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Zinc Finger Nucleases for Site-Specific Correction of Adenosine Deaminase Deficiency

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Zinc Finger Nucleases for Site-Specific Correction of Adenosine Deaminase Deficiency

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Microbiology, Immunology and Molecular Genetics

by

Alok Vishnu Joglekar

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ABSTRACT OF THE DISSERTATION

Zinc Finger Nucleases for Site-Specific Correction of Adenosine Deaminase Deficiency

by

Alok Vishnu Joglekar

Doctor of Philosophy in Microbiology, Immunology and Molecular Genetics

University of California, Los Angeles, 2013

Professor Donald Barry Kohn, Chair

Adenosine Deaminase (ADA) Deficiency is an inherited disorder resulting in immunodeficiency. Currently, gene therapy for ADA Deficiency is performed using retroviral or lentiviral vectors that pose a risk of insertional oncogenesis. In this project, an alternative, potentially safer approach for correcting ADA deficiency was investigated. This approach uses site-specific zinc finger nucleases (ZFNs) to achieve genome editing. In this study, various aspects of using ZFNs at the ADA locus in human hematopoietic cells were investigated. To ensure efficient delivery of ZFNs and donor templates, Integrase-Defective Lentiviral Vectors (IDLVs) were used. The vector design of IDLVs was optimized. The use of Histone Deacetylase Inhibitors was tested in conjunction with IDLVs to enhance their efficiency further. In addition, small molecule inhibitors of DNA-dependent protein kinase (DNA-PK) were tested for their ability to increase the efficiency of gene modification. These studies provide novel findings that are potentially applicable to the entire genome modification field and will benefit current and future work on similar approaches.
The dissertation of Alok Vishnu Joglekar is approved.

Gay M. Crooks

Arnold J. Berk

Benhur Lee

Donald Barry Kohn, Chair

University of California, Los Angeles, 2013
This thesis is dedicated to my family:

Ragini, Aai, Baba and Swarada
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I sincerely apologize if I have missed someone’s name in this rather lengthy acknowledgement. I thank everyone involved for making these 4 years memorable.

-Alok Joglekar
VITA

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C. SELECTED PUBLICATIONS


Alok V. Joglekar, Roger P. Hollis, Gabriela Kuftinec, Shantha Senadheera, Rebecca Chan, Donald B. Kohn (2013) “Integrase-Defective Lentiviral Vectors as a Delivery Platform for Targeted Modification of Adenosine Deaminase Locus” Manuscript under revision at Molecular Therapy.
CHAPTER 1: INTRODUCTION

1.1 Gene Therapy for inherited disorders

Several genetic disorders are caused by one of more mutations in the human genome, leading to abnormal function. This can be due to either loss of an essential function or undesirable gain of function. Genetic defects such as inherited immunodeficiencies (Uribe and Weinberg, 1998), urea cycle disorders (Burton, 2000) can be caused by loss of function of essential enzymes or signaling molecules. Whereas disorders such as sickle cell disease (Stamatoyannopoulos, 1972) are caused by abnormal function of a protein. Gain-of-function mutations such as those in the tumor suppressor protein p53 can cause inherited cancers (Freed-Pastor and Prives, 2012). While many of these defects are inherited and well-studied in the context of pathology, they lack a curative therapeutic approach. In case of inherited immunodeficiencies, the only viable approach is bone marrow transplant (BMT) from an allogeneic donor or a matched unrelated donor. Survival of the patient even after BMT is not guaranteed (Szabolcs, 2010). In case of urea cycle disorders, the only cure is a liver transplant from a matched donor (Moini et al, 2010). Similar to these examples, many other genetic disorders have no other cure than organ transplants. While many of these are caused by missing just one essential component, an entire organ needs to be transplanted to compensate for the loss of function. While organ transplants are successful, lack of donor availability is a critical limiting factor. There are significant risks associated with transplants such as graft rejection and graft-versus-host disease (Sung and Chao, 2013). Due to these reasons, there is a grave need for alternate therapeutic approaches that will not be dependent on availability of a matched donor. The most promising of the therapies that have been studies is ‘gene therapy’.
Gene therapy (GT) is a therapeutic approach to treat genetic disorders by altering a patient’s genomic DNA in a therapeutically beneficial way (Kohn and Kantoff, 1989). GT aims to correct, replace, or introduce gene in order to establish or augment proper genome function. If a disease is caused by loss-of-function mutations, GT aims to introduce a functional copy of the gene, thereby restoring the lost function. If a disease is caused by abnormal function due to a mutation, GT aims to introduce a genetic function to compensate for the abnormal function or to revert the cells to a normal state. For most of the research done on GT, it aims to introduce genetic elements that will restore a lost function. The earliest reports on GT were focused on primary immunodeficiencies. The first reported clinical trial of gene therapy was in 1990, when a child with Adenosine deaminase deficient severe combined immunodeficiency (ADA-SCID) was treated. The patient was immunodeficient due to the lack of ADA enzyme activity. Lymphocytes from this patient were isolated and treated with a retrovirus to introduce a functional copy of ADA and then infused back into the bloodstream. The therapeutic effect of this treatment was short-lived, but without adverse effects (Kohn et al., 1987; Blaese et al., 1995). This paved the way for further gene therapy clinical trials and much advancement is reported since then. To date, clinical GT approaches have focused on the use of retroviral or lentiviral vectors for permanent therapy and Adenoviruses and Adeno-associated viruses for transient therapy. While the transient approaches have shown promise, they are limited due to their short duration of effect. Hence, much of GT research has focused on the use of integrating viruses (reviewed in Young et al., 2006).

1.2 Gene therapy using retro- and lentiviruses

Retroviruses (RVs) and Lentiviruses (LVs) are two types of viral vectors that have been used extensively for GT (Kohn et al., 1987; Kohn, 2007). The RNA genomes of these viruses are
reverse transcribed into double stranded DNA that is imported to the nucleus of the host cell. Upon import to the nucleus, the double stranded DNA integrates into the host cell genome. The integrated provirus can be transcribed to genomic RNAs that are packaged into new virions. The integrated provirus can also be transcribed to mRNAs that are translated to produce viral proteins (Nisole and Saib, 2004). In order to use these viruses for GT, much of the viral genomic components are removed and are replaced by desired genetic elements (Logan et al., 2002). The genes encoding viral proteins are removed from the viral genome and are replaced by transgene expression cassettes. These viral genomes can be packaged into virions by providing the viral proteins *in trans* in the packaging cell lines (Delenda, 2004). The virions containing modified viral genomes, also known as viral vectors, are used to transduce target cells where they integrate as proviruses and act as the source of desired gene function.

Currently, RVs to correct X-SCID, ADA-SCID and X-linked Chronic Granulomatous Disease (X-CGD) are in clinical trials (reviewed in Kohn, 2009). GT using retroviruses has been the most successful in the case of X-linked SCID. The X-SCID patients lack a functional Interleukin-2 receptor common gamma chain (IL2Rg) and hence are immunodeficient (Noguchi et al., 1993). In GT clinical trials, HSCs from X-SCID patients were transduced with retroviral vectors carrying IL2Rg and infused back. In 10/11 cases, the patients successfully recovered their immune function (Gaspar *et al.* 2004; Hacein-Bey-Abina *et al.*, 2002). However, 5/10 patients developed leukemias due to complications arising from the retroviral vectors (Hacein-Bey-Abina *et al.*, 2003; Kohn *et al.*, 2003). The retroviral vectors, despite removal of viral genes, still have their long-terminal repeats (LTRs) intact. Retroviral LTRs have strong enhancer elements that can transactivate cellular genes (reviewed by Nienhuis *et al.*, 2006). In case of X-SCID patients, the retroviral vectors integrated near proto-oncogenes such as LMO2 (Hacein-Bey-Abina *et al.*, 2003).
2002) and activated their oncogenic potential, causing transduced cells to expand clonally and give rise to leukemia. This phenomenon, now known as insertional oncogenesis, has proven to be the most significant drawback of using retroviral vectors for gene therapy. Insertional oncogenesis is observed in retroviral vectors due to their integration profiles (reviewed in Gabriel et al., 2012). Retroviruses, especially gamma-retroviruses such as MoMLV (Moloney Murine Leukemia Virus) have a tendency to integrate into the 5’ upstream regions of actively transcribed genes. Because of this preference for integration, RVs exhibit a strong risk of insertional activation of proto-oncogenes by integrating near their regulatory elements. Due to these drawbacks, RVs are being ‘phased-out’ from GT clinical trials and are being replaced by lentiviruses (Kohn, 2007).

Lentiviruses such as HIV-1 are a class of retroviruses that are distinguished by their ability to infect non-dividing cells (Naldini, 1998). Most of the lentiviral vectors have been based on HIV-1 genome. Lentiviral vectors also lack the viral genes from their genomes, but retain some of the enhancer elements in the LTRs. The transgenes in LVs are usually transcribed by incorporating an internal promoter. However, the genomic integration profile of lentiviruses is potentially safer than retroviruses. LVs tend to integrate into the actively transcribed genes, preferentially downstream of the transcription start site. This reduces the potential for transactivating the genes they integrate into; however, this increases the risk of disrupting the gene. In the studies comparing RVs to LVs, LVs show reduced insertional oncogenesis potential. Therefore, LVs are preferred over RVs for gene therapy (Baum et al., 2004). Currently LVs are in clinical trials for the treatment of beta-thalassemia (Nienhuis and Persons, 2012), X-linked Adrenoleukodystrophy (X-ALD) (Cartier et al., 2009) and X-CGD (Kang and Malech, 2012). The clinical trials for beta-thalassemia and X-ALD have reported successful curative benefits. However, there are reports of
clonal expansion in the beta-thalassemia trial (Cavazzana-Calvo et al, 2010). This emphasizes that point that LVs, although safer than RVs, are not ideal due to the inherent risk of insertional oncogenesis. Hence, there is a need for better, safer methods for GT. One of the most significant advances has been the development of site-specific genome modification technologies. Rather than integrate exogenous viral DNA, these methods aim to site-specifically modify the target gene and achieve therapeutic benefit.

1.3 Site-specific gene modification technology

An alternate approach to insertion of exogenous DNA elements would be to ‘fix’ the endogenous genes. If a disorder is caused by a point mutation, then rather than inserting a viral vector into the genome, it would be beneficial if the point mutation is ‘fixed’ to the normal base-pair. This approach will potentially result in permanent therapeutic gene modification with minimal undesirable changes. To that end, multiple techniques for achieving site-specific genome modification have been developed.

The oldest technology for such modification is that of homologous recombination (HR). This technique utilizes long stretches of DNA homologous to the target gene, but carrying desired changes (Morrow and Kucherlapati, 1993). The DNA fragments are introduced into cells, where they can be used as templates for HR to modify the genome. This technique has been used to create transgenic mice by microinjecting embryos with linear DNA. However, the inherent frequency of HR in cells is remarkably low, reducing the overall efficiency of this process to 1 in $10^6$ events (Cappecchi, 1989). The low efficiency of HR has prompted the use of selection methods, which is undesirable in a GT scenario.
Reports from early 1990s demonstrated that the endogenous process of HR can be stimulated by introduction of double strand breaks (DSBs) in DNA (reviewed in Osman et al, 1998). These studies used rare-cutting endonucleases to introduce DSBs in a targeted manner. Targeted DSBs were able to stimulate HR by 100-1000 fold in cells, greatly improving upon the efficiency (Johnson et al, 2001). The most widely studied rare cutting endonuclease is I-SceI, belonging to a class of endonucleases known as homing endonucleases (reviewed in Hafez et al, 2012). These studies prompted further research on homing endonucleases (HEs). To date, many different types of HEs such as LAGLIDADG HEs, GIY-YIG HEs have been discovered. Homing endonucleases, also known as meganucleases are large DNA-binding proteins that can introduce a staggered DSB into their binding site. The binding specificity of HEs is dictated by the amino acid residues in their DNA binding domains and can be engineered to recognize a given target. However, the DNA binding domains and the endonuclease domains of HEs are not functionally modular. Therefore, engineering HE specificity is very difficult and not easily predictable in-silico (Li et al, 2012). Thus, HEs have seen limited usage in relevant applications, despite a large number of proof-of-concept studies.

A recent advance in the field of site-specific gene modification is the emergence of Zinc Finger Nucleases (ZFNs) (Bibikova et al, 2001). ZFNs are chimeric nucleases that can be engineered to recognize a specific sequence and introduce a DSB. ZFNs consist of dimers of sequence specific zinc finger motifs coupled to FokI endonuclease domains. ZF motifs can recognize specific DNA sequences, usually 3-4 base pairs per motif. Each ZFN monomer is composed of 3-6 ZF motifs that are ‘stitched’ together, which recognize 9-18 base pairs of the half-binding site. If two half-binding sites are present in tandem with a 4-8 base pair spacer between them, two ZFN monomers can recognize them and dimerize. The dimer is able to introduce a staggered DSB at
the spacer between the two half binding sites. Using this mechanism, ZFNs can introduce site-specific double strand breaks (Urnov et al, 2005). The sequence specificity of ZFs is dictated by the amino acid residues at the DNA binding surface. Because individual ZF motifs are not constrained by the neighboring motifs, ZFs can be engineered and linked together in a modular way. The modular nature of ZFNs makes them amenable to directed engineering (Porteus and Carroll, 2005). Majority of the ZFN engineering has been performed at Sangamo BioSciences, Inc. and Sigma-Aldrich, and remains proprietary (Scott, 2005). Open-source methods for ZFN engineering have been developed without great success (Maeder et al 2008). This is largely due to context-dependence of ZF motifs. Although individual ZF motifs can be engineered independently, certain combinations of ZFs are unable to function together. This is a relatively poorly understood phenomenon, especially in open-source methods (Sander et al, 2011). Difficulty to engineer and cost of procuring proprietary ZFNs have been some of the limiting factors for this technology.

A new avenue for site-specific endonucleases is the advent of Transcriptional Activator Like Effector Nucleases (TALENs) (Li et al, 2011; Cermak et al, 2011). TALENs consist of 33-35 amino acid long repeats known as repeat variable diresidue (RVDs) linked to FokI endonuclease. Each RVD can recognize a single base pair specifically, depending on the two central amino acid residues. Different combinations of central residues recognize different base pairs. By linking appropriate RVDs together, and linking them to a FokI endonuclease domain, sequence specific TALENs can be engineered. Unlike ZFNs, there is very little context dependence in TALENs, making them easy to engineer. Due to the simplicity of TALEN engineering, this technology is rapidly becoming popular (Clark et al, 2011). Although ZFNs and TALENs are comparable technologies, ZFNs have been characterized more thoroughly in terms of activity and specificity.
There is a growing dispute in the field with regard to which is better of the two; however, the evidence shown by either sides is ambiguous and often biased (Perez-Pinera, 2012). In this study, we are investigating the use of ZFN technology whereas the use of TALEN technology is a future goal.

1.4 Site-specific modifications using nucleases

As described in the previous section, introduction of double strand breaks at a target site can stimulate DNA repair pathways that can be manipulated with purpose. When a DSB is formed at a site, it rapidly invokes signaling pathways leading to its repair. DSBs can be repaired by two major pathways in cells, ca. Non-homologous end joining (NHEJ) and Homologous recombination (HR) (reviewed in Chapman et al, 2012). A more detailed description of the two pathways can be found in chapter 5. The choice of pathway is dependent on multiple factors, including, but not limited to the phase of cell cycle, cell type and presence of a donor template. NHEJ is usually the dominant pathway mainly due to more rapid activation than HR. However, the pathway choice can be influenced by multiple mechanisms. A schematic of how HR and NHEJ can be used for gene modification is shown in Figure 1.1.
NHEJ involves rapid processing of the two DNA strands at the DSB followed by end filling and ligation. Due to the mechanism of NHEJ, it can often be mutagenic. The nucleolytic processing and end filling can lead to introduction of insertions or deletions at the DSB. If the DSB is formed in the coding region of a gene, repair by NHEJ can lead to disruption of the open-reading frame. By exploiting the error-prone NHEJ pathway, DSB production can be used to disrupt genes. If a site-specific DSB is introduced in the coding region of a gene, that gene can be disrupted, leading to loss-of-function. This aspect of the site-specific modification technology is widely used to create knock-out cell lines, embryos or plants (Liu et al., 2010; Zhang et al., 2011; Cui et al., 2011). The gene disruption strategy has also seen application in gene therapy approaches. The most significant of these is the disruption of the co-receptor CCR5 in order to acquire resistance to HIV infection (Cannon and June, 2011). In these studies, ZFNs were used to target CCR5 and disrupt it in T-cells or HSCs (Holt et al., 2010; Perez et al., 2008). The resulting ΔCCR5 cells are resistant to HIV infection. This approach is currently in clinical trials for HIV-AIDS (Maier et al., 2013). Similarly, many other genes have been targeted for disruption.

HR, on the other hand is an accurate repair mechanism. HR relies on presence of a homologous donor template that is copied to repair a DSB. Because of its dependence on homology, HR is less prone to disruption of a gene. Normally, cells use the sister chromatids as donor templates, whereas if an exogenous DNA fragment is provided, it can be used as well. If a DSB is introduced at a target site in proximity of a mutation, and a donor template identical to the region, but containing the ‘correct’ basepair is provided, the mutation can be corrected. This
approach has been used in proof-of-concept studies to correct the IL2Rg mutations in X-SCID among many others (Lombardo et al, 2007; reviewed in Carroll, 2011).

Along with a DSB, if a donor template containing homology arms flanking a desired cassette is provided, the cassette can be inserted at the DSB. The HR machinery can use the homology arms a guide and copy the region between them as a part of end repair. Thus, this strategy can be used to insert desired changes in a site-specific manner. The efficiency of site-specific insertion is lower compared to correcting a single base pair and decreases with increasing size of the insert (Chen et al, 2011). One of the well-known applications of this strategy is the use of AAVS1 as a safe-harbor (DeKelver et al, 2010).

In this study, we investigated the use of ZFNs to correct ADA deficiency. We explored ZFN mediated correction of a known disease-causing mutation as a proof-of-principle approach for gene therapy for ADA-SCID. We also studied various methods to deliver the ZFNs and donor templates to cells. Additionally, we investigated ways to increase the efficiency of this approach. These studies, taken together, report advances and potential improvements to the field of genome editing, specifically in the context of proof-of-concept approaches.
CHAPTER 2: Zinc Finger Nuclease Mediated Gene Modification of ADA

2.1 Introduction

2.1.1 Adenosine Deaminase deficiency

Adenosine deaminase (ADA) deficiency is an autosomal recessive disorder resulting from loss-of-function mutations of the human ADA gene. ADA is a crucial component of the purine metabolism pathway. ADA catalyzes the conversion of Adenosine (in ATP or dATP) to Inosine. In absence of ADA enzyme activity, intracellular dATP levels rise, leading to pro-apoptotic events. This is particularly detrimental to lymphocytes due to their high rate of proliferation. Hence, patients suffering from ADA deficiency develop severe lymphopenia, giving rise to severe combined immunodeficiency (SCID) (Blackburn and Kellems, 2005). The mutations that cause ADA deficiency are documented to exist throughout the ADA gene on human chromosome 20 (Piirila, 2006).

Patients suffering from ADA-SCID often have less than 5% ADA enzyme activity (Herschfield, 2003). They are characterized by the absence of B-, T- and NK- cells in their lymphatic system. Owing to the profound lymphopenia, ADA-SCID patients succumb to opportunistic infections if not diagnosed and treated early (Aldrich et al., 2000). The current standard-of-care for ADA-SCID is enzyme replacement therapy (ERT). The patients are administered weekly intravenous dosage of Pegylated bovine ADA (PEG-ADA). PEG-ADA treatment temporarily reduces intracellular and extracellular levels of dATP. Patients treated with PEG-ADA show immune recovery and sub-normal lymphocyte counts. A major drawback of ERT is the cost of PEG-ADA, which can rise up to $250,000 per year per patient. Additionally, patients with modest recovery of immune function can develop antibodies to PEG-ADA and can blunt the effect of
ERT (Booth et al., 2009; Chan et al., 2005). Because of these drawbacks, ADA-SCID has been the target of gene-therapy approaches.

### 2.1.2 Gene therapy for ADA-SCID.

ADA-SCID was the first disorder on which gene therapy was performed. In 1990, the first clinical application of gene therapy was reported in which lymphocytes from an ADA-SCID patient were treated with a retrovirus carrying ADA gene and infused back into the patient. Although this therapy was short-lived, it paved the way for further advances in gene therapy. Since then, gene therapy for ADA-SCID has been advanced to clinical trials (Candotti et al., 2012; Gaspar et al., 2011; Aiuti et al., 2009). Current gene therapy approaches focus on using a retroviral vector carrying a functional ADA gene. Hematopoietic stem/progenitor cells are isolated from a patient’s bone marrow and transduced with the retroviral vector. Transduced cells are transplanted back into the patient where they migrate to the bone marrow and generate hematopoietic cells with functional ADA enzyme. This therapy has life-long benefit, but has certain drawbacks. Retroviral vectors insert themselves preferentially in promoter elements of genes. The strong enhancer elements in the retroviral LTRs can trans-activate nearby genes and this could potentially lead to insertional oncogenesis. This has not been observed in ADA-SCID trials so far, but has been observed in X-linked SCID trials where the retroviral vector caused T-cell leukemias (Hacein-Bey-Abina et al., 2003). As a potentially safer approach, lentiviral vectors carrying ADA gene are under investigation for gene therapy for ADA-SCID. Although the integration profile of lentiviruses is safer than that of retroviruses, lentiviruses can potentially cause insertional oncogenesis. Therefore, there is a need for better, more controlled therapeutic approaches for ADA-SCID.
In this study, we investigated the proof-of-principle approach for gene therapy for ADA-SCID. Our approach uses zinc finger nuclease technology to site-specifically correct known mutation in the ADA gene. ZFNs were generated to target a mutation in exon 7 of ADA and were tested for their potential to correct ADA-deficiency in a site-specific manner.

2.2 Results

2.2.1 Zinc Finger Nucleases to target ADA

For this proof-of-principle study, we targeted the human ADA gene (Chr20q12:q13) for ZFN mediated gene modification. To select an appropriate target site for gene modification, an overview of various ADA-SCID mutations was considered. A summary of different mutations and their frequencies is shown (Figure 2.1.1a). ZFNs were developed at Sangamo BioSciences, Inc. to target a single mutation (G216R) (Ozsahin et al, 1997) for site-specific correction. The ZFNs bind and recognize a 36 bp long sequence in the intron 6 of ADA. The ZFN binding site is split into two half binding sites (13 and 18 bp) separated by a 5 bp spacer. A detailed schematic of ZFN binding site and the mutation targeted for modification is shown in Figure 2.1.1b. Initially, two sets of ZFN pairs were generated. The two monomers of each pair were linked to each other by a self-processing 2A peptide from Thosea asigna (Kim et al, 2011). The first pair (Wt) consisted of wild-type FokI endonuclease domains that can homodimerize, whereas the second pair (ELD/KKR) consisted of modified FokI domains that are obligate heterodimers (Miller et al, 2007). If ZFN homodimers are formed, they increase the chances of off-target cutting by ZFNs. However, ELD/KKR pairs can potentially exhibit lower ZFN activity. Thus, we compared both these constructs for their activity as well as toxicity.
Figure 2.2.1: **Zinc Finger Nucleases to target ADA.**

a. Schematic of human ADA gene showing the number of recorded instances of mutations causing ADA-SCID. The numbers below the exon numbers indicate the number of patients known to harbor a mutation in the corresponding exon.

b. ZFN target site at the human ADA locus. ZFN binding sites in the intron 6 of ADA are shown. The lower panel shows the potential site of mutagenesis (in red) causing allelic disruption of ADA.

### 2.2.2 Comparison of Wt and ELD/KKR ZFN constructs
In order to decide which ZFN construct is the best, we transfected HEK293T cells with 500 ng of plasmid carrying the ZFN pairs. We analyzed the cells at 3 days post transfection to determine if the ZFNs were able to induce allelic disruption at the target locus by Surveyor nuclease assay. Both the constructs were able to generate allelic disruption in HEK293T cells. The ELD/KKR construct induced two-fold higher %Allelic disruption compared to the Wt construct (Figure 2.2.2 a). To assess toxicity from the ZFNs indirectly, we stained cells for γH2A.X, which measures overall DSBs in each cell. We classified γH2A.X<sup>Bright</sup> cells as the cells having more DSBs than background levels (Rogakou et al., 1998). As compared to the ELD/KKR construct, the Wt construct showed up to 3-fold more % γH2A.X<sup>Bright</sup> cells, indicating elevated levels of DSB production (Figure 2.2.2 b). This result suggested that the ELD/KKR construct is more active and less cytotoxic as compared to the Wt construct. Hence, we continued with the ELD/KKR construct for further studies.

Figure 2.2.2: Comparison of Wt and ELD/KKR ZFN constructs

a. Allelic disruption of induced by Wt or the ELD/KKR ZFN constructs in K562 cells at 4 days post-nucleofection. Y-axis indicates %Allelic disruption as quantified by
densitometry. X-axis indicates the plasmids used for nucleofection. Bars represent mean ± SD.

b. Fidelity of Wt and ELD/KKR constructs. Y axis indicates %γH2A.X\textsuperscript{Bright} cells in the pool of K562 cells nucleofected with ZFN plasmids and measured at 3 days later. Bars represent mean ± SD.

2.2.3 Optimization of ZFN Activity in K562 cells by nucleofection

We sought to optimize the delivery of ZFNs to K562 by nucleofection of ZFN plasmids. We nucleofected K562 cells with increasing amounts of ZFN and analyzed those 4 days later. Surveyor nuclease assay indicated that there was a dose-dependent increase in %Allelic disruption (Figure 2.2.3 a). In order to confirm site-specific disruption at the target locus, we re-sequenced the target locus by Illumina high throughput sequencing and analyzed them using Integrated Genomic Viewer (IGV). Re-sequencing revealed that upon ZFN mediated DSB production, the target locus was repaired inaccurately by NHEJ. The NHEJ repair introduced insertions as well as deletions (in/dels). Deletions of up to 30 bp and insertions of up to 7 bp were observed. The most frequently observed mutation was duplication of the 5 bp spacer between the two half-binding sites, accounting for more than half of the mutations. Duplication of the spacer can occur if the ends of the DSB are filled without processing, followed by ligation. The distribution of different in/dels observed in K562 cells is shown in (Figure 2.2.3 b).
Figure 2.2.3: **Optimization of ZFN Activity in K562 cells by nucleofection.**

a. Surveyor nuclease assay indicating ZFN activity in K562 cells at 4 days post-nucleofection with ZFN plasmids (left). The numbers at the bottom of the gel indicates %Allelic disruption as quantified by densitometry. A graphical summary is shown in the right panel. Bars represent mean ± SD.

b. In/dels at the ZFN binding site in K562 cells. The X-axis represents the size of in/dels (negative values correspond to deletions) at the ZFN binding site. The Y-axis represents the frequency of each in/del as a percentage of total.
2.2.4 Off-target cutting by ZFNs

After confirming site-specific allelic disruption of ADA intron 6 site by the ZFN pair, we sought to determine the off-target cleavage by the ZFNs. ZFNs can potentially introduce DSBs at sites other than the intended site due to non-specific binding of the ZFs to DNA. Potential off-target sites can be predicted in silico. We used the ‘The ZFN-Site’ algorithm to predict non-specific binding and cleavage by the ZFNs (Cradick et al, 2011). The algorithm predicted two candidates (JMJD2B and SHISA7) for assessing off-target effects, each with a 4 bp mismatch with the ZFN binding site. We performed the Surveyor Nuclease assay to determine if there is any disruption at these loci in K562 cells nucleofected with ZFN plasmids. Out of the two loci, SHISA7 locus failed to amplify, whereas JMJD2B locus revealed no detectable allelic disruption (Figure 2.2.4). This suggests that there is minimal off-target cutting by these ZFNs. However, for comprehensive analysis of off-target effects, high-throughput sequencing based approaches need to be performed.

![Figure 2.2.4](image-url)

a.

<table>
<thead>
<tr>
<th>Site</th>
<th>Sequence</th>
<th>ZFN Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADA</td>
<td>CCAGGTCA</td>
<td>TGGGCTGTTAGTCGAGGAGGGCCCGGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NNNNN</td>
</tr>
<tr>
<td>JMJD2B</td>
<td>TGGGG</td>
<td>GAGGATTTGCCCCGGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NNNNN GGG</td>
</tr>
<tr>
<td>SHISA7</td>
<td>CCCGGGGGCCCTGCCGCCGGGCGGGGGCCGGCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NNNNNN A G</td>
</tr>
</tbody>
</table>

b.

![Image of PCR gel](image-url)
Figure 2.2.4: **Off-target cutting by ZFNs**

a. A summary of *in silico* predicted off-target sites. The two predictions are shown along with mismatches with the intended target.

b. Surveyor nuclease assay for detection of cleavage at the intended target site in ADA (left) and at the predicted off-target site in JMJD2B (right) from K562 cells nucleofected with ZFN plasmids are shown.

### 2.2.5 Nucleofection of ZFNs to HSPCs

Encouraged by the results from K562 cells, we sought to test the ZFN plasmids in primary human umbilical cord blood CD34⁺ hematopoietic stem progenitor cells (CB-CD34⁺). CB-CD34⁺ cells were nucleofected with various amounts of ZFN plasmids and cultured for 7-days. In order to optimize pre-nucleofection conditions, cells were nucleofected immediately after thawing (no-prestim) or pre-stimulated for 18 hours in culture medium supplemented with cytokines (prestim). Surveyor nuclease assay was performed on the genomic DNA from these cells to assess allelic disruption (Figure 2.2.5a). However, the Surveyor nuclease assay revealed that there was apparent mismatch in mock-treated samples, generating a high background signal. This could potentially happen if there is a mixed pool of alleles in the CB-CD34⁺ population. To assess the frequency of allelic disruption without a sequence dependent assay, we subjected these samples to Illumina high-throughput targeted resequencing. Deep re-sequencing of the regions surrounding the ZFN binding site revealed the presence of multiple different in/dels. A summary of the in/dels is shown in Figure 2.2.5b. Duplication of the 5 bp spacer accounted for approximately 45% of the total in/dels. However, the overall frequency of allelic disruption was
about 10-20 fold lower than that in K562 cells. This could be attributed to lower efficiency of nucleofection as compared to K562 cells.

Figure 2.2.5: **Nucleofection of ZFNs to HSPCs**

a. Surveyor nuclease assay performed on human CB-CD34+ HSPCs nucleofected with ZFN plasmids (left). Frequency of allelic disruption in the same samples as measured by high-throughput sequencing is indicated (right).

b. In/dels at the ZFN binding site in CB-CD34+ HSPCs. The X-axis represents the size of in/dels (negative values correspond to deletions) at the ZFN binding site. The Y-axis represents the frequency of each in/del as a percentage of total.
2.2.6 Effect of sequence variation on allelic disruption

To investigate if presence of any sequence variants can lead to the background in the Surveyor nuclease assay, we probed the re-sequencing data. In approximately 36% of the sequence reads, there was a G→A variation in the left-ZFN binding site as indicated by Figure 2.2.6a. This explained the background signal. Since the sequence variation was found in the ZFN binding site, we sought to determine if it had any effect on allelic disruption. To that end, we analyzed the unique in/dels from ZFN treated samples and categorized them as having the normal allele (G) or the variant allele (A). Interestingly, 95% of the in/dels consisted of the normal allele, whereas only 5% of the in/dels consisted of the variant allele (Figure 2.2.6b). This skewing in favor of the normal allele suggests that presence of the sequence variation can diminish ZFN binding and cleavage. However, it did not abolish allelic disruption completely, indicating low levels of ZFN binding to the variant sequence. This presents a significant challenge for the site-specific genome editing strategies, as they need to be modified according to each individual.

In order to avoid these issues, we began to screen umbilical cords for presence of SNPs at the ZFN binding site. Instead of pooling umbilical cord blood from multiple donors and isolating CD34⁺ cells, we processed samples from each donor individually. Before using the CD34⁺ cells for experiments, we screened the CD34⁻ fraction by Surveyor nuclease assay followed by DNA sequencing. This system was set up so that experiments to assess allelic disruption would be performed only on the samples that pass the screen. An example of the screening system is shown in Figure 2.2.6c. The samples that lack a background signal in Surveyor nuclease assay, and lack any sequence variations in the ZFN binding site were chosen for further experiments.
a. Sequence alignment of the left-ZFN binding site from the two sequence variants in CB-CD34+ cells (left). The arrow indicates the position of variation. Allelic disruption at the normal and the variant alleles as measured by high-throughput sequencing. The bars indicate Mean ± SD.

b. A representative results of screening for homozygosity of CB-CD34+ cells at the ADA exon 7 locus. The alphanumeric designations above the bands indicate the identification numbers for each single umbilical cord that was processed individually. PBMC – Peripheral Blood Mononuclear Cells.

Figure 2.2.6: **Effect of sequence variation on allelic disruption**
2.2.7 Gene modification in K562 cells by nucleofection

Encouraged by detection of allelic disruption in K562 cells as well as CB-CD34\(^+\) cells, we investigated the use of this approach for modification of a single base pair. We amplified a 1.3 kbp region surrounding the ZFN binding site to serve as the template for HR. The donor template was modified by site-directed mutagenesis to contain a 2 bp change that results in the creation of an \(Sst\)II restriction site. The \(Sst\)II site replaces an \(Eag\)I restriction site flanking the G216R mutation (Figure 2.2.7a). This allows measurement of gene modification frequencies by restriction fragment length polymorphism (RFLP) analysis. We nucleofected K562 cells with plasmids carrying either ZFNs or the donor templates and analyzed them 4 days later. We amplified a 2 kbp region surrounding the ZFN binding site in order to avoid co-amplification of transfected plasmids, and performed RFLP analysis on the amplicons. Digestion of amplicons with \(Sst\)II showed that only the cells treated with ZFN as well as donor plasmids harbor the \(Eag\)I→\(Sst\)II change (Figure 2.2.7b). Although this demonstrated successful gene modification, the frequency was outside the limits of accurate quantification by densitometry. Therefore, we developed a more sensitive, quantitative PCR based method to measure gene modification frequencies.
Figure 2.2.7: Gene modification in K562 cells by nucleofection

a. Schematic of the ADA exon 7 locus and the donor template is shown in the top panel. The lower panel shows the modified ADA locus along with the locations of the primer binding sites for the RFLP assay.

b. Gene modification in K562 cells by RFLP at 4 days post-nucleofection with plasmids carrying ZFNs and donor templates. RFLP analysis by SsrI digestion of amplicons is shown. The bands with lower molecular mass indicate SsrI digestion products.
2.2.8 qPCR assay for measurement of gene modification

In order to determine the gene modification frequencies accurately, we designed a qPCR assay. We used the 2 kb RFLP PCR as the first round of amplification to preclude co-amplification of donor templates. We developed a second round of PCR specific to the modified SstII site (SstII PCR). Using this PCR, we could amplify gene modification events with at least a 1000-fold higher specificity than unmodified DNA. In order to normalize for the input template, we designed a second round PCR that amplified a region in intron 9 of ADA (i9 PCR) (Figure 2.2.8a). The second round PCRs were made quantitative using SYBR Green in order to determine the gene modification frequencies. A plasmid standard curve was used to interpolate the gene modification frequencies based on the difference between cycles-to-threshold (Ct) values for the SstII and i9 PCRs. The qPCR assay indicated that the gene modification frequencies in K562 cells nucleofected with plasmids were up to 1% (Figure 2.2.8b).

![Diagram of qPCR assay](Image)

![Graph of qPCR analysis](Image)
2.2.8 **qPCR assay for measurement of gene modification**

a. A schematic of the qPCR assay for measurement of gene modification frequencies is shown. The primer pairs for the 1st round amplification (RFLP primers) and the 2nd round amplification (i9 and SstII primers) are indicated by arrows.

b. A representative standard curve for the qPCR assay is shown (left). The Y-axis indicates the difference in Ct values for the SstII and i9 PCRs for each data point. The X-axis indicates the logarithm of % gene modification to the base 2 for each data point. The standard curve is fitted on to a straight line with the linear regression coefficient indicated by \( R^2 \). The right panel shows gene modification in K562 cells by nucleofection as measured by the qPCR assay. The bars indicate mean ± SD, n=4.

2.2.9 **ZFN resistant donor templates**

One of the potential reasons for lower gene modification frequencies could be cleavage of the donor template by the ZFNs. Since the donor template contains the ZFN binding site, it can be cleaved by the ZFNs. This could lead to the donor templates acting as decoys and reducing cleavage at the genomic locus. Alternatively, it could also make the cleaved donor templates unable to participate in HR. The modified locus can also be recut and potentially mutated by the ZFNs. To investigate this phenomenon, we modified the donor templates to be resistant to cleavage by ZFNs. Structure-function studies have shown that ZFN activity is highly sensitive to the length of the spacer between the two half binding sites (Elliott *et al.*, 1998). ZFN activity was reported to diminish greatly if the spacer length was less than 4 bp or more than 8 bp. Therefore, we generated two donor templates, one with the entire spacer deleted (del5), or one with a 2 bp
deletion in the spacer (del2) (Figure 2.2.9a). We tested these two donor templates along with the parent donor template (P) in K562 cells by nucleofection followed by RFLP and qPCR assay to measure gene modification. Both of these constructs were able to act as the donor templates for gene modification. However, there was no significant difference between the three constructs (Figure 2.2.9b).

Figure 2.2.9: ZFN resistant donor templates

a. Schematic of modifications to construct the ZFN resistant donor templates. The ZFN binding sites of the three donor templates are shown on the right. The sites of deletions in the Del2 and the Del5 constructs are indicated.

b. Gene modification induced by the three donor templates in K562 cells by nucleofection. The left panel indicates gene modification by RFLP assay and the right panel indicates gene modification measured by qPCR. The bars represent mean ± SD.
2.3 Discussion

In this part of the project, we determined if ZFN mediated gene modification is a feasible strategy for correction of ADA deficiency in human cells. Based on the reported ADA-SCID mutations, we chose the region in proximity of exons 7-9 for gene modification. The ZFNs were designed using the ZF motif library at Sangamo BioSciences using proprietary methods. After screening multiple ZFN pairs, a pair targeting the intron 6 of ADA was chosen for these studies. After obtaining the ZFN pair from Sangamo BioSciences, we tested the ZFNs and found them to be functional at site-specifically disrupting the locus, with no detectable off-target activity in preliminary testing. The allelic disruption levels were up to 20% in K562 cells following nucleofection of plasmids carrying the ZFNs. These levels are comparable to those obtained by the early reports on using ZFNs. The current ZFN pairs were generated using the 1st generation ZF motif library at Sangamo BioSciences. The more advanced 2nd and 3rd generation libraries have been reported to produce ZFNs with higher activity, with allelic disruption levels reaching up to 70-80%. Usually, the ZFNs generated from an earlier version of a library are iteratively re-optimized using newer libraries. However due to technical and logistic limitations, the ZFNs for the current study were not considered for re-optimization. Nevertheless, these studies are well applicable to the current state of the ZFN construction. This is mainly due to the widespread commercialization of ZFNs by Sigma-Aldrich. Sigma-Aldrich released the CompoZr custom ZFN service in 2010 (Hansen et al, 2012), which is based on the 1st generation ZF library licensed from Sangamo BioSciences. The spread of ZFNs from Sangamo BioSciences is restricted through collaborations, unlike the commercial nature of those from Sigma-Aldrich. Due to this, a large number of currently ongoing studies using ZFNs are based on ZFNs from
Sigma-Aldrich, i.e. based on the 1\textsuperscript{st} generation ZF library. Hence, our proof-of-principle studies should be broadly applicable to those involving commercially obtained ZFNs.

In addition to testing the ZFNs, we constructed donor templates for gene modification at the exon 7 of ADA. We developed a system for measuring gene modification frequencies by RFLP and by qPCR. Using these assays, we could achieve up to 1% gene modification frequencies upon nucleofection of plasmids in K562 cells. There was a potential concern about the ZFNs being able to cleave the donor templates, or the modified locus. We tested ZFN-resistant donor templates and found them to be similar to the unmodified templates. Thus we establish that gene modification frequencies of about 1% as the benchmark for this system.

Gene modification frequency of 1% is low for ZFN mediated gene modification, even with the 1\textsuperscript{st} generation ZFNs. The major reason for the low levels of gene modification is the distance of the modification from ZFN binding site. It is known that the efficiency of I-SceI mediated gene modification reduces with increasing distance from the cleavage site. This phenomenon is mainly due to the length of gene conversion tracts, which is dependent on the extent of end processing at the DSB. It has been reported that the gene conversion tracts can reach up to 400 bp from the DSB. However, the efficiency reduces dramatically within 50 bp and diminishes beyond 100 bp to 10% of that at the cleavage site (Elliott \textit{et al}, 1998). Even though these results were from an I-SceI system, they should be true for any DSB inducing endonucleases, such as ZFNs. In our system, the site targeted for gene modification is 75 bp away from the cleavage site. This would place the modified site towards the outer limits of the gene conversion window. Hence, even with an effective pair of ZFNs, we are limited to lower gene modification efficiencies. However, this has virtually no bearing on the following studies as we are using ZFN mediated modification of ADA as merely a model system. The numbers of gene modification are
too low for this approach to be considered for gene therapy, but we establish this as a reproducible model system for further studies involving other aspects of the ZFN technology.

One of the most significant results of this study was the effect of sequence variations at the ZFN binding site. In preliminary experiments with primary CB-CD34\(^+\) cells, we found a mixed pool of sequence variants. This resulted in a background signal in the Surveyor nuclease assay. This is a very important point to consider while exploring cell types to use for this strategy. K562 cells were homozygous for the allele that was targeted with ZFNs, thereby facilitating clean results from the assay. However, if there are more than one alleles present, it makes the use of Surveyor nuclease assay virtually impossible. The gene modification assay, however, was unaffected by sequence variants as it relies on the modified locus. Therefore, screening must be performed before choosing a cell line for studies involving measurement of ZFN activity. The more important aspect of sequence variation is its effect on ZFN binding and activity. We found that one of the sequence variation occurred at the ZFN binding site. Our sequence data indicated that the variation reduced ZFN binding and cleavage greatly. To overcome this, we established a screening system for individual cell sources so we can preclude any sequence variations in the ZFN binding site. We highly recommend using a similar screening system for primary cell sources to be used in nuclease mediated approaches.

Sensitivity to sequence variation is one of the major drawbacks of site-specific modification technologies. In order to make a universal approach with ZFNs, the binding site should be designed to avoid any SNPs. This may prove challenging for some diseases due to existence of a considerable frequency of disease associated SNPs. The ADA gene itself has 664 total recorded SNPs, 34 of which are in the coding region (dbSNP). The SNP that occurred in the CB-CD34\(^+\) cells has a mean heterozygous frequency of 20\% in the population. This would mean that our
approach might only be half as effective in those individuals. This highlights the importance of adjusting the targeting strategies as per the variations at the locus of interest. ZFNs can be engineered to have an ambiguity in the binding site, so that they can recognize and cleave multiple sequence variants. However, this will reduce the specificity of the ZFNs, making them more likely to cleave unintended targets.

In summary, we established site-specific modification of ADA exon 7 by ZFNs as a model system for further studies. Henceforth, we have used this system for experimental purposes and for exploring avenues to increase its efficiency.
2.4 Materials and methods

2.4.1 Cell culture and maintenance

HEK293T cells (CRL-11268, ATCC) were cultured in Dulbecco’s Minimal Essential Medium (Cellgro, Manassas, VA) supplemented with 10% Fetal Bovine Serum (Gemini Bio-products, West Sacramento, CA) and Penicillin/Streptomycin/L-glutamine (Gemini Bio-products, West Sacramento, CA). K562 Cells (CRL-243, ATCC) were cultured in RPMI1640 (Cellgro, Manassas, VA) supplemented with 10% Fetal Bovine Serum (Gemini Bio-products, West Sacramento, CA) and Penicillin/Streptomycin/L-glutamine (Gemini Bio-products, West Sacramento, CA). Human umbilical cord blood samples were collected from UCLA Ronald Reagan Medical Center and have been deemed exempt from review from IRB as anonymous medical waste. Mononuclear cells (MNCs) were isolated from cord blood by Ficoll-Paque (GE Healthcare Biosciences, Piscataway, NJ) based separation. CD34+ cells were isolated from the MNCs using MACS CD34+ enrichment kit (Miltenyi Biotec Inc, Auburn, CA) and cryopreserved in 90% Fetal bovine serum and 10% DMSO (Life Technologies, Green Islands, NY) until use. Genomic DNA from cells was extracted using PureLink Genomic DNA mini kit (Life Technologies, Green Islands, NY) and quantified using NanoVue (GE Healthcare Biosciences, Piscataway, NJ). Cell counts were measured using a Beckman-Coulter ViCell-XR automated cell counter (Beckman-Coulter, Indianapolis, IN).

2.4.2 Transfection of HEK293T cells

HEK293T cells were transfected with TransIT-293 (Mirus Bio, Milwaukee, WI) transfection reagent according to manufacturer’s protocol. Briefly, plasmid DNA and the transfection reagent were mixed together and incubated for 25 minutes at room temperature. The culture medium on
the cells was replaced by Opti-MEM (Life Technologies, Green Islands, NY). The complexes of DNA and transfection reagent were added to the cells in a dropwise manner. The cells were cultured for 24 hours before replacing the medium with fresh culture medium.

2.4.3 Nucleofection of K562 cells

K562 cells were nucleofected using Nucleofector-4D (Lonza, Walkersville, MD) as per the recommended protocol. Briefly, 1x10^6 cells per sample were centrifuged at 90g for 10 minutes. The pellets were resuspended in 100 µl of Cell line SF solution. Appropriate amounts of DNA were added to the cells and the mixture was nucleofected using the recommended program. The cells were allowed to recover at room temperature for 10 minutes and then supplemented with culture medium and transferred to 6-well tissue culture treated plates (Corning, Corning, NY).

2.4.4 Nucleofection of CD34^+ cells

CD34^+ cells were thawed in Iscove’s modified Dulbecco’s medium (Cellgro, Manassas, VA) supplemented with 20% fetal bovine serum. The cells were nucleofected using Nucleofector-4D (Lonza, Walkersville, MD) as per the recommended protocol. Briefly, 1x10^6 cells per sample were centrifuged at 90g for 10 minutes. The pellets were resuspended in 100 µl of Primary cell P3 solution. Appropriate amounts of DNA were added to the cells and the mixture was nucleofected using the recommended program. The cells were allowed to recover at room temperature for 10 minutes and then cultured in IMDM (Cellgro, Manassas, VA) supplemented with 20% Fetal Bovine Serum (Gemini Bio-products, West Sacramento, CA) and 25 ng/ml Stem Cell Factor (Amgen, Thousand Oaks, CA), 10 ng/ml Interleukin-6 (R&D System Inc, Minneapolis, MN) and 5 ng/ml Interleukin-3 (R&D System Inc, Minneapolis, MN) on 12-well tissue culture treated plates (Corning, Corning, NY).
2.4.5 Staining for γH2A.X

HEK293T cells were dissociated from the culture plate using 0.05% Trypsin-EDTA (Cellgro, Manassas, VA) and centrifuged at 300g for 5 minutes. The cells were fixed by 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 4°C. The cells were pelleted and permeabilized using 1X Perm/Wash buffer (0.05% Saponin (Sigma-Aldrich, St. Louis, MO) and 2.5% fetal bovine serum in Dulbecco’s PBS (Cellgro, Manassas, VA)) for 30 minutes. Cells were pelleted and incubated with 1 µg FITC-labeled anti-γH2A.X (Sigma-Aldrich, St. Louis, MO) in 50 µl 1X Perm/Wash buffer for 30 minutes in dark. The cells were washed 2 times with 1X Perm/Wash buffer and resuspended in DPBS and analyzed by flow cytometry.

2.4.6 Generation of donor templates

For the donor construct, the 1.3kb region surrounding the ZFN binding site of the human ADA gene intron 6 (33133-34488 in NG_007385) was amplified using X7-F1 (5’AGGGACTCCTGCTTACGCG3’) and X7-R2 (5’CTGCTTCTGGCTGGATTTGC3’) and cloned in pCR4-TOPO (Life Technologies, Green Islands, NY). The EagI site was mutated to SstII site using QuickChange Site-directed Mutagenesis Kit (Agilent Technologies Inc., Santa Clara, CA). For the Del2 and Del5 constructs, the pCR4-TOPO-Donor construct was mutagenized using QuickChange Site-directed Mutagenesis Kit (Agilent Technologies Inc., Santa Clara, CA).

2.4.7 Surveyor nuclease assay

Surveyor Nuclease Assay was used to determine ZFN induced site-specific allelic disruption. A 332 bp region surrounding the ZFN binding site was amplified from 200 ng of genomic DNA
using 474F (5’CTACCTGACACATGGTAACTGGCTAATGAG3’) and 474R (5’AATAGAGCCAAGTATGGGAGGAGGCAGTGAGGAGGG3’) using Accuprime Taq Hi-Fi (Life Technologies, Green Islands, NY) under the following conditions: 94\(^\circ\)C for 2 minutes followed by 30 cycles of 94\(^\circ\)C for 30 seconds, 62\(^\circ\)C for 30\(^\circ\)C seconds and 68\(^\circ\)C for 1 minute, followed by a final extension of 68\(^\circ\)C for 5 minutes. The primers used for JMJD2B are 5’CTGGGGTCCACAGTGTGC3’ and 5’CTAGATGCTGGGAAAGGTTTCTGT3’. The primers used for SHISA7 are 5’ GCACGTTGATATCCCGGT3’ and 5’CAACTACGACACGCCGC3’. The PCR product was diluted in 1:2 in 6 \(\mu\)l of 1X Accuprime buffer and subjected to denaturation and reannealing under the following conditions: 94\(^\circ\)C for 10 minutes, followed by cooling to 85\(^\circ\)C at -2\(^\circ\)C per second and cooling to 25\(^\circ\)C at -0.1\(^\circ\)C per second. The reaction mixture was then digested with 1 \(\mu\)l of Surveyor Nuclease (Transgenomic, Inc., Omaha, NE) at 42\(^\circ\)C for 15 minutes. The reactions were resolved on 8% TBE polyacrylamide gels (Life Technologies, Green Islands, NY) at 120V for 45 minutes. The gels were removed from the cassettes and stained with 5 \(\mu\)l of Gel-Green (Phenix Research Products, Candler, NC) in 50 ml of 1X TBE for 15 minutes and imaged on a Kodak Molecular Imaging Station. The gel images were analyzed by densitometry and allelic disruption was determined using the following formula:

\[
\% \text{ Allelic Disruption} = 100 \times (1 - \sqrt{(\text{intensity of the uncut band / total intensity of cut and uncut bands})})
\]

2.4.8 RFLP analysis

Site-specific gene modification was detected by Restriction Fragment Length Polymorphism (RFLP). A 2kbp region surrounding the ZFN binding site was amplified using primers X7-2kb-F
(5’AGACCGTGGTAGCCATTGAC3’) and X7-2kb-R (5’GCCAGGTGTCAAGAAGAGAG3’) and Accuprime Taq Hi-Fi (Life Technologies, Green Islands, NY) under the following conditions: 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 62°C for 30°C seconds and 68°C for 2 minutes, followed by a final extension of 68°C for 5 minutes. The PCR product was purified using PureLink PCR cleanup kit (Life Technologies, Green Islands, NY) and digested using 10 units of SstII (Life Technologies, Green Islands, NY) for 2 hours at 37°C. The digestion products were separated on 0.8% TBE-Agarose gel pre-stained with Gel-Green and imaged on a Kodak Molecular Imaging Station.

2.4.9 Gene modification qPCR

In order to quantify gene modification, a quantitative PCR based assay was used. A set of two PCR reactions were performed using the 2kb PCR product as a template. The first PCR was performed to amplify modified genomes, using primers X7-Fwd (5’TGACACATGTAAGCTTGGCTAATGAG3’) and SstII-Rev (5’CTCACCTCTTTTACTACTCTTCGCG3’). The second PCR was performed to normalize the input template using primers i9-Fwd (5’TCCCCTGGGGCTGT3’) and i9-Rev (5’TGGAAGCTCTCCTCTGTAATG3’). Both of these PCRs were made quantitative using SYBRGreen PCR master mix (Life Technologies, Green Islands, NY) and acquired on ViiA7 Real time PCR system (Life Technologies, Green Islands, NY). Frequency of gene modification was determined using the Ct (cycles to threshold) difference between the two PCRs and using a plasmid standard curve.
CHAPTER 3. Integrase Defective Lentiviral Vectors for Delivering ZFNs and Donor Templates

3.1 Introduction

3.1.1 Methods to deliver ZFNs and Donor templates.

One of the major aspects of implementing the ZFN technology has been delivery of ZFNs and donor templates to target cells. There are several requirements that a delivery system should fulfill in order to be useful. These are: 1) High efficiency of transgene delivery, 2) Low cytotoxicity, 3) Transient nature of delivery, 4) Feasibility of scale-up to clinical scale.

The most commonly used delivery method for delivering ZFNs to cell lines is transfection of plasmids either by lipid-based reagents (Pruett-Miller et al., 2008) or by electroporation (Holt et al., 2010). Lipid-based methods are effective for adherent cell lines; however, they are not efficient for suspension cell lines or primary cells (Unpublished data). Electroporation using the Lonza Nucleofector system has been shown to be highly efficient in suspension cell lines. Nucleofection in primary cells is efficient at delivering transgenes; however it exhibits high levels of cytotoxicity. Thus, nucleofection of primary cells has seen limited success. Nucleofection is not a clinically approved method and has concerns about scalability. Due to these drawbacks, much of the research is focused on using viral vectors for ZFN or donor delivery.

Among the viral vectors, chimeric adenoviral vectors have been studied for delivering ZFNs to primary hematopoietic cells (Maier et al., 2013). The most common adenoviral vector (Ad5/35) is a chimeric vector based on Adenovirus-5 backbone, but containing a chimeric fiber protein derived from Adenovirus-35. Ad5/35 vectors are efficient at transducing the target cells at high
copy numbers, but exhibit high cytotoxicity (M. Hoban, unpublished data). Adeno-associated viruses (AAVs) have also been studied for ZFN delivery. Recent reports have demonstrated efficacy of AAVs for in vivo delivery of ZFNs to murine liver (Li et al., 2011). AAVs have been used as homologous recombination donor templates. However, AAVs are limited by their host cell tropism. Integrase defective lentiviral vectors (IDLVs) have also been studied for delivering ZFN and donor templates with limited success (Lombardo et al., 2007).

3.1.2 Integrase Defective Lentiviral Vectors for ZFN and donor delivery

Integrase-defective lentiviral vectors are lentiviral vectors packaged with a catalytically inactive integrase protein, often harboring a D64V mutation (reviewed in Wanisch and Yáñez-Muñoz, 2009). Upon entering a host cell, IDLV genomes undergo reverse transcription and shuttle to the nucleus in a double-stranded DNA form. In absence of integrase activity, these dsDNA forms exist as episomes. Due to the lack of a replication origin, IDLV proviruses dilute with cell division and hence exist transiently in the nucleus (Nightingale et al., 2006). Episomal IDLV proviruses can act as templates for transcription as well as can act as donor templates for HR. If IDLVs are pseudotyped with a Vesicular Stomatitis Virus Envelop Glycoprotein (VSV-G), they can transduce a wide variety of human cell types. LVs have been approved for clinical trials of gene therapy, so IDLVs have the advantage of clinical feasibility. However, transgene expression from IDLVs is about 10-fold lower than the corresponding integrating LVs (reviewed in Wanisch and Yáñez-Muñoz, 2009).

IDLVs were studied in context of gene modification to deliver I-SceI and were found to be effective (Cornu et al., 2007). IDLVs to deliver ZFNs have been studied before, most significantly by Lombardo et al. IDLVs were found to be efficient for delivering HR donor
templates and have been used in conjunction with nucleofection or Ad5/35s for ZFN-mediated gene editing (Benabdallah et al, 2010). IDLVs have seen limited efficacy for delivering ZFNs. However, the reasons for this phenomenon have not been investigated thoroughly. In this study, we aimed to evaluate the potential of IDLVs to deliver ZFNs and donor templates. We also investigated potential recombination events in IDLV genomes and optimized the vector design of IDLVs to deliver ZFNs.
3.2 Results

3.2.1 IDLVs to deliver ZFN monomers

Initially, we constructed two IDLVs to deliver one ZFN monomer each. The backbone used for these is based on the HIV-1 genome, with all the viral genes removed while keeping only the central polypurine tract (cPPT). The LTRs of the backbone were modified to be self-inactivating (SIN-LTRs) due to a deleting in the U3 region. These IDLVs were designed to express either left or right ZFN monomer, under the control of short, intron-less human elongation factor 1α (EFS) promoter. The ZFN monomers were translationally linked to mCherry using self-processing 2A peptide from Porcine teschovirus 1 (P2A). This was done in order to facilitate assessment of gene expression from IDLVs using flow cytometry. The IDLVs encoded the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) to increase the stability of genomic as well as internal transcripts. A detailed schematic of the vectors (EFS-Single-IDLVs) is shown in Figure 3.2.1a. We packaged these vectors using a 3-plasmid packaging system containing a gag/pol plasmid harboring a D64V mutant catalytically inactive integrase, and pseudotyped them with VSV-G. The vectors were concentrated 2000-2500 fold using tangential flow filtration and the titers were determined using qPCR as published by Cooper et al (Cooper et al, 2011)

We co-transduced K562 cells with the EFS-Single-IDLVs and measured transduction levels by determining vector copy number per cell (VCN) and mCherry expression post-transduction. High levels of mCherry expression indicated robust transduction capability of the two vectors. A time-course analysis of mCherry expression confirmed the transient nature of transgene expression from these IDLVs. At 21 days post-transduction, mCherry+ cells reduced to less than 5% of the population Measurement of VCN from these cells also corroborated robust
transduction with a dose-dependent response (Figure 3.2.1b). We performed Surveyor nuclease assay on transduced cells and found that the IDLVs were able to induce allelic disruption (Figure 3.2.1c). The overall values of allelic disruption were lower than nucleofection; nevertheless, the EFS-Single-IDLVs were successfully able to deliver ZFNs to K562 cells.

Figure 3.2.1: IDLVs to deliver ZFN monomers

a. Self-inactivating lentiviral constructs for delivery of ZFN monomers. \( \Delta U3-R-U5 \) – HIV-1 derived self-inactivating LTR, \( \psi \) – HIV-1 packaging signal, EFS – truncated, intron-less human elongation factor 1\( \alpha \) promoter. ZFN-L and ZFN-R are left- and right-ZFNs respectively, P2A – 2A peptide from Porcine teschovirus-1, WPRE – woodchuck hepatitis virus post-transcriptional regulatory element.

b. Delivery of ZFNs to K562 cells by EFS-Single-IDLVs. Expression of mCherry in K562 cells transduced with EFS-Single-IDLVs at a range of concentrations from \( 2 \times 10^7 \) TU/ml to \( 1.6 \times 10^8 \) TU/ml. Y-axis shows levels of transduction indicated by mCherry expression, X-axis
shows days post transduction (left). Average VCN in transduced K562 cells at days post transduction (center). Bars and data points represent Mean ± SD. Allelic disruption in K562 cells transduced with EFS-Single-IDLVs (right). Representative Surveyor nuclease assay results from K562 cells transduced with various concentrations of EFS-Single-IDLVs are shown. The smaller products, corresponding to the cut bands arising from allelic disruption are indicated by the arrow. Quantified %Allelic disruption is indicated by the numbers under the bands on the gel.

3.2.2 EFS-Single-IDLVs in CB-CD34+ cells

Encouraged by the ability of EFS-Single-IDLVs to deliver ZFNs to K562 cells, we tested them in CB-CD34+ cells. CD34+ cells were isolated from screened umbilical cords and pre-stimulated for 18 hours on retronectin with cytokines. After pre-stimulation, the cells were co-transduced with various concentrations of the EFS-Single-IDLVs. The cells were analyzed at 4-6 days post-transduction by flow cytometry and by qPCR to determine VCN. We demonstrated that the EFS-Single-IDLVs were able to transduce CB-CD34+ cells with high efficiency, indicated by both mCherry expression and VCN. Surveyor nuclease assays showed that despite efficient transduction, there was no detectable allelic disruption in these cells (Figure 3.2.2). We postulated that one of the reasons for this could be inefficient co-transduction with the two vectors, leading to poor co-delivery of two ZFN monomers to the same cell.
Figure 3.2.2: **EFS-Single-IDLVs in CB-CD34+ cells**

Transduction of CB-CD34+ cells with EFS-Single-IDLVs. Expression of mCherry in CB-CD34+ cells transduced with various concentrations of the EFS-Single-IDLVs, measured at 5 days post transduction (left). Y-axis shows %mCherry+ cells, X-axis shows the vector concentrations. Average VCN in CB-CD34+ cells transduced with EFS-Single-IDLVs, measured at 4 days post transduction (center). Y-axis indicates average VCN, X-axis indicates vector concentrations. Data points represent Mean ± SD. Allelic disruption in CB-CD34+ cells transduced with EFS-Single-IDLVs (right). Representative Surveyor nuclease assay results from CB-CD34+ cells transduced with various concentrations of EFS-Single-IDLVs are shown.

### 3.2.3 IDLV to co-deliver two ZFNs

A potential limitation to the use of Single-IDLVs to produce site-specific DNA modification is the requirement to achieve simultaneous co-transduction of cells with two vectors, needed for 1:1 stoichiometry of the two ZFN monomers. In order to avoid co-transduction for efficient delivery, we designed an IDLV construct to deliver both the ZFN monomers from the same vector. We linked two ZFN monomers using the 2A peptide from Thosea asigna (T2A) and linked them to mCherry using P2A. A detailed schematic of the vector (**EFS-Double-IDLV**) is shown in [Figure 3.2.3a](#). We packaged the EFS-Double-IDLV as described previously and tested it in K562 cells. K562 wells were transduced with various concentrations of the vector and analyzed 4 days later by flow cytometry for mCherry expression and by qPCR for VCN. The vector demonstrated efficient transduction capacity; however, it was lower than that of EFS-Single-IDLVs. We performed the Surveyor nuclease assay on transduced cells and found that there was no detectable allelic disruption. At VCN equivalent to the EFS-Single-IDLVs, EFS-Double-
IDLV did not induce allelic disruption (Figure 3.2.3b). We postulated that this unexpected result was due to vector rearrangements. Rearrangements in the HIV-1 genome because of repeat regions have been reported previously. Based on these reports, we hypothesized that vector rearrangements could be occurring in the EFS-Double-IDLV, since the vector contains repeat regions at 5’ and 3’ ends of each ZFN monomer. Vector rearrangements could have deleterious effects for ZFN delivery and we sought to investigate this phenomenon.

Figure 3.2.3: IDLV to co-deliver two ZFNs

a. Schematic of the EFS-Double-IDLV construct. ΔU3-R-U5 – HIV-1 derived self-inactivating LTR, ψ – HIV-1 packaging signal, EFS – truncated, intron-less human elongation factor 1α promoter. ZFN-L and ZFN-R are left- and right-ZFNs respectively, P2A – 2A peptide from Porcine teschovirus-1, T2A – 2A peptide from Thosea asigna, WPRE – woodchuck hepatitis virus post-transcriptional regulatory element. Detailed arrangement of various domains in the ZFN monomers is shown. 3x-FLAG – triple
b. Transduction of K562 cells by EFS-Double-IDLV. Expression of mCherry in K562 cells transduced with EFS-Double-IDLV at a range of concentrations from $2 \times 10^7$ TU/ml to $1.6 \times 10^8$ TU/ml. Y-axis shows levels of transduction indicated by mCherry expression, X-axis shows days post transduction (left). Average VCN in transduced K562 cells at days post transduction (right). Bars and data points represent Mean ± SD.

Representative Surveyor nuclease assay results from K562 cells transduced with various concentrations of EFS-Double-IDLV are shown.

3.2.4 Recombination in Double-IDLV genomes

The two ZFN monomers in the EFS-Double-IDLV constructs have two regions of sequence homology with each other. The 5’ end of the ZFNs contains the triple FLAG-tag and the Nuclear Localization Signal (NLS) that are identical in both the monomers. These are followed by distinct ZF motifs. The 3’ end of the ZFNs contains the FokI domains, which are identical between the two monomers, except at three basepairs at the ELD/KKR mutations. Due to these regions of homology, the EFS-Double construct can potentially undergo recombination. To investigate if the vector is undergoing rearrangements, we designed a PCR based assay for testing vector integrity. We designed a set of primer pairs to amplify overlapping regions of the proviral vector as indicated in Figure 3.2.4.

We performed the PCRs using the primer sets on genomic DNA from K562 cells transduced with either EFS-Single-IDLVs or EFS-Double-IDLV. The plasmid for the EFS-Double construct was used as a positive control. Gel electrophoresis of the amplicons revealed that most of the
vector genome maintained its integrity. However, one the reactions (reaction B) showed unexpected results. Reaction B amplifies across the left-ZFN with an expected amplicon size of 1.3 kbp for Double-IDLV construct, and 400 bp for the EFS-Single-IDLV construct. The PCR from cells transduced with EFS-Single-IDLVs showed the expected 400 bp product. The EFS-Double-IDLV plasmid generated the expected 1.3 kbp product. However, the cells transduced with EFS-Double-IDLV showed a 400 bp product, rather than the expected 1.3 kbp product. This indicated the occurrence of a deletion in the EFS-Double-IDLV genome, particularly in the left-ZFN. We confirmed, by sequencing, that the rearrangement of the vector was causing deletion of the left-ZFN. We hypothesized that the sequence homology between the 5’ ends of the left and the right-ZFNs was responsible for vector rearrangement.

Figure 3.2.4: Recombination in Double-IDLV genomes

Primer pairs for testing integrity of proviral genome of EFS-Double-IDLV. Arrows connected by broken lines indicate primer pairs for reactions, A, B, C, D, E, and ZFN (top). Gel electrophoregrams from reactions A, B, C, D, E and ZFN performed on K562 cells transduced
with IDLVs are shown (bottom). Single – Cells transduced with EFS-Single-IDLVs, Double – Cells transduced with EFS-Double-IDLV, PC – EFS-Double-IDLV construct plasmid, NC – No template control, 1kb – QuickLoad 1kbp ladder. The reaction with aberrant results, reaction B, is highlighted with a solid rectangle.

3.2.5 Modifications to IDLV design

To avoid rearrangements in the EFS-Double-IDLV, we modified the construct in two different ways (Figure 3.2.5a). In one of the constructs, **EFS-Double-dF-IDLV**, we deleted the N-terminal FLAG tag from the left-ZFN to eliminate one region of sequence homology; however the NLS was retained. In the other construct, **EFS-Double-CoOp-IDLV**, we codon-optimized the left-ZFN, including its FLAG tag, NLS, zinc finger motifs and the FokI domain. This resulted in a decrease in the DNA sequence identity between the two ZFNs (summarized in Table 3.2.5), potentially reducing the possibility of recombination. We tested these two constructs by transducing K562 cells with the corresponding integrating lentiviral vectors. The EFS-Double-dF-LV did not induce allelic disruption as assessed by the Surveyor nuclease assay. However, the EFS-Double-CoOp-LV construct did successfully induce allelic disruption (Figure 3.2.5b).

To determine whether the restoration of ZFN activity is due to reduction in recombination, we performed PCR analysis (Reaction B) for vector integrity of the proviral genomes. We performed reaction B on genomic DNA from cells transduced with EFS-Double-LV, EFS-Double-dF-LV or EFS-Double-CoOp-LV. We observed the same results for the EFS-Double-LV construct as before, with the 400 bp product being the major product. However, in case of the EFS-Double-dF and EFS-Double-CoOp constructs, the 1.3 kbp amplicon was the major product.
Densitometry analysis revealed that the 1.3 kbp product was 10-15 fold more abundant in the modified constructs as compared to the unmodified construct. The increase in relative abundance of the 1.3 kbp product suggested a reduction in recombination levels in both the constructs. These data suggest strongly that DNA sequence homology between two ZFN monomers in a given pair can be detrimental to their co-delivery by the same IDLV. However, by introducing DNA sequence changes to reduce homology, we successfully overcame this barrier and achieved delivery of both the ZFN monomers by one construct.

Figure 3.2.5c: Modifying to IDLV design

virus post-transcriptional regulatory element. ZFN-L^{dF} indicates the left-ZFN without N-terminal FLAG tag. ZFN-L^{CoOp} indicates the codon-optimized left-ZFN.

b. Comparison of ability of EFS-Double-LV constructs to induce allelic disruption in K562 cells. Surveyor nuclease assays on K562 cells transduced with EFS-Double-LV, EFS-Double-dF-LV, EFS-Double-CoOp-LV are shown, demonstrating successful allelic disruption only with the Double-CoOp-LV construct. Numbers indicated quantified % Allelic disruption.

c. Vector integrity analysis on the modified IDLV constructs. Gel electrophoregrams of the reaction B (from 3.2.4) performed on K562 cells transduced with the different constructs.
Table 3.2.5: **Comparison of repeat sequences in Double-IDLV and Double-CoOp-IDLV.** Left-ZFN in each construct is compared with Right-ZFN and analyzed for sequence identity using Blast2Seq. Homology between the Left-ZFN for each construct with the Right-ZFN is indicated as % identity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EFS-Double-IDLV</th>
<th>EFS-Double-CoOp-IDLV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identity in FLAG-tag</td>
<td>66/66</td>
<td>58/66</td>
</tr>
<tr>
<td>Identity in NLS</td>
<td>21/21</td>
<td>20/21</td>
</tr>
<tr>
<td>Identity in zinc finger motifs</td>
<td>326/371</td>
<td>272/373</td>
</tr>
<tr>
<td>Identity in FokI domains</td>
<td>585/588</td>
<td>541/588</td>
</tr>
<tr>
<td>Total identity of ZFN-left with ZFN-right</td>
<td>1016/1061 (96%)</td>
<td>901/1063 (85%)</td>
</tr>
<tr>
<td>Longest stretch of identity between ZFN-left and ZFN-right</td>
<td>614 bp</td>
<td>64 bp</td>
</tr>
</tbody>
</table>

3.2.6 **Testing EFS-Double-CoOp as IDLV**

Encouraged by the restored ability of the EFS-Double-CoOp construct as an integrating LV, we determined if the vector is efficient as an IDLV. We packaged the vector as an IDLV as described previously and tested its ability to transduce K562 cells. We compared the efficiency of the vector directly to the EFS-Single-IDLVs. Flow cytometry and qPCR analyses revealed
that the EFS-Double-CoOp-IDLV construct was able to transduce K562 cells robustly. We also performed Surveyor nuclease assay on transduced cells and found that even at comparable VCN, the EFS-Double-CoOp-IDLV was not able to induce allelic disruption (Figure 3.2.6b). The comparison of the fluorescence intensities of mCherry from the two vectors revealed that the expression from EFS-Double-CoOp-IDLV was significantly lower than EFS-Single-IDLVs (Figure 3.2.6c).

**Figure 3.2.6:** Testing EFS-Double-CoOp as IDLV

a. Head-to-head comparison of EFS-Single-IDLVs and EFS-Double-CoOp-IDLV. Expression of mCherry in K562 cells transduced with the ZFN IDLVs at 4 days post...
transduction (left). Y-axis represents percentage of mCherry+ cells and X-axis represents vector concentrations. Average VCN in K562 cells transduced with the ZFN IDLVs at 4 days post transduction (right). Y-axis represents average VCN cells and X-axis represents vector concentrations. Data points represent Mean ± SD.

b. Representative Surveyor nuclease assay results from K562 cells transduced with various concentrations of EFS-Single-IDLVs or EFS-Double-CoOp-IDLV are shown. Quantified %Allelic disruption is indicated by the numbers under the bands on the gel.

c. Comparing mCherry expression from EFS-Single-IDLVs and EFS-Double-CoOp-IDLV. Geometric Mean fluorescence intensity (MFI) of mCherry is plotted on the Y-axis. X-axis shows the concentrations of the IDLVs. Bars represent Mean ± SD.

3.2.7 Optimizing promoters for Double-CoOp-IDLVs

We sought to find a stronger promoter to drive high levels of gene expression from the IDLVs. We screened a panel of viral and cellular promoters for enhanced transgene expression as well as their effect on IDLV titers. The following promoters were tested: CMV (cytomegalovirus immediate early promoter), SFFV (Spleen focus forming virus LTR), MND (Myeloproliferative sarcoma virus LTR with a deleted negative regulatory region), and UBC (human ubiquitin C promoter). We replaced the EFS promoter with each of these promoters in the EFS-Double construct and packaged them as IDLVs. The unconcentrated IDLV preps were compared for their titers and expression levels in K562 cells. All the IDLV constructs yielded approximately equal titers, at 1x10^6 TU/ml, except UBC, which yielded 1x10^5 TU/ml (Figure 3.2.7a). Because of its lower titer, we excluded UBC from subsequent analysis. We transduced K562 cells with various IDLVs at equal concentrations and measured mCherry expression and VCN. We
normalized the mCherry expression by VCN. Upon comparison of mCherry expression per vector copy, we observed that MND promoter resulted in the highest expression in K562 cells (Figure 3.2.7b). Based on this comparison, we chose the MND promoter for driving expression of ZFNs from the IDLVs (MND-Double-CoOp-IDLV). K562 cells were transduced with the IDLV and analyzed for mCherry expression and VCN. MND-Double-CoOp-IDLV was able to transduce K562 cells efficiently. We also observed that MND-Double-CoOp-IDLV expresses mCherry at higher levels as compared to the EFS-Double-CoOp-IDLV (Figure 3.2.7c). We performed Surveyor nuclease assay on transduced cells and found that the MND-Double-CoOp-IDLV was able to induce allelic disruption (Figure 3.2.7d). Despite low levels of allelic disruption, we achieved successful co-delivery of two ZFN monomers from the same IDLV. To our knowledge, this is the first report of an IDLV being able to deliver two ZFN monomers.
Figure 3.2.7: **Optimizing promoters for Double-CoOp-IDLVs**

a. Vector titers obtained from IDLV constructs with different promoters. The vectors titers in TU/ml are shown on the Y-axis. X-axis shows the promoters used in the IDLV constructs.

b. Comparison of mCherry expression from different promoters. Normalized mCherry expression (%mCherry+ cells per vector) are shown on the Y-axis. X-axis shows the promoters.

c. Head-to-head comparison of EFS-Double-CoOp-IDLV and MND-Double-CoOp-IDLV. Expression of mCherry in K562 cells transduced with the ZFN IDLVs at 4 days post transduction (left). Y-axis represents percentage of mCherry+ cells and X-
axis represents vector concentrations. Average VCN in K562 cells transduced with the ZFN IDLVs at 4 days post transduction (right). Y-axis represents average VCN cells and X-axis represents vector concentrations. Data points represent Mean ± SD.

d. Representative Surveyor nuclease assay results from K562 cells transduced with various concentrations of EFS-Double-CoOp-IDLV or MND-Double-CoOp-IDLV are shown. Quantified %Allelic disruption is indicated by the numbers under the bands on the gel.

3.2.8 Testing MND-Double-CoOp-IDLV in primary hematopoietic cells

We sought to test the MND-Double-CoOp-IDLV in primary hematopoietic cells. We transduced activated T-lymphocytes with various concentrations of the IDLV and analyzed them for mCherry expression and VCN. We found the IDLV to be able to transduce T-lymphocytes with high efficiency. However, it was not able to induce detectable allelic disruption (Figure 3.2.8a). We also tested the IDLV in CB-CD34+ cells and observed high levels of transduction. Despite high transduction efficiency, the MND-Double-CoOp-IDLV was not able to induce detectable levels of allelic disruption (Figure 3.2.8b).

We hypothesized that the inability of the IDLVs to induce allelic disruption is due to lower expression in primary hematopoietic cells as compared to K562 cells. Therefore, we tested if the ZFN expression from a lentiviral vector is different between K562 cells, T-lymphocytes and CB-CD34+ cells. We transduced these cell types with EFS-Double-CoOp-LV and measured ZFN expression by western blotting for the FLAG-tag, and VCN by qPCR at 3 days post-transduction. We performed 2-fold serial dilutions on the lysate from K562 cells to obtain a range of VCN equivalents. The western blot revealed that the ZFN expression at equivalent VCN is 10-15 folds
lower in T-lymphocytes and CB-CD34⁺ cells as compared to K562 cells (Figure 3.2.8c). Surveyor nuclease assays on these cells revealed that K562 cells exhibited allelic disruption, whereas T-lymphocytes did not (Figure 3.2.8d). Presence of heterozygosity in CB-CD34⁺ prevented detection of allelic disruption, however, there was no increase in transduced cells over background. This indicates the presence of some mechanism in the primary hematopoietic cells that causes low expression from lentiviral vectors. These concerns are addressed more in detail in Chapter 4.

![Figure 3.2.8: Testing MND-Double-CoOp-IDLV in primary hematopoietic cells](image)

**Figure 3.2.8:** Testing MND-Double-CoOp-IDLV in primary hematopoietic cells

a. Transduction of human T-lymphocytes with MND-Double-CoOp-IDLV. Expression of mCherry in T-lymphocytes transduced with the MND-Double-CoOp-IDLV at 4 days post transduction (left). Y-axis represents percentage of mCherry⁺ cells and X-axis represents vector concentrations. Average VCN in T-lymphocytes cells transduced with the MND-Double-CoOp-IDLV at 4 days post transduction (right). Y-axis represents average VCN cells and X-axis represents vector concentrations. Data points represent Mean ± SD.
b. Transduction of human CB-CD34<sup>+</sup> cells with MND-Double-CoOp-IDLV. Expression of mCherry in CB-CD34<sup>+</sup> cells transduced with the MND-Double-CoOp-IDLV at 4 days post transduction (left). Y-axis represents percentage of mCherry<sup>+</sup> cells and X-axis represents vector concentrations. Average VCN in CB-CD34<sup>+</sup> cells transduced with the MND-Double-CoOp-IDLV at 8 days post transduction (right). Y-axis represents average VCN cells and X-axis represents vector concentrations. Data points represent Mean ± SD.

c. Comparison of ZFN expression from EFS-Double-CoOp-LV in hematopoietic cells. Western blot against FLAG-tag are shown. Equal amounts (50 µg each) of lysates from K562 cells, T-lymphocytes and CB-CD34<sup>+</sup> cells transduced with EFS-Double-CoOp-LV were used for the western blot. VCN corresponding to the lysates is indicated by the numbers.

d. Surveyor nuclease assay results from K562 cells, T-lymphocytes and CB-CD34<sup>+</sup> cells transduced with EFS-Double-CoOp-LV. The numbers indicate quantified %Allelic disruption.

3.2.9 Use of IDLVs as donor templates

In addition to using IDLVs to deliver ZFNs, we investigated the use of IDLVs to deliver donor templates to target cells. We inserted the 1.3 kbp donor template (as described in Chapter 2) in the IDLV construct. The donor template includes introns from ADA, and may potentially undergo splicing. To avoid splicing, the donor template was arranged in reverse direction compared to the vector genome. EFS promoter and WPRE were also added to the construct in
order to increase titers. The resulting construct was packaged as IDLV (Donor-IDLV) and used for subsequent experiments (Figure 3.2.9a).

In order to test if the Donor-IDLV can deliver donor templates to K562 cells, we nucleofected K562 cells with plasmid containing the ZFNs and transduced them with the IDLV 24 hours later. We measured VCN as well as gene modification frequencies from these cells at 4 days post-transduction. Donor-IDLV showed ability to transduce K562 cells efficiently as determined by the VCN. We also observed gene modification frequencies of up to 0.2% using this delivery system (Figure 3.2.9b).

We hypothesized that the low gene modification frequencies are due to low ZFN activity. In order to increase ZFN activity, we increased the amount of ZFN plasmid to be nucleofected in K562 cells and transduced them with a fixed concentration of the Donor-IDLV. We observed a dose-dependent increase in gene modification frequencies with increasing amounts of ZFN plasmid. We could achieve up to 1% gene modification, indicating that the donor delivery by IDLV can yield gene modification levels comparable to plasmids (Figure 3.2.9c).

To investigate if IDLVs could be used to deliver ZFNs and donor templates simultaneously, we co-transduced K562 cells with EFS-Single-IDLVs and the Donor-IDLV. Despite high efficiency of transduction as indicated by VCN, we observed very low levels of gene modification. This could be due to the requirement of co-transduction with three IDLVs. Therefore, we co-transduced K562 cells with MND-Double-CoOp-IDLV and Donor-IDLV. Similar to the triple transduction, these cells were transduced at high levels. However, the gene modification values were not significantly higher (Figure 3.2.9d).
Figure 3.2.9: Use of IDLVs as donor templates


b. Gene modification in K562 cells using Donor-IDLV. Gene modification frequencies in K562 cells nucleofected with 2.5µg of ZFN plasmid and followed by transduction with various concentrations of Donor-IDLV 24 hours later (top). Gene modification frequencies are measured at 4 days post transduction. Y-axis represents % gene
modification, X-axis represents vector concentrations. Average VCN in K562 cells nucleofected with 2.5µg of ZFN plasmid and followed by transduction with various concentrations of Donor-IDLV 24 hours later (bottom). Y-axis represents average VCN at 4 days post transduction, X-axis represents vector concentrations. Bars and data points represent Mean ± SD.

c. Gene modification in K562 cells using Donor-IDLV. Gene modification frequencies in K562 cells nucleofected with various amounts of ZFN plasmid and followed by transduction with 3x10^7 TU / ml of Donor-IDLV 24 hours later (top). Gene modification frequencies are measured at 4 days post transduction. Y-axis represents % gene modification, X-axis represents nucleofection conditions. Average VCN in K562 cells nucleofected with various amounts of ZFN plasmid and followed by transduction with 3x10^7 TU / ml of Donor-IDLV 24 hours later (bottom). Y-axis represents average VCN at 4 days post transduction, X-axis represents nucleofection conditions. Bars and data points represent Mean ± SD.

d. Gene modification in K562 cells with ZFN and donor delivery by IDLVs. Gene modification frequencies in K562 cells co-transduced with various concentrations of EFS-Single-IDLVs or MND-Double-CoOp-IDLV and 3x10^7 TU / ml of Donor-IDLV, measured at 4 days post transduction (left). Y-axis represents gene modification frequencies, X-axis represents concentrations of ZFN-IDLVs. Bars represent Mean ± SD.
3.3 Discussion

In this study, we focused on IDLV-mediated delivery as this method has multiple potential advantages over other delivery methods: (i) IDLVs pseudotyped with VSV-G can efficiently transduce virtually every mammalian cell type, (ii) IDLVs exhibit low cytotoxicity, (iii) IDLVs can deliver transgenes in a transient manner. One of the most important advantages of IDLVs is that lentiviral vectors have already been used in clinical trials for gene therapy. Methods to scale-up production of lentiviral vectors are well established for clinical trial scale vector preparations and IDLVs production would use similar methods. In summary, IDLVs potentially offer advantages over other methods of gene delivery.

IDLVs were previously shown to deliver ZFNs and donor templates successfully, although at relatively low efficiency in primary cells (Lombardo et al., 2007). Studies using IDLVs have reported efficacy delivering one ZFN per vector, requiring co-transduction by two vectors to ensure ZFN delivery. The use of IDLVs for delivering two ZFNs in the same vector has shown lack of efficacy, although the reasons for it have not been investigated thoroughly. In this study, we identified potential concerns with using IDLVs to deliver ZFNs and donor templates and demonstrated a significant improvement in the design and usage of IDLVs for ZFN delivery and. We tested IDLVs to deliver either one or two ZFNs. The IDLVs delivering one ZFN per vector, EFS-Single-IDLVs, were able to deliver ZFNs to cell lines. However, they exhibited lower efficiency of activity in primary cells, corroborating previous reports using IDLVs.

Transduction with two IDLVs has potential drawbacks as a ZFN delivery system, especially in the stoichiometry of the two ZFN monomers. The optimal stoichiometry of ZFN monomers is 1:1, which might not be attained with co-transduction of two IDLVs. To ensure the production of
equivalent amounts of both ZFN monomers, we linked them with 2A peptides. We constructed an IDLV to co-deliver two ZFNs (EFS-Double-IDLV). However, we found that despite comparable rates of transduction, EFS-Double-IDLV failed to deliver functional ZFNs. We further investigated this phenomenon and found that the IDLV underwent recombination due to regions of sequence identity between the two ZFNs. The presence of repeat regions was reported previously to induce recombination in the HIV genome (Fuentes et al., 1996; Basu et al., 2008). The mechanism of recombination mediated by genomic repeats is thought to occur during reverse transcription in an inter-molecular fashion, often giving rise to deletion of the regions between the two repeats. We found that in the EFS-Double-IDLV construct, the recombination events were potentially due to the FLAG-tags and NLSs present in both ZFN constructs. This intra-vector recombination is clearly an important factor limiting the use of IDLVs for ZFN delivery, as most of the ZFN architectures include the FLAG-tag as part of the open reading frame and all have incorporated NLS into the ZFN protein to mediate their nuclear import for access to the genome as well as the FokI endonuclease sequences. Occurrence of repeat sequences in vectors is also a potential concern for viral delivery of transcription activator-like effector nucleases (TALENs). TALENs are comprised of 33-35 amino acids long Repeat-Variable Di-residue (RVD) repeats (Cermak et al., 2011) and would be expected to be highly prone to recombination if used in viral vectors. To deliver either ZFNs or TALENs via viral vectors, it is essential to develop measures to reduce recombination.

In order to minimize recombination, we modified the EFS-Double-IDLV construct in two ways. One of the modifications, codon-optimization of the sequence of one left-ZFN in the EFS-Double-CoOp construct, minimized the recombination, leading to successful ZFN delivery from the same vector. The EFS-Double-CoOp construct, when packaged as IDLV, did not exhibit
allelic disruption in K562 cells. We replaced the EFS promoter with the MND promoter to boost expression in hematopoietic cells. The resulting construct, MND-Double-CoOp-IDLV, was able to induce allelic disruption in K562 cells. The MND-Double-CoOp-IDLV was tested in primary T-lymphocytes and CB-CD34+ cells, but failed to induce allelic disruption. This could potentially be due to insufficient expression of the ZFNs from the IDLV genomes.

Gene expression from IDLVs is lower than other delivery methods and might not be sufficient for every system. The main reasons for low gene expression could be: 1) Low vector copy number per cell, 2) Limited persistence of vector copies and 3) Reduced expression per vector copy. We observed that we could get vector copy numbers of up to 50 per cell. In the experiments with the integrating vectors, an average VCN of 1 per cell was sufficient to achieve detectable allelic disruption by ZFNs. However, since the expression from IDLVs is 10-100 times lower, we anticipate that copy numbers of 10-100 per cell would be sufficient for ZFN delivery. Nevertheless, in primary hematopoietic cells, even at VCN values in that range, we did not detect allelic disruption. VCN can be increased by sequential repeated transductions. Unfortunately, certain primary cell types, especially HSPCs begin to differentiate if cultured ex vivo for longer than 2-3 days. At equivalent numbers of vector copies, the expression of ZFNs was markedly lower in T-lymphocytes and CB-CD34+ cells as compared to K562 cells. The reasons for obtaining lower expression in primary cells are not entirely clear and beyond the scope of this paper. Gene expression from IDLVs can possibly be enhanced by treating transduced cells with proteasome inhibitors. Combining multiple ways to increase gene expression from IDLVs can potentially increase their efficacy as a delivery method for ZFNs.

In addition to delivering ZFNs, we demonstrated that IDLVs could also deliver donor templates efficiently. We used an IDLV to deliver donor templates in conjunction with ZFN delivery by
either nucleofection or IDLVs. Using nucleofection to deliver ZFNs, we could achieve levels of gene modification with Donor-IDLV comparable to those obtained by nucleofection of donor plasmid. We showed that the use of IDLVs for donor delivery could be advantageous as it permits use of high amounts of ZFN plasmids if nucleofection is used for ZFN delivery. However, co-delivery of ZFNs and donors by IDLVs is not as efficient as using nucleofection for ZFN delivery. Analysis of vector copy numbers indicates that when a fixed amount of ZFN plasmid is nucleofected, there is an optimal value of Donor-IDLV VCN, which maximized gene modification, whereas, at the same amount of Donor-IDLV, with increasing ZFN plasmids, it is possible to increase the efficiency of gene modification. We also demonstrated gene modification using IDLV delivery of both ZFNs and donor templates. However, the absolute values were much lower than those obtained by ZFN delivery by nucleofection were. This could be a compound effect of the lower efficiency of the IDLVs combined with the requirement of co-transduction of two or three vectors. Hence, based on our results, we can speculate that ZFN expression is the greater limiting factor in the context of IDLV delivery.

Although we demonstrate successful use of IDLVs for ZFNs, their efficiency is sub-optimal in primary hematopoietic cells. Further improvements in vector constructs may be necessary to increase the efficiency of IDLVs for ZFN delivery. We also showed that IDLVs could work efficiently as donor templates. These results highlight the promise of IDLV based gene delivery of ZFN mediated site-specific genome modification.
3.4 Materials and methods

3.4.1 Cell lines and culture

K562 Cells (CRL-243\textsuperscript{TM}, ATCC) were cultured in RPMI1640 (Cellgro, Manassas, VA) supplemented with 10% Fetal Bovine Serum (Gemini Bio-products, West Sacramento, CA) and Penicillin/Streptomycin/L-glutamine (Gemini Bio-products, West Sacramento, CA). Human umbilical cord blood samples were collected from UCLA Ronald Reagan Medical Center and have been deemed exempt from review from IRB as anonymous medical waste. Mononuclear cells (MNCs) were isolated from cord blood by Ficoll-Paque (GE Healthcare Biosciences, Piscataway, NJ) based separation. CD34\textsuperscript{+} cells were isolated from the MNCs using MACS CD34\textsuperscript{+} enrichment kit (Miltenyi Biotec Inc, Auburn, CA).

Adult human peripheral blood mononuclear cells were obtained from the Center for AIDS Research Virology Core Lab that is supported by the National Institutes of Health award AI-28697 and by the UCLA AIDS Institute and the UCLA Council of Bioscience Resources. PBMCs were activated using Dynabeads\textregistered Human T-Activator CD3/CD28 (Life Technologies, Green Islands, NY) for 3 days and cultured subsequently with RPMI1640 (Cellgro, Manassas, VA) supplemented with 10% Fetal Bovine Serum (Gemini Bio-products, West Sacramento, CA) and Penicillin/Streptomycin/L-glutamine (Gemini Bio-products, West Sacramento, CA) and 5 ng/ml Interleukin-2 (R&D System Inc, Minneapolis, MN). HEK293T (CRL-11268\textsuperscript{TM}, ATCC) and HT-29 (ATCC HTB-38\textsuperscript{TM}) cells were maintained in DMEM (Cellgro, Manassas, VA) supplemented with 10% Fetal Bovine Serum (Gemini Bio-products, West Sacramento, CA) and Penicillin/Streptomycin/L-glutamine (Gemini Bio-products, West Sacramento, CA). Genomic DNA from cells was extracted using PureLink Genomic DNA mini kit (Life Technologies,
Green Islands, NY) and quantified using NanoVue (GE Healthcare Biosciences, Piscataway, NJ). Cells were monitored for mCherry expression on BD-LSRFortessa or BD-LSRII flow cytometers (Becton-Dickinson, Franklin lakes, NJ). Cell counts were measured using a Beckman-Coulter ViCell-XR automated cell counter (Beckman-Coulter, Indianapolis, IN).

3.4.2 **Generation of vector constructs**

The ZFNs targeting human ADA intro 6 were produced at Sangamo Biosciences Inc. Single ZFN IDLV coding sequence was constructed using spliced-overlap PCR. ZFN-P2A was amplified from pVAX-ZFN using primers Flag-Fwd (5’ATGGACTACAAAGACCATTGACGG3’) and P2A-Fok-Rev (5’CTCCGCTTCCGGATCTCTTTGCTCGGAAGTTGATCTCGCCGTTGTT’). P2A-mCherry was amplified from pmcherry (Clontech Laboratories, Inc., Mountain View, CA) using primers P2A-mCh-Fwd (5’CATGTATCCTCCTCGCCCTTGCTCACCATGGATCCCCGGTA CCG3’) and mCh-Rev (5’ CGCGGAATTCCGACTTGTACAGCTCGTCCATGC3’). ZFN-P2A-mCherry was amplified using the Flag-Fwd and mCh-Rev primers using an equimolar mixture of ZFN-P2A and P2A-mCherry amplicons as a template. ZFN-P2A-mCherry amplicons were cloned into the EcoRI site of pCCL-EFS-x-WPRE. For Double-IDLV, ZFN-L-T2A was amplified using Flag-Fwd and T2A-Fok-Rev (5’CCTAGGGCCGCGATTCTCCTCACCAGTCACCACAGCATGTTAAAGACTTCTCTGCCCCTC TCGCCGCCCAGATCTGAGTTGATCTCGCGGTTGTT3’); T2A-ZFN-R-P2A-mCherry was amplified from pCCLc-EFS-ZFN-R-P2A-mCherry-WPRE using T2A-Flag-Fwd (5’AGAATCCGCCGCCCTTAGGATGGACTACAAAGACCATTGACGG3’) and mCh-Rev. ZFN-L-T2A and T2A-ZFN-R-P2A-mCherry were cloned into pCCLc-EFS-x-WPRE using EcoRI and AvrII. Double-dF-IDLV constructs were made from Double-IDLV constructs using InFusion
HD cloning system (Clontech Laboratories, Inc., Mountain View, CA). The 3xFLAG-tag (5’-DYKDHDGDYKHDIDYKDDDDK-3’) was deleted from the left-ZFN, but was kept intact in the right-ZFN. Double-CoOp-Constructs were designed using GeneArt gene synthesis system (Life Technologies, Green Islands, NY). The left-ZFN, including 3xFLAG-tag, NLS, zinc finger motifs and FokI domain was codon optimized. The codon-optimized region was synthesized in overlapping 500bp gBlocks fragments (Integrated DNA Technologies, Coralville, IA). The gBlocks were fused using spliced overlap PCR using primers Frag1-Fwd (5’AGGTGTCGTGACGCGGGATCTCGA3’) and Frag3-Rev (5’CCTAGGGCCAGGTTCTCTTCCAC3’) and cloned into Double-IDLV constructs using XhoI-BmgBI (New England Biolabs, Ipswich, MA). For the vectors with different promoters, InFusion HD cloning system was used (Clontech Laboratories, Inc., Mountain View, CA). For Donor-IDLV constructs, the previously described 1.3 kb donor template was amplified using X7-F1-Eco (5’CGCGGAATTCAGGGACTCCTGCTTCCTATGCG3’) and X7-R2-Eco (5’CGCGGAATTCCTGCTTCTGGCTGTGATTTGC3’) and cloned in the reverse orientation into pCCLc-EFS-x-WPRE using EcoRI.

3.4.3 **Vector packaging and titer determination**

For lentiviral vector production on a small scale, 5x10⁶ HEK293T cells were seeded on poly-L-Lysine (Sigma) coated petri dishes (VWR) in culture medium. The cells were transfected 24 hours later using TransIT-293 with 5 µg pCMV-R8.2int(-), 1 µg pCAGGS-VSVG and 5 µg of transfer plasmid according to manufacturer’s protocol. At 16 hours after transfection, the cells were incubated with the culture medium with 10 mM sodium butyrate (Sigma) and 20 mM HEPES (Life Tech) for 8 hours. The culture medium was replaced with fresh medium with 20 mM HEPES (Life Tech). The cell-free supernatant was harvested 48 hours later and filtered.
through 0.45 micron filters (??). The viral supernatants were stored at -80°C. For vector production and concentration on a large scale as well titer determination by qPCR, the protocols demonstrated in Cooper et al were used.

3.4.4 Transduction of cell lines and primary cells with IDLVs

K562 cells, 1x10^5 per sample, were centrifuged at 90g for 10 minutes and resuspended in 50 µl of culture medium per sample. Appropriate dilutions of viruses were made in culture medium in 50 µl. The diluted virus was added to cells in 48-well tissue culture treated plates (Corning, Corning, NY). Two days post transduction, the cells were transferred to 6-well tissue culture treated plates (Corning, Corning, NY) with 2 ml of culture medium.

CB-CD34+ cells were pre-stimulated on Retronectin (Clontech Laboratories, Inc., Mountain View, CA) coated 48-well non-tissue culture treated plates (Corning, Corning, NY) with X-Vivo15 (Lonza, Walkersville, MD) supplemented with 50 ng/ml Stem Cell Factor (Amgen, Thousand Oaks, CA), 50 ng/ml Flt3-Ligand (R&D System Inc, Minneapolis, MN) and 50 ng/ml Thrombopoietin (R&D System Inc, Minneapolis, MN) for 18 hours at 1x10^5 cells/ml . CB-CD34+ cells were transduced with appropriate dilutions of the virus in X-VIVO15. At 48 hours post-transduction, cells were transferred to tissue culture treated 24-well tissue culture treated plates (Corning, Corning, NY) and maintained in IMDM (Cellgro, Manassas, VA) supplemented with 20% Fetal Bovine Serum (Gemini Bio-products, West Sacramento, CA) and 25 ng/ml Stem Cell Factor (Amgen, Thousand Oaks, CA), 10 ng/ml Interleukin-6 (R&D System Inc, Minneapolis, MN) and 5 ng/ml Interleukin-3 (R&D System Inc, Minneapolis, MN).

Activated T-lymphocytes were resuspended to disrupt clumps and 1x10^5 cells per sample were centrifuged at 300g for 5 minutes. The cell pellets were resuspended in 50 µl culture medium
with 10 ng/ml rhIL2. Virus dilutions were made in culture medium without IL-2 and added to the cells to achieve a final rhIL2 concentration of 5 ng/ml. The transductions were performed in 48-well tissue culture treated plates (Corning, Corning, NY). Two days post transduction, the cells were transferred to 12-well tissue culture treated plates (Corning, Corning, NY) in 1 ml of culture medium with 5 ng/ml rhIL2.

3.4.5 Nucleofection followed by transduction

K562 cells were nucleofected using Nucleofector 4DTM (Lonza, Walkersville, MD) as per the recommended protocol. Briefly, 1x106 cells per sample were centrifuged at 90g for 10 minutes. The pellets were resuspended in 100 µl of Cell line SF solution. Appropriate amounts of DNA were added to the cells and the mixture was nucleofected using the recommended program. The cells were allowed to recover at room temperature for 10 minutes and then supplemented with culture medium and transferred to 6-well tissue culture treated plates (Corning, Corning, NY). At 24 hours post-transduction, 1x10^5 cells per sample, were centrifuged at 90g for 10 minutes and resuspended in 50 µl of culture medium per sample. Appropriate dilutions of viruses were made in culture medium in 50 µl. The diluted virus was added to cells in 48-well tissue culture treated plates (Corning, Corning, NY). Two days post transduction, the cells were transferred to 6-well tissue culture treated plates (Corning, Corning, NY) with 2 ml of culture medium.

3.4.6 Western blot for analysis of ZFN expression

ZFN expression was monitored by western blotting for FLAG-tag. Lysates from cells transduced with the ZFN vectors were produced using Denaturing cell extraction buffer (Life Technologies, Green Islands, NY) supplemented with Complete Mini protease inhibitor tablets (Roche Applied Science, Indianapolis, IN). Cells were centrifugated at 500g for 10 minutes at 40C and the
supernatant was aspirated. The cell pellets were resuspended in 50 µl lysis buffer per 1x106 cells and incubated on ice for 45 minutes with frequent vortexing. The lysate was centrifuged at 16,000g for 20 minutes at 4°C. The supernatant was transferred to fresh tubes and stored at -20°C until further use. The protein quantities were estimated using BCA assay (Thermo scientific, Rockford, IL) and read on a Tecan Infinite M1000 Microplate Reader (Tecan, Morrisville, NC). Equal amounts of protein lysate (40 µg per lane) combined with Laemelli sample buffer (BioRad, Hercules, CA) were loaded on 4-12% bis-tris SDS-PAGE gels (Life Technologies, Green Islands, NY). The gel was run at 150V for 1 hour in 1X MOPS-SDS running buffer (Life Technologies, Green Islands, NY) in a Novex gel electrophoresis unit (Life Technologies, Green Islands, NY). Prism Ultra (Abcam, Cambridge, MA) protein standards were loaded for size determination. The proteins were transferred from the gel to a PVDF membrane (Life Technologies, Green Islands, NY) in 1X Novex transfer buffer with 10% methanol (Life Technologies, Green Islands, NY). The transfer was carried out at 30V for 1 hour at room temperature. After the transfer, the membrane was blocked using 5% non-fat milk in PBS-Tween20 for 30 minutes at room temperature. After blocking, the membrane was incubated with 1:1000 diluted Mouse anti-FLAG primary antibody (Sigma-Aldrich, St. Louis, MO) overnight at 4°C. The membrane was then washed three times with PBS-Tween20 and incubated with 1:5000 dilution of Goat anti-mouse IgG-HRP secondary antibody (Sigma-Aldrich, St. Louis, MO) for 3 hours at room temperature. The membrane was washed again three times with PBS-Tween20. The membrane was incubated at room temperature with ECL2 reagent (Thermo scientific, Rockford, IL) for 5 minutes and imaged on Typhoon FLA 9500 phosphorimager (GE Healthcare Biosciences, Pittsburgh, PA).
4.1 Introduction

4.1.1 Chromatin and histone modifications

In eukaryotic cells, DNA is bound by positively charged histone proteins in structures called nucleosomes. Nucleosomes comprise of a histone tetramer containing one molecule each of H2A, H2B, H3 and H4, bound to ~220 bp of DNA. The inter-nucleosomal region is bound by histone H1. Apart from structural role in nuclear organization, Histones play an important role in regulation of transcription of the bound DNA. Each of the histones consists of an N-terminal tail that has several residues that can undergo post-translation modifications. Histone modifications, especially on H3 and H4, play an important role in regulating transcription of bound DNA (reviewed in Berger, 2002). Two major types of post-translational modifications are known to regulate transcription; Methylation on Lysine and Arginine and Acetylation on Lysine. Methylated residues, on histones can act as recruitment points for transcriptional activators or repressors (reviewed in Shilatifard, 2006). Histone methylation can play different roles and can be associated with either active or inactive chromatin. Methylation is brought about by HMTs (histone methyltransferases) and can be reversed by HDMs (histone demethylases) (reviewed in Black et al, 2012). On the other hand, acetylated residues decrease the net positive charge on histones, thereby reducing the electrostatic binding with DNA, making it more accessible. Therefore, histone acetylation is usually associated with active chromatin. Histone acetylation is carried out by HATs (Histone acetyltransferases) and can be reversed by HDACs (Histone deacetylases) (reviewed in Annunziato and Hansen, 2012). A summary of histone modifications and their modifying enzymes is provided in Figure 4.1.1.
Figure 4.1.1: Post-translational modifications of human histone proteins and the enzymes responsible for them (Kouzarides, 2007).

Post-translational histone modifications are carried out by several classes of enzymes. A summary of known histone modifications and the enzymes responsible for them is shown in Figure 4.1.1. These modifications can modulate the accessibility of the DNA bound to the histones. In addition to modifying the accessibility, histone post-translational modifications are
responsible for recruiting transcriptional regulators, such as activators and repressors, as well as chromatin remodeling proteins. Histone acetylation and deacetylation play an important role in transcriptional regulation. There is a constant turnover of histone acetylation mediated by HATs and HDACs. Histone acetylation at a promoter is often an indicator of the activity from that promoter. Active promoters are enriched in H3K9Ac and other acetylation marks, whereas silent promoters are depleted for these marks. Promoter activation and silencing is largely carried out by HATs and HDACs. In viral infection of cells, viral DNA can also be modified by HDACs and HATs in order to modulate transcription from it (reviewed in Kouzarides, 2007; Banister and Kouzarides, 2011).

4.1.2 Viral DNA and Chromatin

In cells infected with DNA viruses such as Adenovirus-5 (Ad5), Herpes Simplex Virus (HSV), and Cytomegalovirus (CMV), the viral DNA exists as double stranded DNA. Viral dsDNA undergoes chromatinization immediately upon entry into the nucleus (Knipe et al, 2013, Takacs et al, 2010). Chromatinization of viral DNA enables regulation of its transcription by histone modifications. Viral chromatin can be regulated by cellular HMTs, HATs and HDACs. A summary of viral chromatin regulation is provided in Figure 4.1.2.

![Viral chromatin modifying complexes](Lieberman, 2008)
One of the mechanisms of viral chromatin regulation, especially promoter silencing, is through POD complexes. Once in the nucleus, viral DNA localizes to POD protein complexes that include promyelocytic leukemia-associated (PML) protein and transcriptional repressors Sp100 and DAXX (Murges et al., 2001). DAXX is shown to recruit HDAC1 and HDAC2 to POD complexes. DAXX and Sp100 contribute to transcriptional repression of viral DNA by POD complexes. Viral proteins such as HSV protein ICP0 and CMV protein pp71 target the POD complex proteins for degradation. Viruses lacking these proteins are transcriptionally inactive and are marked by histone deacetylation and condensed chromatin.

Retroviruses, including lentiviruses, enter the nucleus as double-stranded DNA enclosed in the pre-integration complexes (PICs). PICs are known to interact with multiple chromatin associated proteins including LEDGF/p75 and SWI/SNF chromatin remodeling proteins (Cherepanov et al., 2003; Agbottah et al., 2006). DNA from PICs is potentially susceptible to heterochromatinization. However, because DNA from PICs integrates efficiently into the host genome, chromatinization of PICs has not been well characterized. Recent reports suggest that HIV-1 DNA is heterochromatinized while in PICs. HIV-1 silencing is a well-studied phenomenon that gives rise to viral latency (Dahabieh et al., 2013; Schulze-Forster et al., 1990). In latent HIV-1 infection, the provirus is enriched for silencing chromatin marks and thus transcriptionally inactive. This is of concern for the use of lentiviral vectors (LVs). Studies have shown that proviral DNA from LVs can be susceptible to silencing upon integration (Haas et al., 2003). In case of IDLVs, the viral DNA remains in an episomal form and hence is prone to epigenetic silencing. Reports from Pelascini et al (Pelascini et al., 2013) suggest that IDLV genomes are enriched in transcriptionally repressive chromatin marks. Silencing of episomal genomes could significantly reduce gene expression from IDLVs. Therefore, we sought to
modulate silencing of IDLV genomes using small molecules. Since epigenetic silencing is carried out predominantly by HDACs, we investigated the use of HDAC inhibitors (HDACi) for enhancing transgene expression from IDLVs.

4.1.3 **HDAC Inhibitors and HIV**

Histone Deacetylase Inhibitors (HDACis) are small molecule compounds that can inhibit the catalytic activity of one or more classes of HDACs (reviewed by Drummond *et al.*, 2005). HDACis are known to have widespread effects on cellular transcription as well as on HIV-1 transcription (reviewed in Rasmussen *et al.*, 2013; Matalon *et al.*, 2011). Among various HDACis, VPA, Trichostatin A (TSA), Vorinostat (VST) and Givinostat (GST) are undergoing studies for HIV reactivation. These HDACis were originally discovered as agents to treat unrelated diseases. VPA is used as an anti-convulsant and mood-stabilizing drug (Lance and Anthony, 1975); TSA is used as an anti-fungal antibiotic (Vanhaecke *et al.*, 2004); VST is currently marketed for treatment of cutaneous T-cell lymphoma (CTCL) (Duvic *et al.*, 2007) and GST is used as salvage therapy for multiple malignancies (Passamonti *et al.*, 2012). Studies have shown that HDACis can reactivate silenced HIV-1 proviruses (Matalon *et al.*, 2011). Valproic acid (VPA) was shown to be a potent agent for reactivation of HIV-1 in human CD4+ T-cells. Treatment of latently infected T-cells with VPA activated viral production and thus made the virus-producing cell sensitive to anti-retroviral therapy (ART). This approach is under investigation for eradicating HIV reservoirs in patients. Combined treatment with ART and VPA was shown to be effective in flushing out latent HIV reservoirs in patients. Reactivation of latent HIV by HDACis was shown to be due to reversal of silencing epigenetic marks (Matalon *et al.*, 2011). Based on these results, we hypothesized that HDACis can be used to reverse or prevent silencing of lentiviral genomes, specifically in the context of IDLVs. A recent report showed that treating cells with TSA and
Sodium butyrate increased expression from IDLVs by reducing silencing marks (Pelascini et al, 2013). We are investigating if this effect can be exploited for increasing the efficiency of IDLVs for ZFN delivery.
4.2 Results

4.2.1 Effect of HDAC inhibitors on GFP expression from IDLVs

In order to test if treatment by HDAC inhibitors can influence gene expression from IDLVs, we transduced K562 cells with an IDLV encoding an eGFP reporter under the control of the MND promoter (MND-eGFP-IDLV) in the presence of various concentrations of the HDAC inhibitors Valproic Acid (VPA) and Sodium Butyrate (NaBu). GFP expression from the cells was measured by flow cytometry at 4 days post-transduction, at which point the treatment with HDAC inhibitors was stopped. The cells treated with both the HDAC inhibitors showed an increase in GFP expression. The effect of NaBu was the highest at 0.5 mM, whereas that of VPA was the highest at 1 mM. VPA also showed a higher increase in GFP expression as compared to NaBu. The cells treated with NaBu did not proliferate after withdrawal of treatment (Figure 4.2.1). The cells treated with VPA did recover from the treatment and resumed proliferation. These preliminary results showed that HDACis could be potentially used to increase gene expression from IDLVs. Since VPA showed less severe effect on cell proliferation and a higher increase in GFP expression than NaBu, we investigated the use of VPA for further experiments.

Figure 4.2.1: Effect of HDAC inhibitors on GFP expression from IDLVs
Effect of HDACi on GFP expression from MND-eGFP-IDLV in K562 cells (left). The Y-axis represent geometric mean fluorescence intensity of GFP in K562 cells at 4 days post-transduction. The concentration of each HDACis is indicated on the X-axis. Effect of HDACis on cell expansion (right). The Y-axis shows total viable cell count. The X-axis shows days post-transduction. The arrow indicates the time point at which HDACi treatment was withdrawn.

4.2.2 Optimization of VPA concentrations for K562 cells

We sought to optimize the concentration of VPA in order to maximize its effects on IDLV expression. K562 cells were transduced with MND-eGFP-IDLV at 4x10^8 TU/ml and treated with various concentrations of VPA, ranging from 0.1 mM – 10 mM. The cells were assessed 3 days later by flow cytometry for GFP expression. VPA treatment was withdrawn at day 3 and the cells were cultured until 9 days post-transduction, followed by flow cytometry. Geometric mean fluorescent intensity (GeoMFI) at 3 days post-transduction indicated a dose-dependent increase in GFP expression with increasing concentrations of VPA. The highest increase in GFP GeoMFI was seen in cells treated with 1 mM VPA. This effect was reversible, as the expression of GFP returned to background levels once the treatment was withdrawn, indicated by GeoMFI at day 9 post-transduction (Figure 4.2.2a).

Although VPA treatment induced an increase in GFP expression in transduced cells, it severely affected the cell expansion as well as viability. At 3 mM and 10 mM, cell counts were below the limit of detection, indicating severe toxicity of VPA. At 1 mM, cell expansion was significantly limited; however, the viability was slightly affected (Figure 4.2.2a). This indicated that out of the various VPA concentrations, 1 mM is the optimal concentration.
We sought to optimize the VPA concentration further by examining a narrow interval around 1 mM. To that end, we transduced K562 cells with MND-eGFP-IDLV and treated them with 0.5 mM – 2.5 mM VPA. The cells were analyzed at 3 days post-transduction by flow cytometry. GeoMFI of GFP indicated a dose-dependent increase in GFP expression, corroborating previous results. However, at 1.5 mM of VPA, the GFP expression declined, indicating that 1 mM is the optimal concentration (Figure 4.2.2b). K562 cells treated with 2 mM and 2.5 mM VPA exhibited significant toxicity, indicated by viable cell counts below the detection limit. Based on these results, we treated cells with 1 mM VPA for subsequent experiments.

Figure 4.2.2: Optimization of VPA concentrations for K562 cells

a. Testing VPA concentrations from 0.1 mM to 10 mM in K562 cells. Effect on GFP expression (left), as measured by GFP GeoMFI at 3 days post-transduction (black bars). GFP geoMFI at 9 days post-transduction is indicated by solid grey bars Y-axis represents fold-expansion in 3-
days. Effect on cell viability (right). Y-axis represents % viable cells at 3 days post-transduction. Bars represent Mean ± SD, n=3.

b. Testing VPA concentrations from 0.5 mM to 2.5 mM in K562 cells. Effect on GFP expression (left), as measured by GFP GeoMFI at 3 days post-transduction. Y-axis represents GFP geoMFI. Effect on cell viability (right). Y-axis represents % viable cells at 3 days post-transduction. Bars represent Mean ± SD, n=3.

4.2.3 Kinetics of VPA action

We sought to determine the period in which VPA exerts its effects of gene expression from IDLVs. We transduced K562 cells with MND-eGFP-IDLV at 4x10^8 TU/ml and treated them with 1 mM VPA. We analyzed the cells for GFP expression everyday up to 4 days post-transduction. The GFP expression in untreated cells increased with time, reaching a plateau at 4 days post-transduction. The increase in GFP expression due to VPA treatment was evident at 2 days post-transduction, and reached the maximum a day later. This indicated that VPA treatment for 3 days was required for maximal increase in transgene expression from IDLVs (Figure 4.2.3a). The cell viability was not affected at any time point, however, the cells expansion as reduced as compared to untreated cells (Figure 4.2.3a).

In order to determine the duration of VPA treatment needed to obtain the increase in expression from IDLVs, we performed a series of time-course experiments, as illustrated by Figure 4.2.3b. We transduced K562 cells with MND-eGFP-IDLV in presence of VPA and stopped the treatment at various time points (VPA-removal series). Alternately, we transduced K562 cells with MND-eGFP-IDLV in absence of VPA, and started the treatment at various time points
(VPA-addition series). We analyzed the cells for GFP expression at 4 days post transduction. The cells in the VPA-addition series indicated that the effect of VPA treatment decreased with each day of delay in VPA addition. If VPA was added at 2 days or later post-transduction, it did not induce an increase in GFP expression. The cells in the VPA-removal series indicated that the effect of VPA treatment was absent if it was removed before 2 days post-transduction (Figure 4.3.2b). Taken together, these two series suggest that treatment of K562 cells with VPA for 2 days post-transduction is both necessary and sufficient for increasing GFP expression.

Figure 4.2.3: Kinetics of VPA action.
a. Time-course of VPA action on K562 cells transduced with MND-eGFP-IDLV. Effect of VPA on GFP expression (left). Y-Axis represents GFP geoMFI in cells treated with VPA (black bars) or in untreated cells (white bars). Effect of VPA on cell viability (center). Y-Axis represents % viable cells upon treatment with VPA (black bars) or no VPA (white bars). Effect of VPA on cell expansion (right). Y-Axis represents fold-expansion of treated with VPA (black bars) or in untreated cells (white bars). X-axes represent days post-transduction. Bars represent Mean ± SD, n=3.

b. Necessary and sufficient duration of VPA treatment in K562 cells. Schematics of the addition series (top left) and the removal series (top right) are shown. The time of VPA addition is indicated by ‘VPA’, the time of VPA removal is indicated by ‘remove’. The arrows indicate time of transduction and of flow cytometry. The red text inside the arrows indicates the day post-transduction of either addition or removal of VPA. The effect of various times of VPA addition and removal on GFP expression (bottom). Y-axis represents GFP geoMFI in cells treated with VPA at 4 days post-transduction. Bars represent Mean ± SD, n=3.

4.2.4 Effects of pre-treatment of cells with VPA

We sought to determine if treating K562 cells with VPA prior to transduction would result in similar effects on expression from IDLVs. We treated K562 cells with 1 mM VPA for up to 3 days before transduction. Equal cell numbers from each sample were transduced with MND-eGFP-IDLV. The cells were incubated for 3 days either in presence or in absence of 1 mM VPA. The cells were analyzed by flow cytometry for GFP expression at the end of the treatment. We found that if VPA treatment was withdrawn post-transduction, pre-treatment for up to 3 days produced a slight increase in GFP expression (up to 1.7 fold). However, if VPA treatment was
continued post-transduction, GFP expression from the IDLV increased by up to 3.5-4 fold. This effect was dependent on the duration of pre-treatment. With longer pre-treatment times, fold-increase in GFP expression was higher (Figure 4.2.4a). However, the cells suffered from severe reduction in proliferation. Although cell viability was not significantly affected, cell expansion decreased with increasing duration of pre-treatment. At 3 days of pre-treatment, the cells expansion reduced by half (Figure 4.2.4b). Due to this reduction in cell expansion, pre-treatment with VPA was not continued for further studies.

Figure 4.2.4: Effects of pre-treatment of cells with VPA

a. a. Effect of pre-treatment on GFP expression from MND-eGFP-IDLV in K562 cells. Schematic of pre-treatment (left). The time of VPA addition is indicated by ‘VPA’, the arrows indicate time of transduction and of flow cytometry. The red text inside the
arrows indicates the days pre-transduction. GFP expression from K562s pre-treated with VPA (right). Y-axis represents GFP geoMFI in cells treated with VPA at 4 days post-transduction. White bars indicate cells not treated with VPA post-transduction, Black bars indicate cells treated with VPA post-transduction. Bars represent Mean ± SD, n=3.

b. Effect of pre-treatment on cell expansion in K562 cells. Y-Axis represents fold-expansion of cells treated with VPA post-transduction (black bars) or in untreated cells (white bars). X-axes represent days of pre-treatment. Bars represent Mean ± SD, n=3.

4.2.5. Use of VPA to enhance ZFN delivery by IDLVs

Based on the encouraging data using MND-eGFP-IDLV, we hypothesized that VPA can be used to enhance ZFN delivery by IDLVs. To test this hypothesis, K562 cells were transduced with $4 \times 10^7$ TU/ml MND-Double-CoOp-IDLV (Chapter 3) and treated with 1 mM VPA for 4 days. The cells were harvested for genomic DNA and protein lysates at the end of the incubation. We measured mCherry expression by flow cytometry and performed western blots to measure ZFN expression. We performed Surveyor nuclease assay to determine ZFN activity. The cells treated with VPA showed 2.5-3 fold increase in mCherry expression, corroborating the previous results. Western blot using anti-FLAGtag antibody revealed that the ZFN expression per cell was 2-2.5 fold higher in VPA treated cells as compared to untreated cells, in agreement with mCherry expression. To test whether this increase in ZFN expression resulted in an increase in ZFN activity, we performed Surveyor nuclease assay on the cells. The cells treated with VPA revealed 2-2.5 fold increase in allelic disruption as compared to untreated cells (Figure 4.2.5a). These results show that treatment of cells with VPA can enhance ZFN delivery by IDLVs.
We sought to determine if the effect of VPA treatment is seen at different concentrations of the ZFN-IDLV. Hence, we transduced K562 cells with the MND-Double-CoOp-IDLV at concentrations from $1 \times 10^7$ TU/ml to $8 \times 10^7$ TU/ml and treated them with VPA. At 3 days post-transduction, we measured mCherry expression by flow cytometry and allelic disruption by Surveyor nuclease assay. VPA treatment induced a 2-2.5 fold increase in mCherry expression at all vector concentrations. The increase in mCherry expression also correlated with increased allelic disruption in VPA treated cells (Figure 4.2.5b). These results confirm our finding that VPA treatment can be used to increase ZFN expression by IDLVs.

Figure 4.2.5: Use of VPA to enhance ZFN delivery by IDLVs

a. Effect of VPA treatment on mCherry expression from MND-Double-CoOp-IDLV in K562 cells (left). Y-axis indicates mCherry geoMFI at 4 days post-transduction. Effect of VPA treatment on ZFN expression as measured by western blot (center). Anti-FLAG western blots from K562 cells transduced with MND-eGFP-IDLV and treated with VPA.
or untreated. The numbers below the bands indicate normalized expression levels. Effect of VPA treatment on ZFN activity as measured by Surveyor nuclease assay (right). Y-axis represents % Allelic disruption at day 4 post-transduction. Bars represent Mean ± SD, n=3.

b. Effect of VPA treatment on mCherry expression from MND-Double-CoOp-IDLV in K562 cells at various vector concentrations (left). Y-axis indicates mCherry geoMFI at 4 days post-transduction and X-axis represent vector concentrations. White bars indicate untreated cells, black bars indicate cells treated with VPA. Effect of VPA treatment on ZFN delivery from MND-Double-CoOp-IDLV in K562 cells at various vector concentrations (left). Y-axis indicates allelic disruption at 4 days post-transduction and X-axis represent vector concentrations. White bars indicate untreated cells, black bars indicate cells treated with VPA. Bars represent Mean ± SD, n=3.

4.2.6 Comparison of FDA-approved HDACis for enhancement of gene expression from IDLVs

Encouraged by previous results involving the use of VPA, we sought to test if other FDA-approved HDACis show a similar effect on transgene expression. To that end, we compared Vorinostat (VST), Givinostat (GST) and Trichostatin A (TSA) with VPA for their effect on IDLVs. These drugs are FDA-approved and are in various stages of clinical use as anti-leukemia agents. In order to assess their efficacy, we transduced K562 cells with MND-eGFP-IDLV and treated them with various concentrations of each drug, ranging from 1 nM – 1 µM. We measured GFP expression by flow cytometry and cell viability at 4 days post-transduction. We used cells treated with 1 mM VPA as a benchmark for comparison of the inhibitors.
All the three HDACis tested induced an increase in GFP expression from MND-eGFP-IDLV. The optimal concentration of VST was 1 µM, whereas that of GST and TSA was 300 nM, based on increase in GFP expression (Figure 4.2.6a). A comparison of the three HDACis is shown in Figure 4.2.6b. Based on the ability to enhance GFP expression, VST and GST were superior to VPA, whereas TSA was inferior to VPA. The effect of all the HDACis on cell expansion was similar to each other at the same concentrations. At the concentrations where GFP expression was maximal, TSA had the least effect on cell expansion, with 1.5 fold more expansion than VPA. On the other hand, VST and GST reduced the cell expansion further as compared to VPA at their optimal concentrations (Figure 4.2.6b). These results indicate that the FDA-approved HDACis also exert a decelerating effect on cell cycle in K562 cells.

Figure 4.2.6: Comparison of FDA-approved HDACis for enhancement of gene expression from IDLVs.
a. Testing the effect of Vorinostat (left), Givinostat (center) and Trichostatin A (right) on GFP expression from MND-eGFP-IDLV. Y-axes represent GFP geoMFI at 4 days post-transduction. X-axes represent the concentrations used. Bars represent Mean ± SD, n=3.

b. Comparison of VST, GST and TSA on GFP expression (left). Y-axis represents fold increase in GFP expression in treated cells at 4 days post-transduction. Black bar in each series indicates the best concentration of each drug, white bar indicates untreated cells, grey bars indicate VPA-treated cells. Effect of the HDACis on cell expansion (right). Y-axis represents fold-expansion in K562 cells at 4 days post-transduction normalized to untreated cells. The arrows indicate the concentrations of each drug that induce the highest increase in GFP expression. Bars represent an average of 3 replicates normalized to the average of untreated cells.

4.2.7 Effect of FDA-approved HDACis on ZFN delivery by IDLVs

We sought to determine if treatment with VST or GST would enhance ZFN delivery by IDLVs. We transduced K562 cells with 4x10^7 TU/ml of MND-Double-CoOp-IDLV and treated them with the different HDACis at their respective optimal concentrations. We measured mCherry expression from these cells by flow cytometry 4 days later. Surveyor nuclease assay was performed to determine ZFN activity in these cells. Flow cytometry revealed that all three of the HDACis led to increased mCherry expression from the IDLV in K562 cells, as compared to VPA. VPA treatment induced a ~3-fold increase in expression, corroborating previous results. TSA treatment induced ~6-fold increase in expression whereas VST treatment induced ~10-fold increase. GST treatment resulted in the highest increase in expression out of all the drugs, reaching up to 20-fold increase. According to Surveyor nuclease assays, all the four drugs were able to induce an increase in allelic disruption (Figure 4.2.7a). Based on mCherry expression,
these drugs were more effective than VPA. However, the FDA-approved HDACis had a more severe effect on cell expansion than VPA, with 2-4-fold lower cell expansion. This effect on cell expansion could possibly due to cytotoxicity of high levels of ZFN expression. These results provide further evidence for the possibility of using HDACis to boost ZFN delivery from IDLVs.

Figure 4.2.7: Effect of FDA-approved HDACis on ZFN delivery by IDLVs

a. Effect of VST, GST and TSA on mCherry expression from MND-Double-CoOp-IDLV in K562 cells (left). Y-axis represents mCherry geoMFI at 4 days post-transduction. Effect of VST, GST and TSA on ZFN delivery from MND-Double-CoOp-IDLV in K562 cells
(right). Y-axis represents allelic disruption in K562 cells at 4 days post-transduction. Bars represent Mean ± SD, n=3.

b. Effect of the HDACis on cell expansion (right). Y-axis represents fold-expansion in K562 cells at 4 days post-transduction normalized to untreated cells. Bars represent an average of 3 replicates normalized to the average of untreated cells.

4.2.8 Effect of HDACi treatment on IDLVs in primary hematopoietic cells

To determine if HDACis are effective in primary hematopoietic stem/progenitor cells (HSPCs), we transduced human umbilical cord blood CD34⁺ cells (CB-CD34⁺ cells) with MND-Double-CoOp-IDLV and treated them with the different HDACis. We measured mCherry expression from these cells by flow cytometry 4 days later. Surveyor nuclease assay was performed to determine ZFN activity in these cells. VPA, VST and GST had a moderate effect on mCherry expression from CB-CD34⁺ cells, by up to 1.5-fold increase as compared to untreated cells. TSA showed a greater effect, resulting in 2.5-fold increase over untreated cells (Figure 4.2.8). However, all the HDACis reduced the cell expansion, similar to the results in K562 cells. Out of these drugs, VPA was the least static, reducing expansion by 60%, whereas VST, GST and TSA reduced the cell expansion by more than 80% (Figure 4.2.8). These results would indicate that out of the four HDACis that were compared, VPA is the ideal one as it induced an increase in gene expression while exerting a moderate effect on cell expansion. A more detailed study optimizing the concentrations of each HDACi for use in CB-CD34⁺ cells is required.
Figure 4.2.8: **Effect of HDACi treatment on IDLVs in primary hematopoietic cells**

Effect of VST, GST and TSA on mCherry expression from MND-Double-CoOp-IDLV in CB-CD34⁺ cells (left). Y-axis represents % mCherry⁺ cells at 4 days post-transduction. Bars represent Mean ± SD, n=3. Effect of the HDACis on cell expansion (right). Y-axis represents fold-expansion in CB-CD34⁺ cells at 4 days post-transduction normalized to untreated cells. Bars represent an average of 3 replicates normalized to the average of untreated cells.

4.2.9 **Effect of HDACis on the cell cycle**

To elucidate the mechanism by which HDACis exert their effect on the IDLVs, we first investigated their effect on the cell cycle. We tracked the proliferation of K562 cells with CFSE (Carboxy-Fluorescein Succinimidyl Ester). CFSE is incorporated into the cell membrane upon staining and is diluted symmetrically with each cell division. Hence, based on reduction of CFSE fluorescence, cell divisions can be tracked. Therefore, we labeled K562 cells with CFSE and then transduced them with MND-Double-IDLV in presence or absence of 1 mM VPA. At 3 days post-transduction, we performed flow cytometry on these cells to measure CFSE fluorescence and analyzed the cells with ModFit for proliferation.
In the untransduced and untreated cell population, ~65% of cells had undergone 3 divisions and ~28% cells had undergone 2 divisions. Treatment with VPA skewed this ratio towards 2 cell divisions, with ~57% as compared to 35% at 3 divisions. This indicated that VPA was impeding cell division. In the transduced and untreated population, the ratio was slightly skewed towards 2 divisions. However, in the transduced and VPA-treated cell population, the ratio was highly skewed towards 2 cell divisions, with 81% of the cells having undergone only 2 divisions (Figure 4.2.9). This indicated that treatment of cells with VPA along with viral transduction reduced the rate of cell division. This was important because IDLVs are diluted with cell division and a reduction in rate of cell division can increase the persistence of IDLV genomes.

Figure 4.2.9: Effect of VPA on cell cycle in K562 cells
Representative analyses of cell proliferation in K562 cells at 3 days post-transduction, as measured by CFSE-labeling. The data were analyzed by ModFit and the cell populations were divided into 3-cell divisions or 2-cell divisions, as indicated by green and orange respectively. NT – not transduced.
4.3 Discussion

In this study, we investigated the possibility of epigenetic modulation of gene expression from IDLVs. Previously published reports suggest that IDLVs undergo epigenetic silencing carried out by Histone deacetylases (HDACs) (Pelascini et al., 2013). Therefore, we assessed if inhibiting HDAC activity by small molecules could prevent or reverse epigenetic silencing of IDLVs. For this purpose, we first tested two commonly used HDAC inhibitors (HDACis), Valproic Acid (VPA) and Sodium butyrate (NaBu). We assessed the effect of these HDACis on GFP expression from the MND-eGFP-IDLV in K562 cells. Preliminary results indicated that VPA was able to increase GFP expression by 2.5-3 folds without permanent cytotoxicity. We optimized the concentration of VPA to be used in K562 cells and found it to be 1 mM. Therefore, we continued further studies with 1 mM VPA as the treatment of choice.

We performed kinetics analyses of the effect of VPA on MND-eGFP-IDLV. We found that VPA treatment exhibited its effect on IDLVs at 2 days post-transduction, with the maximal effect 24 hours later. This indicated that VPA requires at least 48 hours to exert its effect on IDLVs in K562 cells. We performed experiments to dissect the temporal requirements of VPA treatment in K562 cells. Using a series of experiments involving various time points for VPA addition, we concluded that the presence of VPA is essential for the first 2 days post-transduction. Using a series of experiments involving various time points for withdrawal of VPA treatment, we concluded that 2 days of treatment is sufficient for the increase in GFP expression. These two sets of experiments combined together suggest that in K562 cells, treatment with VPA from 0-2 days post-transduction is both necessary and sufficient to achieve an increase in GFP expression from MND-eGFP-IDLV. We also tested if treating cells with VPA prior to transduction is sufficient for its effect. We found that pre-treating cells with VPA does not induce an increase in
GFP expression if VPA treatment is discontinued post-transduction. On the other hand, pre-treatment of cells increases GFP expression even further if VPA treatment is sustained. However, due to severe reduction in cell proliferation, pre-treatment was deemed not highly effective for the effects of VPA.

Our kinetic analyses indicate that the first 48 hours post-transduction are critical for the effects of VPA in K562 cells. The first 24 hours post-transduction are presumably required for the viral life cycle to complete. Transgene expression is usually not detectable at 24 hours post-transduction. The second day post-transduction may represent the peak period of transgene expression before the genomes are diluted with cell division. Hence, it is conceivable that the first 48 hours would be crucial both for the cells and for the virus to attain equilibrium between silencing and transcription. It is possibly during these 48 hours that the cellular epigenetic machinery silences most of the IDLV proviruses, while some escape the silencing and continue transcription. Therefore, inhibition of HDACs during these critical 48 hours is important for preventing the silencing of IDLVs. This could represent a window of opportunity for ensuring high levels of transgene expression. Finding the window of efficacy would be important in the case of stem/progenitor cells, especially because of their tendency to differentiate in vitro.

Next, we determined if the ability of VPA to increase transgene expression from IDLVs could be applied for enhancing delivery of ZFNs targeting hADA by IDLVs. In the previous chapter, we optimized the lentiviral architecture for delivery of ZFNs. We tested the effect of VPA treatment on ZFN delivery to K562 cells by MND-Double-CoOp-IDLV. We found that treating cells with VPA increased ZFN expression in K562 cells and this increase correlated with increased allelic disruption at the target locus. These results show for the first time that use of HDAC inhibitors can be used to enhance ZFN delivery by IDLVs. This is an important finding, as IDLVs have
been deemed not ideal for ZFN delivery due to low expression from them. Our previous results established that ZFN expression from IDLVs is insufficient for genome editing. Improvement in gene expression from IDLVs by HDACis can potentially make them effective candidates for ZFN delivery. In the case of using HDACis on primary cells, we showed preliminary results that corroborated with those from K562 cells. We did not characterize the cytotoxicity of HDACis in detail, but it is conceivable that they would be less toxic to normal primary cells as compared to leukemia cell lines. However, if HDACis were to be used on primary cells, it would be ideal if they were already FDA-approved. Hence, we investigated three FDA-approved HDACis, Vorinostat (VST), Givinostat (GST) and Trichostatin-A (TSA) for their efficacy on IDLVs. We found that VST and GST were more effective than VPA in increasing transgene expression. We also found that the FDA-approved HDACis were all able to increase gene expression from ZFN-IDLVs. This is the first report of use of these drugs for modulation of gene expression from IDLVs. These results are encouraging for potential use of these drugs in pre-clinical studies. Several studies are underway to achieve ZFN mediated genome editing in primary cells. One of the most important aspects of these studies is the delivery of ZFNs to the cells. IDLVs have known to be the least cytotoxic of all the delivery methods; however, their use is limited by low gene expression. Our findings indicate that using FDA-approved HDACis can be a viable strategy to overcome the limitations of IDLVs. LVs have the advantage of having a ‘track-record’ for clinical gene therapy use. If FDA-approved HDACis were truly effective in increasing expression from IDLVs, their use would prove to be along a relatively easier path to the clinic.

Following the successful use of HDACis, we sought to dissect the mechanism by which they act on IDLVs. We propose three possible phenomena acting in concert to result in increased
transgene expression from IDLVs. These are: i) Reduced epigenetic silencing of IDLVs, ii) Global transcriptional upregulation in cells, iii) Increased persistence of IDLV genomes due to reduced cell proliferation. Reduced epigenetic silencing was thought to be the primary mechanism of action of HDACis. A recent report, published during our studies, showed that treatment with TSA and NaBu increases the presence of active chromatin marks on the IDLV genomes. This report also stated that the mechanism for the effect of TSA and NaBu on IDLVs is based solely on epigenetic de-silencing, as indicated by the model proposed (Figure 4.3.1). Although our results are in agreement with theirs, we propose that the effects of HDACis on cell cycle and global transcriptional upregulation also play an important role.

Figure 4.3.1: Working model for epigenetic regulation of IDLVs (Pelascini et al, 2013)
Our experiments with cell cycle analysis indicated that treatment of cells with HDACis reduces the rate of cell division. If an exponential decay in IDLV genomes is considered as a mimic for the physiological rate, reduced cell division rate can result in a higher vector copy number per cell. Even at normal transcriptional levels, increased VCN can contribute to elevated transgene expression. Therefore, we speculate that a fraction of the effect of HDACis is due to increased vector persistence. Upregulation of global transcription by HDACis is a well-documented phenomenon (Liu et al., 2006). We propose that increased transcription from IDLVs could also be due to this global upregulation. However, it would be difficult to distinguish this phenomenon from reduced IDLV silencing with the existing assays for epigenetic analysis. Another effect of HDACis could be the increased accessibility of genomic DNA (Rahman et al., 2004). Due to increased histone acetylation, DNA is more loosely bound around the histones. This increases the accessibility to DNA to proteins. This phenomenon could potentially play a role in ZFN-mediated gene editing. Treatment with HDACis could make the target locus more accessible to ZFNs, thereby increasing their efficiency. However, reports have suggested that even the heterochromatin can be targeted with ZFNs (Hockemeyer et al., 2008). Thus, this effect may be insignificant for our purposes.

In summary, we demonstrate successful use of HDACis for enhancing transgene expression from IDLVs. We also show the application of this phenomenon to improve the ability of IDLVs to deliver ZFNs to target cells. Although the mechanism of action needs to be elucidated further, we show the potential in using HDACis for ZFN mediated gene editing. By using FDA-approved HDACis, we demonstrate the feasibility of this approach at a pre-clinical level. These studies will provide an important and feasible strategy to make ZFN based gene editing more efficient, especially in clinically relevant cell types.
4.4 Materials and methods

4.4.1 Cell lines and culture

K562 Cells (CRL-243TM, ATCC) were cultured in RPMI1640 (Cellgro, Manassas, VA) supplemented with 10% Fetal Bovine Serum (Gemini Bio-products, West Sacramento, CA) and Penicillin/Streptomycin/L-glutamine (Gemini Bio-products, West Sacramento, CA). Human umbilical cord blood samples were collected from UCLA Ronald Reagan Medical Center and have been deemed exempt from review from IRB as anonymous medical waste. Mononuclear cells (MNCs) were isolated from cord blood by Ficoll-Paque (GE Healthcare Biosciences, Piscataway, NJ) based separation. CD34+ cells were isolated from the MNCs using MACS CD34+ enrichment kit (Miltenyi Biotec Inc, Auburn, CA). Genomic DNA from cells was extracted using PureLink Genomic DNA mini kit (Life Technologies, Green Islands, NY) and quantified using NanoVue (GE Healthcare Biosciences, Piscataway, NJ). Cells were monitored for mCherry expression on BD-LSRFortessa or BD-LSRII flow cytometers (Becton-Dickinson, Franklin lakes, NJ). Cell counts were measured using a Beckman-Coulter ViCell-XR automated cell counter (Beckman-Coulter, Indianapolis, IN).

4.4.2 Transduction of cell lines and primary cells with IDLVs

K562 cells, 1x10^5 per sample, were centrifuged at 90g for 10 minutes and resuspended in 50 μl of culture medium, with or without HDACi, per sample. Appropriate dilutions of viruses were made in culture medium in 50 μl. The diluted virus was added to cells in 48-well tissue culture treated plates (Corning, Corning, NY). At two days post transduction the cells were transferred to 6-well tissue culture treated plates (Corning, Corning, NY) with 2 ml of culture medium with or without HDACi.
CB-CD34+ cells were pre-stimulated on Retronectin (Clontech Laboratories, Inc., Mountain View, CA) coated 48-well non-tissue culture treated plates (Corning, Corning, NY) with X-Vivo15 (Lonza, Walkersville, MD) supplemented with 50 ng/ml Stem Cell Factor (Amgen, Thousand Oaks, CA), 50 ng/ml Flt3-Ligand (R&D System Inc, Minneapolis, MN) and 50 ng/ml Thrombopoietin (R&D System Inc, Minneapolis, MN) for 18 hours at 1x10^5 cells/ml. CB-CD34+ cells were transduced with appropriate dilutions of the virus in X-VIVO15 with or without the HDACis. At 48 hours post-transduction, cells were transferred to tissue culture treated 24-well tissue culture treated plates (Corning, Corning, NY) and maintained in IMDM (Cellgro, Manassas, VA) with or without HDACis supplemented with 20% Fetal Bovine Serum (Gemini Bio-products, West Sacramento, CA) and 25 ng/ml Stem Cell Factor (Amgen, Thousand Oaks, CA), 10 ng/ml Interleukin-6 (R&D System Inc, Minneapolis, MN) and 5 ng/ml Interleukin-3 (R&D System Inc, Minneapolis, MN).

4.4.3 Cell cycle analysis by CFSE

For cell cycle analysis, cells were labeled with CFSE (Life Technologies, Green Islands, NY). Cells were resuspended in CFSE solution and incubated at 37°C for 10 minutes. The cells were washed with DPBS twice and resuspended at 1x10^5 cells per 50 μl for transduction. Labeled cells were transduced as stated above. The cells were analyzed by flow cytometry at 3 days post-transduction. Cell proliferation was analyzed by ModFit-LT (Verity Software House, Topshem, ME).

4.4.4 Western blot for analysis of ZFN expression

ZFN expression was monitored by western blotting for FLAG-tag. Lysates from cells transduced with the ZFN vectors were produced using Denaturing cell extraction buffer (Life Technologies,
Green Islands, NY) supplemented with Complete Mini protease inhibitor tablets (Roche Applied Science, Indianapolis, IN). Cells were centrifuged at 500g for 10 minutes at 40°C and the supernatant was aspirated. The cell pellets were resuspended in 50 µl lysis buffer per 1x10^6 cells and incubated on ice for 45 minutes with frequent vortexing. The lysate was centrifuged at 16,000g for 20 minutes at 40°C. The supernatant was transferred to fresh tubes and stored at -20°C until further use. The protein quantities were estimated using BCA assay (Thermo scientific, Rockford, IL) and read on a Tecan Infinite M1000 Microplate Reader (Tecan, Morrisville, NC). Equal amounts of protein lysate (40 µg per lane) combined with Laemelli sample buffer (BioRad, Hercules, CA) were loaded on 4-12% bis-tris SDS-PAGE gels (Life Technologies, Green Islands, NY). The gel was run at 150V for 1 hour in 1X MOPS-SDS running buffer (Life Technologies, Green Islands, NY) in a Novex gel electrophoresis unit (Life Technologies, Green Islands, NY). Prism Ultra (Abcam, Cambridge, MA) protein standards were loaded for size determination. The proteins were transferred from the gel to a PVDF membrane (Life Technologies, Green Islands, NY) in 1X Novex transfer buffer with 10% methanol (Life Technologies, Green Islands, NY). The transfer was carried out at 30V for 1 hour at room temperature. After the transfer, the membrane was blocked using 5% non-fat milk in PBS-Tween20 for 30 minutes at room temperature. After blocking, the membrane was incubated with 1:1000 diluted Mouse anti-FLAG primary antibody (Sigma-Aldrich, St. Louis, MO) overnight at 40°C. The membrane was then washed three times with PBS-Tween20 and incubated with 1:5000 dilution of Goat anti-mouse IgG-HRP secondary antibody (Sigma-Aldrich, St. Louis, MO) for 3 hours at room temperature. The membrane was washed again three times with PBS-Tween20. The membrane was incubated at room temperature with ECL2 reagent (Thermo scientific,
Rockford, IL) for 5 minutes and imaged on Typhoon FLA 9500 phosphorimager (GE Healthcare Biosciences, Pittsburgh, PA).
CHAPTER 5. Use of DNA-PKcs Inhibitors to Increase Efficiency of Gene Modification

5.1 Introduction

5.1.1 DNA Damage and Repair

The DNA of a cell can be damaged by environmental as well as metabolic reasons. DNA can be damaged in multiple different ways, by chemically modifying base pairs, by introducing breaks in the double helix or by crosslinking bases. Cellular DNA repair mechanisms can safeguard against this by repairing the lesions. The DNA repair machinery of the cells consists of an elaborate set of molecular mechanisms to detect and reverse DNA lesions. The interplay between genotoxic stress and DNA-repair in cells leads to repair of majority of the lesions. However, if lesions are not repaired or repaired inaccurately, it can results altered function of the genome. Cells with DNA damage can undergo apoptosis or senescence or can undergo genomic rearrangements. Out of the various kinds of DNA lesions, double-strand breaks (DSBs) are particularly hazardous to cells. DSBs are lesions in which both the strands of the DNA double helix are severed in a blunt or a staggered manner. DSBs can lead to genomic rearrangements if not repaired immediately (reviewed in Thompson, 2012). Various genotoxic agents such as Etoposide and ionizing radiation can cause DSB formation leading to severe consequences (Sinkule, 1984). DSBs can also form because of stalled replication forks and as a part of resolution of inter-strand crosslinks. Site-specific nucleases such as ZFNs and TALENs also introduce DSBs, and are potential genotoxic agents. Formation of DSBs invokes DSB repair pathways in the cell. Cellular DSB response consists of two major pathways, Homologous recombination (HR) and Non-homologous end joining (Mao et al, 2008). A detailed schematic of the two pathways is shown in Figure 5.1.1.
DSBs are detected by ATM or ATR complexes that recruit a phosphorylated form of histone H2A.X to the DSB. If the DSB is to be repaired by the HR pathway, the Mre11-Rad50-Nbs1 complex (MRN complex) is recruited to it (Cohn and D’Andrea, 2008). The ends of the DSB are
resected by the exonuclease activity of Mre11 to expose single stranded DNA (ssDNA) (Bernstein and Rothstein, 2009). Newly resected strands are coated by RPA to prevent secondary structures. RPA also recruits Rad51-BRCA1 complexes as well as Rad52 and Rad54. Rad51 displaces RPA on the ssDNA, which leads to homology search mediated by BRCA1, Rad52 and Rad54. Once a homologous template is found, the ssDNA invades the double helix of the template and anneals to its complement. Complementary strands of the ssDNA are synthesized by DNA polymerases with help from transient unwinding of the DNA by RecQ. The completion of DNA synthesis results in Holliday junctions that are resolved by resolvases to separate the template DNA and the newly repaired DNA (Saleh-Gohari and Helleday, 2004). Through this process of using a template for DNA repair, HR repairs DSBs in an accurate manner (Yokota et al, 2009).

NHEJ on the other hand, is a faster process to repair DSBs. If a DSB is to be repaired by NHEJ, Ku70/Ku80 complexes are recruited to it (Gullo et al, 2006), along with DNA-PKcs. DNA-PKcs phosphorylates and activates multiple target proteins that facilitate rapid repair of the DSB (Callen et al, 2009). The ends of the DSB are processed exonucleolytically by Artemis to expose short stretches of ssDNA. The complementary strands of the ssDNA are synthesized by Pol μ and Pol λ, with transient unwinding by RecQ. The newly synthesized double stranded ends are ligated by the Lig4/XRCC4/XLF complex to repair the end (Nagaraju et al, 2009). This mode of DNA repair can often be mutagenic because it involves template-independent strand synthesis. The extent of exonucleolytic end processing followed by strand synthesis can result in insertions and deletions being introduced at either end of the DSB.

Although it is mutagenic, NHEJ pathway results in rapid DSB repair and hence is the preferred pathway in cells. The propensity of a DSB to be repaired by NHEJ or by HR in a cell depends on
multiple factors, dictated mainly by the protein composition of the nucleus. Therefore, the choice of DSB repair pathway is influenced by the cell type and stage of differentiation, e.g. Mouse embryonic stem cells are more efficient at HR than mouse primary cells. The choice of pathway is also dependent on the phase of cell cycle (Figure 5.1.2). The efficiency of HR increases in S and G2/M phases, presumably due to availability of the sister chromatid as the repair template.

Figure 5.1.2: Propensity of cellular DSBs to be repaired by NHEJ and HR as a function of cell cycle (Mao et al, 2008)

The choice of DSB repair pathway becomes important in the context of nuclease-mediated gene editing. Nucleases such as ZFNs and TALENs introduce site-specific DSBs that are repaired by either HR or NHEJ. Either of these pathways can be exploited to introduce intended modifications. NHEJ can be used to introduce insertions and deletions, thereby disrupting the target DNA. HR, on the other hand, can be used to correct a sequence or to insert a cassette in a site-specific manner. However, because NHEJ is the prominent pathway of DSB repair, the efficiency of HR mediated modification is lower than NHEJ mediated disruption. If the choice of DSB repair pathway can be influenced to favor HR, the efficiency of HR mediated gene modification can be increased. To tilt the balance of DSB repair towards HR, proteins that regulate HR positively can be targeted for overexpression or for increasing activity. On the other
hand, the proteins that positively affect NHEJ can be targeted for knock-down or for inhibition of activity. In this study, we investigated the effects of regulating NHEJ on the efficiency of genome modification. We examined if the inhibition of DNA-PKcs activity by small molecules can be used to regulate NHEJ.

5.1.2 DNA-PKcs activity and its modulation by small molecule inhibitors.

As described previously, DNA-PKcs is one of the first proteins to be recruited to a DSB for NHEJ mediated repair (Shrivastav et al, 2009). One of the specialized roles of DNA-PKcs is in V(D)J recombination where NHEJ is required to complete the recombination. Cells lacking DNA-PKcs activity are deficient in NHEJ. Mutations in the prkdc gene in mice and humans leads to Severe Combined Immunodeficiency (SCID) due to lack of productive V(D)J recombination. DNA-PKcs is a 469 kDa protein encoded by ‘prkdc’ gene (Gapud and Sleckman, 2011). DNA-PKcs forms the catalytic subunit of the Ku70/Ku80/DNA-PKcs complex that has DNA-dependent protein kinase activity. These complexes can autophosphorylate DNA-PKcs that results in activation and dissociation of DNA-PKcs. The Ku70/Ku80/DNA-PKcs complex is thought to act as a scaffold for recruitment of the effectors of NHEJ, namely Artemis and the Lig4/XRCC4/XLF complex (Mao et al, 2009). DNA-PKcs can also phosphorylate these effectors, activating them for NHEJ repair. The central role of DNA-PKcs makes it a critical regulator of the NHEJ pathway. Inhibition of DNA-PKcs activity can result in increased toxicity from DNA damage. Transient inhibition of DNA-PKcs by small molecules can worsen toxicity from DSB inducing agents. This effect was exploited as a strategy to potentiate chemotherapy against cancer. Many chemotherapeutic agents, such as Etoposide, Cisplatin and Cyclophosphamide induce DNA damage. If DNA-PKcs is inhibited, cells become more
susceptible to toxicity from chemotherapy. Small molecule inhibitors of DNA-PKcs were discovered as way to potentiate chemotherapy by these agents (Tavecchio et al, 2012).

Currently, three small molecule inhibitors of DNA-PKcs are commercially available. DMNB, a modified form of vanillin was discovered in a screen for small molecules to abrogate the effects of Cisplatin on lymphoma cells (Kim et al, 2009). DMNB was found to be 50-100 fold more potent than vanillin for sensitizing Cisplatin chemotherapy. Nu7026 was also discovered in a screen of small molecules to sensitize chemotherapy. It was found to inhibit DNA-PKcs specifically and with high affinity. Treatment with Nu7026 was found to sensitize K562 cells to Topoisomerase II poisons such as Etoposide (Li et al, 2012). Nu7441 was discovered in a screen of substituted variants of Nu7026 based on structure-activity relationships. Nu7441 was found to be highly specific to DNA-PKcs with nanomolar affinities. It was found to potentiate effects of chemotherapy and radiotherapy in hepatocellular carcinoma cells. Nu7741 is currently in pre-clinical testing for chemosensitization in cancer chemotherapy (Tavecchio et al, 2012).

We investigated if these DNA-PKcs inhibitors can be used to inhibit NHEJ transiently for gene modification purposes. We hypothesized that by inhibiting NHEJ, we can increase the efficiency of HR mediated gene modification. Previous reports have shown high efficiency of HR mediated gene insertion in a prkdc−/− mouse model (S. Rahman, unpublished data). Based on these reports, we hypothesized that in the context of gene modification of ADA, DNA-PKcs inhibitors can be used to increase efficiency.
5.2 Results

5.2.1 Preliminary testing of DNA-PKcs inhibitors

We performed preliminary tests to assess if DNA-PKcs inhibitors can be used to enhance the efficiency of ZFN mediated gene correction. K562 cells were nucleofected with plasmids encoding the ZFNs or the donor templates. The cells were treated with Nu7441, Nu7026 or DMNB at their respective 50% inhibitory concentrations. The cells were collected three days later and genomic DNA was isolated from them. Assays to quantify gene modification and allelic disruption were performed using the genomic DNA.

Surveyor nuclease assays indicated that K562 cells nucleofected with ZFN plasmids and treated with DMNB or Nu7026 did not exhibit increased allelic disruption as compared to the vehicle control (DMSO). However, the cells treated with Nu7441 showed lower allelic disruption compared DMSO. This indicated that the inhibition of DNA-PKcs by Nu7441 was able to reduce NHEJ mediated allelic disruption. In the cells nucleofected with plasmids encoding ZFNs and donor templates, the treatment with DMNB or Nu7441 did not affect the frequency of gene modification. Treatment with Nu7441 induced an increase in gene modification as compared to treatment with DMSO. This indicated that by inhibiting NHEJ, we were successfully able to increase the frequency of gene modification in K562 cells (Figure 5.2.1). Based on these results, we used Nu7441 for subsequent experiments.
Figure 5.2.1: Preliminary testing of DNA-PKcs inhibitors

Effect of DNA-PKcs inhibitors on allelic disruption (left) and gene modification (right) in K562 cells, 3 days post-nucleofection. The X-axes show the inhibitor used or DMSO, the vehicle control. The Y-axis shows the frequency of allelic disruption as measured by Surveyor nuclease assay (left) or the frequency of gene modification as measured by qPCR (right). Bars represent Mean ± SD, n = 4.

5.2.2 Effect of Nu7441 on allelic disruption

We sought to optimize the concentration of Nu7441 for modulating the NHEJ mediated repair following ZFN mediated cleavage. To that end, we nucleofected K562 cells with 2.5 µg, 5 µg or 10 µg of plasmid encoding ZFNs followed by treatment with various concentrations of Nu7441. The cells were cultured in presence of Nu7441 and analyzed 3 days later by Surveyor nuclease assay. For K562 cells nucleofected with 2.5 µg Treatment with Nu7441 at concentrations between 1 nM and 10 nM showed an increase in allelic disruption followed by a decrease. At concentrations higher than 10 nM, allelic disruption levels remained largely steady. The same trend was also seen in K562 cells nucleofected with 5 µg or 10 µg of the plasmid (Figure 5.2.2).
These results did not corroborate with the previous results. This could be due to the semi-quantitative nature of the Surveyor nuclease assay.

Effect of Nu7441 on allelic disruption of ADA exon 7 in K562 cells at 3-days post-nucleofection. The Y-axes show % Allelic disruption as measured by the Surveyor nuclease assay. The X-axes show the concentration of Nu7441 in nmoles/liter. The amount of ZFN plasmids nucleofected is shown above each plot. Bars represent Mean ± SD, n =3.

5.2.3 Effect of Nu7441 on gene modification

To assess the effect of Nu7441 on ZFN-mediated gene modification, we used the previously described system of nucleofection of K562 cells followed by transduction with the Donor-IDLV. We nucleofected K562 cells with 2.5 µg, 5 µg or 10 µg of plasmid encoding ZFNs and transduced them with the Donor-IDLV 24 hours later. We cultured the cells in presence of various concentrations of Nu7441 for 3 days and analyzed them for gene modification.

Gene modification frequencies indicate that at 1 nM, there was no apparent effect of Nu7441 treatment. At 3 nM, the effect of Nu7441 was apparent only in the cells nucleofected with 10 µg of plasmids. These cells exhibited a slight increase in gene modification compared to untreated
cells. Cells treated with 10 nM Nu7441 also did not show changes in gene modification frequencies (Figure 5.2.3). These results were contradictory to the previous results that we had obtained. The confounding patterns suggested that these results could be due to artefacts of the low efficiency of gene modification. We hypothesized that the effects of Nu7441 could potentially be more apparent in an efficient ZFN-mediated gene modification system.

Figure 5.2.3: **Effect of Nu7441 on gene modification.**

Effect of Nu7441 on gene modification of ADA exon 7 in K562 cells at 3 days post transduction with Donor-IDLV. Pairwise comparison of various concentrations of Nu7441 (1 nM, top left; 3 nM, top right; 10 nM, bottom) with vehicle control (0 nM) are shown. The Y-axes show % gene modification as measured by qPCR. X-axes show the amount of ZFN plasmid nucleofected. Bars represent Mean ± SD, n =3.

5.2.4 **Effect of Nu7441 on gene modification of beta-Globin**
In order to assess the effects of Nu7441 in a system with higher gene modification efficiency, we used the beta-globin gene modification by ZFNs as a system. In this system, the ZFNs target the human beta-globin gene for site-specific double strand break production. A donor template that introduces a HhaI restriction site can be used to achieve gene modification. The frequency of gene modification can be measured by RFLP using HhaI. This system can yield gene modification efficiencies of up to 40% in K562 cells (M. Hoban, unpublished data).

We nucleofected K562 cells with plasmids carrying the ZFNs targeting beta-globin and transduced them with a beta-globin Donor-IDLV 24 hours later. The cells were cultured for 3 days in presence of various concentrations of Nu7441 and analyzed by RFLP and Surveyor nuclease assay. RFLP analysis revealed that in the cells treated with 1 nM or 3 nM Nu7441, there was no difference in gene modification frequencies as compared to untreated cells. The cells treated with 10 nM Nu7441 showed a decrease in gene modification efficiency as compared to untreated cells (Figure 5.2.4). These results indicate that in this system, Nu7441 failed to homologous recombination mediated gene modification.
Figure 5.2.4: **Effect of Nu7441 on gene modification of beta-globin.**

Effect of Nu7441 on gene modification of beta-globin in K562 cells at 3 days post transduction with Donor-IDLV. Pairwise comparison of various concentrations of Nu7441 (1 nM, top left; 3 nM, top right; 10 nM, bottom) with vehicle control (0 nM) are shown. The Y-axes show % gene modification as measured by RFLP. X-axes show the amount of ZFN plasmid nucleofected. Bars represent Mean ± SD, n =3.
5.3 Discussion

In this study, we investigated the use of small molecule inhibitors of DNA-PKcs to increase the efficiency of ZFN-mediated gene modification. We used a previously established system of gene modification of exon 7 of ADA using ZFNs to evaluate the efficacy of the inhibitors. Under this hypothesis, ZFNs would introduce a site-specific DSB that can be repaired by HR or by NHEJ. As the desired modification of the locus is based on HR, discouraging NHEJ would increase its efficiency. Therefore, we hypothesized that by using these inhibitors we can increase the frequency of gene modification of ADA exon 7.

To test this hypothesis, we compared the three inhibitors, DMNB, Nu7026 and Nu7441, for their efficacy on gene modification of ADA. We found that out of these, Nu7441 showed a reduction in NHEJ as indicated by allelic disruption. Corroborating with this result, it showed an increase in HR as indicated by gene modification. DMNB and Nu7026 did not exhibit an effect. Hence, we chose Nu7441 for further characterization. In these experiments, we used nucleofection of plasmids for delivery of ZFNs and donor templates. As described previously, nucleofection can be highly toxic to cells. In order to avoid further toxicity by nucleofection, we chose a combination of nucleofection for plasmids for delivery of ZFNs and IDLVs for delivery of donor templates.

We nucleofected K562 cells with plasmids encoding ZFNs, treated them with a range of concentrations of Nu7441 and assayed them for allelic disruption. Across a 100-fold concentration range of Nu7441, there was no significant effect on allelic disruption. However, we did observe a trend where allelic disruption increased slightly at 1 nM and decreased at 3 nM and 10 nM of Nu7441. It is possible that the inherent subjectivity in the Surveyor nuclease assay
could influence the observed values of allelic disruption. Thus we are limited by the lack of a more sensitive and reproducible assay for measuring allelic disruption.

In order to assess the effects of Nu7441 on gene modification, we treated K562 cells with Donor-IDLV in addition to nucleofected plasmids and cultured them with various concentrations of Nu7441. We analyzed the cells for gene modification using a more sensitive and accurate qPCR based assay. However, we did not see any effect on gene modification frequencies by Nu7441 treatment. These results did not corroborate with the preliminary results. We hypothesized that this may be due to the lower efficiency of the ADA gene modification system. Hence, we tested Nu7441 in the beta-globin gene modification system. Beta-globin gene modification system was previously established in our laboratory to achieve efficiencies of up to 40-45% in K562 cells.

Under this system, K562 cells were nucleofected with plasmids carrying ZFNs and transduced with and IDLV carrying the donor template. We treated the K562 cells with a range of concentrations of Nu7441 and analyzed them for gene modification. However, like the ADA locus, gene modification at the beta-globin locus did not show any increase in efficiency. These results taken together indicated that Nu7441 was not effective in increasing gene modification efficiency.

The reasons for the lack of efficacy could be multiple. The most important reason could be potential cross talk between NHEJ and HR through DNA-PKcs. In order for a DSB to be repaired by HR, the initial response proteins, including DNA-PKcs must dissociate from it to allow the HR pathway to take over (Davidson et al, 2013). A recent study by Tavecchio et al showed that DNA-PKcs must be autophosphorylated in order to dissociate from the DSB. They showed that due to Nu7441 mediated inhibition, DNA-PKcs remained bound to DSBs and this
phenomenon led to reduced HR (Tavecchio et al., 2012). These studies, which were published after our studies were completed, could provide a compelling explanation for our results. The inhibitory effect of Nu7441 on both NHEJ and HR could make it ineffective for altering ZFN mediated gene editing outcomes.

This does raise the question of inconsistency within the preliminary results and definitive results. We speculate that in our preliminary findings that showed Nu7441 being effective; gene-editing frequencies were artificially skewed. The solvent for the three inhibitors, DMSO, is known to exhibit cytotoxicity. We speculate that our preliminary results could be skewed because of cytotoxicity from DMSO. We used 1000 fold lower DMSO in the subsequent studies that did not show an effect. The toxicity from the nucleofection process could be contributing to the results. Another important reason for skewing of the results could be the toxicity from the drug itself. Nu7441 was initially used to sensitize leukemia cells towards DSB producing agents. In this study, we were using erythroleukemia cell line K562 and treating with a DSB producing agent, ZFN. Therefore, elevated toxicity from ZFNs by Nu7441 treatment is not entirely unexpected. A better system to assess toxicity would be to use primary cells for gene modification. However, for both the gene modification systems under investigation, efficacy in primary cells is very limited. Based on the results in K562 cells, the use of Nu7441 was not an attractive strategy to increase gene modification efficiency.

In summary, we tested the use of Nu7441 for increasing gene modification efficiency by altering the balance between NHEJ and HR. Our findings suggest that although theoretically it should be possible, the possible negative effects of Nu7441 on HR preclude it from being effective. Alternative options to modulate the DSB repair pathways should be considered for this purpose.
CHAPTER 6: Discussion

6.1 ZFNs for correction of ADA deficiency

In this study, we investigated the use of ZFN-mediated genome editing as a strategy to correct human ADA deficiency. We designed ZFNs and donor templates to modify exon 7 of ADA in a site-specific manner. The overall strategy was to introduce single base pair changes in exon 7 in order to correct a mutation that causes ADA deficiency. We tested the feasibility of this strategy in K562 human erythroleukemia cell line and in human primary CD34+ hematopoietic stem/progenitor cells. The efficiency of ZFN mediated cutting that we could establish in K562 cells was up to 25% (as measured by Surveyor nuclease assay) using nucleofection to deliver ZFN-encoding plasmids. Using the same delivery method, we could achieve up to 1% allelic disruption in human CD34+ cells. Using nucleofection for both ZFNs and donor templates, we could achieve up to 1.5% gene modification in K562 cells, whereas, the gene modification frequencies in CD34+ cells were undetectable (Chapter 2). In the context of gene modification of ADA, the therapeutically relevant level of gene modification is 8-10%, based on expression studies from ADA-deficient patients as well as from studies on gene therapy for ADA. If the goal for ZFN-mediated gene modification is to achieve 8-10% frequency in bone-marrow derived CD34+ cells, then significant advances need to be made from the current system. There are multiple factors that limit the efficiency of gene modification in our system. These factors are elaborated upon in the following discussion.

One of the most important factors is the efficiency of the ZFNs. The efficiency at which a given ZFN pair introduces site-specific DSBs directly dictates the possible efficiency of gene modification at that locus. Therefore, to achieve therapeutically relevant levels of gene modification, an efficient pair of ZFNs is essential. One of the main reasons for the low gene
modification frequencies observed in our studies is lower efficiency of the ZFN pair. The frequencies of allelic disruption that we obtained are on par with the current commercial ZFN architecture (commercialized by Sigma-Aldrich). While this level of efficiency is sufficient to be easily detectable, it is not high enough for gene therapy purposes. Currently, much of the commercialization of the ZFN technology is geared towards generating custom-modified research models such as reporter cell lines and knock-out mice. For these purposes, 10-15% allelic disruption or 1-2% gene modification is usually sufficient. However, for gene therapy approaches, higher frequencies are essential. Hence, if the current approach were to be extended to a pre-clinical setting, further improvements in the ZFNs to target this locus are required.

Another important factor that can influence the efficiency of gene modification is the distance from the ZFN cleavage site. Previous reports have shown that the frequency of gene modification decreases with increasing distance from the double strand break. The length of gene conversion tracts that are formed as a part of HR is limited to 90-120 bp. At distances over 50 bp from the DSB, there is a dramatic decrease in gene modification efficiency (Elliott et al, 1998). In corroboration with these reports, our study reveals low gene modification efficiencies at 75 bp away from the DSB. In order to achieve high efficiencies, a ZFN pair that introduces DSBs in proximity of the targeted mutation is desirable. However, because of the constraints of ZFN design, it may not always be possible to design ZFNs to target an ideal location. This is a major caveat of this technology. Due to this shortcoming, the newer site-specific modification technology involving TALENs might be the ideal candidate for such approaches.

Other factors influencing the efficiency of this approach are the delivery of the ZFNs and donor templates to the target cells and the balance between the two main pathways of DSB repair. These are discussed in the following sections.
6.2 Delivery of genome editing reagents to target cells

There are multiple different ways to deliver transgene payload to a particular cell type. These include Adenoviruses, Adeno-associated viruses, Lentiviruses as well as non-viral delivery methods such as lipofection and electroporation. In this study, we focused on using Integrase defective lentiviral vectors (IDLVs) for delivering ZFNs as well as donor templates for gene modification.

We tested IDLVs for efficient delivery of ZFNs and donor templates to K562 cells and primary hematopoietic cells. Our results indicated that IDLVs are suitable vectors for delivering donor templates. Although we reported successful use of IDLVs to deliver ZFNs to K562 cells, we uncovered certain problems with their use in primary cells. One of the major findings was the propensity of recombination in IDLVs. We found that if the IDLVs are designed to carry two genes that are homologous, the vectors underwent rearrangements. We successfully overcame this problem by increasing sequence diversity between the two payloads. To date, this is the first report of successful delivery of two ZFNs from the same vector. In spite of the successful results, we uncovered factors that make IDLVs less than ideal for delivering ZFNs. The major drawback of IDLVs for ZFN delivery is low expression from them. Even though IDLVs can transduce primary cells at high levels, the ZFN expression from them is not sufficient to induce high efficiency of genome modification.

Our results, in agreement with published reports, indicate that ZFN delivery to cells is a crucial component of gene modification. High levels of ZFN protein expression are required to achieve efficient modification. For this purpose, alternative delivery methods would be ideal. Vectors such as Adenoviral vectors allow very high copy numbers, thereby allowing high ZFN levels per
cells. Non-viral methods such as electroporation of plasmid DNA or mRNA also allow high expression levels. However, these approaches exhibit significant potential toxicity towards the cells. Therefore, there is still a great need for vectors that can deliver transgenes to primary cells. Although IDLVs meet the criteria of low cytotoxicity, transient delivery and high gene transfer efficiency, they are limited by low gene expression. A way to overcome this issue might be the use of integrating lentiviral vector that can later be excised. Gene expression from lentiviral vectors is ~10 fold greater than IDLVs (Nightingale et al, 2006). However, the possibility of insertional oncogenesis precludes their use for ZFN delivery. If LVs are modified to incorporate loxP sites, they could be excised using Cre recombinase. This approach has been used for multiple purposes, most significantly in epigenetic reprogramming to generate induced pluripotent stem cells. Such an approach could potentially be used for delivery of ZFNs, although this introduces a further complication of delivering Cre to the cells. Another way to improve IDLVs as a ZFN delivery method is to increase expression from them. We explored a small molecule based approach to achieve this.

6.3 Modulation of gene expression from IDLVs

We investigated the use of HDAC inhibitors for enhancing ZFN delivery from IDLVs. HDACis are known to modulate global gene expression as well as to reactivate latent HIV proviruses. We tested if HDACis can be used to ‘activate’ gene expression from IDLVs. The small molecule HDACi, VPA, was found to be effective in increasing ZFN expression from IDLVs by 2-3 fold. We tested FDA-approved HDACis, VST, GST and TSA, and found them to be effective for increasing ZFN expression from IDLVs. Our results are encouraging for further testing of these drugs for use in ZFN-mediated genome editing.
Although we showed a functional effect of HDACis on ZFN-delivery by IDLVs, the exact mechanism by which this effect is exerted needs to be elucidated. A recent report has postulated vector de-silencing as the mechanism for this effect (Pelascini et al., 2013). However, our results indicate that the mechanism for the activity of HDACis is a complex combination of vector de-silencing, cell cycle impedance and global transcriptional upregulation. Further studies need to be performed in order to determine the exact mechanism. However, due to the limits of biological assays, it could be difficult to unlink these three phenomena from each other.

One of the significant effects of HDACis might be on the differentiation state of the cells. We investigated their use in hematopoietic cells, especially HSPCs. HDACis, due to their ability to alter gene expression patterns, can affect the differentiation of cells. Although reports suggest that treatment with VPA maintains stem-ness of murine HSPCs (Walasek et al., 2012), it needs to be seen if this holds true for human HSPCs. If HDACis were to be applied to pre-clinical studies for gene modification, their effect on maintaining stem-ness of HSPCs needs to be investigated. VPA and other HDACis, notably NaBu, have been used in epigenetic reprogramming. These indicate that use of HDACis could potentially be a feasible strategy for gene modification of ESCs or iPSCs using IDLVs.

Although we were able to enhance the delivery of ZFNs by IDLVs, the overall gene modification efficiency in our system remained low due to limitations mentioned in Section 6.1. However, we also investigated an alternate approach to increase gene modification.

6.4 Altering cellular DNA repair

The efficiency of gene modification depends on the efficiency of the HR pathway in a cell. By default, a DSB is repaired by NHEJ. Therefore, HR mediated gene modification is a much less
efficient process compared to NHEJ mediated gene disruption. We investigated if altering the cellular balance between these two pathways would provide a viable strategy to increase gene modification efficiency.

To this end, we investigated the feasibility of small-molecule inhibition of DNA-PKcs, a key protein in NHEJ, as a way to increase HR mediated repair. Although the preliminary results were encouraging, they were deemed as artifacts of the low gene modification frequencies. We also tested this approach in a higher efficiency model system, gene modification of beta-globin, and found it to be ineffective. There could be multiple possible reasons for failure of this approach. The most significant of these might be a strong cross talk between HR and NHEJ involving DNA-PKcs (Tavecchio et al, 2012). A recent report showed that inhibition of DNA-PKcs resulted in inhibition of NHEJ as well as HR. These results could potentially explain our findings.

Despite the unsuccessful use of DNA-PKcs inhibitors, altering the cellular DSB repair balance could be a feasible strategy. If key regulators of NHEJ and HR are identified, the levels of these could be altered by over-expression or knock-down. By modulating levels of key regulatory proteins, the balance of DSB repair could potentially be tilted in favor of either of the two pathways, depending on the desired outcome. However, this would introduce the added complexity of requiring another transgene delivery. It is conceivable that small changes in regulatory proteins could lead to larger changes in DSB repair. Therefore, with highly efficient transgene delivery methods, modulation of specific proteins in addition to ZFN-mediated genome editing would be possible. These approaches can be trumped by other ways to increase the efficiency of ZFN-mediated genome editing. However, in the case of studies where there are limited options, modulating DNA repair may provide a way to increase the efficiency.
6.5 Concluding remarks

In summary, we performed a proof-of-concept study to achieve genetic correction of ADA-deficiency in human cells. We tested the feasibility of ZFN-mediated gene modification as a way to correct mutations in the ADA gene. Although we reported insufficiently low efficiency of this process, we shed light on multiple factors that led to it. One of the major factors was the delivery of ZFNs to target cells. In order to provide a solution for this issue, we focused on the use of IDLVs. We found problems regarding genome rearrangements in IDLVs and managed to solve them successfully. The major limitation of IDLVs as per our findings was the expression of transgenes. We examined if HDAC inhibitors can be used to enhance expression from IDLVs and found them to be effective in cell lines as well as primary cells. We also investigated the use of inhibitors of NHEJ as a way to increase gene modification efficiency and found it to be ineffective.

Through these studies, we report incremental advances to the field of ZFN-mediated gene modification. The findings reported in this study can be applied to strategies for editing of other genomic loci. Our findings about IDLVs, concerning vector rearrangements and ways to enhance expression, have implications beyond this field. These studies will provide a base for further improvement in delivery methods for genome editing reagents. These studies also provide a foundation for developing ways to making genome-editing technologies more efficient.
CHAPTER 7: Bibliography


