human Jurkat CD4+ Cell Line and Primary CD4+ Cells by CRISPR-Cas9 Genome-Editing Technology. *Mol Ther Nucleic Acids* **12**, 267-274, doi:10.1016/j.omtn.2018.05.012 (2018).
230 Xu, L., Yang, H., Gao, Y., Chen, Z., Xie, L., Liu, Y., Liu, Y., Wang, X., Li, H., Lai, W., He, Y., Yao, A., Ma, L., Shao, Y., Zhang, B., Wang, C., Chen, H. & Deng, H. CRISPR/Cas9-Mediated CCR5 Ablation in Human Hematopoietic Stem/Progenitor Cells Confers HIV-1 Resistance In Vivo. *Mol Ther* **25**, 1782-1789, doi:10.1016/j.ymthe.2017.04.027 (2017).
231 Xu, L., Wang, J., Liu, Y., Xie, L., Su, B., Mou, D., Wang, L., Liu, T., Wang, X., Zhang, B., Zhao, L., Hu, L., Ning, H., Zhang, Y., Deng, K., Liu, L., Lu, X., Zhang, T., Xu, J., Li, C., Wu, H., Deng, H. & Chen, H. CRISPR-Edited Stem Cells in a Patient with HIV and

Acute Lymphocytic Leukemia. *N Engl J Med* **381**, 1240-1247, doi:10.1056/NEJMoa1817426 (2019).

232 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. *Antimicrob Agents Chemother* **59**, 5297-5305, doi:10.1128/AAC.00949-15 (2015).

233 Fedorov, Y., Anderson, E. M., Birmingham, A., Reynolds, A., Karpilow, J., Robinson, K., Leake, D., Marshall, W. S. & Khvorova, A. Off-target effects by siRNA can induce toxic phenotype. *RNA* **12**, 1188-1196, doi:10.1261/rna.28106 (2006).

234 Judge, A. D., Sood, V., Shaw, J. R., Fang, D., McClintock, K. & MacLachlan, I. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat Biotechnol* **23**, 457-462, doi:10.1038/nbt1081 (2005).

235 Grimm, D., Streetz, K. L., Jopling, C. L., Storm, T. A., Pandey, K., Davis, C. R., Marion, P., Salazar, F. & Kay, M. A. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**, 537-541, doi:10.1038/nature04791 (2006).

236 Park, S. B., Uchida, T., Tilson, S., Hu, Z., Ma, C. D., Leek, M., Eichner, M., Hong, S. G. & Liang, T. J. A dual conditional CRISPR-Cas9 system to activate gene editing and reduce off-target effects in human stem cells. *Mol Ther Nucleic Acids* **28**, 656-669, doi:10.1016/j.omtn.2022.04.013 (2022).

237 Qi, C., Jia, X., Lu, L., Ma, P. & Wei, M. HEK293T Cells Are Heterozygous for CCR5 Delta 32 Mutation. *PLoS One* **11**, e0152975, doi:10.1371/journal.pone.0152975 (2016).

238 An, D. S., Qin, F. X., Auyeung, V. C., Mao, S. H., Kung, S. K., Baltimore, D. & Chen, I. S. Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol Ther* **14**, 494-504, doi:10.1016/j.ymthe.2006.05.015 (2006).

239 Sun, C. P., Wu, T. H., Chen, C. C., Wu, P. Y., Shih, Y. M., Tsuneyama, K. & Tao, M. H. Studies of efficacy and liver toxicity related to adeno-associated virus-mediated RNA interference. *Hum Gene Ther* **24**, 739-750, doi:10.1089/hum.2012.239 (2013).

240 Makinen, P. I., Koponen, J. K., Karkkainen, A. M., Malm, T. M., Pulkkinen, K. H., Koistinaho, J., Turunen, M. P. & Yla-Herttuala, S. Stable RNA interference: comparison of U6 and H1 promoters in endothelial cells and in mouse brain. *J Gene Med* **8**, 433-441, doi:10.1002/jgm.860 (2006).

241 Spanevello, F., Calistri, A., Del Vecchio, C., Mantelli, B., Frasson, C., Basso, G., Palu, G., Cavazzana, M. & Parolin, C. Development of Lentiviral Vectors Simultaneously Expressing Multiple siRNAs Against CCR5, vif and tat/rev Genes for an HIV-1 Gene Therapy Approach. *Mol Ther Nucleic Acids* **5**, e312, doi:10.1038/mtna.2016.24 (2016).

242 Brake, O. T., Hooft, K., Liu, Y. P., Centlivre, M., Jasmijn von Eije, K. & Berkhout, B. Lentiviral Vector Design for Multiple shRNA Expression and Durable HIV-1 Inhibition. *Mol Ther* **16**, 557-564, doi:10.1038/sj.mt.6300382 (2008).

243 Gao, Z., Harwig, A., Berkhout, B. & Herrera-Carrillo, E. Mutation of nucleotides around the +1 position of type 3 polymerase III promoters: The effect on transcriptional activity and start site usage. *Transcription* **8**, 275-287, doi:10.1080/21541264.2017.1322170 (2017).

244 Putzbach, W., Gao, Q. Q., Patel, M., van Dongen, S., Haluck-Kangas, A., Sarshad, A. A., Bartom, E. T., Kim, K. A., Scholtens, D. M., Hafner, M., Zhao, J. C., Murmann, A. E. &

Peter, M. E. Many si/shRNAs can kill cancer cells by targeting multiple survival genes through an off-target mechanism. *Elife* **6**, doi:10.7554/eLife.29702 (2017).

245 Gao, Q. Q., Putzbach, W. E., Murmann, A. E., Chen, S., Sarshad, A. A., Peter, J. M., Bartom, E. T., Hafner, M. & Peter, M. E. 6mer seed toxicity in tumor suppressive microRNAs. *Nat Commun* **9**, 4504, doi:10.1038/s41467-018-06526-1 (2018).

246 Batista Napotnik, T., Polajzer, T. & Miklavcic, D. Cell death due to electroporation - A review. *Bioelectrochemistry* **141**, 107871, doi:10.1016/j.bioelechem.2021.107871 (2021).

247 Rao, S., Amorim, R., Niu, M., Temzi, A. & Mouland, A. J. The RNA surveillance proteins UPF1, UPF2 and SMG6 affect HIV-1 reactivation at a post-transcriptional level. *Retrovirology* **15**, 42, doi:10.1186/s12977-018-0425-2 (2018).

248 Kosicki, M., Allen, F., Steward, F., Tomberg, K., Pan, Y. & Bradley, A. Cas9-induced large deletions and small indels are controlled in a convergent fashion. *Nat Commun* **13**, 3422, doi:10.1038/s41467-022-30480-8 (2022).

249 Wang, H., Perrault, A. R., Takeda, Y., Qin, W., Wang, H. & Iliakis, G. Biochemical evidence for Ku-independent backup pathways of NHEJ. *Nucleic Acids Res* **31**, 5377-5388, doi:10.1093/nar/gkg728 (2003).

250 Wang, H. & Xu, X. Microhomology-mediated end joining: new players join the team. *Cell Biosci* **7**, 6, doi:10.1186/s13578-017-0136-8 (2017).

251 Sfeir, A. & Symington, L. S. Microhomology-Mediated End Joining: A Back-up Survival Mechanism or Dedicated Pathway? *Trends Biochem Sci* **40**, 701-714, doi:10.1016/j.tibs.2015.08.006 (2015).

252 Bhattacharya, D. & Van Meir, E. G. A simple genotyping method to detect small CRISPR-Cas9 induced indels by agarose gel electrophoresis. *Sci Rep* **9**, 4437, doi:10.1038/s41598-019-39950-4 (2019).