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Eradication of the latent HIV viral reservoir: Targeted disruption of the integrated HIV provirus using engineered meganucleases and quantitation of the latent HIV reservoir using multiplex ddPCR

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Abstract

Eradication of the latent HIV viral reservoir: Targeted disruption of the integrated HIV provirus using engineered meganucleases and quantitation of the latent HIV reservoir using multiplex ddPCR

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The major barrier to HIV cure is the establishment of a long-lived latent viral reservoir that is not affected by combination ant-retroviral therapy and is not cleared by the immune system. When HIV positive patients on combination anti-retroviral therapy with undetectable viremia interrupt therapy, viremic rebound occurs within two weeks of discontinuing cART. This viral rebound is due to stochastic reactivation of HIV transcription and replication in the long-lived viral reservoir. HIV cure necessitates eradication of this long-lived reservoir.

To eradicate the HIV proviral reservoir, we are using HIV-specific engineered meganucleases. Mutations in essential HIV genes can disrupt the integrated provirus and could prevent reactivation from latency. I demonstrate that engineered meganucleases can introduce

mutations in an integrated HIV provirus. The HIV-specific engineered meganucleases also cleave and introduce mutations in genomic off-target sites. We show that a second generation of an HIV-specific meganuclease developed using structure guided protein engineering, has an improved off-target toxicity profile and retains activity at the HIV target site. I further demonstrate that expression of the three prime repair exonuclease-2 (Trex2) in combination with either the meganuclease or fusion megaTALs increases the frequency of mutations at the HIV target site. The compact size of meganucleases and the increasing ease with which they can be redesigned to recognize new DNA sequences makes meganucleases an attractive tool for HIV cure applications.

HIV cure also requires precise quantitation of the viral reservoir. Current assays used to measure the HIV viral reservoir are imprecise and tend to either over or underestimate the size of the functional reservoir. In the setting of both structured and analytical treatment interruptions it is critical that the reservoir is measured accurately so that patients do not discontinue cART in the setting of large functional reservoirs. I describe here a multiplex ddPCR assay that can simultaneously detect up to six viral genes in the same reaction. The multiplex ddPCR assay gives an estimate of the completeness of the integrated provirus, which can be used to measure levels of functional integrated HIV provirus. I hypothesize that this multiplex ddPCR assay gives a truer estimate of the size of the functional HIV reservoir.

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DEDICATION

To Gash, Joan, Connie and Lynn, and to the memory of my mother, Grace Kabayundo

Chapter 1. INTRODUCTION

1.1 THE HIV/AIDS PANDEMIC

In June of 1981, the U.S. Centers for Disease Control and Prevention's Morbidity and Mortality Weekly Report (MMWR) published five cases of previously healthy gay men with pneumocycstis carinii pneumonia (PCP) in Los Angeles(1,2), which rarely seen in healthy people. Later that year, several cases of Kaposi Sarcoma, also rare in immune-competent patients, were reported in previously healthy adults in California and New York(3,4). In 1982, the CDC termed this disease the Acquired Immune Deficiency Syndrome (AIDS). AIDS was subsequently identified in recipients of transfusions, especially hemophiliacs but was also identified in infants, children and women(5).

The agent responsible for AIDS, the Human Immunodeficiency Virus (HIV) was discovered in 1983(6). By 1985, HIV was found to have spread to over 50 countries worldwide. In a number of African countries such as Zaire (now Democratic Republic of Congo, DRC) and Uganda, there was epidemiological evidence to suggest that HIV and AIDS had been responsible for patient deaths several years before AIDS was described in the United States. In Zaire, Peter Piot and colleagues in 1983 reported 38 cases of patients with AIDS within a three-week period(7). The majority of these patients, 32(84%) had opportunistic infections including Kaposi Sarcoma as had been previously described in the US and Europe. Importantly, this group also showed that most of these cases were a result of heterosexual transmission. Serwadda and colleagues also reported cases of patients dying from an HIV-like illness in Uganda in 1985(8). In Uganda, and in neighboring countries, AIDS was called the "SLIM disease" owing to the severe cachexia and wasting associated with syndromic HIV/AIDS.

HIV patients were found across the globe and the HIV epidemic is still unrelenting. In the early days of the epidemic there were no drugs for the illness and the disease was not well understood. New infections from HIV continued to grow, as did deaths from HIV/AIDS. Deaths from HIV/AIDS peaked in 2004 after more HIV positive patients were initiated on combination anti-retroviral therapy (cART) through such initiatives as the Global Fund and the President's Emergency Fund for AIDS Relief (PEPFAR), a program started by the U.S. government to enable provision of cART to developing countries(9-11). Presently, in 2016, an estimated 37 million people worldwide are living with HIV. The majority of these HIV positive patients (70%) are in Sub-Saharan Africa. Importantly, however, by mid-2015 an estimated 15.8 million HIV positive patients (42% of all positive patients) were on combination anti-retroviral therapy which prevents progression to AIDS. However, despite these successes, in 2014 there were 1.2 million deaths from HIV/AIDS and an estimated 2 million new HIV infections(12).

1.2 ORIGINS AND DIVERSITY OF HIV-1

Human immunodeficiency virus-1 is a result of a zoonotic transmission of the chimpanzee virus, SIVcpz, to humans. SIVcpz is a recombinant virus derived from the Simian immunodeficiency virus of red-capped mangabeys (SIV rcm) and Simian immunodeficiency virus of mona, moustachied and greater spotted nose monkeys (SIV gsn/mus/mon). SIV gsn/mus/mon and SIVrcm are thought to have recombined in chimpanzees to give rise to the SIVcpz found in chimpanzees. The recombinant SIVcpz virus is then thought to have been transmitted from chimpanzees to humans as HIV-1. Chimpanzees have been established as the intermediate hosts of both pandemic HIV-1 (group M) and non-pandemic HIV-1 (group N)(13-15). HIV-1 group P was transferred from western lowland gorillas (Gorilla gorilla gorilla) to

humans and group O was transferred from chimpanzees to gorillas and then eventually to humans(16,17). HIV-2 is a result of a transmission of Simian immunodeficiency virus from sooty mangabey monkeys (SIV smm)(18-20).

Pandemic HIV-1 (group M) is thought to have originated in the present day Democratic Republic of Congo (DRC), and Southern Cameroon in the Congo Basin region in West Africa. Evidence for the origin of HIV from these regions is from both primate studies as well as studies of archived samples in the DRC. An analysis of archival specimens taken from patients in the DRC in 1959 and 1960 found that there was already significant sequence variation and divergence between the HIV virus isolated from these two archival specimens(21,22). The sequence diversity between the viruses isolated from these two specimens allows us to estimate the introduction of HIV into humans to the turn of the 20th century, around 1908 (between 1884 and 1924). HIV-2 and other HIV-1 subtypes, N, O and P, were all introduced into humans between 1900 and 1970(23).

More evidence for the emergence of the HIV-1 pandemic from the Congo basin is from sequence analysis of the virus circulating in the Republic of Congo and the DRC. Analysis of these sequences showed very high genetic diversity in the HIV-envelope in areas surrounding Kinshasa, Brazzaville, Mbuyi-Mayi and Ponte-Noire(24,25). HIV is then thought to have spread along trading routes, waterways, and railway lines to major towns, which were heavily populated and probably contributed to amplification of the epidemic. HIV then spread from these regions to the rest of the world.

HIV has extensive genetic diversity. Analysis of 2996 genomic sequences established that at the nucleotide level, there is an estimated 50% difference between HIV-1 and HIV-2, 37.5% between different HIV-1 groups, about 15% between different HIV-1 subtypes, 8.2%

within HIV-1 subtypes and almost 1% intra-patient diversity(26). HIV-1 group M can further be subdivided into 10 different phylogenetic subgroups/clades A to K, and up to 20 circulating recombinant forms that result from co-circulation of more than one clade in a region. Within group M, interclade genetic variability is 15% in the gag region and 25% in env (27). All the different HIV subtypes have different geographical distributions probably resulting from human movements and migrations. Almost all the HIV groups and subtypes are found in Africa. In west and central Africa, HIV-1 clades A, C, D, F, G, H, J, K and recombinant forms CRF02_AG, CRF01 are found to be widely circulating (28).

Nearly all the different HIV types and subtypes have been found circulating in West Africa. HIV-2 has been described in several West African countries where it has largely been confined(29,30). In these countries, HIV-2, which is less pathogenic, is gradually being replaced by the more pathogenic HIV-1(28,31). HIV-1 clades N, O and P have also been isolated from West Africa in patients living in or originally habituated in Cameroon(32-35). In Southern Africa, clade C predominates while in East Africa the dominant subtypes are A, C and F. HIV-1 clade B is the most common HIV-1 group M clade in the Americas (North and Latin), Western Europe and Oceania. In Eastern Europe and central Asia, clade A is the predominant circulating viral subtype while in East Asia, clades B and F are the dominant subtypes.

The extensive diversity of the HIV virus has important ramifications for both HIV drug management and HIV vaccine design. The high mutation rates of the virus mean that the virus may readily develop resistance to the currently used anti-retroviral drugs and there is evidence to suggest development of mutations that confer resistance to the current therapeutics, although this is less frequent now with highly efficacious cART. Additionally, vaccine design has to take into account both the diversity of the circulating viral subtypes but also the intra-patient evolution

that happens once a patient is infected with the virus. The vaccine, as should HIV-specific endonucleases, should also offer coverage for the prevalent subtypes and should tolerate the on-going evolution within the target antigenic epitopes, although even a single clade vaccine would be very useful in preventing infections and probably provide a platform for development of vaccines for other clades.

1.3 HIV LIFE CYCLE

The HIV virus is an enveloped positive sense single stranded RNA virus with a pseudo-diploid genome. The viral capsid is icosahedral and each virion contains two positive sense RNA strands. The viral genome, which is approximately 9.7kb, encodes several proteins that are important both in viral replication and disease pathogenesis. The gag gene encodes structural proteins Matrix (MA), Capsid (CA), Nucleocapsid (NC) and P6. The Pol gene encodes the enzymatic proteins Protease (PR), Reverse transcriptase (RT) and Integrase (IN) which are critical proteins in the viral replication cycle. The viral Env protein is processed into gp120 and gp41 that are important in viral entry into cells. The virus also encodes two regulatory proteins (tat and rev) that are important in viral replication as well as a variety of accessory proteins; vpu, vif, vpr and nef. While the accessory proteins are not absolutely critical to viral replication, they are important in viral pathogenesis and usually work by antagonizing host defense mechanisms.

The viral envelope proteins, gp120 and gp41 both participate in HIV viral cell entry and membrane fusion with susceptible cells. The primary cellular receptor of HIV is the CD4 cell receptor and the main co-receptors for HIV entry are chemokine receptor-5 (CCR5) and the C-X-C chemokine receptor-4 (CXCR4). A number of other co-receptors have been identified including CCR2b, CCR3, CCR8, Bonzo, US28 and GPR15. The nature of interactions and role

of these co-receptors in HIV membrane fusion and entry are not well understood(36). The HIV envelope glycoprotein gp120 interacts strongly with the CD4 cell receptor. Binding of gp120 to the CD4 receptor induces conformational changes in the structure of the envelope trimer and exposes binding epitopes for the co-receptor, either CCR5 or CXCR4, which leads to membrane fusion by the gp41 protein. The CD4 receptor is necessary but not sufficient to promote membrane fusion and cellular entry. The viral capsid contains both the positive sense viral RNA and a viral reverse transcriptase that has RNA dependent polymerase activity, RNaseH activity and DNA-dependent polymerase acrivity for second strand synthesis. The viral ssRNA then undergoes reverse transcription to double stranded viral DNA that is shuttled to the nucleus. The RT can use either of the strands for reverse transcription and template switching can lead to recombination of the strands and subsequent introduction of both insertions and deletions(37). The viral reverse transcriptase enzyme is also error prone and introduces errors during reverse transcription. The high error rate of the reverse transcriptase, the template switching and the high multiplicative capacity of HIV in addition to chronicity of HIV infection are some of the reasons that contribute to the extensive diversity of the virus. The chronicity of infection allows for accumulation of individual mutations during the lifetime of the patient. Recombination during reverse transcription also combines mutations from different packaged RNA strands and these can be passed on to new virions, including mutations that improve viral fitness.

Following reverse transcription and shuttling to the nucleus, the viral integrase enzyme then catalyzes insertion of the viral double stranded DNA randomly into open chromatin genomic DNA. The pre-integration complexes also contain several host proteins such as p75 (LEDGF), HMGA2, and INI1 that play important roles in integration. LEDGF is thought to shuttle the viral pre-integration complex to integration sites (38).

The integrated viral DNA (HIV provirus) can then be transcribed to produce new virions or may remain latent in host cells where it can lie transcriptionally silent for many years. Several host transcription factors such as NFAT and NF-κB are important in HIV viral RNA synthesis. Initial transcription of the provirus results in short transcripts(39). However, one of the earliest transcripts is tat. Tat forms complexes with p-TEFB (positive transcription elongation factor B) and several host cyclins. This complex then binds to the viral TAR region (transactivation region) and stabilizes the RNA II polymerase complex and leads to transcription elongation. The viral Rev protein then leads to cytoplasmic export of full-length transcripts where translation of the viral proteins occurs. Some of the full-length viral RNA transcripts become genomic viral single stranded RNA. Following virion assembly, the immature virions bud off the plasma membrane. Maturation of the virion after budding is facilitated by the viral protease. In the absence of active protease, the immature virion is non-infectious(40).

Each of the key steps in the viral replication cycle is a target of anti-retroviral therapy and several drugs have been described targeting these steps. At the entry step there are both entry and fusion inhibitors that target gp120 and gp41. The fusion inhibitor enfurvitide is an inhibitor of gp41 and membrane fusion. There are several drugs that block the CCR5 co-receptor such as maraviroc that prevent interaction of the virus with the co-receptor and subsequent cell entry. The broadest range of drugs available against HIV are those drugs that inhibit the viral reverse transcriptase. There are several classes of drugs that inhibit the viral reverse transcriptase and these include the nucleoside reverse transcriptase inhibitors (NRTIs) such as zidovudine (Azidothymidine, AZT), which was the first anti-retroviral drug to be approved. Other nucleoside analogues include emtricitabine (FTC), zalcitabine (DDC), stavudine (D4T), lamivudine (3TC) and didanosine (DDI). Nucleoside analogues are metabolized by addition of a

phosphate group to nucleotides. Another group of drugs is the nucleotide analogue reverse transcriptase inhibitors (NRTIs) such as tenofovir (TDF) and Adefovir (ADV). When nucleotide or nucleoside analogues are incorporated into the viral DNA during reverse transcription, they lead to irreversible termination of transcription. The other class of reverse transcriptase inhibitors is the non-nucleoside reverse transcriptase inhibitors (NNRTIs) which include Nevirapine (NVP) and Efavirenz (EFV). The NNRTIs bind away from the active site and change the conformation of the active site of reverse transcription leading to termination of reverse transcription.

There are several integrase inhibitors such as Raltegravir that prevent integration. Maturation inhibitors (also called Protease inhibitors or PIs) inhibit the activity of the viral protease and prevent conversion of the mature virion into infectious viral progeny. Protease inhibitors include the drugs Nelfinavir, Lopinavir, Amprenavir, Atazanavir and numerous others(41).

1.4 NATURAL HISTORY AND PATHOGENESIS OF HIV-1 INFECTION

On initial infection, viral replication rapidly takes place with detectable viremia and sero-conversion occurring within about two weeks of infection. The target of the HIV virus is CD4+ T cells, primarily, activated CD4+ T cells which have high expression levels of the CD4 receptor and the CCR5 co-receptor. The proportion of CD4+ T cells that express CCR5 in the gut is higher than in peripheral blood and other secondary lymphoid organs(42). The dynamics of these CD4+ T cells mirror the dynamics of the virus. The initial rise in HIV viremia is accompanied by a rapid fall to a set point, which is sustained. The viral set point varies from patient to patient and is affected by both patient factors such as the HLA subtype and the immune response to the virus, as well as viral fitness characteristics(43-46). The viral set point is associated with the rapidity of disease progression to AIDS. The CD4+ T cells, which are the target of HIV

infection, initially contribute to viral control and help in lowering the viral load. The CD4+ cell count progressively declines until a so-called inflection point, which is accompanied by rapid decline in the cell count(47). The rapid decline of CD4+ cell count is accompanied by a rise in HIV viremia and heralds the onset of opportunistic infections. This is clinical AIDS for which common opportunistic infections include Pneumocystis jiroveci pneumonia and Kaposi Sarcoma, two diseases whose description in HIV patients in California led to the first description of AIDS. Other opportunistic infections include a variety of AIDS-defining malignancies such as Non-Hodgkin Lymphoma(NHL) and Cervical cancer(48). AIDS is also characterized, in the untreated patient, by cachexia, CMV reactivation including retinitis, lymphadenopathy, tuberculosis, toxoplasmosis and several other viral, bacterial and parasitic infections that take advantage of the depleted immune system(49,50).

The average rate of progression to AIDS in the untreated patient is estimated to be between 5 to 10 years although some patients may progress to AIDS within as few as two years and is dependent on many factors such as the immune system and the viral set point(51,52). On the other end of the spectrum are patients who have long periods of sustained HIV suppression with undetectable viremia with or without high CD4+ cell levels. These patients are called elite controllers. Another group of patients, known as long term non-progressors (LTNPs) have long periods of sustained high CD4+ cell counts. LTNPs may have high or low viral loads and have been shown to have long periods before progression to AIDS(53). Elite controllers and LTNPs comprise between 1 to 5% of all HIV infected patients. Another group of patients, the viremic controllers, have low level viremia off cART and independently comprise about 6% of all HIV-infected patients(54). Elite controllers and viremic controllers are collectively known as HIV controllers (HIC). Patients have been shown to maintain HIV control for a median of 4.1 years

from a French cohort of HIV positive patients with some patients attaining more than 10 years of viremic control(55). The controller status is established early during primary infection and has been suggested to be associated with development of potent antibodies and immune control of the virus although this is not well described(56). Decline in the CD4+ cell count is associated with viral blips in these patients that achieve long-term control of HIV viremia. These groups of patients that either spontaneously control HIV viremia or achieve long-term preservation of the immune system are important in understanding HIV pathogenesis and immune control of HIV.

Death from HIV/AIDS results from a variety of opportunistic infections such as tuberculosis, cryptococcal meningitis, toxoplasmosis, and HIV associated malignancies that are all a consequence of the severely depleted immune system.

1.5 COMBINATION ANTIRETROVIRAL THERAPY FOR HIV

The first drug approved for HIV was Zidovudine in 1987 and for a while, it was used as monotherapy for HIV, although monotherapy led to rapid development of drug resistance and disease progression. Several antiretroviral drugs were approved in the early 1990s but it wasn't until 1995 that triple combination antiretroviral therapy was shown to lead to drastic reduction of HIV viremia to undetectable levels(57,58). These findings led to the adoption of combination anti-retroviral therapy (cART) for widespread use in management of HIV in 1996. Single or dual therapy for HIV results in development of resistant mutations and viral rebound. HIV has a high mutation rate and consequently the likelihood of developing resistant mutations with single or dual therapy is high(59). Combination anti-retroviral therapy (cART) uses a combination of efficacious anti-retroviral drugs that target different steps of the HIV replication cycle. The multi-step targeting reduced the likelihood of simultaneous development of drug mutations to all

three drugs in the cocktail and enabled control of HIV replication, although resistance to all classes of antiretroviral drugs has been reported. The introduction of effective cART led to dramatic improvements in the quality of life of HIV positive patients and slowed the progression to AIDS. Combination anti-retroviral therapy was effective in controlling viral replication, and viral load in many patients who were adherent to medication dropped to undetectable levels. The introduction of cART in 1996 brought a raging epidemic under control and many countries saw a rapid decline in transmission events (incidence of HIV) and a reduction in incidence of opportunistic infections. In the US and Europe, cART led to a steady reduction in incidence of many AIDS-associated cancers such as Kaposi Sarcoma and Non-Hodgkin Lymphoma(60,61). Combination antiretroviral therapy did not roll out to many resource-limited settings until 2004 through the President's Emergency Fund for AIDS Relief (PEPFAR) and funding from such organizations as the Global Fund for HIV/AIDS, Malaria and Tuberculosis, the Gates Foundation and UNAIDS. This expanded access to cART has led to reductions in HIV transmissions worldwide although the reduction in opportunistic infections has not been as not dramatic in developing countries as in developed countries(62,63).

HIV DYNAMICS ON ANTI-RETROVIRAL THERAPY

On initiation of cART, HIV decays in a multiphasic pattern(64). Treatment initiation leads to rapid viral clearance and a precipitous drop in HIV viremia over the first two weeks of treatment. This first phase of viral decay is thought to be due to elimination of cell free virus (half-life of ~ 6 hours) and near-complete blockade of ongoing viral replication and the rapid turnover of HIV-infected activated CD4+ T cells. Activated CD4+ T cells have a half-life of approximately two to three days and are cleared from the circulation rapidly(58,65). This rapid

first phase decay suggests that large numbers of activated CD4+ T cells are infected every day and that the blockade of ongoing viral replication prevents infection of these cells leading to the decline in HIV viremia. First phase decay requires effective blockade of viral replication and cannot be achieved on monotherapy. On monotherapy, there is rapid development of resistance mutations and rebound viremia.

The first phase of HIV decay gives way to a slower second phase of viral decay that has a half-life estimated at four weeks. The virus in this phase of decay is thought to be from cells that are relatively long-lived such as macrophages and longer lived T cells (66,67). It has been estimated that viral clearance from the compartments that contribute to first and second phase viral decay takes about four years(68). During effective cART, the first and second phase decay give way to third phase decay, which has also been termed as viral persistence phase. This third phase is characterized by persistent low level viremia. Although patients usually have undetectable viremia, they frequently experience breakthrough viremic blips. Persistent low-level viremia is thought to result from HIV infected long lived memory CD4+ T cells(69). Memory CD4+ T cells have a half-life of 39.5 to 43.5 months and it has been estimated that at the decay rate using current antiretroviral therapy, it would require almost 65.7 to 74 years to clear this compartment of virus(70,71). Recent publications have also suggested that this phase of decay may still be characterized by on-going viral replication and evolution in tissue reservoirs that have sub-optimal drug concentrations such as lymph nodes or other drug privileged sites like the brain and gonads although the evidence for this is inconclusive(72,73). Maintained on suppressive therapy, patients will have undetectable viremia unless there is development of resistance mutations in which case patients can be switched to a different drug

regimen. Importantly, even on suppressive therapy it is still possible to isolate drug resistant virus despite the undetectable viremia(74).

1.6 HIV RESERVOIRS AND VIRAL REBOUND

Current cART therapies are quite effective at controlling viral replication and patients are able to live with undetectable viremia for long periods. However, interruption or discontinuation of cART inevitably results in viremic rebound. After discontinuing therapy, viremic rebound occurs within one to two weeks(75). The viral rebound is thought to result from the long-lived viral reservoir in latently infected resting memory CD4+ cells and characteristics of the reservoir affect both the rebound time and rebound viremia. Several factors are thought to influence the rapidity of viral rebound. Both the size of the viral reservoir and the viral set point before cART initiation influence the rebound time. Seeding of the reservoir occurs early in infection and several studies have shown that early initiation of cART is associated with reduced size of the viral reservoir and is associated with longer periods of virologic remission(76-80).

An HIV cure will require the elimination of the latent viral reservoir, although modeling studies suggest that a 2 to 4 log reduction may lead to long-term virologic remission without cART(81,82). Elimination of the latent HIV viral reservoir requires an understanding of the reservoir, including both its size and location, i.e. cell types or tissue compartments that harbor the latent viral reservoir and are responsible for viremic rebound in patients on cART with undetectable viremia. The most important HIV viral reservoir compartment is thought to be comprised of resting memory CD4+ T cells, which harbor the majority of the reservoir(83-85). Latent HIV infected resting memory CD4+ cells do not express activation markers such as

NFAT and NF-κB and have low nucleotides pools, all factors that favor HIV latency. The integrated HIV provirus may also undergo several chromatin modifications that hinder transcription and protein expression(86). As a result of these post-integration controls and modifications, the virus in these cells is latent and does not express any viral proteins and is therefore invisible to the immune system. Low-level replication of the virus replenishes the viral reservoir. Drug resistant clones can also exist within the integrated viral reservoir and can be reactivated even when on therapy(87). The HIV viral reservoir has also been described in other types of memory T cells such as transitional memory, effector memory and naïve memory cells, as well as in macrophages although the precise role of these cells in reservoir maintenance are still being determined. What is important however, is that to achieve long-term virologic remission without cART, we need to first prevent expansion of the cells in the latent reservoir pool, and then achieve significant reduction in the size of the viral reservoir or eliminate the reservoir altogether.

1.7 MEASURING THE HIV VIRAL RESERVOIR

Curative therapies for HIV require accurate quantification of the HIV viral reservoir size. The viral outgrowth assay (VOA) is often considered to be the gold standard assay for HIV reservoir. The VOA is a limiting dilution assay based on activation of HIV latently infected cells with phytohemagglutinin (PHA) which leads to global CD4 cell activation and subsequently viral transcription. The latently infected cells are co-cultured with irradiated peripheral blood mononuclear cells (PBMCs) or molt cells and activation of HIV transcription by PHA leads to HIV production and infection of new cells. HIV replication is detected using a p24 ELISA assay (88).

Other reservoir quantification methods are based on polymerase chain reaction and detect a segment of the integrated HIV viral DNA. Both qPCR and ddPCR have been used to quantify the HIV viral reservoir.

All the assays currently used to quantify the HIV viral reservoir are only approximate and are not a true reflection of the size of the reservoir. It has been estimated that about 89.2% of all integrated HIV proviruses are defective with large deletions in some segments (89). Moreover, not all intact replication competent viruses are induced to produce virus when stimulated with PHA during the virus outgrowth assay. Quantification of the reservoir using PCR based assays measures total viral DNA and will likely capture defective replication incompetent viruses that are inconsequential to disease reconstitution or viral rebound and disease pathogenesis. Using the VOA, replication competent viruses that are not activated to produce virus will not be captured by the assay. Both assays give us an idea of what the reservoir looks like but to truly cure HIV we must be able to accurately measure the viral reservoir. PCR based assays over-estimate the true reservoir size while the VOA under-estimates reservoir size.

Additionally, there is poor agreement between the current assays used to measure the HIV viral reservoir. Using the VOA assay, the frequency of HIV latently infected resting memory CD4 cells is estimated at 1 in every million cells ($1/10^6$) while using PCR-based assays, the reservoir is estimated at about 300 per million cells ($300/10^6$) (90). The Tat/rev induced limiting dilution assay (TILDA) is an RN-based assay for quantifying the latent viral reservoir that combines both stimulation and PCR measurement. In the TILDA assay, reservoir cells are stimulated using phorbol 12-myristate 13-acetate(PMA) and ionomycin, a combination that yields more stimulation of latent reservoir cells than PHA. Subsequently PCR

is performed to detect tat/rev messenger RNA transcripts. The TILDA assay gives higher estimates of reservoir size than QVOA and is more comparable to DNA PCR measurements of the reservoir. However, although the TILDA assay is based on measurement of RNA transcripts, it detects a single viral gene segment and cannot differentiate between partial and complete viral RNA transcripts (91).

There is need to develop new assays that can more accurately quantify the HIV viral reservoir. We propose using multiplex droplet digital PCR (ddPCR) to measure the viral reservoir. Multiplex ddPCR allows for simultaneous detection of several HIV DNA segments that can give an idea about the completeness of the integrated HIV provirus and ability to produce viable viral progeny.

1.8 HIV RESERVOIR ERADICATION AND CURE STRATEGIES

In 1999, the case of the now famous "Berlin patient" was described in the New England Journal of Medicine. The article described the case of a male patient with Acute Myeloid Leukemia (AML) and underlying HIV infection who achieved long term virologic control following two allogeneic stem cell transplants from a donor homozygous for CCR5 delta-32 mutation(92). The patient had been followed up for 20 months and had undetectable viremia by the time of publication. Almost 8 years since the transplant, the patient still has undetectable viremia and is considered cured of HIV. The persistent HIV viral reservoir is a barrier to cure and durable virologic remission can only be achieved through eradication or reduction in size of the long-lived HIV viral reservoir. In the "Berlin patient" it would appear that the depletion of the viral reservoir through a bone marrow conditioning regimen and subsequent transplant with

the CCR5 delta-32 deleted cells achieved sufficient control of HIV replication, and led to sustained virologic control that is still maintained to date.

Mathematical models estimate that a significant reduction in reservoir size could lead to almost lifelong virologic remission without cART. Conway and Perelson studied the dynamics of immune mediated control of viral infection in cART experienced patients and estimate that a two-log reduction in the size of the reservoir could lead to year-long virologic remission without cART(82,93). Another modeling paper from the Siliciano lab that looked at efficacy of latency reversing agents in combination with efficacious cART, estimates that a six-log reduction in the size of the viral reservoir could lead to a 30-year virologic remission(94). Several strategies are currently being studied to reduce the size of or eradicate the HIV viral reservoir. These strategies include early ART initiation, bone marrow transplant, CCR5 modification, use of latency reversing agents, use of immune modulating agents and targeted proviral disruption using engineered endonucleases(95). These strategies are reviewed below.

EARLY ART INITIATION

Early ART initiation prevents reservoir establishment and several studies have demonstrated that early treatment results in a diminished reservoir size and could lead to long-term virologic remission. The Visconti cohort comprises patients who initiated HIV treatment early during HIV primary infection and received treatment for up to five years, after which treatment was discontinued. These patients have been documented to have achieved sustained virologic remission off cART with one teenager achieving virologic remission for 12 years with no adverse effects in health(77,96). Another study that infected rhesus macaques with SIV and then initiated treatment after 3, 7, 10 and 14 days found that animals that were initiated on

treatment at 3 days had longer periods of virologic remission when cART was discontinued(80). Inevitably, however, all these animals rebounded.

In 2013, Deborah Persaud and colleagues reported a case of an infant who started cART within 30 hours of a normal spontaneous vaginal delivery. The child, famously called the "Mississippi baby" was lost to follow-up and stopped cART at 18 months. The infant had no detectable viremia for 27 months(97). Similar cases where cART was initiated early in life in the "Canada baby" (within 24 hours of life) and the "Milan baby" (within 12 hours of life)(98) were not nearly as successful with viral rebound occurring within two weeks despite having been on therapy for 3 years(99). The virus in the "Mississippi baby" did eventually rebound but the duration of virologic remission in this infant was promising. Early cART initiation preserves the immune system, prevents development of opportunistic infections, and advancements in drug design and safety mean that cART is now more tolerable(100). Such successes with early cART initiation could also lead to re-evaluation of structured treatment interruptions. Although structured treatment interruptions (STIs) have been reported to lead to reduced life expectancy when initiated at lower CD4+ cell count, the immune preservation achieved with early ART initiation could potentially be combined with STIs without severe detriment to the immune status and life expectancy of the patient(101). The demonstrated efficacy of early ART initiation has led to the World Health Organization recommending early initiation of HIV treatment.

LATENCY REVERSING AGENTS

The integrated HIV provirus undergoes a variety of epigenetic modifications and chromatin remodeling, all of which are important in maintaining latency of the provirus. It has been reported that HIV-1 infection leads to marked increase in methylation, particularly

H3K9me3 and H3K27me3 methylation that have been shown to be important in transcriptional repression(102,103). It has also been shown that histone deacetylases and histone acetyltransferases accumulate in the promoter region of HIV and that histone modifications contribute significantly to transcriptional silencing(104). A variety of other chromatin and epigenetic modifications that act cooperatively to regulate and maintain HIV transcriptional latency have been reported in numerous publications(86,105-110). HIV latency is the major barrier to HIV eradication and reversal of latency could lead to targeting of the reactivated virus using antiretroviral therapy. Consequently, several strategies have been designed to overcome HIV latency including Histone Deacetylase (HDAC) inhibitors, Protein Kinase C (PKC) activators, and demethylases. All these are collectively termed latency reversing agents (LRAs) and some of the key studies on use of latency reversing agents are summarized below.

A perfect latency-reversing agent would activate integrated HIV transcription in all infected cells without inducing global immune cell activation. The replicating virus and infected cell would then be targeted both by the immune system and anti-retroviral therapy. Cytokines such as tumor necrosis factor-alpha (TNF-α) were shown very early to be able to activate HIV transcription. When TNF-α binds to its receptor, it initiates a signaling cascade that leads to activation of the NF-κB domain in the promoter region in the long terminal repeats (LTR) and consenquently activates HIV transcription(111,112). Additionally, TNF-α has been shown to cooperate with both IL-10 and HIV tat to promote HIV replication(113,114). TNF-α could serve as a therapeutic agent in two ways. In the first instance, TNF-α inhibition could prevent HIV replication and could be used as an adjunct to cART. However, TNF-α is an important cytokine and blockade of TNF-α may lead to some opportunistic infections such as tuberculosis(115). In the second instance, TNF-α or TNF-α agonists could be used to activate HIV-1 transcription and

several studies have demonstrated the ability of TNF-α to activate viral transcription(116,117). However, TNF-α is a strong pro-inflammatory cytokine and leads to widespread inflammation and immune activation such as is seen in patients with HIV/AIDS and this is detrimental to the host(118).

Several other clinical trials were conducted and designed to activate resting memory $CD4^+$ T cells using a variety of cytokines such as IL-2, interferon-γ and antibodies against the human CD3 receptor. All these trials had severe side effects and therapy was discontinued, and although patients in some cases had no detectable viremia while on treatment, viremia rebounded after stopping treatment(119,120). There are currently clinical trials underway using Interferon-α, IL-5, and IL-7, cytokines that are involved in regulation of T-cell physiology and further refinement of T-cell activation based therapies could still be useful in reservoir eradication studies. Challenges associated with modulation of cytokines to promote HIV reactivation led to a search for pharmacological compounds that can be used to induce HIV transcription without widespread immune activation. Several compounds have been tested *in vitro*, in animal models and in modest clinical trials in HIV-infected adults.

HDAC inhibitors were some of the earliest pharmacological agents used to activate HIV transcription. HDAC inhibitors activate HIV transcription without the global T-cell activation and inflammation seen in cytokine based therapies. HDAC inhibitors are routinely combined with combination antiretroviral therapy in what has been called the "shock and kill, or kick and kill" strategy. The HDAC inhibitors activate HIV replication and the virus is subsequently targeted with cART. Numerous HDAC inhibitors have entered clinical trials including Valproic acid, Trichostatin A (TSA), Panobinostat, Vorinostat, givinostat, belinostat and Romidepsin. HDAC inhibitors have been well tolerated by patients and demonstrated activation of HIV

transcription, but there was no change in the size of the reservoir after multiple dose administrations(121-126). Further study of these agents could still yield valuable insights into viral reservoir eradication.

Other classes of pharmacological latency reversing agents used in the "shock and kill" approach are Protein Kinase C (PKC) agonists such as bryostatin-1 and prostratin, and bromodomain inhibitors such as JQ1. PKC agonists activate T-cells without the global activation phenotype observed with cytokine therapies(127,128). Bromodomain inhibitors work on bromodomain proteins such as Brd4 that inhibit HIV tat and repress HIV transcription(129,130). Both classes of latency reversing agents; those that modify chromatic and increase NF-κB activation and those that activate Tat expression work cooperatively to reverse latency.

Recently, some groups have also used gene-editing enzymes to activate the latently integrated HIV provirus. A catalytically inert CRISPR/Cas9 endonuclease fused to a transcriptional activation domain has been demonstrated to lead to activation of viral transcription by the integrated HIV provirus(131-133). Since this activation is sequence specific it is likely to by-pass the global activation seen with immune modulators and could potentially be more effective than HDAC inhibitors, although delivery of CRISPR/Cas9 endonucleases to the latently infected cells is still a major challenge.

The "shock and kill" strategy will likely require more than one class of LRA to achieve significant HIV re-activation and subsequent reduction in reservoir size following targeting with cART. Several combinations of HDAC inhibitors and either PKC agonists or bromodomain inhibitors are being studied. Synergistic action has been found in cell culture but there are no

conclusive studies in patient populations yet(134). This is still an area of intense study and will likely continue to be for several years.

IMMUNO-MODULATORY STRATEGIES

Immuno-modulatory strategies that seek to boost the body's immune system are also being studied as possible approaches to eliminating the latent viral reservoir. Elite controllers have lower HIV associated DNA levels than non-controllers and lessons from elite controllers who suppress HIV viremia without cART are informative in the design of an immune strategy to eliminate the long-lived viral reservoir. Studies have shown that $CD8^+$ T cells from elite controllers are able to suppress HIV viral replication (135) and that elite controllers have a higher frequency of HIV-specific memory B cells than non-controllers (136). Both of these factors might be important for immune mediated control of HIV viral replication in these elite controllers. Studies, however, have shown that cytolytic CD8+ T cells from patients on long-term suppressive cART do not clear latently-infected cells even when HIV transcription is activated with HDAC inhibitors(137). This is likely due to an absence of antigen stimulation within patients who are aviremic. Further studies have shown that *ex vivo* prestimulation or priming of patients' CD8+ T cells can restore killing and lead to clearance of latently infected cells(138). Cells were stimulated with gag peptides and IL-2, or with HIV-1 loaded dendritic cells and then re-infused into the patient. These cells showed significant killing activity of latently infected cells and reduced the half-life of latently infected cells from 34.2 days to 3.6 days. In elite controllers, prestimulation further strengthened the cytolytic activity of their immune cells(139). The use of latency reversing agents in patients on suppressive cART has been premised on having a robust immune system that can clear the reactivated cells and the

finding that cytolytic cells in suppressed patients do not actually kill infected cells is likely to lead to renewed efforts to modulate the immune system to create a synergistic killing effect. Prestimulation of CD8+ T cells and cytolytic killing activity could be combined with latency reversing agents and combination therapy to produce durable virologic remission.

The use of the immune system for HIV replication control and eradication has particularly benefited from the discovery of HIV broadly neutralizing antibodies (bNAbs). HIV bNAbs have been isolated from multiple patients and have been shown to have broad neutralizing potency *ex vivo* against different HIV sub-types. One of the earliest bNAbs to be isolated was the b12 antibody which targets the CD4 binding site of the HIV gp120 protein(140). Subsequently several other second generation bNAbs were isolated including VRC01 and PG9/PG16 which have broader neutralization potencies than first generation bNAbs(141).

Broadly neutralizing antibodies have shown protective efficacy when administered to SHIV-infected macaques. In a study using PGT121 and 3BNC117, Barouch and colleagues demonstrated rapid clearance of peripheral HIV viral DNA, consistent with ADCC-mediated clearance of HIV infected cells (142). Another demonstration of the efficacy of bNAbs was shown using 3BNC117 to prevent acquisition of SHIV. Two rhesus macaques were passively immunized with 3BNC117 and 24 hours later inoculated with an infectious dose of SHIV. The antibody prevented SHIV acquisition in both animals (143). In the same paper, the authors describe the use of 3BNC117 as immunotherapy for SHIV chronically-infected macaques and administration of the bNAbs resulted in reduction in viremia in these animals. Escape variants against several of these bNAbs including VRC01 have been described(144,145) and combinations of multiple bNAbs targeting different HIV epitopes may be required to effectively control HIV replication and clear the HIV reservoir.

Optimal combinations that comprise triple or quadruple bNAbs have been described as having better neutralizing potency than single bNAbs (146). Also, the protective efficacy of 3BNC117 in the setting of analytical treatment interruption in a clinical trial involving 13 patients with virus sensitive to 3BNC117 was recently shown. They showed that they could delay viral rebound by between 7 and 10 weeks in patients treated with infusion of 3BNC117(147). This group showed protection against a specific viral variant (sensitive to 3BNC117) but until we can identify broadly-neutralizing antibodies that neutralize all possible HIV variants, they will likely be used in combination with other HIV cure strategies such as latency reversing agents and cART. Recently an engineered CD4 immunoglobulin was described that was designed to bind to conserved epitopes of the HIV envelope. This so-called eCD4-Ig is a fusion of a CD4-Ig and a CCR5 mimetic peptide and was shown to have better efficacy than bNAbs in protecting macaques from multiple SHIV challenges(148). Although the bNAbs and other immunotherapeutic approaches may not be able to clear latently infected reservoir cells, since they express no immunogenic epitopes, they could be combined with other approaches to prevent continued replication of the virus and further infection of new cells. Such combination strategies will likely be required for complete HIV reservoir eradication.

TRANSPLANT, CELL AND GENE THERAPY

Blood and bone marrow transplants for HIV cure

To date, the “Berlin patient”, Timothy Brown, remains the only person to have sustained durable virologic remission after stopping HIV targeted therapy and it is generally assumed he is cured of HIV. The “Berlin patient” received two stem cell transplants but he is by no means the only patient to receive a stem cell transplant for HIV cure. The earliest such transplants were

performed in the early days of the epidemic before the introduction of cART(149). Patients who were transplanted had co-morbid malignancies such as acute lymphoblastic leukemia and in some cases Kaposi Sarcoma. In this setting, nearly all patients who received transplants died, usually as a result of opportunistic infections(150-152). Allogeneic bone marrow transplants were also carried out in HIV patients following the approval of Zidovudine in 1987. However, there was almost uniform viral rebound and early mortality in all these patients(153-155).

There have been several other patients who received bone transplants in the era of cART but none so successful as the "Berlin patient". Two HIV-positive patients, anecdotally called the "Boston patients", underwent allogeneic bone marrow transplant and initially had undetectable viremia, but both had virologic rebound at 12 weeks and 32 weeks respectively post treatment interruption(156). Questions of course abound as to why the transplant in the "Berlin patient" was so effective in controlling HIV replication and none of the successive transplants were nearly as effective. The key aspect of the transplants Timothy Brown received seems to have been that the bone marrow was from a donor homozygous for the CCR5 delta-32 deletion. Several studies have shown that these cells can be resistant to infection by CCR5-tropic HIV variants and this could have played a key role in preventing viral relapse in the "Berlin patient"(157,158).

There are other patients that have received transplants with cord blood cells from donors homozygous for the CCR5 delta-32 deletion. Unfortunately, all the patients who received these transplants died, either from relapse of the underlying malignancy, infection or from graft versus host disease(159,160). Transplant with CCR5 delta-32 deleted cells seems to have no effect on CXCR4 tropic viruses as evidenced by a patient who was transplanted with these cells experiencing rapid viral rebound with a CXCR4-tropic virus (161). Allogeneic bone marrow

transplant for HIV is promising but still significantly risky and can only be performed in resource-rich settings and in patients who have both HIV and malignancies. However, it is possible that gene editing strategies that target the CCR5 receptor could create a population of HIV resistant cells that could reconstitute the patients' immune system and delay or prevent progression to AIDS. Additionally, patients could be given a mini-transplant or autologous transplant with their own modified CCR5 cells, which could have significantly lower side effects and mortality than a full allogeneic transplant (162). These strategies are areas of active investigation and several of them have been brought to the clinical trial stage.

CCR5 Receptor modification

Chemokine receptor 5 (CCR5) was cloned in 1996 and later described as a critical co-receptor for M-tropic HIV(163). Polymorphisms in the CCR5 receptor have been identified and these patients live without significant loss in cellular function. In HIV, the critical polymorphism is a homozygous 32bp pair deletion in the CCR5 gene that confers resistance to the M tropic HIV strain. The homozygous CCR5 delta 32bp deletion occurs in the Caucasian population at a frequency of about 0.1%(163).

Ex vivo knockout of CCR5 using genome-editing techniques bypasses the need for donors with a homozygous CCR5 delta 32bp gene modification, which is extremely rare, and can create a population of HIV resistant cells in the patient. When engineered endonucleases bind to target DNA sequences, they introduce double strand breaks, which can be repaired either through non-homologous end joining (NHEJ) or through homologous directed repair (HDR)(164,165). NHEJ is error prone and can introduce mutations in target sequences. These mutations may lead to frame shifts or other inactivating mutations in the target sequences.

In HDR, a homology repair template is provided and the DNA double strand break repair is repaired according to the sequence of the homology repair template. HDR can be used to introduce specific inactivating sequences. For CCR5 knockout, both strategies can be used to 'delete' the CCR5 receptor in order to create HIV resistant cells and several groups have demonstrated CCR5 disruption using engineered endonucleases (166).

Pablo Tebas and colleagues were one of the first groups to use CCR5 modified CD4+ cells in patients (167). They modified the CCR5 receptor in autologous CD4+ cells from 12 patients using zinc finger nucleases (ZFNs). ZFNs are comprised of zinc finger proteins which are modular DNA binding scaffolds fused to the cleavage domain of the non-specific type IIs restriction enzyme, Fok1(168). The Fok1 restriction enzyme requires dimerization of two Fok1 domains for cleavage to occur and the ZFNs typically will have left and right halves. ZFNs can theoretically be engineered to recognize almost any DNA sequence because of their modular nature. Tebas et al. designed ZFNs targeting the CCR5 receptor and demonstrated first that the use of ZFN-modified cells was safe in patients but also importantly demonstrated that use of these modified cells leads to reduction in HIV DNA. Multiple other trials using ZFN modified CD4+ T cells to treat HIV patients are currently on going (169).

Other genome editing platforms including the meganucleases, the clustered regularly interspaced palindromic repeats and associated proteins (CRISPR/Cas9), and the transcription activator like effector nucleases (TALENs) have all been used to target the CCR5 receptor with the view to creating HIV resistant cells that can later be re-infused into patients(168,170-173).

To have a meaningful effect using CCR5 modified cells however, there would be a need to reconstitute the patient's bone marrow with HIV resistant CCR5 modified cells, in essence to replicate the transplant in the "Berlin patient". Several groups have now shown modification of

CD34+ hematopoietic stem cells to knock out the CCR5 receptor using ZFNs. Transplantation of the modified CD34+ hematopoietic stem cells in mouse models of HIV showed selective expansion of the HIV resistant CC5 modified cells(174). Other groups have demonstrated disruption of the CCR5 receptor in induced pluripotent stem cells(175) and have also demonstrated selective resistance to HIV in modified cells. The advantage of using CD34+ hematopoietic and the induced pluripotent stem cells is that they have the ability to reconstitute the patients' marrow and create a population of mature HIV resistant CD4+ cells which can consistently be replenished by the CCR5 modified stem cells. The CCR5 modified cells likely have a survival advantage over the unmodified cells and the hope is that after sufficient reconstitution, the patient can be withdrawn from cART without adverse consequences. Recently a group described long-term engraftment of ZFN modified CCR5 hematopoietic stem cells in a non-human primate model(176) and this will have great implications for the management of HIV using gene modification strategies.

Modification of HIV cellular receptors to engineer HIV resistance has mostly focused on the CCR5 receptor because the CD4 receptor and the CXCR4 co-receptor are critical to immune cell functions including interaction with MHC class II for CD4(177,178) and cellular migration for CXCR4(179). Some groups however have shown successful CXCR4 modification using ZFNs and CRISPR/Cas9 (180-183). The challenge for CCR5 modification may be the emergence of X4 variants in patients who have received CCR5 modified cells, although this has not been observed with small molecule inhibitors of CCR, and is therefore less unlikely to happen with CCR5 modified cells. The HIV-1 X4 variants use CXCR4 as a co-receptor and will not be affected by CCR5 modification. However, as seen from Tebas and colleagues, patients

can achieve reduction in HIV DNA with transplant of CCR5 modified cells and in the case of the "Berlin patient" significant virologic remission without cART of almost 10 years.

Targeted mutagenesis of the integrated HIV provirus

Genome editing techniques can also be used to introduce inactivating mutations into the integrated HIV provirus that lead to the production of non-viable viral progeny. During HIV infections, the animal hosts of viral pathogens produce several lentiviral restriction factors to counteract HIV infection. One of these restriction factors is the APOBEC3 family of proteins that are packaged in new virions and deaminate the viral cytidine (C) to uracil (U). During reverse transcription uracil is recognized as Thymine (T) and is transcribed to Adenine (A) instead of the Guanine (G) present in the original viral genome(184,185). The accumulation of C to T mutations reduces the replicative fitness of the virus and can prevent infection and transmission of the virus(186). Consequently, HIV-1 has developed mechanisms to evade APOBEC proteins. HIV encodes vif which binds APOBEC and targets it for proteosomal degradation(187). Importantly however, we can use engineered endonucleases to introduce similarly inactivating mutations into the integrated HIV provirus to reduce replicative fitness of the virus and this could potentially lead to long-term virologic control without cART.

Four classes of engineered endonucleases have been used to introduce mutations in the integrated HIV provirus(188). Zinc finger nucleases, which have been used extensively to knockout the CCR5 receptor, have also had numerous applications in targeted disruption of the integrated HIV provirus. A number of groups have demonstrated mutagenesis of the target HIV provirus sequence in 293T cells, the integrated HIV provirus using CD4 T cells lines, and in primary cell models(189,190). De Silva Feelixge and colleagues demonstrated that with targeted

disruption of the integrated HIV provirus using zinc finger nucleases, there is the potential to develop resistant mutations(191). The escape variant was resistant to re-cleavage by the same endonuclease but could be cleaved by ZFNs targeting other regions of the HIV genome. Importantly, this escape variants was still susceptible to antiretroviral therapy.

TALENs, much like ZFNs, are also modular DNA binding scaffolds fused to the cleavage domain of the type IIS restriction endonuclease Fok1(192,193). Each TALEN is made up of identical DNA binding scaffolds that differ at amino acid positions 12 and 13. Positions 12 and 13 are termed the repeat variable diresidues (RVDs). Each modular DNA binding component recognizes one nucleotide and TALENs can be assembled to recognize any DNA sequence(194,195). TALENs have had numerous applications in targeted disruption of the integrated HIV provirus. Strong et al. designed TALENs targeting conserved regions of the HIV TAR region and demonstrated that TALENs introduced mutations in the integrated provirus(196). Other groups have described TALENs targeting several different HIV target regions and all were able to introduce mutations at their target sites(197,198).

CRISPR/Cas9 endonucleases comprise a nuclease, Cas9, and a single guide RNA that is comprised of two RNA sequences, a crRNA, that is complementary to target DNA sequences and tracrRNA that binds to the Cas9. For DNA cleavage to occur, the Cas9 nuclease must interact with specific DNA sequences called the protospacer adjacent motif (PAM) that is adjacent to the target DNA sequence complementary to the crRNA. The PAM sequence is specific to different types of Cas9 proteins. Retargeting CRISPR/Cas9 to new DNA sequences is very facile as it involves changing the guide RNA, and this system is now used for many genome-editing applications(199-203). Multiple groups have used CRISPR/Cas9 endonucleases to introduce mutations in target HIV sequences. Ebina et al. showed that they could introduce

mutations in the integrated HIV provirus in Hela and Jurkat cells using guides targeting the HIV 5'LTR region (204). Kaminski et al. also recently demonstrated excision of long segments of the integrated HIV sequences in experiments in mouse models using multiplexed guides in the HIV 5'LTR and gag regions (205). A number of other groups have also demonstrated cleavage of target HIV sequences using CRISPR/Cas9 either on HIV plasmids or on integrated proviral DNA(198,206). A major challenge to use of CRISPR/Cas9 endonucleases in HIV-cure is delivery to target cells. However, the discovery of smaller Cas9 proteins such as that from the *Staphylococcus aereus* which can be packaged in an Adeno-associated viral (AAV) vector are likely to make delivery of the CRISPR/Cas9 endonucleases easier(207)

Engineered endonucleases have also been used to modify both viral restriction factors and proteins involved in viral replication. These strategies could prevent viral replication and can be used synergistically with other cure strategies. Voit et al. have also demonstrated the use of ZFNs to introduce a TRIM-5α reading frame into the CCR5 locus(208). TRIM-5α is a lentiviral restriction factor that recognizes the incoming viral capsid and targets it for proteosomal degradation(209). Voit et al. took advantage of the HDR pathway to introduce a TRIM-5α motif into the CCR5 locus and showed that this prevented HIV infection. TALENs have been used to disrupt LEDGF/p75, a host protein that interacts with the viral integrase, plays a role in lentiviral integration and is also thought to play a role in virion assembly and packaging(210). TALENs mediated knockout of LEDGF/p75 (PSIP1) and severely impaired HIV-1 viral replication (172,211).

The major challenge associated with the use of engineered endonculeases for targeted HIV-disruption is the potential for off-target cleavage activity(212,213). Several approaches have been described to reduce off-target cleavage activity such as high fidelity Cas9

proteins(214,215), and paired nicking Cas9 proteins (nickases) that require dimerization (216)..Another reported challenge is the development of escape mutants that are resistant to specific guide RNAs because of mutations arising from NHEJ repair(217,218). In these instances, the solution is to use guide RNAs targeting different HIV gene segments; combination nuclease targeted disruption is in a sense similar to combination antiretroviral therapy. Concerted disruption of multiple gene targets will make it harder to generate a virus with significant replicative fitness. The prospects for use of CRISPR/Cas9 in HIV targeted disruption remain very exciting despite these challenges.

Meganucleases are compact DNA binding proteins that introduce double strand breaks in target HIV sequences which are subsequently repaired by error prone NHEJ as described above(219). Our group and others have demonstrated cleavage of target HIV DNA sequences using engineered meganucleases. We demonstrated through transfection of HEK293T cells that we could obtain mutations in almost 40% of all target HIV sequences using HIV-specific engineered megaTALs(220).

My thesis focuses on the use of engineered meganucleases to disrupt the integrated HIV provirus. I demonstrate that engineered meganucleases can cleave and introduce mutations in target HIV sequences. Further, I demonstrate that engineered meganucleases have significant off-target cleavage activity and describe mechanisms to reduced off-target cleavage and improve target sequence specificity. I also describe an approach to quantifying the HIV reservoir using multiplex droplet digital PCR.

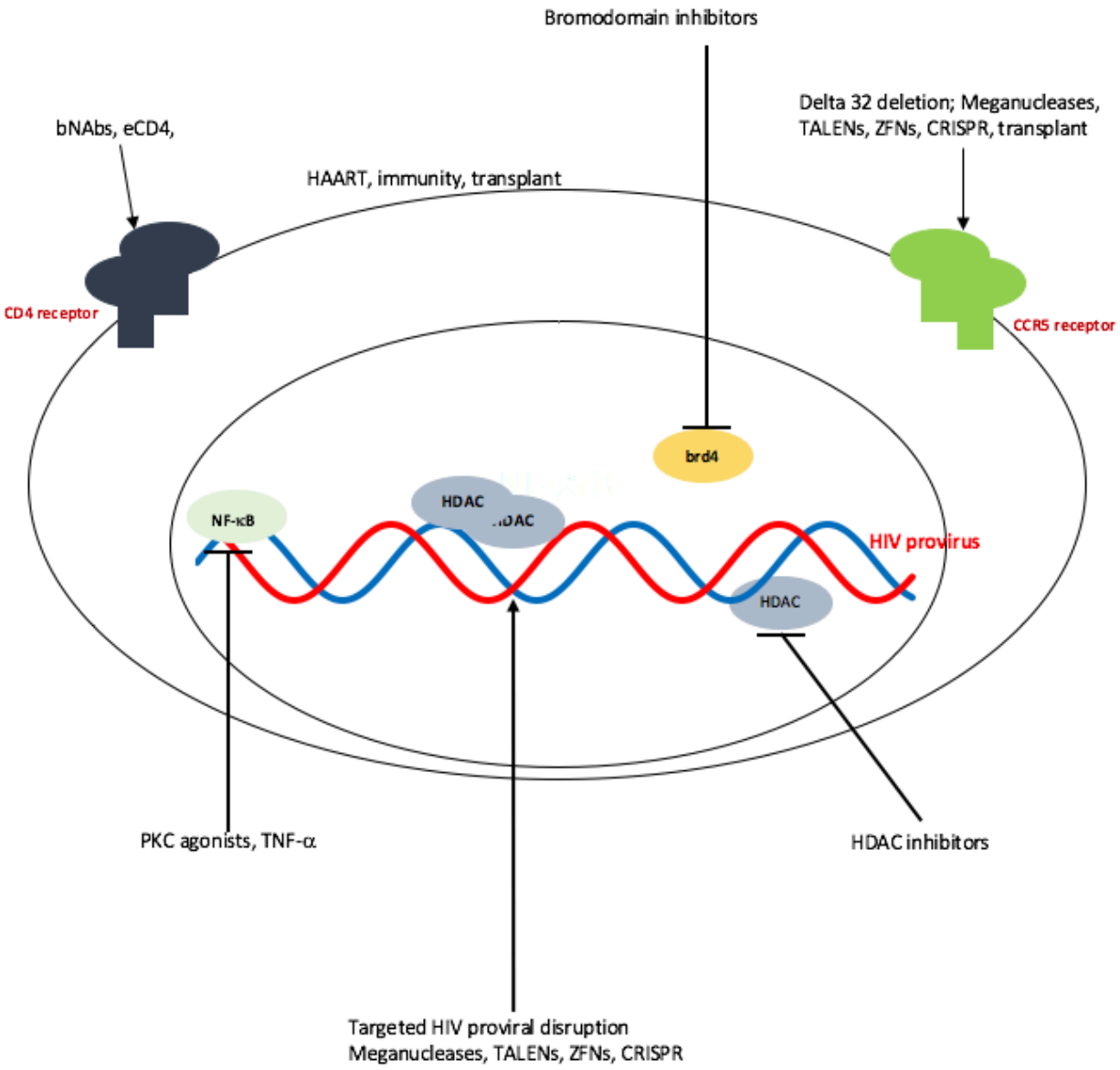


Figure 1.1. **Schematic of HIV Cure strategies.**

Early cART initiation, immunity and transplant reduce the size of the reservoir/infected cells, CCR5 disruption prevents HIV entry along with strategies to target CD4/env interaction such as use of bNAbs and engineered CD4 antibodies. HDAC inhibitors, bromodomain inhibitor, PKC agonists and TNF-α activate HIV transcription which can be target by cART and the immune system. Engineered endonucleases disrupt the HIV provirus (121,221).

Chapter 2. MATERIALS AND METHODS

2.1 PLASMIDS

Plasmids eOnuHIVInt_v1 and cognate megaTALs eOnuHIVInt_v1_5.5mT, eOnuHIVInt_v1 _6.5mT, and eOnuHIVInt_v1_7.5mT were obtained from Dr Sandrine Boissel and Dr Andrew Scharenberg (Seattle Children's Research Institute) and Dr. Jordan Jarjour (Pregenen - now Bluebird Bio) and have been described in detail previously(220). Each plasmid, in the backbone of a lentiviral vector, expresses an I-*OnuI* derived meganuclease, re-engineered to recognize a 22-nucleotide sequence in the HIV pol gene, and the reporter BFP. Plasmid eOnuHIVInt_v1_5.5mT, eOnuHIVInt_v1 _6.5mT, and eOnuHIVInt_v1_7.5mT contain a fusion of a TAL-effector domain and a meganuclease, a hybrid enzyme that has been termed a megaTAL as previously described (222,223). The megaTAL eOnuHIVInt_v1_5.5mT contains 5.5 RVD repeats specific for a sequence adjacent to the meganuclease target site while eOnuHIVInt_v1 _6.5mT, and eOnuHIVInt_v1_7.5mT contain 6.5 and 7.5 RVD repeats respectively. The wild type I-*Onu*I plasmid, called wt-Onu here, was obtained from Dr Barry Stoddard at the Fred Hutchinson Cancer Research Center. The plasmid eOnuHIVInt_v2, is an improved version the HIV-specific meganuclease eOnuHIVInt_v1 and was made by iterative structure guided engineering to improve specificity. The new improved enzyme, eOnuHIVInt_v2, was then fused to TAL repeats with 7.5 RVDs to create a new megaTAL, eOnuHIVInt_v2_7.5mT with better target sequence recognition.

HIV plasmids, pDHIV3 and pDHIV3-GFP, were provided by Dr. Vicente Planelles and have been previously described (224).

2.2 CELLS

HEK 293T cells (ATCC# CRL-3216) were cultured in DMEM supplemented with 10% FBS. SupT1 CD4+ T cells (ATCC# CRL-1942) were grown in RPMI supplemented with 10% FBS. Primary CD4+ T cells were isolated by negative selection from PBMCs obtained from healthy adults using a CD4 cell isolation kit (Militenyi Biotech), cultured in RPMI supplemented with 10% FBS and L-Glutamine, and activated using CD3/CD28 beads (Life technologies) as previously described (191).

2.3 YEAST SURFACE DISPLAY CLEAVAGE

The tethered flow cleavage assay was performed by expressing the HIV specific meganuclease on the yeast surface as a fusion with the yeast surface protein AgaII as previously described (225,226). A biotinylated and fluorophore labeled target dsDNA oligo was tethered to the enzyme via an HA tag. The C-terminus of the meganuclease has a FITC tag and the dsDNA oligo has an APC tag. Upon meganuclease mediated cleavage, the dsDNA oligo will lose the APC fluorescence. In the absence of cleavage, there is co-fluorescence in both the APC and FITC channels. The shift in fluorescence represents the amount of cleavage of the target dsDNA oligo by the enzyme.

2.4 GENE DISRUPTION ANALYSIS

HEK 293T cells were seeded in a 12-well plate at $2.5x10^5$ cells/well and each well was co-transfected with 0.5ug of an env-defective replication deficient HIV construct that expresses GFP (pDHIV3-GFP) and 1ug of eOnuHIVInt_v1, eOnuHIVInt_v1_7.5mT, or eOnuHIVInt_v2_7.5mT. Transfection was performed using polyethyenimine (PEI) as previously described (220). Media was changed at 18 hours post transfection, and at three-days post

transfection, the cells were harvested and gDNA extracted using the Qiagen DNeasy blood and tissue kit per manufacturer's instructions. Extracted DNA was used for mutation analysis using the mismatch cleavage assay, and subsequently for sequencing.

2.5 MISMATCH CLEAVAGE ASSAY

Genomic DNA from cells was extracted using the Qiagen DNEasy Blood and Tissue kit. We analyzed on-target cleavage by amplifying the meganuclease target site from the resulting genomic DNA using HIV_Int forward primer (TAGCAGGAAGATGGCCAGTA) and HIV_Int reverse primer (TCCTGTATGCAGACCCCAAT) and Phusion DNA polymerase (NEB). The mismatch cleave assay was performed using T7 endonuclease I (New England Biolabs). Briefly, the PCR amplicons were heated to 95^{o}C to denature the DNA strands and then slowly cooled to room temperature to allow homoduplex and heteroduplex formation. The PCR amplicons were then treated with T7 endonuclease 1 and incubated at 37^{o}C for 30 minutes. The T7 endonuclease I cleaves any heteroduplexes in the samples creating two fragments. The resulting cleavage bands were visualized on a 3% Agarose gel and quantified using ImageJ software (227). Off-target cleavage analysis was performed using T7 endonuclease I and the primers listed in table S2 of the appendix. The mismatch cleavage assay is illustrated in figure 2.1 below.

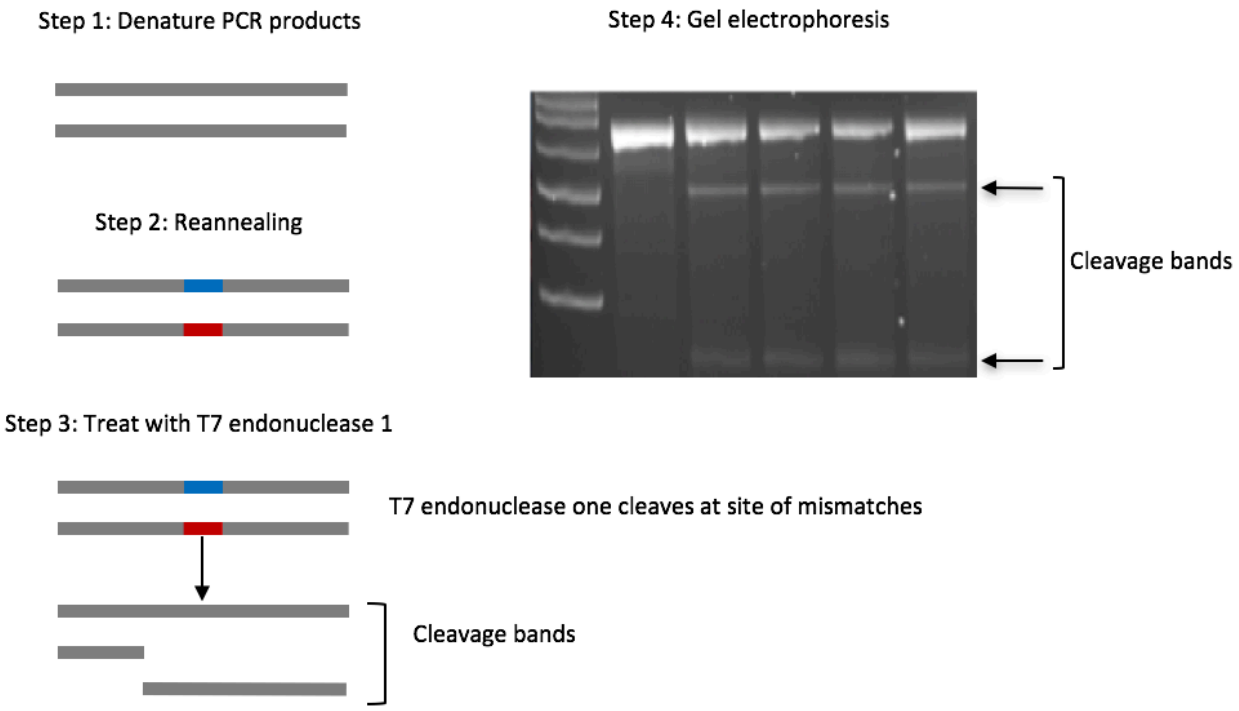


Figure 2.1. DNA mismatch cleavage assay.

Schematic of the DNA mismatch cleavage assay. **Step 1**, PCR amplicons of the target sequence are denatured by heating to 95C, **Step 2**, the denatured DNA is then slowly cooled to room temperature to allow re-annealing. **Step 3**, the DNA is then digested with the T7 endonuclease 1 which cuts at the site of mismatches (red/blue). **Step 4**, the resulting cleavage bands can be separated by gel electrophoresis(220,228).

2.6 PRODUCTION OF HIV

DHIV3 is a replication-deficient env-deficient viral clone derived from NL4-3. DHIV3-GFP similarly is replication incompetent but contains a GFP expression cassette in place of HIV *nef*. Virus was made by transfecting HEK 293T cells with pDHIV3 (229) and pLET-LAI (a CXCR4

tropic envelope expressing plasmid) using a previously described protocol (191). Viral supernatant was harvested 72 hours post transfection, filtered using a 0.45µm filter, and titered using the TZM-BL assay as previously described (230).

2.7 LENTIVIRAL VECTOR PRODUCTION

To make the lentiviral vectors, the vector plasmids eOnuHIVInt_v1, eOnuHIVInt_v1_5.5mT, eOnuHIVInt_v1 _6.5mT, eOnuHIVInt_v1_7.5mT and eOnuHIVInt_v2_7.5mT were transfected into HEK 293T cells. Cells were co-transfected with the packaging plasmid psPAX2 and the VSV-G envelope expressing plasmid pMDG2. Cell growth media was changed at 18 hours post transfection. The viral supernatant was collected at 72 hours post transfection and filtered using a 0.45µm filter. Lentiviral vectors were concentrated by centrifuging for 8 hours at 8000g and the resulting pellet was re-suspended in media concentrating the virus by 100x. Lentiviral vectors were titered by infecting SupT1 CD4+ T cells and performing flow cytometry at 3-days post infection to assess transduction levels using BFP as a proxy for enzyme transduction.

2.8 VECTORS AND CELLULAR TRANSDUCTION

SupT1 CD4+ T cells were seeded in 6 well plates (5×10^5 cells/well) and infected with the CXCR4 tropic DHIV3 virus at a multiplicity of infection (MOI) of 2 infectious units/cell. At 24 hours post infection with DHIV3, cells were infected with lentiviral vectors expressing the HIV-specific engineered meganuclease or megaTALs. The efficiency of transduction was measured after 72 hours by flow cytometry using BFP expression as a surrogate for meganuclease or megaTAL expression. We then measured p24 expression to assess the efficiency of DHIV transduction or GFP expression for DHIV3-GFP.

2.9 OFF-TARGET SITE ANALYSIS

We used the web based off-target site prediction program PROGNOS(231) to identify the closest matched genomic sites to the 22-nucleotide target sequence of the HIV-specific meganuclease. For the 10 closest matched targets sites we performed the mismatch cleavage assay and yeast display cleavage assay as described above. We also amplified these genomic loci using primers listed in Appendix table 2, and performed Illumina sequencing for evidence of endonuclease mediated off-target cleavage as previously described (191).

2.10 IN VITRO CELL TOXICITY ANALYSIS

HEK 293T cells were transfected with eOnuHIVInt_v1, eOnuHIVInt_v1_5.5mT, eOnuHIVInt_v1 _6.5mT, eOnuHIVInt_v1_7.5mT or eOnuHIVInt_v2_7.5mT as described above. Toxicity was assessed at 48 hours post transfection by flow cytometry. Briefly, transfected cells were trypsinized and stained with Propidium Iodide (BD biosciences) and then flow cytometry was performed to identify cells positive for PI. Toxicity in SupT1 CD4+ cells was assessed after transduction with the HIV-specific meganuclease, eOnuHIVInt_v1, and megaTALs, eOnuHIVInt_v1_7.5mT and eOnuHIVInt_v2_7.5mT. At 3-days post transduction cells were harvested and stained with Annexin V and Propidium Iodide using the apoptosis detection kit (BD biosciences). Analysis was performed using a LSRII flow cytometer (Becton Dickinson) and FloJo software version 10.0.8 (TreeStar).

2.11 ILLUMINA SEQUENCING AND ANALYSIS

For Illumina sequencing, PCR amplicons containing target HIV sequence and sequences of 11 of the closest matched predicted off-target sites were generated using the primers listed in Appendix A. PCR amplicons were column purified using a Zymo Research DNA clean and

concentration kit (Zymo Research Corp, Irvine, California). Adapter sequences of the 16S ribosomal RNA gene (16S rRNA) were then ligated to the PCR amplicons diluted to 1ng/ul. NexteraXT reactions were then performed according to the manufacturer's instructions (Illumina) with 1.25ul sample volumes. Sequencing libraries were then amplified and barcoded using 14 cycles of PCR with the NexteraXT Index Kit (Illumina)(220). Samples were quantified by Bioanalyzer (Agilent) and diluted such that we achieved about 200,000 reads per sample using a MiSeq sequencer (Illumina).

Raw reads were processed using the Galaxy suite, Trimmomatic and Cutadapt as previously described(220). Reads were then mapped to either the HIV target sequence to determine on-target mutation frequency, or mapped to the predicted off-target sequences to determine the off-target mutation rates. For mutations, both insertions and deletions were quantitated at each site.

2.12 CLONAL SEQUENCING

For clonal sequence analysis, we PCR amplified the HIV target site in different treatment conditions using the HIV_Int forward primer (TAGCAGGAAGATGGCCAGTA), the HIV_Int reverse primer (TCCTGTATGCAGACCCCAAT) and Phusion DNA polymerase (NEB). PCR amplicons were cloned into the Zero Blunt Topo PCR cloning vector (Invitrogen) and subsequently transformed using OneShot Top10 chemically competent *E. coli*(Invitrogen). For each condition, we picked 94 individual colonies for sequencing from each enzyme treatment condition.

2.13 DROPLET DIGITAL POLYMERASE CHAIN REACTION (DDPCR)

To quantify the HIV reservoir, we designed multiple primers and probes covering different regions of the HIV genome. For the SHIV ddPCR assay, we designed six sets of primers and probes based on the sequence of the SHIV-1157 ipd3n4 plasmid (232) that covered the 3'-LTR (3'LTR_F – 5'- AGCAGGTAGAGCCTGGGTGTT -3', 3'LTR_probe – 5'-VIC-CTAGACTCTCACCAGCACTTGGCCGG -BHQ-3', 3'LTR_R – 5'-CTTTAAGTAAGCAAGCGTGGAGTCA -3'), 5'-LTR (5'LTR_F – 5'-ACGGCTGAGTGAAGGCAGTAA -3', 5'LTR_probe – 5'-VIC-CGGCAGGAACCAACCACGACG -BHQ-3', 5'LTR_R – 5'-GACCCGCGCCTTTATAGGA -3'), gag (gag_F – 5'- GCAGAGGAGGAAATTACCCAGTAC -3', gag_probe – 5'-FAM-CAATTTTACCCA GGCATTTAATGTT -BHQ-3', gag_R – 5'-CAATTTTACCCAGGCATTTAATGT T -3'), vpu (vpu_F – 5'-TCCTTGGGATGTTGATGATCTG -3', vpu_Probe – 5'-FAM- TGCTACAGAAAAATTGTG -MGBNFQ-3', vpu_R – 5'- CATACAGGTACCCCATAATAGACTGTGA -3'), pol (pol_F – 5'-CCCGACCAATCCATACAACAC-3', pol_Probe – 5'-FAM- CCCACATTTGCTATAAAG-MGBNFQ-3', pol_R – 5'- GCATTCTCCATTTGTTCTTATCCTTT-3'), and env (env_F – 5'-GGCTGCTCTGGAAAACTCATCT-3', env_probe – 5'-FAM-CACCACTGCTGTGCCTTGGAACGA-BHQ-3', env_R – 5'-TCATGTTCTCCCAAATATCTGTTTG-3'). Initial validation of the SHIV ddPCR assay was performed using SHIV plasmid and subsequently the assay was performed on tissue samples from SHIV infected macaques. We created two multiplex assays 3'-LTR/gag/env and 5'-LTR/pol/vpu that took advantage of both excitation channels as well as amplitude of the different primer probe sets. Droplet digital PCR reactions were performed using the QX100 Droplet

Digital PCR System (Bio-Rad Laboratories, Hercules, CA) using the primers and probes listed above and the Bio-Rad Supermix for Probes (no dUTP). The ddPCR Supermix for Probes (no dUTP) was mixed with 900nM of primers and varying concentrations of probes (3'-LTR, 5'-LTR and vpu - 250nM, gag – 375nM, and env, and po – 500nM), 5ul of template DNA and water to make a 20µl total reaction volume. Reactions were package into droplets using a droplet generator per the manufacturer's instructions and then PCR thermocycling was performed using the following conditions: 95 °C for 10 minutes, 40 cycles of 94 °C for 30 seconds and 60 °C for 1 min, followed by 10 minutes at 98 °C and a 4 °C hold. After thermocycling, the reactions were analyzed using a Bio-Rad QX100 droplet reader per the manufacturer's instructions. The results were the analyzed using QuantaSoft software (Bio-Rad Labotories).

2.14 MACAQUE TISSUE SAMPLES

Tissue samples for validation of the SHIV multiplex ddPCR assay were obtained from Dr Hans Peter Kiem and Dr Christopher Peterson at the Fred Hutchinson Cancer Research Center and comprise lymph node biopsies from SHIV-infected macaques undergoing different experimental therapies including cART and bone marrow transplant for reservoir eradication. Genomic DNA was extracted from lymph node biopsies using a Qiagen DNeasy kit (Qiagen) per the manufacturer's guidelines and used for multiplex ddPCR assay validation.

2.15 STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism. For cell toxicity analysis we performed a Kruskal_wallis test to detect differences between the different treatment conditions. We also performed a two sided t-test analysis to determine P values for pairwise comparison

between treatment groups. We used Stata 13 (Stata corp) to determine the Pearson's correlation co-efficient between qPCR and ddPCR quantification of macaque SHIV samples.

Chapter 3. TARGETED DISRUPTION OF THE INTEGRATED HIV PROVIRUS USING MEGANUCLEASES

3.1 INTRODUCTION

The four genome editing platforms; Meganucleases, ZFNs, TALENs and the CRISPR/Cas9 RNA-guided endonucleases have all been used for a wide variety of genome editing applications including therapies for chronic persistent viral infections such as HSV, HPV, EBV and HBV(233), All these gene editing platforms have also been used to target the integrated HIV provirus as potential curative therapies(168). When engineered endonucleases bind to target HIV sequences, they induce double strand breaks, which are preferentially repaired by non-homologous end-joining (NHEJ). NHEJ is error prone and can lead to mutations at the site of the double strand break(234). If these mutations are in key genes in the integrated HIV provirus, they can lead to inactivating mutations and prevent reactivation from latency. My thesis focuses on the use of engineered meganucleases (also called homing endonucleases) to disrupt the integrated HIV provirus, prevent reactivation from latency, and in essence cure HIV or lead to long-term virologic remission off cART.

Meganucleases are selfish genetic elements that propagate their own DNA through cleavage and modification of target DNA sequences(235). Several classes of meganucleases have been characterized including the LAGLIDADG, HNH, GIY-YIG, His-Cys box, PD-(D/E)xK and EDxHD families(219). The LAGLIDADG family of meganucleases contains a LAGLIDADG motif, is the best characterized and the most sequence specific of the meganucleases. They are small, compact bifunctional proteins that recognize long DNA sequences (12 to 40 nucleotides long), and both bind to and cleave DNA(235,236). Together with collaborators we have re-

engineered a LAGLIDADG meganuclease, I-*Onu*I, to recognize and cleave HIV DNA sequences. The LAGLIDADG meganuclease, I-*Onu*I, was isolated from the microfungi, *Ophiostoma Novo-Ulmi*, which causes Dutch elm disease, and recognizes a 22-nucleotide DNA sequence(237,238). This meganuclease has been re-engineered to recognize a sequence in the HIV-pol gene region that encodes for integrase.

Therapeutic application of engineered endonucleases requires exquisite specificity and ideally no off-target activity. The HIV specific engineered meganuclease has been fused to a TAL effector domain to create a fusion megaTAL that has been shown to have better on target activity than the isolated meganuclease(222,223). Transcription activator-like effectors (TAL effectors) are modular DNA-binding scaffolds comprised of indentical repeats (Repeat Variable Diresidues or RVDs) which differ only at positions 12 and 13(195). Each repeat recognizes a single nucleotide and they can be assembled in a modular fashion to recognize any sequence. We demonstrate here that both the HIV specific meganuclease and the fusion megaTALs cleave and introduce mutations in HIV DNA sequences.

We also demonstrate that these HIV specific meganucleases and megaTALs have significant cleavage activity at predicted off-target sites, and cause cellular toxicity when compared to the untreated cells. Using yeast surface display and iterative structure-guided engineering we have generated an improved version of the HIV-specific meganucleases. We demonstrate that this improved version of the HIV-specific meganuclease and its cognate megaTAL have reduced off-target cleavage activity while retaining activity at the HIV target site.

3.2 RESULTS

MEGANUCLEASE TARGET SITE

The LAGLIDADG meganuclease, I-*Onu*I, was engineered to create an HIV-specific enzyme, eOnuHIVInt_v1 that recognizes a 22-nucleotide sequence in the HIV *pol* gene in the integrase coding region (Fig 3.1a). LAGLIDADG meganucleases are made of overlapping amino acid modules each of which contacts three nucleotides. Mutation of these modules on a scaffold of I-OnuI can retarget the native meganuclease to recognize and cleave a new DNA sequence(239). The I-OnuI was redesigned to recognize and cleave a 22-target nucleotide HIV sequence. Mutations at this site have been reported previously to lead to production of non-viable progeny(240). The HIV-specific engineered meganuclease was fused to TAL effector domains with varying numbers of RVDs to create three new enzymes eOnuHIVInt_v1_5.5mT, eOnuHIVInt_v1 _6.5mT, and eOnuHIVInt_v1_7.5mT that recognize respectively an additional 6, 7 and 8 nucleotides increasing the length of the target sequence from 22 nucleotides to 28, 29 and 30 for the different megaTALs (Fig 3.1b).

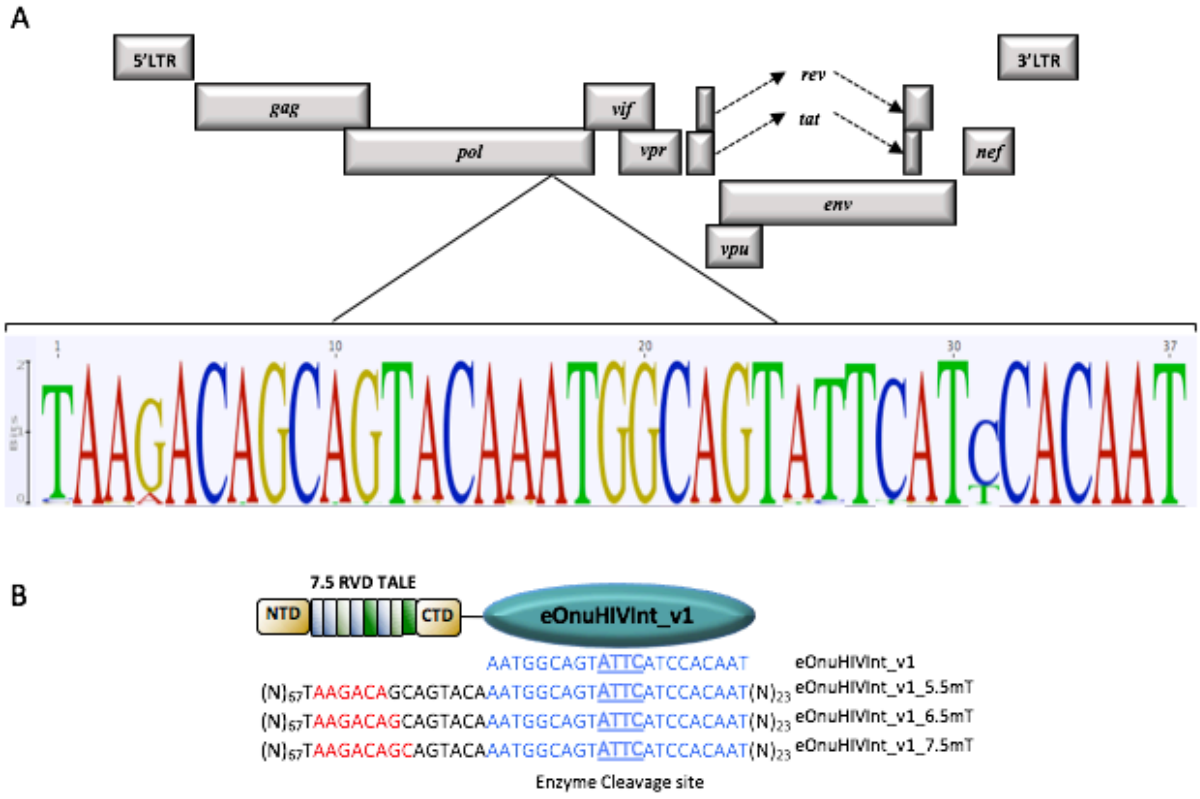


Figure 3.1. Sequence and conservation of meganuclease target site

Conservation plot of the megaTAL target sequence from an alignment of 2635 sequences present in the Los Alamos National Laboratory HIV database. The sequence coordinates within the HIV-1 HXB2 genome sequence annotation are 5675-5711 and are found in the integrase-coding region of the HIV-1 *pol* gene. **B,** Schematic of the megaTAL showing the TAL domain and the meganuclease domain. The meganuclease recognizes 22 nucleotides (blue) and the TAL domain binds to 6, 7, or 8 nucleotides depending on the RVD length (shown in red). The four nucleotide 3'-overhang created following cleavage by the meganuclease (ATTC) is hown underlined.

HIV-SPECIFIC MEGANUCLEASES CLEAVE TARGET HIV SEQUENCES

To determine whether these HIV-specific engineered meganucleases can cleave target HIV sequences, HEK293T cells were co-transfected with plasmids expressing the enzymes; eOnuHIVInt_v1_6.5mT or eOnuHIVInt_v1_7.5mT, along with the plasmid pDHIV3, which contains the HIV cleavage target of the HIV-specific enzymes. To improve the efficiency of mutagenesis we also co-transfected the cells with a plasmid expressing the 3'-exonuclease, Trex2, which is a DNA end-processing enzyme. When LAGLIDAGG meganucleases cleave target DNA, they create four-nucleotide 3'-prime overhangs. Trex2 is able to remove these overhangs prior to NHEJ-mediated repair, and this improves the rate of mutagenesis by preventing precise repair of the target sequence (241). At three days post-transfection, genomic DNA was extracted from transfected HEK293T cells and used to assess the presence of mutations by a variety of techniques. First we used the mismatch cleavage assay to determine whether mutations were present at the target site. The presence of cleavage bands suggested there were mutations at the target site as shown in Figure 3.2a. We used imageJ to estimate the frequency of mutations in the transfected cells (Figure 3.2a). Wild type I-*Onu*I was used as an experimental control. Using the mismatch cleavage assay we saw mutation rates ranging between 4% and 14% for the different enzymes in the presence or absence of Trex2 (data not shown for eOnuHIVInt_v1 and eOnuHIVInt_v1_5.5mT). To confirm the presence of mutations at the target site, we performed both clonal sequencing and next-generation Illumina sequencing. For samples transfected with either eOnuHIVInt_v1_6.5mT +/- Trex2 or eOnuHIVInt_v1_7.5mT +/- Trex2 and we obtained mutation rates ranging from 16% to 45% (Figure 3.2b) for the different meganucleases. These mutation rates were also confirmed using a previously published ddPCR mutation detection assay that was developed in the laboratory

(220). The mutation profile at the target site sequence varied from single nucleotide deletions and insertions to complex multiple nucleotide deletions (Figure 3.2c).

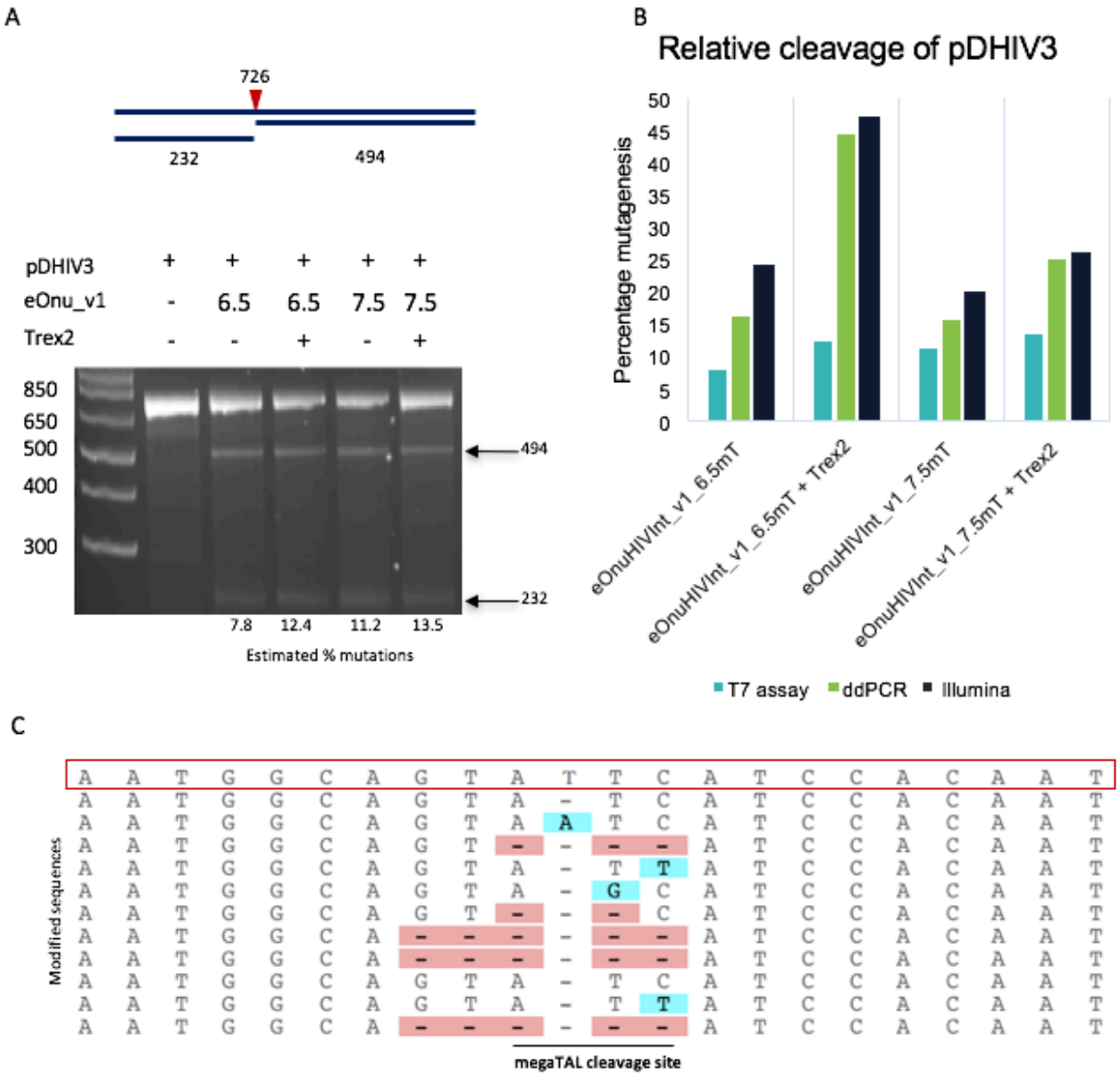


Figure 3.2. HIV-specific engineered endonucleases cleave target HIV sequences.

A, Mismatch cleavage assay of PCR amplicons from HEK293T cells. The schematic shows the expected cleavage bands which are visible upon agarose gel electrophoresis of T7 endonuclease I treated PCR amplicons. All the enzymes mutated the target HIV sequences with or without

treatment with Trex2. The enzymes eOnu_v1 represents eOnuHIVInt_v1, 6.5 represents eOnuHIVInt_v1_6.5mT, and 7.5 represents eOnuHIVInt_v1_7.5mT. Relative mutation rates, as assessed using ImageJ, are indicated for each treatment. **B,** cleavage efficiency of the different enzymes is shown for different methods of mutation detection including the mismatch cleavage/surveyor assay (cyan), clonal sequencing (green) and Illumina sequencing (blue). **C,** examples of target site mutations found after clonal sequencing of the samples treated with eOnuHIVInt_v1_7.5mT.

CLEAVAGE ACTIVITY AT PREDICTED OFF-TARGET SITES

For any therapeutic applications, the HIV-specific engineered meganucleases have to be highly specific for target HIV sequences and have minimal off-target cleavage effects. To determine the potential for off-target cleavage, we used the online off-target site prediction software PROGNOS(231) to identify the closest matched human genome sites by homology to the 22-nucleotide HIV target sequence for the engineered meganuclease eOnuHIVInt_v1. We identified 30 genomic sites that had between 2 and 5 nucleotide differences from the target HIV sequences (Figure 3.3a). In gDNA extracted from treated cells we did not detect any cleavage bands using the mismatch cleavage assay (Figure 3.3b). To identify low frequency mutations we Illumina sequenced PCR amplicons from cells that were transfected with eOnuHIVInt_v1, and eOnuHIVInt_v1_7.5mT. We detected both mutations and insertions at all 11 of the predicted off-target sites, which suggests that both eOnuHIVInt_v1, and eOnuHIVInt_v1_7.5mT cleave the predicted off-target sites (Figure 3.3c). The statistically significant mutation rates with eOnuHIVInt_v1, were at off-target 7 of 0.005% of all sequences (p=0.002). In cells treated with eOnuHIVInt_v1_7.5mT, mutation rates at off-target sites 8 (0.0018, p=0.00) and off-target site 30 (0.005, p=0.001) were statistically significant compared to untreated cells.

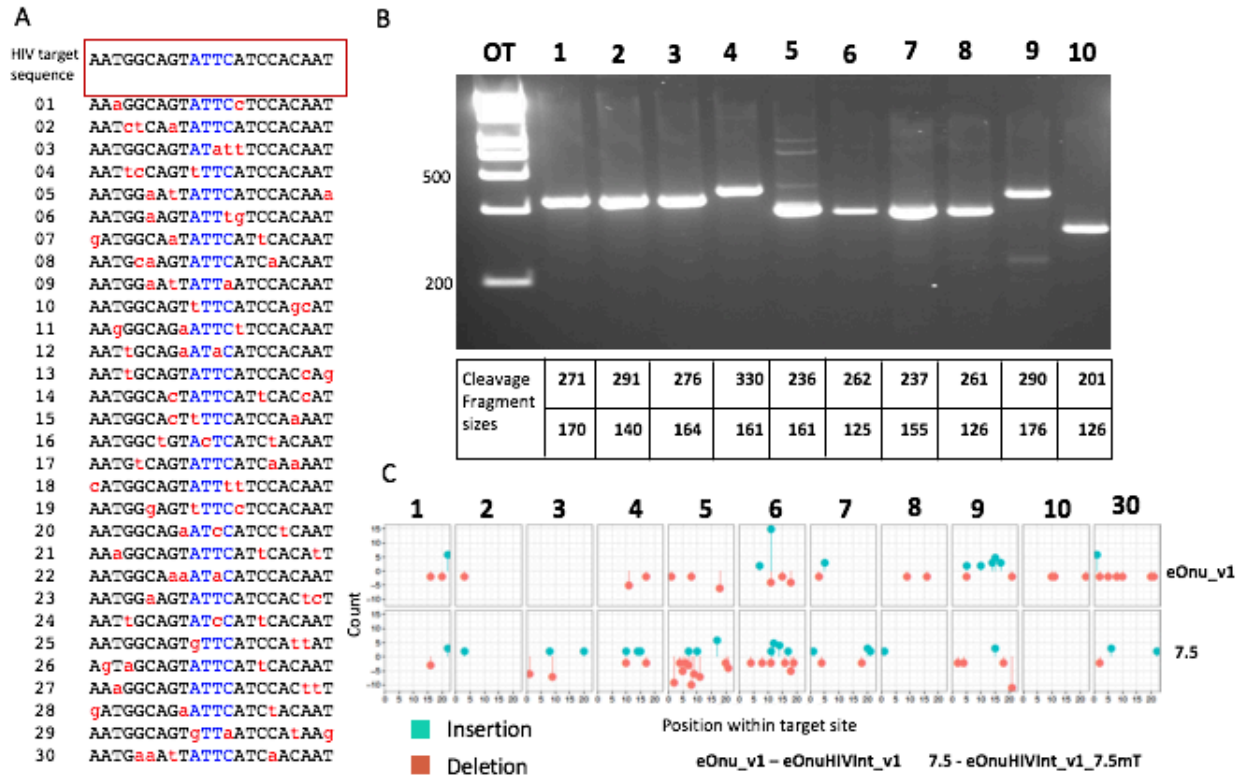


Figure 3.3. Engineered meganucleases cleave off-target genomic DNA sequences.

A, predicted off-target sites obtained using PROGNOS for the HIV-specific engineered meganuclease. Nucleotide differences are highlighted in red while the central four nucleotides, the site of endonuclease cleavage, are shown in blue. **B,** Mismatch cleavage assay for 10 predicted off-target sites. No predicted cleavage bands were detected in cells treated with eOnuHIVInt_v1. **C**, Illumina sequencing of PCR amplicons for predicted off-target sites 1-10, and 30. PCR amplicons of 11 of the predicted off-target sites were sequenced and analyzed for presence of mutations. Mutations (both insertions (green) and deletions(orange)) were detected in cells treated with both eOnuHIVInt_v1 and eOnuHIVInt_v1_7.5mT.

ONE-OFF SPECIFICITY ANALYSIS OF HIV-SPECIFIC MEGANUCLEASE, eOnuHIVInt_v1

In collaboration with Dr. Barry Stoddard and Dr. Abigail Lambert at the Fred Hutchinson Cancer Research Center, we performed a yeast surface display (YSD) cleavage analysis of the HIV-specific meganuclease, eOnuHIVInt_v1. To perform this the enzyme was fused to the yeast surface protein Aga2 and expressed on the yeast surface. A biotinylated fluorophore labeled target dsDNA oligo was then tethered to the enzyme via a streptavidin-phycoerythrin (PE) conjugate and an HA tag that is fused to the N-terminal end of the yeast expressed meganuclease. The 3'-end of the target DNA oligo has the fluorophore A647. We performed a one-off cleavage analysis, which measures the tolerance of the enzyme for single nucleotide polymorphisms at each position in the target DNA sequence.

To perform a one-off analysis of the 22-nucleotide target of the enzyme, 66 dsDNA oligos containing all the possible single nucleotide changes were each tethered to the enzyme. Cleavage of the dsDNA oligos was assessed by flow cytometry in the presence of Calcium or magnesium ions. In the presence of calcium ions, the enzyme binds the dsDNA oligo but there is no cleavage and both flourophores are expressed (SAV-PE-blue, A647-red) and co-linear fluorescence is seen as showed in Figure 3.4b. In the presence of magnesium ions, if the enzyme tolerates a particular mutation/nucleotide substitution, there is cleavage and a loss of Y-axis fluorescence (loss of A-647-red)(Figure 3.4a). The HIV-specific meganuclease, eOnuHIVInt_v1 tolerates many nucleotide substitutions across the 22-nucleotide target sequence, and the specificity is particularly promiscuous in the 5'-terminal end of the target where nearly all the substitutions increase the level of target sequence cleavage. Target specificity is better in the 3'-

terminal end of the HIV-target sequence, and in the central four nucleotide site of cleavage where minimal tolerance for substitutions was seen. The promiscuity of the meganuclease in the one-off specificity analysis suggests that this meganuclease will likely have widespread off-target cleavage effects when used in therapeutic applications.

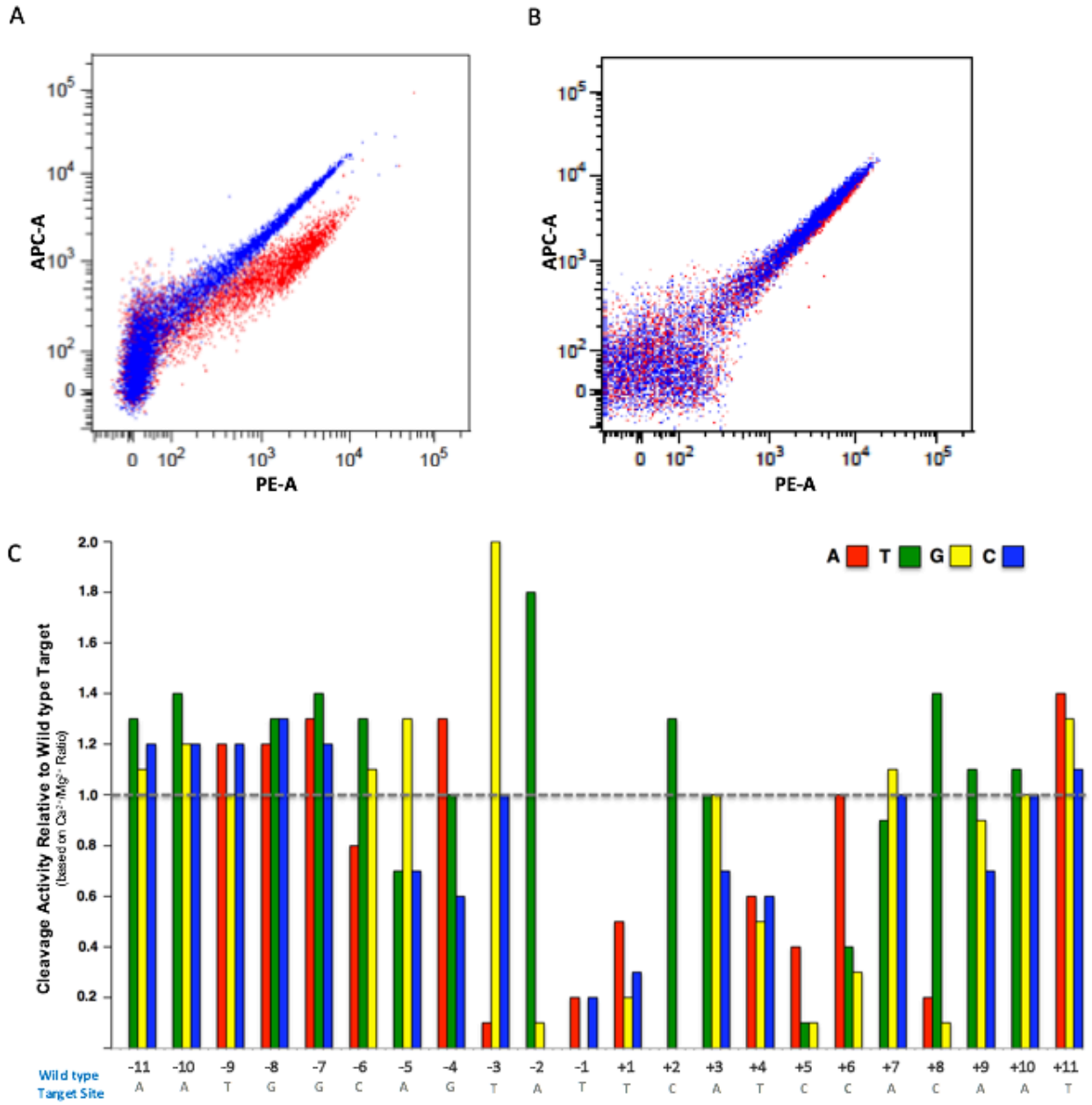


Figure 3.4. Yeast surface display cleavage analysis of eOnuHIVInt_v1.

(A, B) Schematic of the flow cytometry cleavage analysis for a meganuclease expressed on the yeast surface and fused to AgaII. In the presence of a nucleotide substitution that is tolerated by the enzyme, the dsDNA oligo will be cleaved leading to loss of Y-axis fluorescence (**A**). In the presence of a nucleotide substitution that is not tolerated by the enzyme, there is no cleavage and co-linear fluorescence (**B**). **C,** Graph showing a one-off analysis of eOnuHIVInt_v1 and subsequent nucleotide tolerance at each of the 22 nucleotides within the meganuclease target sequence. The target DNA sequence is displayed at the bottom and cleavage of each dsDNA oligo is relative to cleavage of the wild type target sequence shown as a dotted line at 1.0. Regions with bars above the cleavage of the target HIV sequence show single nucleotide substitutions that are better tolerated than the native nucleotide in the target sequence.

STRUCTURE GUIDED IMPROVEMENT OF MEGANUCLEASE SPECIFICITY

Based on the one-off specificity of the HIV-specific meganuclease, eOnuHIVInt_v1, together with the Stoddard lab, we generated a new version of the HIV-specific meganuclease, eOnuHIVInt_V2, that was made by a multi-step iterative process. The initial focus of the structure redesign was to improve target specificity for the 5'-end of the HIV-target sequence. This was largely because the enzyme is very non-specific in this region as shown in figure 3.4 above. To improve the specificity of the enzyme, seven amino acid residues in eOnuHIVInt_v1 were randomized; 4 residues (positions 26, 28, 30 and 80) were completely randomized while 3 amino acid residues (positions 44, 70 and 82) were partially randomized (numbering of residues is according to the crystal structure of I-OnuI, pdb id; 3QQY(237)). The basis of randomization and subsequent protein engineering was the one-off specificity analysis of eOnuHIVInt_1 which

demonstrated which areas of the enzyme were not specific for the HIV target site. The relative cleavage of the dsDNA residues was higher for those with substititions in the 5'-end of the dsDNA target which is recognized by the N-terminal end of the enzyme. Additionally, the selection was also targeted to those predicted off-target sites that were cleaved by the meganuclease in the tethered assay. The completely randomized residues could potentially be any amino acid while the partially randomized residues were constrained to specific acid residues. Amino acid residues at position 44 were constrained to asparagine (N), lysine (K), serine (S) or arginine (R), position 70 amino acids could be isoleucine (I), threonine (T), valine (V) or alanine (A), and position 82 amino acids could be T, R, A or G. At position 26 a leucine (L) in I-*Onu*I became glycine (G) in eOnuHIVInt_v1, and in eOnuHIVInt_v2. At position 28, arginine (R) in I-*Onu*I became tyrosine (Y) in eOnuHIVInt_v1, and in eOnuHIVInt_v2. At position 30, arginine (R) in I-*Onu*I became tyrosine (Y) in eOnuHIVInt_v1, and Isoleucine (I) in eOnuHIVInt_v2. At position 44 glycine in I-*Onu*I became arginine in eOnuHIVInt_v1, and lysine in eOnuHIVInt_v2. At position 70 alanine in I-*Onu*I became threonine in eOnuHIVInt_v1, and reverted to alanine in eOnuHIVInt_v2. At position 80 lysine in I-*Onu*I became histidine in eOnuHIVInt_v1, and isoleucine in eOnuHIVInt_v2. At position 82 threonine in I-*Onu*I became serine in eOnuHIVInt_v1, and reverted to threonine in eOnuHIVInt_v2. The amino acid differences between I-*Onu*I, eOnuHIVInt_v1 and eOnuHIVInt_v2 are shown in the amino acid alignment below (Figure 3.5).

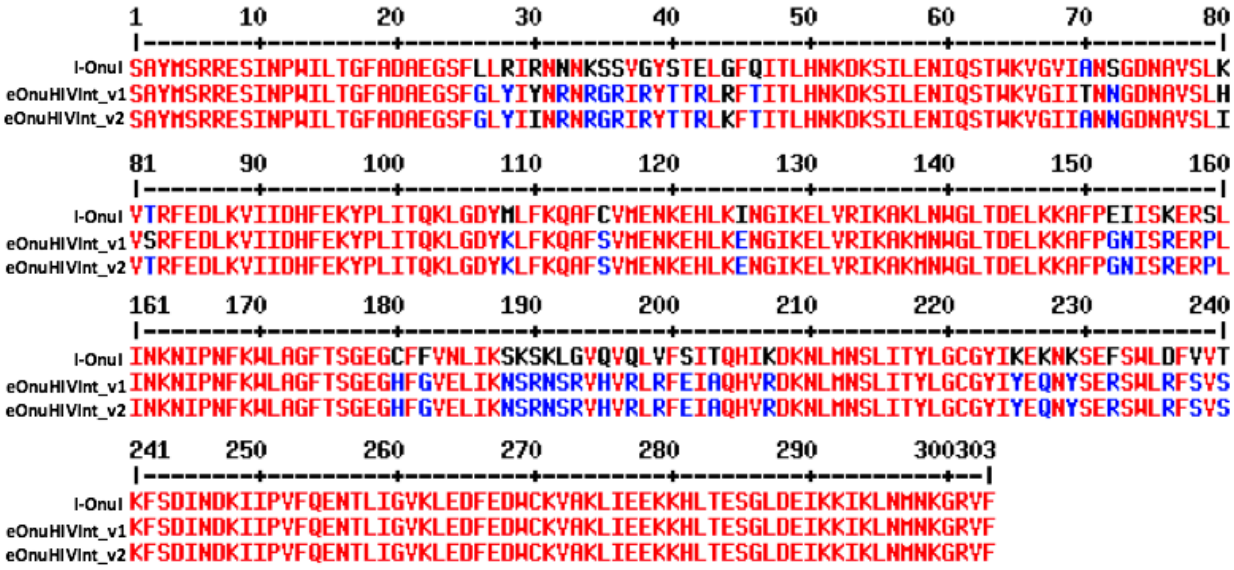


Figure 3.5. Amino acid sequence alignment of HIV-specific engineered meganucleases. Amino acid sequences are shown for I-*OnuI* (NCBI Accession: 3QQY_A) and I-*OnuI* derived HIV-specific meganucleases eOnuHIVInt_v1 and eOnuHIVInt_v2 that were generated through multiple iterative engineering steps to improve specificity. Amino acid differences between the different meganucleases are color coded in blue or black.

COMPARISON OF CLEAVAGE OF eOnuHIVInt_v1 and eOnuHIVInt_v2 USING YEAST SURFACE DISPLAY

To determine whether the new redesigned HIV-specific meganuclease has improved specificity relative to eOnuHIVInt_v1, both enzymes were expressed on the yeast surface as a fusion to the yeast surface protein AgaII as described above. We first decided to perform a one-off specificity analysis of eOnuHIVInt_v2 (as with eOnuHIVInt_v1 in figure 3.4c above) to compare tolerance for single nucleotide polymorphisms across the 22-nucleotide target sequence of the meganuclease. The 66 dsDNA oligos with every possible single nucleotide substitution

from the 22-nucleotide target of the meganuclease were tethered to eOnuHIVInt_v2 via an HA tag. Flow cytometric analysis was then performed in the presence of magnesium or calcium ions as described above to quantify cleavage and tolerance for single nucleotide substitutions.

The new improved enzyme, eOnuHIVInt_v2, shows better target specificity in the one-off analysis than eOnuHIVInt_v1 (Figure 3.6). The overall specificity for the 22-nucleotide HIV target sequence improved as the tolerance for single nucleotide substitutions at multiple sites decreased based on the difference in cleavage of the dsDNA oligos with single nucleotide substitutions between eOnuHIVInt_v1 and eOnuHIVInt_v2. The specificity in both the 5' and 3'-terminal ends improved, with the greatest specificity seen at the 3'-terminalus. The specificity within the central 4 nucleotides was also greatly improved. The increase in specificity across the entire length of the target sequence suggests reduced binding to non-specific DNA targets.

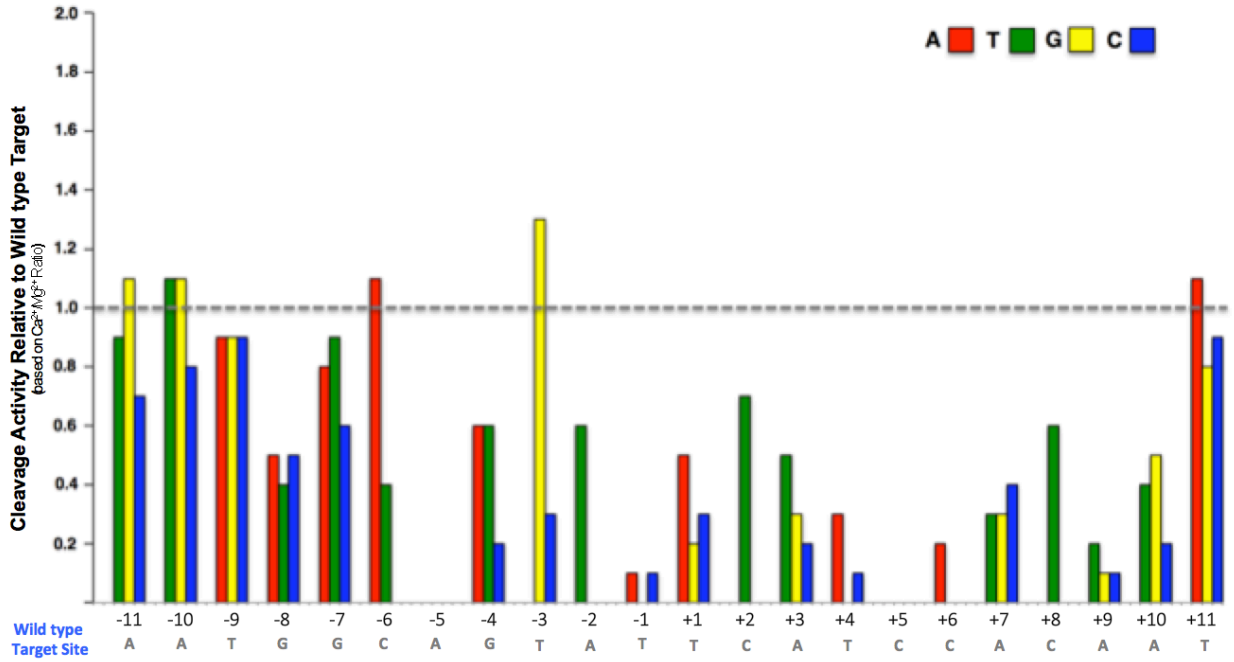


Figure 3.6. One-off specificity analysis of eOnuHIVInt_v2.

Tolerance for single nucleotide substitutions is shown for each of the 22 nucleotides within the target sequence of the HIV-specific evolved meganuclease. The target DNA sequence is displayed at the bottom x-axis. If a particular single nucleotide substitution is favored relative to the target sequence, cleavage of the dsDNA oligo containing that oligo is higher than that of the target dsDNA oligo and will have a bar over the target sequence cleavage (dotted line). Conversely, a substitution that is not favored will have cleavage less than of the target sequence and will be below the dotted line.

We next wanted to use yeast surface display to determine whether eOnuHIVInt_v1 and eOnuHIVInt_v2 are able to cleave any of the predicted off-target sites identified using PROGNOS (Figure 3.3a). We ordered dsDNA oligos for the 30 closest matched predicted off-target cleavage sites and tethered these to each of the enzymes as described above. HIV-specific meganuclease, eOnuHIVInt_v1 was able to cleave 8 of the predicted targets at levels of more than 40% relative to cleavage of the native HIV target sequence. The improved enzyme, eOnuHIVInt_v2 showed less cleavage activity at the off-target sites. There was reduced cleavage across all of the predicted off-target sites except for off-targets 3, 5 and 6 (Figure 3.7).

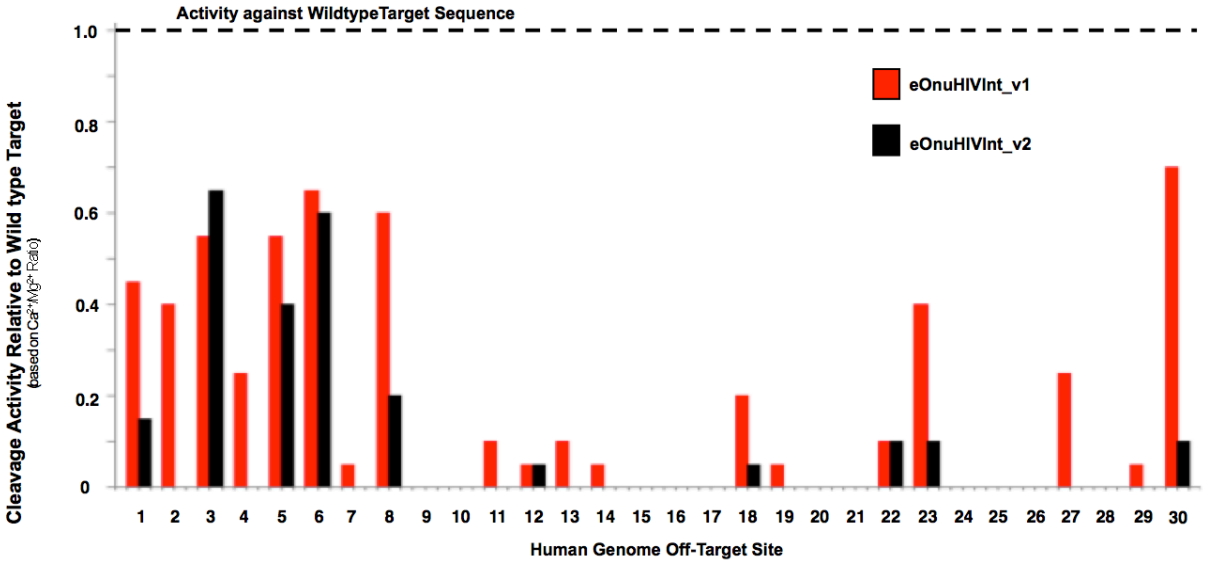


Figure 3.7. Yeast surface display cleavage analysis of predicted off target sites.

Double stranded DNA oligos for the 30 predicted off-target sites were tethered to the meganucleases eOnuHIVInt_v1 and eOnuHIVInt_v2 fused to the yeast surface protein AgaII. Cleavage was assessed using the tethered flow assay, and cleavage of each of the oligos was compared to cleavage of the target HIV sequence (indicated by the dotted line). Cleavage activity of the meganucleases at the predicted off-target sites is shown. Cleavage activity by eOnuHIVInt_v1 at some of the predicted off-target sites is greater than 40% of wild type. Off-target cleavage activity for eOnuHIVint_v2 was significantly improved at most of the sites that are cleaved by eOnuHIVInt_v1.

IN VITRO CLEAVAGE ACTIVITY PROFILE FOR eOnuHIVInt_v1 and eOnuHIVInt_v2

In the previous experiments, we demonstrated the activity of the yeast expressed meganuclease on target HIV sequences tethered to the enzyme. There was reduced cleavage activity of the target DNA sequence by the re-designed meganuclease eOnuHIVInt_2 compared to eOnuHIVInt_v1 on the basis of the relative shift in A647 fluorescence (Fig 3.8a). The HIV-specific engineered meganucleases eOnuHIVInt_v1 and eOnuHIVInt_v2 were subsequently

fused to TAL effector domains with 7.5 RVDs to create, respectively eOnuHIVInt_v1_7.5mT and eOnuHIVInt_v2_7.5mT, using a previously described method (223). To determine the *in cellulo* activity of the HIV-specific megaTALs, we transfected HEK293T cells with plasmids expressing the megaTALs and the pDHIV3 plasmid. At three days post-transfection, genomic DNA was extracted for subsequent mutation analysis. We first performed a mismatch cleavage assay to detect cleavage bands that would be suggestive of mutations at the target site. We detected cleavage bands in PCR amplicons from both experimental conditions, eOnuHIVInt_v1_7.5mT and eOnuHIVInt_v2_7.5mT, which suggests that both enzymes cleave target HIV sequences (Fig 3.8.b). We subsequently performed Illumina sequencing using the same genomic DNA samples and demonstrated the presence of mutations in both experimental conditions (Fig 3.8c). In cells transfected with the redesigned meganuclease, eOnuHIVInt_v2_7.5mT, we saw mutations rates of 2.16% of all sequences compared to 8.65% with the original HIV-specific meganuclease, eOnuHIVInt_v1_7.5mT. The mutations were present across the entire 22-nucleotide meganuclease target sequence and the profile is similar in cells modified by eOnuHIVInt_v1_7.5mT and eOnuHIVInt_v2_7.5mT.

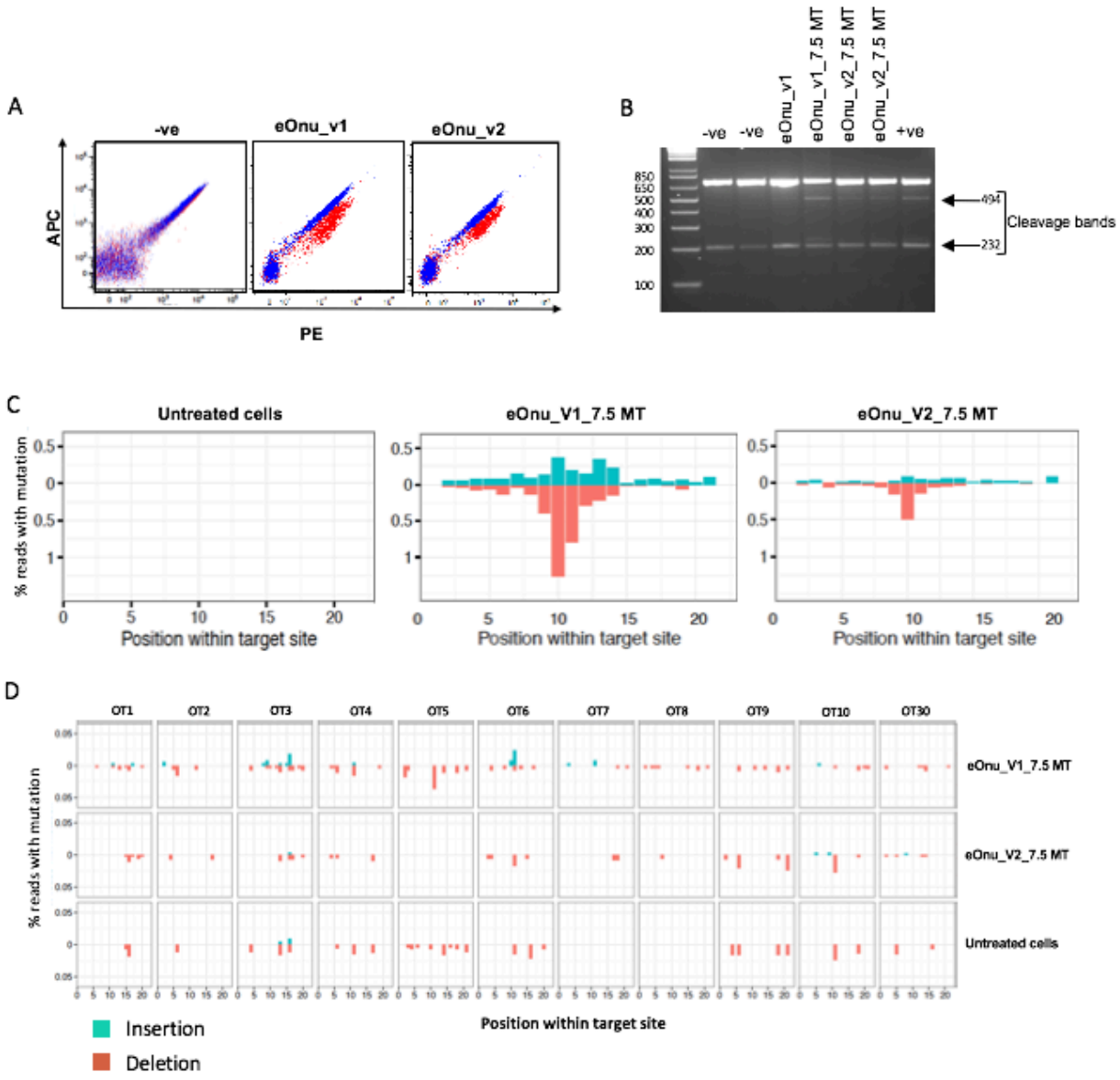


Figure 3.8. *In vitro* cleavage activity of HIV-specific engineered megaTALs.

A, Tethered flow cleavage assay for eOnuHIVInt_v1 and eOnuHIVInt_v2. Both enzymes are expressed on the yeast surface and can cleave a permissive tethered target sequence. **B,** Mismatch cleavage assay of gDNA from HEK293T cells transfected with pDHIV3 and plasmids expressing either eOnuHIVInt_v1, eOnuHIVInt_v1_7.5mT or eOnuHIVInt_v2_7.5mT. At 72 hours post transfection PCR amplicons containing the target HIV sequenced were used in the mismatch cleavage assay, and the presence of cleavage bands shows mutations are present at the target HIV sequence. **C,** Mutation rates for on-target cleavage of the HIV target site by Illumina

sequencing. Insertions (Green) are shown above the y-axis, and deletions (red) below the y-axis. Relative mutation frequencies (% reads with mutation) are indicated for each nucleotide site within the target sequence. **D,** Cleavage rates at predicted off-target sites by Illumina sequencing. Mutation profiles are shown for enzymes eOnuHIVInt_v1_7.5mT and eOnuHIVInt_v2_7.5mT across the 22-nucleotide target sequence of each of the 11 predicted off-target sites (1 to 10 and 30) The off-target mutation levels for eOnuHIVInt_v1_7.5mT appear higher than those of eOnuHIVInt_v2_7.5mT.

IN CELLULO CLEAVAGE OF PREDICTED OFF-TARGET SITE SEQUENCES

Therapeutic application of engineered endonucleases to HIV cure will require high HIV target sequence specificity and minimal off-target cleavage. To determine the off-target cleavage levels of the new improved megaTAL eOnuHIVInt_v2_7.5mT, we first determined the closest matched human genomic sites by homology using PROGNOS (Figure 3.3a). We then transfected HEK293T cells with plasmids containing either eOnuHIVInt_v1_7.5mT or eOnuHIVInt_v2_7.5mT, and extracted genomic DNA from the transfected cells at 5 days post transfection. Illumina sequencing was performed using PCR amplicons of the 11 closest matched genomic sites using primers shown in Appendix A. We also performed Illumina sequencing of PCR amplicons of the 11 closest matched predicted off-target sites using gDNA extracted from SupT1 CD4+ T cells infected with megaTALs expressing lentiviral vectors. In both cell lines we demonstrated mutations rates above background at several of the predicted off-target sites (figure 3.8c). In HEK293T cells, we demonstrated off-target cleavage rates that range between 0.02% and 0.12% for the different predicted off-target sites. At off target site 3 (mutation rate= 0.09%, P=0.0130), off-target site 5 (mutation rate=0.12%, P=0.0096), and off-target site 8 (mutation rate=0.03%, P=0.0130), the off-target cleavage rates were statistically significant when compared to the untreated cellular controls. The mutation profile at the predicted off-target sites was similar to that seen at the target HIV sequence. However, eOnuHIVInt_v1_7.5mT has higher overall on-target cleavage activity than eOnuHIVInt_v2_7.5mT, and therefore has a higher therapeutic ratio.

TOXICITY OF ENGINEERED HIV-SPECIFIC MEGANUCLEASES

To determine the toxicity of HIV-specific endonucleases towards treated cells we infected SupT1 CD4+ T cells with VSV-G pseudotyped lentiviral vector expressing the engineered meganucleases, eOnuHIVInt_v1, eOnuHIVInt_v1_7.5mT or eOnuHIVInt_v2_7.5mT as described above. To assess cell viability/toxicity, SupT1 CD4+ T cells were stained at three days post infection according to the manufacturer's instructions using the FITC Annexin V apoptosis detection kit (BD biosciences), which contains, Annexin V and PI. Lentivirus transduced cells express BFP as proxy for meganuclease transduction. Viability was analyzed for cells that expressed BFP in the experimental conditions, and the percentage cell viability was determined as the fraction of BFP expressing cells that were both Annexin V and BFP negative for the experimental control. In the control uninfected SupT1 CD4+ T cells, cell viability was the fraction of cells that were negative for both Annexin V and PI (Figure 3.9a). In the untreated SupT1 CD4+ T cells, viability was 85.4%, whereas cells treated with eOnuHIVInt_v1 with eOnuHIVInt_v1_7.5, and the new improved megaTAL eOnuHIVInt_v2_7.5mT showed cell viabilities of 42%, 57.6% and 74.4% . The difference seen between the two megaTALs using the Wilcoxon rank sum test was statistically significant (P=0.01).

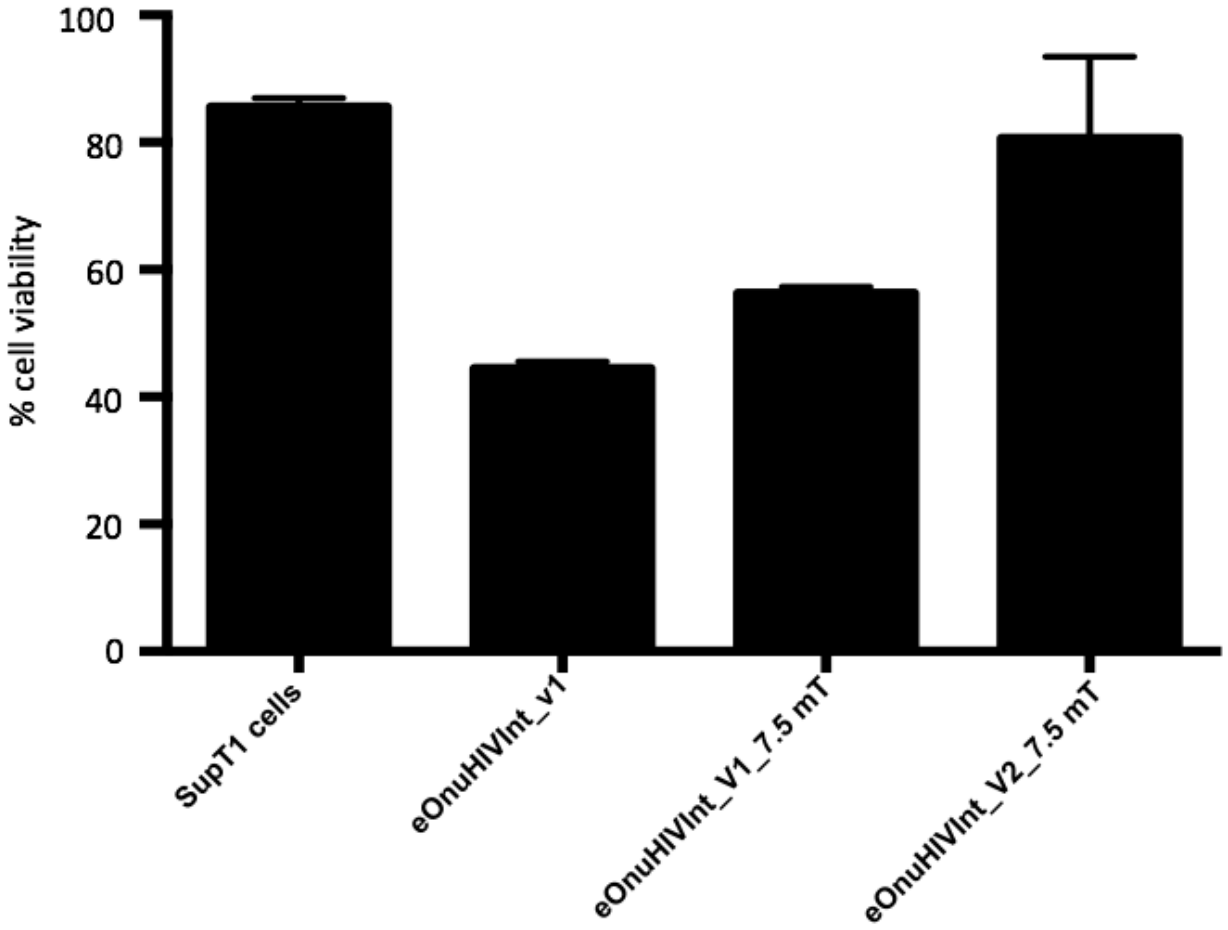


Figure 3.9. Cell viability in meganuclease treated cells.

SupT1 CD4+ T cells were transduced with VSV-G pseudotyped lentiviral vector expressing either eOnuHIVInt_v1, eOnuHIVInt_v1_7.5mT or eOnuHIVInt_v2_7.5mT. At 3 days post infection, the cells were harvested and stained with Annexin V and PI, and flow cytometry analysis was performed using BFP expressing cells. Percentage cell viability for each of the experimental conditions was calculated as the percentage of cells that were negative for both Annexin V and PI, and is represented graphically.

3.3 DISCUSSION

Meganucleases are small compact proteins and are deliverable in several gene delivery vectors such as Adeno-associated virus (AAV) vectors which makes them an attractive option for gene disruption. The major challenges, however, for the use of meganucleases in gene disruption are the complexity of engineering required to retarget the meganuclease to a new DNA sequence, and the possibility of off-target cleavage effects. This study sought to characterize the activity of HIV specific engineered meganucleases on target HIV sequences and homologous off-target sites within the human genome. We wanted to determine whether engineered meganucleases efficiently cleave target HIV proviral sequences and to characterize activity at predicted off-target sites. Additionally, we wanted to reduce off-target cleavage activity of the HIV-specific meganucleases and megaTALs through structure guided engineering, although this was at the expense of on-target cleavage activity.

We used a LAGLIDADG meganuclease, I-*Onu*I that has been engineered to recognize a 22-nucleotide sequence in the HIV pol gene. The engineered meganuclease eOnuHIVInt_v1 was fused with TAL effectors of varying RVD lengths to improve both activity and specificity. We demonstrated that the engineered meganuclease, eOnuHIVInt_v1, and the related fusion megaTALs, eOnuHIVInt_v1_5.5mT, eOnuHIVInt_v1_6.5mT, and eOnuHIVInt_v1_7.5mT, all cleave target HIV sequences with HIV-target site mutation rates ranging from 10% to 40% when measured by Illumina sequencing. The mutation rates detected at the HIV target sequence increased as the number of RVDs present in the megaTALs increased. We also showed that concurrent treatment with the 3'-exonuclease Trex2 markedly improves mutation rates at target

sites leading, in some cases, to a 2-fold increase. This suggests that DNA end-processing enzymes will likely be important in improving success rates of gene editing therapies.

For therapeutic application, it is important to characterize the off-target cleavage activity of the HIV-specific engineered meganuclease. In transfection experiments in HEK293T cells, we show that eOnuHIVInt_v1 and eOnuHIVInt_v1_7.5mT, the HIV specific engineered meganuclease and its megaTAL with 7.5 RVDs, cleave and introduce mutations at predicted off-target sites. While we have not characterized the effect of insertions and deletions at the predicted off-target sites, the presence of these mutations suggested that there was a need to improve the specificity profile of the engineered meganuclease for target HIV sequences and further reduce its off-target cleavage activity.

In collaboration with Dr. Barry Stoddard and Dr. Abigail Lambert at the Fred Hutchinson Cancer Research Center, we sought to characterize the mutational tolerance of the HIV-specific engineered meganuclease eOnuHIVInt_v1 using a yeast surface display cleavage assay. Using this tethered flow assay, we demonstrated that the meganuclease tolerates multiple single nucleotide substitutions in the 22-nucleotide recognition sequence. We further demonstrated that the meganuclease also significantly cleaves several predicted off-targeted sequences in this assay. We subsequently generated a new improved version of the HIV specific meganuclease, eOnuHIVInt_v2. eOnuHIVInt_v2, has an improved one-off specificity profile when compared to eOnuHIVInt_v1, and also has less cleavage activity at predicted off-target sites. The improved one off-specificity profile suggests that this redesigned meganuclease has better discrimination between target and off-target sites, and would have less off-target cleavage activity.

Importantly, the new enzyme, eOnuHIVInt_v2 and its megaTAL eOnuHIVInt_v2_7.5mT retain in *cellulo* cleavage activity at their HIV target sequence in both HEK293T cells and SupT1

CD4+ T cells, and have lower cleavage activity at the predicted off-target sites in both these cell lines. The new improved HIV specific engineered meganuclease, eOnuHIVInt_v2, has lower cleavage activity at its HIV target sequence than eOnuHIVInt_v1, although the its mutagenic potential can be rescued by co-transfection or treatment of cells with end-processing enzymes such as Trex2. The off-target cleavage activity observed with the HIV-specific engineered meganucleases is consistent with off-target cleavage activity seen in other applications of meganucleases or other nuclease platforms(212,242). MegaTAL nucleases and CRISPR/cas9 endonucleases have collectively been found to exhibit the lowest levels of off-target cleavage activity(243), and could therefore, be the most adaptable to clinical applications. Sequential improvements in off-target cleavage activity through structure guided engineering could lead to better enzymes that are more tolerable in clinical applications. However, it is important to determine a cut-off between target and off-target cleavage activity at which an endonuclease would be considered safe for use in therapeutic applications.

Our data suggest that engineered meganucleases could be used as viable options in HIV cure strategies. We also show that through iterative engineering, we can improve specificity of the enzyme and subsequently reduce off-target cleavage, both of which are vital for application of these engineered meganucleases in patient populations. As has been previously shown, the 3'-exonuclease Trex2, improves the efficiency of meganuclease-mediated mutagenesis(241). We show here that addition of Trex2 improves mutation frequency by over 2 fold. Trex2 can be added to improve the efficiency of meganuclease mediated disruption of the integrated HIV provirus. We also demonstrate, in agreement with previous observations(222), that fusion megaTALs have improved activity relative to the parental meganuclease. We postulated and subsequently showed that meganucleases and fusion megaTALs can be engineered to recognize

unique HIV DNA sequences and cleave that sequence, resulting in the introduction of mutations in these DNA sequences. If these mutations are introduced in key genes for viral replication, they could lead to functional HIV eradication. Key issues such as off-target toxicity can be addressed through structure guided nuclease engineering to improve target specificity paving the way for therapeutic application of engineered endonucleases in human subjects, although additional challenges such as delivery to target cells still remain unaddressed.

Chapter 4. ESTIMATION OF THE SIZE OF THE HIV VIRAL RESERVOIR USING MULTIPLEX DDPCR

4.1 INTRODUCTION

The major barrier to HIV eradication is the establishment of a long-lived reservoir with latently integrated virus that is resistant to clearance by combination anti-retroviral therapy. The best-characterized reservoir comprises resting memory CD4+ T cells that have a half-life of 39.5 to 43.5 months and would take between 67 and 74 years to clear from circulation on current cART regimens(70,71). Importantly, the integrated HIV provirus in resting memory CD4+ cells is transcriptionally silent, and resting memory CD4+ T cells do not express any viral epitopes so are resistant to clearance by cytotoxic T cells(244). This transcriptionally silent virus can however be activated to replicate through a variety of mechanisms to produce viable progeny and reconstitute infection. When suppressive cART in HIV positive patients with undetectable viremia is interrupted or discontinued, viral rebound occurs within about two weeks(75). This viral rebound is due to activation of the long-lived latent reservoir in resting memory CD4+ T cells and other less well described cell types. HIV cure and eradication will require clearance of the viral HIV viral reservoir.

To successfully cure HIV, we need to be capable of accurately quantifying the latent viral reservoir. The gold standard assay for reservoir quantification is the viral outgrowth assay (VOA) which has been described extensively(90,245). The viral outgrowth assay is a limiting dilution assay that is dependent on activation of viral replication using phytohemagglutinin (PHA). However, recent studies suggest that a substantial proportion (>90%) of intact replication competent virus is not induced to produce virus following maximal activation by PHA(89). The

VOA, therefore, tends to under-estimate the reservoir. Other reservoir quantification assays are based on polymerase chain reaction (PCR). PCR-based assays amplify a segment of the viral genome and will detect all viral DNA in the cell. The Siliciano laboratory has shown that a large percentage of the integrated proviruses are defective with large mutations and not replication competent(89). PCR-based assays amplify all HIV DNA in the sample regardless of how complete the genome is, including 1 and 2-LTR circles that are replication incompetent. Consequently, PCR-based reservoir quantification assays over-estimate the viral reservoir. The Tat/rev induced limiting dilution assay (TILDA) is a hybrid RNA-based assay that combines both stimulation and PCR measurement. Reservoir cells are stimulated using phorbol 12-myristate 13-acetate(PMA) and ionomycin, a combination that yields more stimulation of latent reservoir cells than PHA. Subsequently PCR is performed to detect tat/rev messenger RNA transcripts. The TILDA assay gives higher estimates of reservoir size than QVOA and is more comparable to DNA PCR measurements of the reservoir, although it detects a single viral gene segment and cannot differentiate between partial and complete viral RNA transcripts (91). Reservoir eradication studies will require both precise quantification of the reservoir and distinction between intact replication competent virus and defective proviruses that cannot produce viable viral progeny.

Droplet digital PCR (ddPCR) technology gives absolute quantification of target DNA without the need for a linear standard curve(246). HIV viral quantification by ddPCR is equivalent to qPCR, although a number of publications have showed ddPCR to be more accurate and precise than qPCR(247-249). Multiplex ddPCR allows for simultaneous detection of several DNA fragments in a sample and has been used previously to differentiate between HHV6-A and B(250), and to detect CMV and adenovirus in the background of an internal plasmid

control(251). Similarly, multiplex ddPCR can be used to accurately quantify the HIV viral reservoir. We propose to use multiplex ddPCR to give a better estimate of the functional HIV reservoir. Here we demonstrate that by designing and multiplexing several ddPCR assays targeting different segments of the proviral genome, we can evaluate the integrated proviral DNA for genome 'completeness' which can be used to estimate the fraction of the replication competent and functional viral reservoir. Using samples from SHIV-infected macaques we demonstrate that we can detect up to six viral regions and that there is differential quantification of different gene segments. This assay could be used to give a rapid estimate of which gene segments are likely to be deleted in the integrated provirus and those with potentially inactivating mutations resulting from either endonuclease mediated treatment or APOBEC induced mutagenesis.

4.2 RESULTS

VALIDATION OF ddPCR PRIMERS AND PROBES USING PLASMIDS

Initial validation of the multiplex ddPCR assay for SHIV quantitation was performed using a SHIV 1157-ipd3n4 containing plasmid. The choice of SHIV for validation was largely motivated by the availability of a cohort of SHIV-infected macaques undergoing different experimental therapies. We initially wanted to determine whether all the six assays performed equivalently using plasmid as template. We then wanted to develop a multiplex strategy that would enable simultaneous detection of multiple SHIV gene segments. We first performed individual reactions with each of the primer/probe sets to optimize the ddPCR reactions. For these individual reactions, we used a known quantity of SHIV plasmid linearized with EcoRI and determined whether we could amplify the plasmid with each primer/probe set. All six primer

probe sets amplified the plasmid equivalently (Figure 4.1a, 4.1b). We next selected combinations of different primer/probe sets to make a multiplex assay. We developed 2 multiplex assays for SHIV quantitation by multiplexing three sets of primers/probes per reaction. The multiplex assays were developed by combining a probe with a VIC fluorophore with two probes in the FAM channel at different concentrations so that we could achieve different amplitudes for those probes in the same channel (Figure 4.1 c and d). In each assay, we wanted to demonstrate that each primer and probe set performed equivalently. Summation of the droplets that are positive for each primer probe set, that is the single positives, double positives and triple positives for each probe set, and comparing each of the three assays gave us equivalent quantification using the SHIV 1157-ipd3n4 plasmid that contains equal copies of each gene.

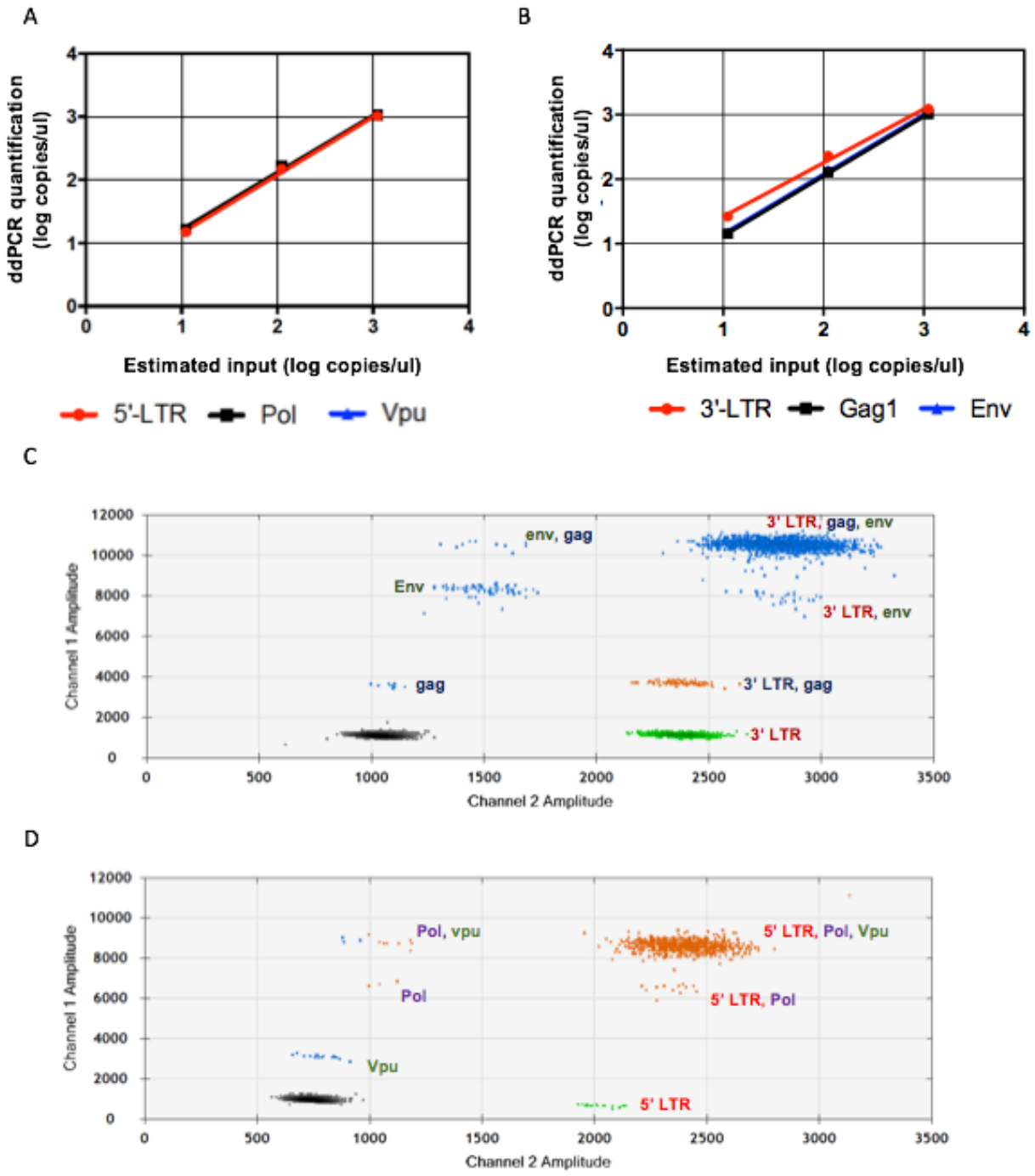


Figure 4.1. Validation of SHIV multiplex ddPCR assay on plasmid.

A, B, Comparison of six SHIV assays on SHIV plasmid 1157-ipd3n4. SHIV plasmid DNA was linearized with EcoRI and a known quantity of plasmid was used to validate the six ddPCR

assays. Titers obtained for each assay were compared to ensure that all assays are giving equivalent quantitation of SHIV plasmid, which contains all the six target genes. Panel A, comparison titers from assays in one triplex assay of 5'-LTR, pol and vpu, and panel B, comparison of titers obtained with second triplex assay of 3'-LTR, gag, and env. C, Multiplex ddPCR assay of 3'-LTR, gag and env assays showing separation of different droplet populations. The single positive droplets, the double positive and the triple positive droplets all cluster separately and can be quantified individually. D, as described for C, a multiplex ddPCR assay of 5'-LTR, pol and vpu assays.

LACK OF INHIBITION OF ddPCR QUANTIFICATION USING GENOMIC DNA

To determine whether genomic DNA interferes with quantitation of the SHIV plasmid, we added between 400ng-800ng of unlinearized macaque genomic DNA from LLC-MK2 cells (ATCC# CCL-7) per reaction. We performed endpoint PCR using the thermocycling conditions listed in the methods above. SHIV plasmid titers obtained with and without macaque genomic DNA were equivalent suggesting that macaque genomic DNA does not interfere with the reaction. In the presence of macaque genomic DNA, we were also able to achieve good separation between the different primer/probe sets (for individual gene segments) in the multiplex assays as shown for the gag and env assays below (Figure 4.2).

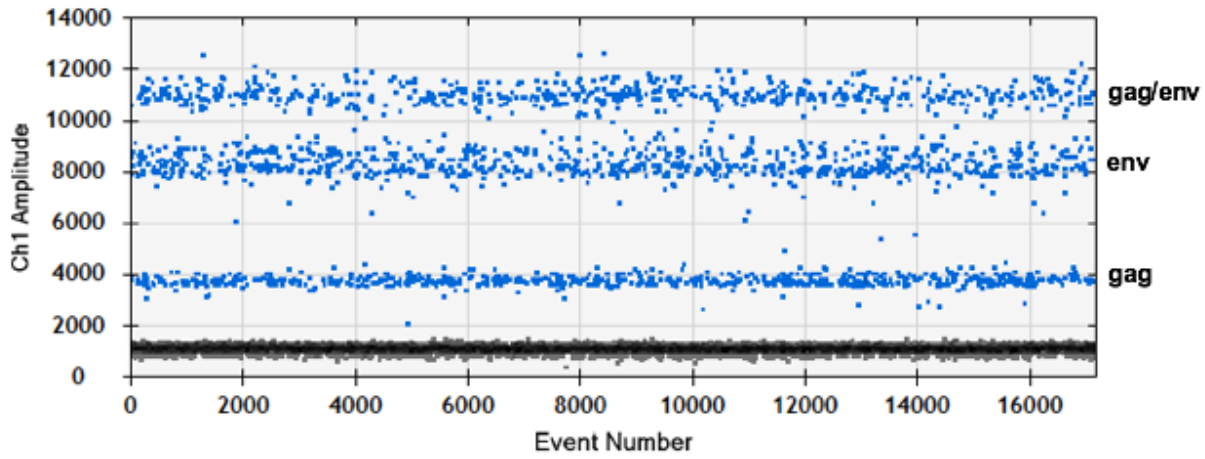


Figure 4.2. Effect of genomic DNA on ddPCR assay quantification.

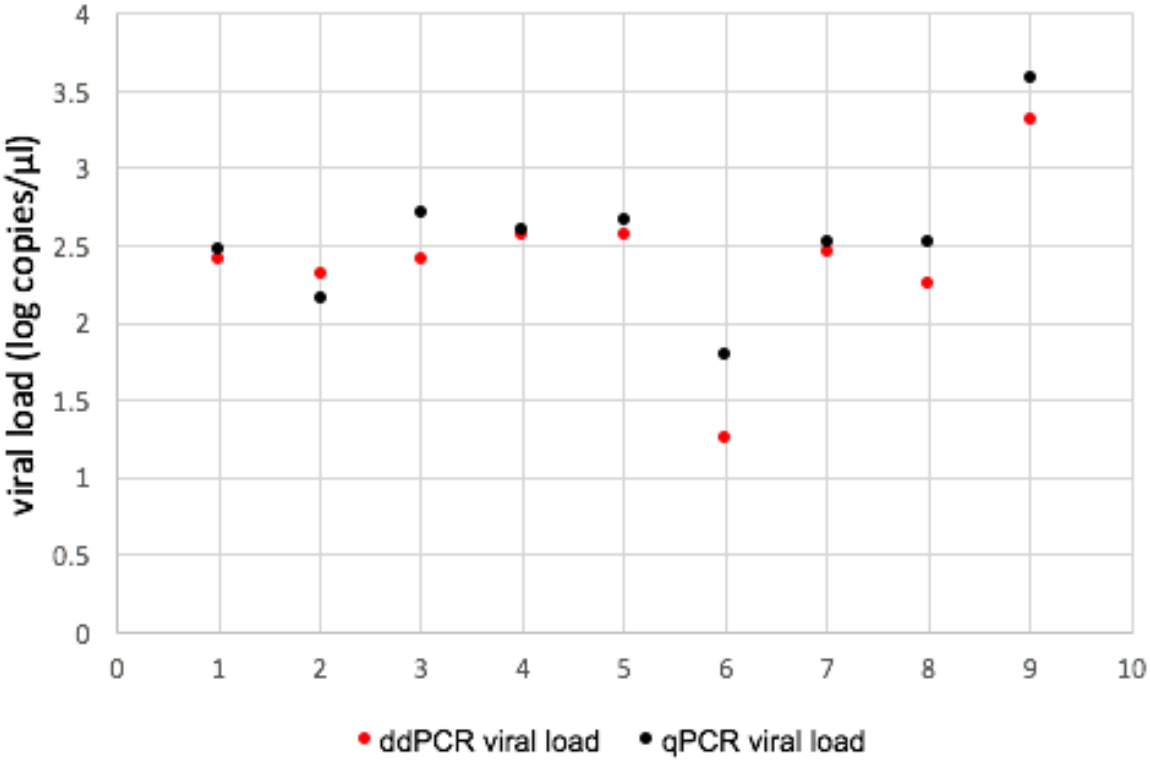
To determine the effect of genomic DNA on quantification of SHIV plasmid DNA using the multiplex ddPCR assay, we performed the multiplex reaction in the background of 400ng-800ng of macaque genomic DNA extracted from LLC-MK2 cells. The panel shows that even in the background of macaque genomic DNA, there is still sufficient clustering of different droplets to identify DNA positive for each of the three assays in the multiplex SHIV assay. This figure demonstrates the gag and env assays, both of which are in the FAM channel.

COMPARISON OF ddPCR TO qPCR FOR DETECTION OF HIV/SHIV DNA

After validating and establishing the multiplex assay using the SHIV plasmid, we performed the assay on samples from SHIV-infected macaques. We performed the two multiplex assays on nine lymph node biopsy samples from SHIV infected macaques. First we wanted to determine whether the multiplex ddPCR assay performed as well as an established clinical qPCR. We quantified the virus using the multiplex ddPCR assay and compared the titers from the gag ddPCR primer/probe set (same primer/probe set used in the multiplex assay) with a qPCR assay performed using the same set of gag primers and probe. For all 11 tissue samples, the SHIV titers

obtained using either the qPCR assay or the multiplex ddPCR assay were within half a log of each other. There was a positive correlation between the qPCR and ddPCR assays (Pearson's correlation co-efficient=0.92(p=0.0004) and the R^2=0.853, which again suggests that the multiplex ddPCR assay is equivalent to a clinical SHIV qPCR assay performed using the same primers and probes.

A


viral load (log copies/µl)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
1
2
3
4
5
6
7
8
9
10
ddPCR viral load
qPCR viral load

B

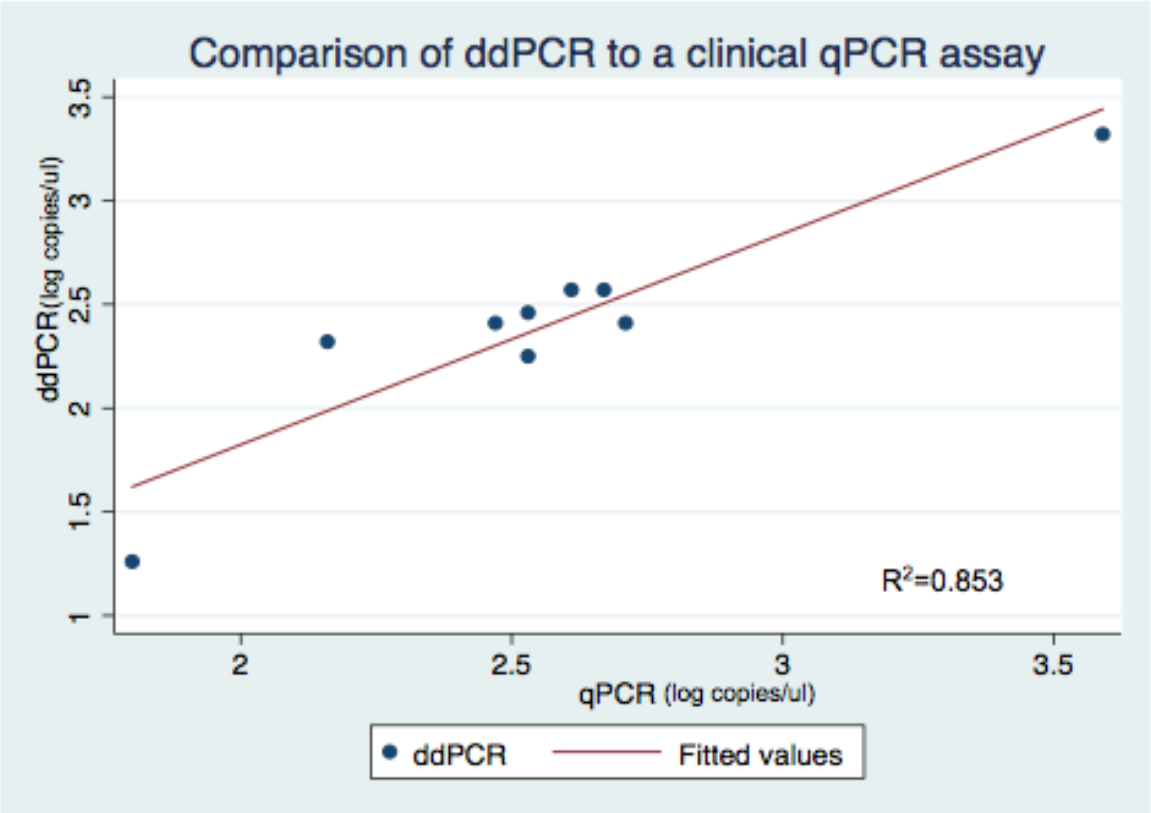

Comparison of ddPCR to a clinical qPCR assay
ddPCR(log copies/ul)
1
1.5
2
2.5
3
3.5
qPCR (log copies/ul)
2
2.5
3
3.5
R²=0.853
ddPCR
Fitted values

Figure 4.3. Comparison of ddPCR titers to qPCR titers.

A, Graph shows comparison of titers obtained using the gag ddPCR assay to those obtained using a clinical qPCR assay. The clinical qPCR assay uses the same primers and probes as the ddPCR assay. The titers obtained by the two assays are equivalent. The numbers 1 to 9 represent animals from which the lymph node biopsies were obtained. **B,** Graph demonstrates a positive correlation between the gag ddPCR SHIV assay and the gag clinical qPCR assay (R^2=0.853, R=0.92(p value=0.004))

ESTIMATING SIZE OF THE FUNCTIONAL VIRAL RESERVOIR USING A SHIV MACAQUE MODEL OF INFECTION

To measure the proportion of the integrated SHIV provirus that had a complete genome and was therefore likely to be replication competent, we performed the two multiplex assays on DNA from lymph node biopsies of SHIV infected macaques. We then calculated the titers for each primer/probe set in the two multiplex assays. We took the 3'-LTR primer/probe set as the reference assay and compared the titers from all the other five assays to the titers obtained using the 3'-LTR primer/probe set. We found that compared to plasmid, the quantification by the individual assays is not equivalent with some primers/probe sets having higher or lower titers compared to the 3'-LTR in different samples. For example, looking at sample 2162 in Figure 4.4a below, with the 3'-LTR primer/probe set as the reference, the relative quantification by the other five assays is less than that obtained using the 3'-LTR assay (figure 4.4a). The relative quantification by the env assay in this sample is about 40% less than by the 3'-LTR assay. This would suggest that the env gene (region amplified by the env assay) has 40% deletions relative to the 3'-LTR. In samples 2260 and 2384, quantification by several assays is greater than by the

reference 3'-LTR which suggests that the 3'-LTR probably has deletions relative to those genes. In sample 2260, titers by the Pol assay are 10% higher than the 3'-LTR titers, while in sample 2384 the Pol titers are about 40% higher than 3'-LTR. This again suggests that these gene fragments are more abundant that the 3'-LTR which might result from large deletions in the 3'-LTR segment of the integrated SHIV virus. The multiplex ddPCR assay allows us to simultaneously detect different gene segments of the SHIV provirus and to estimate the percentage of replication competent virus. The estimate of the intact replication competent provirus would correspond to the lowest measured titers. In sample 2162, the size of the intact replication competent reservoir would be 62% of the titers measured using the 3'-LTR assay. The 62% represents the relative quantification using the env assay, which again suggests that env is deleted relative to the 3'-LTR gene. Similarly, all the other samples would be measured to the reference 3'-LTR assay. In sample 2260, the lowest titers are obtained using the env assay, which are equivalent to 84% of the titers using the 3'-LTR assay. Regardless of relative increase in abundance of the gag fragment compared to the 3'-LTR, the deletions in env would render the virus non-viable. The viable reservoir in 2384 is 84% of the 3'LTR, which corresponds to the titers using the gag assay. The functional reservoir size is 44% in 2310, 64% in 1671, and 46% in 2078, all corresponding to titers measured using the vpu assay. In samples 1582 and 1764, the relative reservoir size is 97% and 38% respectively and corresponds to pol titers. The vpu, pol and env regions were consistently less abundant than the 3'-LTR, gag and 5'-LTR. Sample 1582 is unique because we did not obtain any values for the vpu assay using ddPCR although we did not perform a qPCR assay for the vpu region. This might suggest that there is no functional reservoir in this animal and is something that warrants further investigation by repeating the assay or sequencing the virus from this animal. The results from this multiplex ddPCR provides

further evidence that using a singleplex assay for HIV reservoir quantification is inadequate. Singleplex assays measure one gene region and will tend to overestimate the reservoir. A multiplex assay will give a more accurate estimate of the intactness of the integrated proviral reservoir.

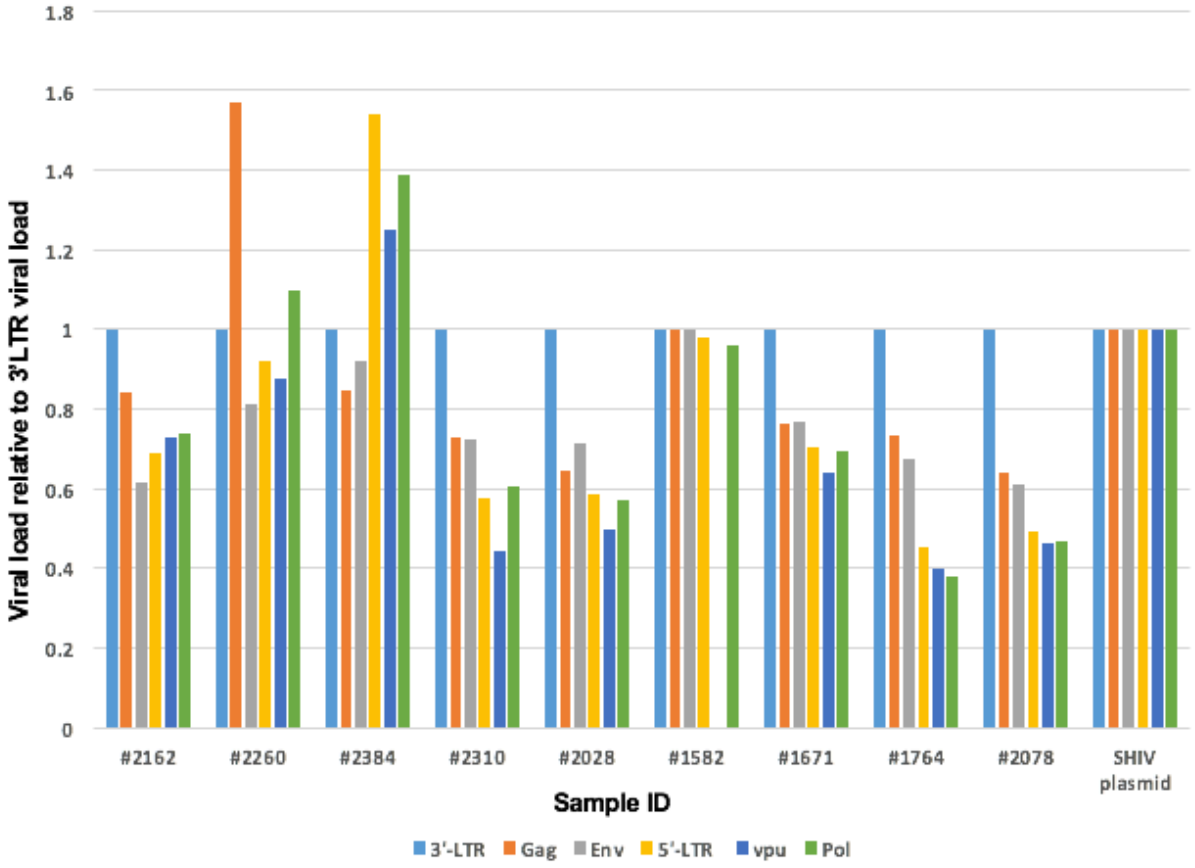


Figure 4.4. Relative quantification of SHIV viral reservoir using multiplex ddPCR. Genomic DNA extracted from lymph node biopsies of macaque infected animals was used to quantify the SHIV proviral reservoir. In each run, a SHIV sample was run as a quantitation control. For each sample, we performed the two multiplex ddPCR assays. The titers obtained for each individual assay represents the sum of the single positive, double positive and triple positive droplets. All five assays were then compared to the 3'-LTR assay, which was used as reference

assay, and depicted graphically. Titers for all six assays using plasmid was equivalent. In the macaque tissue samples there are differences between the different assays, which may suggest mutations or deletions are present at the sites amplified by those specific assays.

4.3 DISCUSSION

In this study, we sought to develop an assay to estimate the size of the functional HIV proviral reservoir. Accurate quantification of the HIV viral reservoir is critical to reservoir eradication strategies and HIV cure. We used samples from SHIV-infected macaques as a model to develop two multiplex assays each comprising three assays in separate gene regions to cover a total of six genes within the integrated SHIV provirus. Using these six assays in 3'-LTR, 5'-LTR, gag, pol, vpu and env we show that we can amplify and detect each of the six viral segments. In initial validation experiments using plasmid, we show that we can achieve sufficient separation between the different populations of droplets that are positive for each of the different combinations of assays representing the single positive, double positive and triple positive droplets. We show that when using a plasmid template these different assays all perform equivalently and have relatively equal amplification and detection. We further demonstrate that host genomic DNA does not interfere with quantification of viral DNA.

The two multiplex assays were then performed using lymph node biopsy samples from SHIV-infected macaques. We show that in samples from infected macaques, where the six ddPCR assays have similar titers using plasmid, different gene levels are found in each of the 9 macaque samples. We compared the values to the 3'-LTR assay as a reference assay and show that relative to the 3'-LTR assay some viral gene segments are more abundant than the 3'-LTR,

but for the most part, the majority are less abundant than the 3'-LTR. Our findings suggest that in those assays where we have lower levels than in the 3'-LTR assay, there could be deletions in the gene segments amplified by these specific assays. This possible pattern of deletions mirrors previous observations where sequencing was performed on HIV provirus from HIV-infected patients on cART(89). In our results, we founnd that the assays in vpu, pol and env regions tend to have viral loads compared to the 3'-LTR, gag and 5'-LTR. One explanation for the lower titers is that the vpu, pol and env regions are preferentially deleted in the integrated SHIV provirus relative to the other regions. Another explanation, however, could be the presence of mutations in those specific regions that abrogate primer and/or probe binding and therefore, lead to under quantification of specific viral gene segments. This is particularly important in a virus like HIV, which has a high mutation rate and significant genetic diversity even within an individual patient. A multiplex ddPCR assay for quantification of the viral reservoir needs to address the wide diversity in the integrated provirus and to overcome this diversity challenge, and our multiplex assays were designed to target highly conserved regions in each of the genes. The SHIV isolate used for these primate studies was clonal and the primers and probes were designed based on the SHIV plasmid sequence. Translation of this assay to HIV reservoir quantitation will require careful consideration of genetic diversity.

Further validation of this assay in HIV-positive patients is underway and will be needed to ensure that we are accurately capturing the entire reservoir. This validation will probably require comparison of the ddPCR assay with sequencing of the reservoir to ensure that each segment that is tagged as deleted in the ddPCR assay is truly deleted and that the low titers in the ddPCR assay are not due to mutations in the SHIV provirus.

The multiplex assay also needs to be compared to the other assays currently being used to quantitate the viral reservoir. Multiple reports have shown discordance between single-plex ddPCR assays, traditional qPCR assays and the VOA (90). We hypothesize that the multiplex ddPCR assay will give a more accurate reflection of the HIV reservoir and will perform favorably in comparison assays.

A multiplex ddPCR assay which allows simultaneous detection of up to six viral gene segments could be used to estimate the size of the intact HIV viral reservoir that is consequential to disease pathogenesis and HIV cure efforts. In this assay, titers from each component of the multiplex assay can be compared and the size of the viral reservoir will likely correspond to the gene fragment that has the lowest levels. It is assumed that the regions that are not amplified have deletions and are replication incompetent.

Chapter 5. PERSPECTIVES AND FUTURE DIRECTIONS

My thesis work has focused on the use of engineered meganucleases for HIV cure and eradication. I have demonstrated that we can cleave and introduce inactivating mutations in key HIV genes using engineered meganucleases. I have also demonstrated that the frequency of mutations at target sites is increased with the addition of the DNA end-processing enzyme, Trex2. Additionally, for therapeutic application, engineered meganucleases have to be able to distinguish between target and off-target sites. I have shown that engineered meganucleases significantly cleave target HIV sequences and demonstrate that structure-guided engineering can lead to improvements in target specificity and diminish off-target cleavage activity while retaining activity at the HIV-target site. In the future, we would like to test the effect of HIV-specific meganucleases using a primary cell model of HIV latency(252), and on samples from HIV-infected patients.

In the second part of my thesis, I describe a multiplex ddPCR assay that can be used to estimate the size of the functional SHIV reservoir. We show using lymph node samples from SHIV infected macaques that this assay can be used to amplify and simultaneously detect up to six viral gene segments. Going forward, we would like to compare this multiplex ddPCR assay to already established assays including QVOA and the TILDA assay.

Similar to our work, several groups have recently demonstrated cleavage of HIV viral sequences using engineered endonucleases. This is a big step in HIV cure and eradication strategies, but several key questions remain to be answered before we can truly achieve HIV cure. Can we deliver engineered endonucleases to a sufficient proportion of the reservoir cells that harbor the integrated HIV provirus? What proportion of reduction in reservoir size is required for HIV cure? How will HIV cure be measured? What are the economics of HIV cure?

Are these therapies scalable and applicable in resource-limited settings where the largest percentage of HIV positive patients lives? I will discuss these questions and what the future holds for HIV cure research.

5.1 DELIVERY OF ENGINEERED ENDONUCLEASES TO HIV RESERVOIR CELLS

To be effective in curing HIV, engineered endonucleases will need to be delivered to target HIV infected cells. Most of the studies that have looked at endonuclease mediated disruption of the HIV reservoir have been performed *in vitro*, but there are several strategies currently being studied that could be used to deliver engineered endonucleases to the HIV reservoir cells that sustain HIV infections *in vivo*. Both viral and non-viral methods of gene delivery have been used for targeted endonucleases. The non-viral methods include polycationic polymer or liposome delivery of DNA, and direct protein delivery. The non-viral delivery methods face significant challenges in delivering DNA to the nucleus. If a DNA payload is delivered to the cytoplasm it can be degraded before it can reach the nucleus and express its transgene. These methods have been successfully used *ex vivo* and in some cases *in vivo*, as liposomal delivery was used in clinical trials for cystic fibrosis with transgene expression being transient(253,254). Nevertheless, non-viral gene delivery vectors will likely be important for transient expression of endonucleases, which might be important for HIV cure.

Viral vectors are an attractive option for delivery of engineered endonucleases. They take advantage of the natural pathogenesis of the virus, and have their viral genes replaced with the gene-editing platform of interest that they deliver instead. Three viral gene delivery vectors are commonly used for gene delivery; adeno-associated virus (AAV), adenovirus and lentiviral vectors, although numerous other viral vectors such as vaccinia, HSV and lentivirus have also

been used in clinical trials (255). All three viral vectors are currently used broadly for gene delivery however extensive application to HIV cure will require overcoming immunogenicity associated with the wild type viruses. Additionally, some viral gene delivery vectors have been shown to integrate in the genome which can be deleterious(256,257), although recent advances in design of the viral gene vectors has made them safer for *in vivo* use. Our lab (Jerome lab) is currently working with AAV as a potential delivery vehicle for HIV-specific engineered meganucleases, although there is still need to determine whether these delivery vehicles can deliver endonucleases to a significant proportion of reservoir cells.

5.2 SAFETY OF ENGINEERED ENDONUCLEASES

In my thesis, I have demonstrated that HIV-specific engineered meganucleases cleave and introduce mutations in off-target genomic sequences. The potential for engineered endonucleases to cleave off-target sites has been characterized before with other engineered endonucleases(242,258,259) and this is likely to be a major limitation to the therapeutic application of engineered endonucleases to HIV cure. Potential side effects from use of gene therapy can arise from a number of different points; immunogenicity to the gene delivery vector, insertional mutagenesis usually associated with viral vectors and oncogenic mutagenesis from cleavage of genomic off-target sites by an endonuclease. Several reports are documented in the literature of such toxicity. A group in France that successfully treated 11 children for X-linked SCID later identified leukemia in two of the children associated with insertional mutagenesis of the retroviral vectors used in this therapy(260,261). An 18-year-old patient with ornithine transcarbomylase (OTC) deficiency also died after gene therapy, which was likely related to immunogenicity of the adenoviral vector that was used to deliver the OTC (262). Gene therapy

based cures for HIV, a disease for which effective, albeit lifelong, treatment exists, will need to overcome these legacies and safety concerns. Already several groups are addressing these challenges through design of better more specific endonucleases, and safer viral and non-viral gene delivery vectors.

5.3 THE FUTURE: WHAT WILL HIV CURE LOOK LIKE?

The only patient cured of HIV to date is the "Berlin patient" who had serial allogeneic stem cell transplants from a CCR5 delta 32 homozygous donor. Subsequent patients who received transplants had viral rebound after variable durations of undetectable virus. What was unique about the transplant in the "Berlin patient"? The key unique characteristic was that he received CCR5 delta 32 deleted cells, which could have played a significant role in his recovery from HIV. It is likely in all these subsequent patients that the transplant conditioning regimens were insufficient to clear the entire reservoir, and that even after transplant there remained a small number of cells capable of producing viable virus. It seems that the key difference then is that the "Berlin patient" was transplanted with the CCR5 delta 32 cells that could resist HIV infection and prevent re-establishment of infection. This of course is also the principle behind transplant with *ex vivo* engineered CCR5 delta 32 modified cells; a population of HIV resistant cells could prevent HIV re-establishment of infection. This is one image of what HIV therapy could look like in the future. Patients could have detectable virus but would also have an intact immune system based on HIV resistant CCR5 delta 32 modified cells.

Additionally, recent efforts have also focused on the use of broadly neutralizing antibodies to control HIV infection. Patients who received infusions of bNAbs had durable virologic remission off cART although they did eventually rebound. Application of broadly

neutralizing antibodies for HIV control will probably in the future require multiple passive immunizations with these monoclonal antibodies.

In the face of all these efforts targeting the HIV reservoir, it is important to develop strategies to measure the residual HIV reservoir and in essence measure HIV cure. Measurement of the reservoir is critical because it is important to determine at what point it is safe to stop cART and declare a patient cured. These levels will probably be established through clinical trials involving structured and analytical treatment interruptions. This, however, requires an assay that can accurately quantify the replication-competent reservoir. The multiplex ddPCR assay we developed can quickly give an estimate of the intact reservoir through simultaneous detection of up to six gene segments. The challenge, however, is that if this assay is used to measure the reservoir in patients treated with engineered endonucleases, small mutations are almost indistinguishable from an intact viral genome that has naturally occurring mutations. We have previously described a droplet digital PCR mutation detection assay that can accurately detect as little as a single nucleotide deletion/substitution in meganuclease/megaTAL mediated HIV-sequence disruption and this could be applied to all quantify disruption with all the endonuclease platforms used in HIV-sequence disruption studies(220). However, there is need to develop assays that can accurately quantify the reservoir for all the currently studied therapeutic approaches.

5.4 ECONOMICS OF HIV CURE: TRANSLATION TO RESOURCE LIMITED SETTINGS

An estimated 70% of all patients living with HIV/AIDS live in resource limited settings, particularly in Sub-Saharan Africa(263), and any HIV cure therapeutics need to be accessible to the populations most afflicted by the disease. My thesis project looked at gene therapy as a

potential therapeutic approach for HIV cure, and although we have demonstrated that engineered endonucleases can cleave target HIV sequences, gene therapy has to be made affordable before we can realistically talk of HIV eradication. One of the only commercially available gene therapies glybera, a treatment for lipoprotein lipase deficiency, has so far been used to treat only one patient. Part of the reason for this is the prohibitive cost of this therapy. A course (up to 23 doses) of glybera is priced at US $ 1 million dollars(264) and is therefore unaffordable to almost the entire population. There is need to make gene therapies that are broadly affordable especially considering that we already have cART that has become widely available and enables most patients to live productively. Recently licensed gene therapies such as Imlygic from Amgen that is used to treat melanoma have significantly reduced costs(265), but at US $65,000 dollars it is still not easily affordable. Moreover, patients with HIV will likely require multiple doses to eradicate the viral reservoir(266), which reinforces the need for affordable therapies.

Other possible HIV cure therapies such as transplant are likely to be even more expensive that gene therapy. Coupled with possible side effects due to immune-depletion, and graft versus host disease (GVHD), which can sometimes be fatal, allogeneic stem cell transplant is not an attractive therapy for HIV cure except in those patients with underlying malignancies.

However, monoclonal or polyclonal therapy based on HIV bNAbs, and the use of latency reversing agents in combination with cART could be made available in many resource-limited settings. Many monoclonal antibodies are already used for cancer management in developing countries and strategies can be designed to make these therapies, if they are successful in eradicating the HIV reservoir, affordable in the regions that are most affected by HIV.

On a final note, any HIV curative therapy, when it is eventually licensed, can and should be made available to patients anywhere in the world. An example is taken from the early days of

cART when it was thought impractical to avail life-saving drugs in resource-limited settings partly because of cost limitations but also because of safety concerns. These drugs were eventually made available and now an estimated 40% of HIV patients in resource-limited settings are on cART(263). Special mechanisms can be set up to make future lifesaving HIV cure therapies available to HIV positive patients in resource-limited settings.

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VITA

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EDUCATION:

2004-2009	Makerere University, School of Medicine, Bachelor of Medicine and Bachelor of Surgery
2008	International Exchange Student, Karolinska Institute, Stockholm, Sweden
2010-2011	MSc, Global Health, Duke University, Durham, NC
2012-2016	Doctoral Program in Molecular and Cellular Biology, University of Washington, Seattle, WA

POSTGRADUATE TRAINING:

2009-2010	Intern, Mulago National Referral Hospital, Kampala, Uganda.
2010	Advanced HIV Treatment and Management, Makerere University, Kampala, Uganda
2010	Advanced Pediatric HIV/AIDS Care and Management, Mildmay, Kampala, Uganda

HOSPITAL APPOINTMENTS:

2011-	Medical Officer, Uganda Cancer Institute, Kampala, Uganda.
2012	Visiting Clinical Scientist, Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA

HONORS AND AWARDS:

2001	Award of Excellence, National Mathematics Contest, Ordinary Level, Kampala, Uganda
2003	Award of Excellence, National Mathematics Contest, Advanced Level, Kampala, Uganda
2006	International Travel Award, Physicians for Human Rights, International AIDS Conference, Toronto, Canada
2008	International Travel Award to Abuja, Nigeria (Africa Health Financing Summit)
2011	Best Doctor Award, Uganda Cancer Institute, Kampala, Uganda.
2012	International travel grant, International CML Foundation
2015	Merck Serono Innovation cup, 1st runner up (Innovations for emerging markets), Darmstadt, Germany

LICENSURE:

2010- Uganda Medical Practicing License (No. 13680)

PROFESSIONAL ORGANIZATIONS:

2006-2010 International AIDS Society
2011- Uganda Society for Health Scientists
2015 - American Association for the Advancement of Science (AAAS)

PROFESSIONAL SERVICE: NATIONAL/INTERNATIONAL:

2011-2012 Study Physician, ACTG 5263, NIH Funded study comparing two drug regimens for Epidemic Kaposi Sarcoma
2012 Mortality and Morbidity Audits, Uganda Cancer Institute

TEACHING ASSIGNMENTS

2010 Graduate Research Assistant, Duke University, Durham, NC
2011 Graduate Teaching Assistant in Global Health Ethics, Duke University, Durham, NC
2011-2012 Introduction to Clinical Oncology (5th Year Medical Students, Makerere University)
2013 Biology of Cancer (Summer 2013, Uganda Cancer Institute)
2013 Viruses and Cancer (Summer 2013, Uganda Cancer Institute/Makerere University)
2015 AIDS-associated Malignancies (University of Washington, Department of Global Health, Epi 530)
2015 Graduate Teaching Assistant, University of Washington (Dr Michael Emerman)

SPECIAL RESPONSIBILITIES: LOCAL:

2006 Editor, Students for Equity in Health Care Handbook on HIV Stigma and Discrimination
2008 Contributor, Physicians for Human Rights White Paper on Human Resources for Health
2008-2009 Co-author, National Library of Medicine community health education handbooks (malaria, diarrhea, Malnutrition) in Uganda

VOLUNTARY AND COMMUNITY SERVICE:

2006-2011 Member, Action Group for Health, Human Rights and HIV/AIDS (AGHA-Uganda)
2006-2011 Member, Physicians for Human Rights (PHR) – USA
2006-2011 Founder and member, Students for Equity in Health Care (SEHC-Uganda)
2008-2009 Volunteer student and clinician, Mifumi Trust, Uganda

MANUSCRIPTS

1. Erin M. Scherer, Robin A. Smith, Cassandra A. Simonich, **Nixon Niyonzima,** Joseph J. Carter, Denise A. Galloway, Characteristics of Memory B Cells Elicited by a Highly Efficacious HPV Vaccine in Subjects with No Pre-existing Immunity, **PLoS Pathogens, 10(10) e100446 (16 October 2014)**

2. Christopher De Boer, **Nixon Niyonzima**, Jackson Orem, John Bartlett, S. Yousuf Zafar, Prognosis and delay of diagnosis among Kaposi's sarcoma patients in Uganda: a cross-sectional study, **Infectious Agents and Cancer, 9:17 (20 May 2014).**

3. Simon Ndira, Daniel Ssebadduka, **Nixon Niyonzima**, Nelson Sewankambo, Julia Royall, Tackling malaria, village by village: A report on a concerted information intervention by medical students and the community in Mifumi, Eastern Uganda, **African Health Sciences (November, 2014)**

4. Ruth Hall Sedlak, Shu Liang, **Nixon Niyonzima**, Harshana de silva Feelixge, Daniel Stone, Pavitra Roychoudhury, Nick Weber, Anqi Cheng, Amalia Margaret, Roger Bumgarner, Keith R. Jerome, Digital detection of endonuclease mediated mutations in HIV, **Scientific Reports,** January, 2016

5. Mahreen Ameen, **Nixon Niyonzima,** Anisa Mosam, Management of Kaposi Sarcoma, (invited review), **Community Dermatology, April, 2016**

6. Daniel Stone, **Nixon Niyonzima**, Keith R Jerome**,** Genome editing and the next generation of antiviral therapy, **Human Genetics, June, 2016**

7. Fred Okuku, Manoj P Menon, Rachel Kansiime, Jason Barrett, Warren Phipps, **Nixon Niyonzima,** Matthew Ulrickson, Jackson Orem, and Corey Casper, Tele-Oncology: A joint, Web-Based, Clinical Conference between the Uganda Cancer Institute and the Fred Hutchinson Cancer Research Center, ASCO, Journal of Global Oncology **(invited article, under review)**

ABSTRACTS:

1. Christopher De Boer, **Nixon Niyonzima**, John Bartlett, S. Yousuf Zafar, Prognosis, diagnostic delay, and patient characteristics associated with diagnostic delay among Kaposi Sarcoma (KS) patients in Uganda; presented at the ASCO 2013 meeting

2. HS De Silva Feelixge, HL Pietz, **N Niyonzima,** P Roychoudhury, JT Schiffer, D Stone, KR Jerome, Targeted Disruption of Essential HIV Proviral Genes using Zinc Finger Nucelases, **Cell and Gene Therapy for HIV Cure Conference, Seattle, USA, 2014**

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4. Harshana De Silva Feelixge, **Nixon Niyonzima**, Harlan Pietz, Martine Aubert, Dan Stone, Keith Jerome, Targeted Disruption of Essential HIV-1 Proviral Genes by Rare-Cutting Endonucleases, Conference on Reroviral and Opportunistic Infections (CROI**), Seattle, USA 2015**

5. **N Niyonzima**, AR Lambert, D Stone, BL Stoddard, KR Jerome, Off-target cleavage activity of an HIV-specific engineered meganuclease, **Cell and Gene Therapy for HIV Cure Conference, Seattle, USA, 2015**

6. Fred Okuku, Manoj P Menon, Jason Barrett, Warren Phipps, **Nixon Niyonzima,** Matthew Ulrickson, Jackson Orem, and Corey Casper, Tele-Oncology: A joint, Web-Based, Clinical Conference between the Uganda Cancer Institute and the Fred Hutchinson Cancer Research Center (AORTIC, 2015)

INVITED PRESENTATIONS:

2006	Bold Solutions to the Health Workforce Crisis in Africa, International AIDS Conference, Toronto, 2006
2008	Public Health Development & Financing Strategy Development Conference, Abuja, Nigeria
2012	Management of Kaposi Sarcoma in resource-limited settings, Kilimanjaro Christian Medical Center (KCMC), Moshi, Tanzania
2012	Inaugural Colloquium on CML in Africa, Ocean Road Cancer Institute, Dar es Salaam, Tanzania

APPENDIX A

Table 1: HIV target site amplification primers

Target site	Forward primer	Reverse primer
AATGGCAGTATTCATCCACAAT	TAGCAGGAAGATGGCCAGTA	TCCTGTATGCAGACCCCAAT

Table 2: Predicted off-target cleavage site amplification primers

OT number	Off-target site sequence	Forward primer	Reverse primer
1	AAaGGCAGTATTCcTCCACAAT	CCTGTGTTGGCTTTTCTCCACC	CTTCCTGAGGGTCTCTTGAATGTC
2	ATTGTGGATGAATAtTGagATT	GTGCCTTCTCTTCAAATGTGGCC	CCCTCATAAGCATAAAGCAGCCC
3	AATGGCAGTATattTCCACAAT	GCCCCCAAGCTAGAAAGTGC	GCCAAAAGTATCCTGAAAAGGTCAGTACT
4	ATTGTGGATGAAaACTGgaATT	GTTCATCATGGCTGTAAGCAGAATTCTGCA	CCTCCACTCTACTGACAAATTCCC
5	tTTGTGGATGAATAaTtCCATT	CCCAGCCTAGGCAAACACGA	CTGGTGGGAGTGTAAAATGGTGC
6	AATGGaAGTATTtgTCCACAAT	CACCGAACAATGAAGGGACAGAG	TCCGTGAGGGTCTCTGTCTTG
7	ATTGTGaATGAATAtTGCCATc	GAAAGATGAAGGCTGAGAAGGGC	GGGTATAGGCACAACGTGAACAAC
8	ATTGTtGATGAATACTtgCATT	CAAAGACAGGGAAAACTGATCTATAGTTTCAGAG	CTAACCAAAAGAACACCAGCATTACTGATA
9	ATTGTGGATtAATAaTtCCATT	GGTCCTGACCATTGGAATTTTGCC	CGGTGAACCATTCAGCTTTCCC
10	ATgcTGGATGAAaACTGCCATT	CATCACTGTCCCCAGGGAAC	AGTTTGCTTGCTGGCTTCTGTCC
11	ATTGTGGAaGAATtCTGCCcTT	GGCTTTGAGTCACAGAAAGTCCC	CCAGCCTGAGCAACATAGGAAAAC
12	ATTGTGGATGtATtCTGCaATT	GAATAGCCATTGCACTCCAGCTTG	CATCTGAGGTAGCCCTCGTG
13	cTgGTGGATGAATACTGCaATT	GTGACAAAGCAAGACTCTGCCTC	GCAATGTTCTCTCACCCTCTACTG
14	AATGGCAcTATTCATtCACcAT	CCCTGGCTTCACAACCACCAA	ATACTTGCTGACTGGGCTGCCAC
15	ATTtTGGATGAAaAgTGCCATT	AGTTCAGGCTGACGAGTGGC	GCTCCTTCTATGTGTCAAGGTCTG
16	AATGGCtGTAcTCATCtACAAT	CTGCGTGGCTTGTTTCACTTGAC	GCACTAGATCTCTTGGTCTTCCTG
17	ATTtTtGATGAATACTGaCATT	CCCCTAAGTGAAGACAACTGCC	GGTTGGCAAGGAGAGAAAGAAGAG
18	cATGGCAGTATTttTCCACAAT	CATTGCCATGTACCACGGCAG	TGGGATTACAGGCGTGAGCCACT
19	AATGGgAGTtTTCcTCCACAAT	CCTTGAAGAGATCCTTCACATCCC	CCCAAGACTGGGCAATTCCAAAAG
20	ATTGaGGATGgATtCTGCCATT	GTCTGTTCCATGGAACCCACTC	CCCAAAGTAAGGGATTTTGGACGG
21	AAaGGCAGTATTCATtCACAtT	GCCGTCTGTACCAACTGATACC	GGGAGTGGAAGTGCTGATTTTTGC
22	ATTGTGGATGtATttTGCCATT	GTCTGGGAAGGGCTTTATTTCACC	GCAACCTCAATTCCATCTTCCCAC
23	AgaGTGGATGAATACTtCCATT	CCTAGTCCAGCATGAGCTCATC	CAGGGCTCATGGACTTAAGGTAG
24	AATtGCAGTATcCATtCACAAT	GGCTTTCGACTATGGATCTGACC	CGTGGTACCTTAGCTAACTGGTG
25	AATGGCAGTgTTCATCCAttAT	CCTTGACCTCACAAAGTGCTAGGA	GCCTTCACTACTGTCAAGGCAATC
26	AgTaGCAGTATTCATtCACAAT	CCCAAACCTCAGCATCATGCAG	CTTCCTTCAGAGTAGAGGAGGG
27	AAaGGCAGTATTCATCCACttT	CCTTACCTTTACCTTCCTACTGTATTCTCC	GCAGTCTGTGGTGTTTGACATCC
28	gATGGCAGaATTCATCtACAAT	CTGCTCACAATGACTATGGACTCG	GGGATACTCCAGGCTGGATG
29	AATGGCAGTgTTaATCCAtAAg	GGTAGTGGAATAGGGAATGAAGAGTGAG	GTGGTTTGACAGATCCATCCTGC
30	ATTGTtGATGAATAaTttCATT	GCGTAGGCTGGGCATGGATT	ATCCAGGGTTTCAGGTCAGTGTC

Table 3: Cleavage activity of engineered endonucleases at on and predicted off-target sites

	HIV target	OT1	OT2	OT3	OT4	OT5	OT6	OT7	OT8	OT9	OT10	OT30
eOnu_v1_7.5 MT	8.65	0.03	0.03	**0.09∗**	0.04	**0.12∗**	0.06	0.02	**0.03∗**	0.04	0.02	0.02
eOnu_v2_7.5 MT	2.16	0.03	0.01	0.03	0.02	0.00	0.03	0.02	0.01	0.05	0.04	0.02

∗ statistically significant from untreated cells