# Critical Parameters for Genome Editing Using Zinc Finger Nucleases

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**Abstract:** The possibility to make precise modifications to the genome at high frequency holds tremendous potential for biotechnology, conventional drug development and gene therapy. Homologous recombination is a powerful method for introducing such modifications in organisms such as mice. However, in mammals and plants, the frequency of gene modification by homologous recombination is quite low, precluding the therapeutic use of this methodology. In the past few years, tremendous progress has been made in overcoming one of primary barriers to efficient recombination, namely the introduction of a targeted double-strand break near the intended recombination site. This review will discuss the advances in engineering custom zinc-finger nucleases and their application in stimulating homologous recombination in higher eukaryotic cells at efficiencies approaching 1 in 2 cells.

**Key Words**: Homologous recombination, targeted mutagenesis, gene therapy, animal models, protein-DNA interactions.

## INTRODUCTION

An important goal of molecular and genetic research is the implementation of gene therapy to fight genetic diseases and degenerative disorders [1, 2]. However, major obstacles must be resolved before successful gene delivery can achieve these important goals. First, there needs to be efficient ways to deliver engineered genes to human patients. Second, such introduced genes need to target the correct cells. Third, genetically-engineered constructs need to be consistently targeted to a precise location and preferably be controlled by the physiologic signals of the host organism. For example, random insertion of a transgene or vector can lead to insertional mutagenesis and/or ocogene activation. The failure to resolve these significant obstacles can result in toxicity and death.

Within the past 5 years, engineered zinc finger nucleases (ZFN) have emerged as an alternative approach to exogenous gene delivery strategies (Fig. **1**) [3]. This approach exploits the normal molecular processes of the cell to induce or correct deleterious genetic mutations. Specifically, the introduction of a double strand break (DSB) in chromosomal DNA has been shown to stimulate the appearance of mutations at the break site by the process of non-homologous end joining (NHEJ). In addition, if appropriate homologous repair DNA is present, a DSB can dramatically stimulate the precise insertion of the repair information at the break site by the process of homologous recombination (HR). Such intentional DSBs can be directed by engineered zinc finger DNA-binding proteins, which can be designed to recognize specific DNA sequences. Attaching a nuclease to the zinc fingers allows them to cleave DNA and generate a targeted DSB. In principle, this approach can be used to correct the underlying mutations contributing to genetic diseases.

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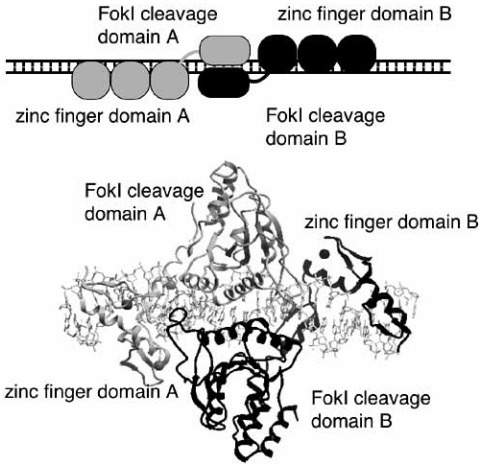


**Fig. (1).** A zinc finger nuclease (ZFN) heterodimer. Each monomer (gray or black) consists of an N-terminal zinc finger DNA-binding domain and a C-terminal FokI nuclease cleavage domain. Monomer A is designed to bind a 9-12 bp sequence on the lower strand of the DNA, while monomer B binds a 9-12 bp sequence on the top strand. The C-terminal cleavage domains dimerize and cleave in a 5-6 bp region of DNA between the two zinc finger binding sites. Even accounting for a small variance the size of the cleavage region, the 18-24 bp of combined sequence recognition should be sufficient to target a DSB to a unique site in the human genome.

The ZFN technology represents a convergence of essentially three parallel areas of research: 1) homologous recombination, 2) engineered nucleases, and 3) engineered zinc fingers.

### 1) Homologous Recombination

In a classical gene targeting experiment, such as might be used to obtain "knock-out" mice, a long linear targeting vector would be prepared that would contain 5-14 kb of homology to the targeted chromosomal locus [4]. Introduction of

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this targeting vector into mouse embryonic stem cells would typically result in gene modification by homologous recombination at a rate of $10^{-6}$ correct targeting events per cell (1 in 1,000,000 cells). A frequent additional complication would be a 100-1000-fold excess of non-targeted vector integration. These concerns prompted the development of methods that would select for vector insertion but against non-homologous insertion. In the early 1990s, experiments by several groups demonstrated that a rate-limiting step in HR was the creation of a DSB in the acceptor molecule (chromosome). However, at that time there was no general method to make targeted DSBs at any desired location. Virtually all such experiments were performed using one natural homing endonuclease, I-SceI, which had a long 18-bp recognition site. To examine I-SceI-mediated stimulation of HR, an I-SceI cleavage site needed to be pre-inserted into the target locus. The "uncatalyzed" rate of classical gene targeting (1 in 1,000,000 cells) could be stimulated about 2-3 orders of magnitude (1 in 10,000 cells) in the cells of yeast [5], *Xenopus* oocytes [6], mice [7], or other cell type. However, without a "targetable" cleavage reagent, the field could not move beyond this "proof-of-concept" stage.

**2) Engineered Nucleases**

To make a targetable nuclease, conceptually one could attach the cleavage domain of a natural nuclease to a DNA-binding domain. Non-specific nucleases, such as *Staphylococcus* nuclease, required strict co-factor control of catalysis to prevent total degradation of DNA, and were thus poor candidates for use in cells [*8*]. Most restriction enzymes and homing endonucleases were also unusable for this purpose because the cleavage and DNA-binding activities were contained within the same domain, and were thus inseparable. However, a subset of restriction enzymes, Type IIS, had separate cleavage and binding domains. Srinivasan Chandrasegaran was first to retarget the cleavage domain of the Type IIS enzyme FokI to the DNA-binding homeodomain of *Drosophila* ultrabithorax protein [9]. He subsequently attached the FokI cleavage domain to the C2H2 zinc fingers of Zif268, and later to engineered zinc finger proteins that had been designed by Jeremy Berg [10]. These were the first ZFNs. Dana Carroll, in collaboration with Chandrasegaran, made significant contributions by defining the optimal target site and nuclease configurations [11]. The ZF-FokI chimeras were shown to require the dimerization of two cleavage domains for activity. When the shortest possible protein linker was used between the ZFs and FokI cleavage domains, the optimal target site configuration was found to be two everted zinc finger binding sites, separated by a 6 bp spacer (although a 5 bp spacer was later shown to be functional [12]). Carroll and Chandrasegaran subsequently demonstrated the stimulation of mutations and HR using the dimeric ZFN in a *Xenopus* oocyte model system [11, 13].

**3) Engineered Zinc Fingers**

The methodology that makes ZFN possible is the ability to make custom DNA-binding proteins to a wide spectrum of DNA sequences. Carl Pabo was the first to solve the structure of a C2H2 zinc finger (Zif268) bound to DNA [14]. This structure showed a relatively simple recognition motif of three amino acids contacting three DNA bases, suggesting that DNA recognition might be reprogrammed by simply changing the identities of these three residues. He also was first to describe the use of phage display as a method to select for amino acid combinations that displayed new binding specificities [15]. Pabo later developed an improved "sequential" selection methodology that overcame a technological barrier caused by interactions between domains [16]. Based on these and other seminal contributions by Carl Pabo, Jeremy Berg, Sir Aaron Klug, and others, Carlos Barbas was able to successfully develop a "modular assembly" strategy that transformed the technology into a generally useful tool for researches outside the zinc finger field. These methods were based on a large set of zinc fingers that had been optimized to recognize specific 3 bp sequences [17-19]. The fingers or "modules" could be assembled in virtually any order necessary to recognize an extended DNA sequence. This modular assembly methodology enabled any researcher to make custom DNA-binding proteins by simply assembling the predefined modules required to bind a desired DNA sequence. Although other elegant selection and assembly methods would be subsequently described [20], the ease of modular assembly allowed it to become the most commonly used and best characterized methodology for engineering zinc finger proteins. At the time of this writing, there are three published PCR-based [21-23] and one plasmid-based [24] methods for the modular assembly of zinc fingers. There are two web-based tools to aid in the design of engineered zinc finger proteins and nucleases, one by Barbas [25] and the other by the Zinc Finger Consortium [26].

***Modern ZFN Technology***

In 2003, Dana Carroll and, separately, Matt Porteus and David Baltimore, demonstrated highly efficient HR (1% or 1 in 100 cells) in *Drosophila* flies and human cell lines, respectively [27, 28]. F1 progeny from treated embryos displayed the expected phenotypic modifications, demonstrating that the genetic modifications were permanent and heritable. Carroll and co-workers also showed ZFN could target mutations in *C. elegans* [29], and, in collaboration with Gary Drews, showed ZFN could target mutations in *Arabidopsis* [30]. In 2005, Dan Voytas [31], Toni Cathomen [32], and a commercial entity, Sangamo Biosciences [12], reported the use of ZFN to stimulate HR in plants (Voytas) and human cell lines (Cathomen, Sangamo). The Sangamo study in particular was highly significant for several reasons. Primary among these was that they observed HR at an endogenous locus (IL2Rgamma) at a frequency of 18%, meaning they could induce a correct targeting event in 1 in 5 cells. This achievement presented the ZFN technology as a serious candidate for corrective gene therapy. They observed no cytotoxicity and no non-targeted integration of the repair vector. The authors suggested the reason for their improved HR rate was due to their manipulation of cellular factors (ie: arrest at G2/M), and that the lack of toxicity might have been due to the use of 4 ZFs per ZFN monomer instead of the more typical 3 fingers. However, the following year Dana Carroll achieved similar HR frequencies (15%) at the *rosy* locus in *Drosophila* flies without cell manipulation and with a non-toxic 3-finger ZFN [33]. However, compared to Dana Carroll's methods, the Sangamo methods were simpler, involving the simple transfection of two ZFN expression plas-

mids and a circular repair plasmid with a modest 1.5 kb of homology to the target gene. As was the case with the modular assembly of zinc fingers, the apparent ease of Sangamo's methods suggested that highly-efficient gene editing could be accessible to any researcher. Improved delivery methods (described below) have enabled Sangamo and their collaborators to achieve recombination frequencies as high as 50% (1 in 2 cells) [34].

### *Many Organisms, Many Genes*

As shown in Table **1**, proof-of-concept ZFN-mediated HR has been reported on synthetic target sites in *Xenopus* oocytes, tobacco plants, and human HEK293 cell lines [11, 28, 31, 32]. At endogenous loci, ZFN-induced *mutagenesis* has been demonstrated in *Drosophila* flies, *C. elegans* worms, and *Arabidopsis* plants [13, 30, 35], and *HR* at two loci in *Drosophila* flies *(yellow and rosy*) and two in human cells (IL2Rgamma and CCR5) [12, 27, 33, 34]. In work presented at the 2007 symposium of the American Society for Gene Therapy, successful ZFN-induced genome editing was shown at several other endogenous loci in human and non-human cells. Sangamo has announced plans to introduce the first ZFN in a Phase I clinical trial by the end of 2007.

## POTENTIAL, POSSIBLE APPLICATIONS

There are three primary capabilities provided the ZFN technology that are currently inaccessible by any other method: 1) targeted mutagenesis by non-homologous end joining, 2) targeted gene correction by homologous recombination, 3) targeted delivery of a transgene to a "safe harbor" or endogenous promoter.

### 1) Targeted Mutagenesis to Create Model Organisms

The robust methods that exist for making knock-out mice have had tremendous impact on modern genetics. However, no such similar gene targeting technology exists for any other organism. In flies, worms, corn, wheat, etc…, mutant

**Table 1. Cell Types Targeted by Zinc Finger-Mediated Mutagenesis or Homologous Recombination**

| Year | Organism | Locus | Event | Frequency[a] | Reference |
|---|---|---|---|---|---|
| 2001 | *Xenopus* oocytes | artificial | recombination | >95% [b] | [11] [d] |
| 2002 | *Drosophila* flies | *yellow* | *mutagenesis* | 0.5% [c] | [13] [d] |
| 2003 | *Drosophila* flies | *yellow* | recombination | 1%[c] | [27] [d] |
| | Human HEK293 cell lines | artificial | recombination | 3%[c] | [28] |
| 2005 | Human HEK293 cell lines | artificial | recombination | 1%[c] | [32] |
| | Tobacco plants | artificial | recombination | 10% | [31] |
| | *Arabidopsis* plants | artificial | *mutagenesis* | 20% | [30] [d] |
| | Human K562 cell lines<br>Primary CD4+ T-cells | IL2Rgamma<br>IL2Rgamma | recombination<br>recombination | 18%<br>5% | [12] [e] |
| 2006 | *Drosophila* flies | *yellow*<br>*rosy*<br>*yellow*<br>*rosy* | recombination<br>*mutagenesis* | 5%<br>15%<br>2%<br>14% | [33] [d] |
| | *C. elegans* worms | *nowhere* [f] | *mutagenesis* | *20%* | [29] [d] |
| 2007 | Human HEK293 cell lines | artificial | recombination | 10% | [39] |
| | Human K562 cell lines | IL2Rgamma<br>IL2Rgamma | recombination<br>*mutagenesis* | 12%<br>45% | [42] [e] |
| | Human K562 cell lines<br>Jurkat cell lines<br>Human embryonic stem cells | IL2Rgamma<br>CCR5<br>CCR5 | recombination<br>recombination<br>recombination | 30%<br>50%<br>5% | [34] [e] |

[a] If multiple frequencies were reported, only the highest are listed here.

[b] unique aspects of this study were the injection of purified ZFN rather than ZFN expression in the cell, and a plasmid-based single-strand annealing HR assay rather than gene conversion assay using an exogenous repair DNA at a chromosomal locus.

[c] nuclease activity limited due to demonstrated toxicity.

[d] study involving Dana Carroll.

[e] study involving Sangamo Biosciences.

[f] *nowhere* is a chromosomal site located more than 1 kb from any known gene.

alleles are still generated by random mutagenesis followed by selection for phenotype and/or genotype. In livestock birds and mammals, gene modifications can also be accomplished by mutagenesis or HR in allogeneic fibroblasts, from which transgenic animals can be derived through the laborious process of somatic cell nuclear transplantation. The ability to target mutations and other genetic modifications using ZFN-mediated gene editing in these organisms would represent a significant technological breakthrough. ZFNs generate mutations by the well-characterized mechanism of NHEJ (Fig. **2A**). When a DBD is created, most cell types will attempt to repair the damage by rejoining the DNA strands. This could result in perfect repair of the junction. However, frequently the ends of the DNA are modified before ligation, resulting in small to moderately sized insertions or deletions (indels) at the breakpoint. The frequency and spectrum of such indels is dependant on a variety of factors such as cell type, competing pathways for resolution of the break (ie: HR), cell cycle status, and experimental conditions. The frequency of NHEJ damage observed by the application of ZFNs can be as high as 45%, making this an extremely efficient method for targeted mutagenesis (Table **1**). The ZFN-stimulated NHEJ approach to making gene knock-outs for the study of gene function in animals and plants could also be used to knock-out genes for therapeutic purposes in humans.

Potential therapeutic knock-out targets include the genomes of infectious agents such as tuberculosis or HIV, cellular genes required by infectious agents such as the CCR5 co-receptor for HIV, dominant negative mutant alleles that cause disease such as in Huntington's disease, or genes required for disease progression such as the multi-drug resistance gene (MDR1) in cancer. Competing methodologies in this area of gene therapy typically target RNA molecules, such as antisense and RNA interference (RNAi). Since many RNA transcripts are made from each DNA gene, targeting DNA genes using ZFNs should be more efficient. In addition, the ZFN-directed modifications are permanent and heritable, and therefore should not require periodic readministration or life-long expression of the therapeutic transgene.

### 2) Targeted Gene Correction for Gene Therapy

The recent demonstrations of highly-efficient gene targeting (10-50%) further suggest possible therapeutic applications. Gene therapy attempts to correct or augment hereditary deficiencies that give rise to disease. The traditional gene therapy approach is to introduce an exogenous, wild-type cDNA of the mutant allele. Such gene *replacement* therapy typically involves gene delivery by integrating or non-integrating viral vectors, or a variety of non-viral methods (reviewed in [1, 2]). However, the fundamental aim of all gene replacement therapies is to introduce a functional transgene. This approach is limited due to potential non-natural gene expression, and the need for the transgene to be expressed over the entire lifetime of the individual. Moreover, random insertion of a transgene or vector can lead to insertional mutagenesis and/or oncogene activation, as was reported recently in a gene therapy trial for X-linked SCID [2]. A better therapeutic approach would be to correct the genetic mutations that give rise to the disease. Such corrections could be achieved by ZFN-mediated HR. ZFNs stimulate HR at a desired locations in the genome by creating targeted DSBs, thus overcoming a critical slow step in the HR process. Unlike traditional gene replacement therapy, the ZFN would not require long-term expression, and the modifica-

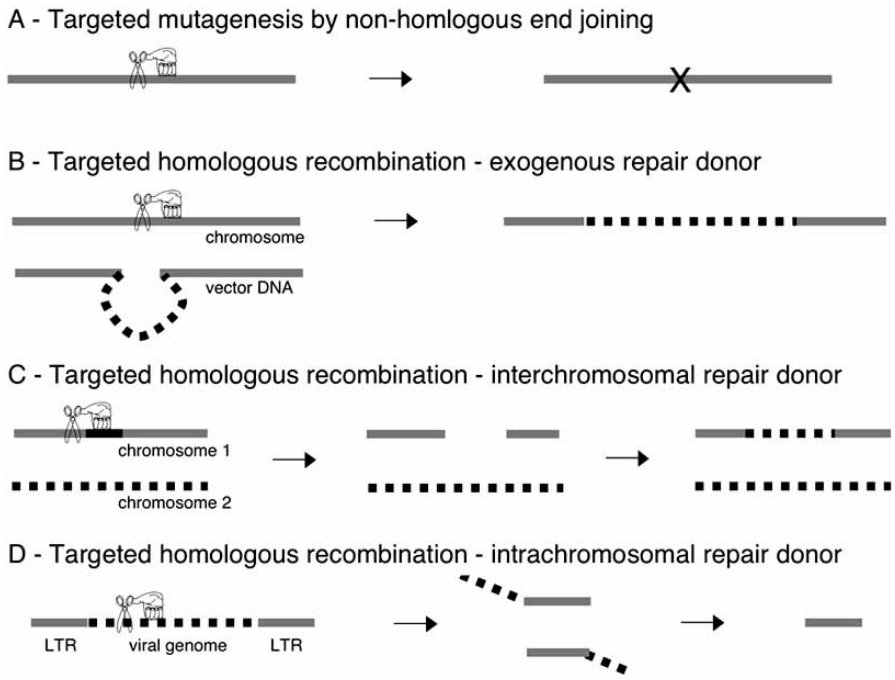


**Fig. (2).** Therapeutically-relevant outcomes of a targeted DSB. A) A DSB directed by a ZFN (depicted as a hand with scissors) can be repaired by NHEJ, leading to insertions and deletions at the breakpoint. In the presence of a repair donor DNA with appropriate homology to the targeted region, the DSB can also be repaired by HR. The repair donor may be B) added exogenously in the experiment, C) exist on the homologous chromosome, or D) exist on the same chromosome, as in the case of retroviral provirus (shown) or tandem repeats.

tion would be permanent and heritable by all progeny cells. In principle, mutations that give rise to genetic diseases could be corrected *in situ*, restoring a normal gene product with endogenous regulation and splicing.

Unlike NHEJ, HR additionally requires the presence of a repair template DNA containing the genetic information to be inserted flanked by DNA arms that are homologous to the target site (Fig. **2B**). Virtually all experiments thus far have supplied an exogenous repair template, which could be either a linear fragment [27] or a circular plasmid [12]. It has been shown that the genetic information to be introduced can range from a single base substitution to an 8 kb insertion, flanked by approximately 1 kb of homology with the target site on each side [34, 36]. The larger fragments resulted in reduced recombination efficiencies in some cases, but up to 50% target gene insertion was observed in other experiments. The ability to target the insertion of a large fragment is highly significant. In many cases, mutations in disease-related genes can occur in any exon, which in genomic DNA can be spread across tens or hundreds of thousands of bases. Targeting insertion of a whole cDNA could circumvent the need of designing ZFNs to correct mutations in every exon. The cDNA would be under the control of the endogenous promoter, thus allowing physiological regulation of the transgene. The repair template DNA can, in principle, also come from endogenous sources, such as the homologous chromosome or repeated sequences on the same chromosome (Fig. **2C** and **D**). For example, integrating retroviruses such as the human immunodeficiency virus (HIV) have a genomic structure that contains two long terminal repeats (LTRs). A DSB targeted between these repeats should stimulate recombination of the repeats, resulting in the loss of the intervening viral genome. The ability to perform highly-efficient corrective gene therapy would represent a significant technological breakthrough.

Potential therapeutic gene correction/insertion targets include recessive monogenic genetic disorders, among which fragile-X, cystic fibrosis, and Duchenne muscular dystrophy are the most common. Early-stage investigations of potential ZFN-based therapies have been reported for X-linked SCID, sickle cell anemia, cystic fibrosis and myotonic dystrophy [12, 23, 37], and the Sangamo website lists several more (www.sangamo.com). However, most diseases have a genetic component and are thus candidates for some form of gene therapy. For example, about 70% of all gene therapies in clinical trial target cancer, despite the obvious concerns regarding the required efficiency of gene transfer [2]. As these more established gene transfer therapies continue to break new ground, the permanent and heritable gene correction therapy offered by ZFNs will likely find application for an increasing number of diseases.

### 3) Targeted Delivery of a Transgene to a "Safe Harbor" or Endogenous Promoter

For applications such as the generation of transgenic animals and plants or the ectopic expression of a therapeutic gene, the goal is not the modification of an endogenous gene but the targeted and highly efficient insertion of an exogenous one. ZFN-mediated HR could direct efficient integration at a "safe harbor", a gene that could act as an easily-detectable marker to identify the successful insertion event but the disruption of which would not otherwise harm the host. The human chemokine receptor 5 (CCR5) gene has been examined for this purpose [23, 34]. It codes for an easily detectable cell surface receptor on cells of hematopoietic lineage. Homozygous null mutants appear to be well tolerated in humans [38]. Using ZFN designed to cleave in the CCR5 gene, a large transgene could be precisely integrated in 5-50% of various cell types [34]. Other genes that might be suitable as safe harbors included the *tyrosinase* locus, the disruption of which affects pigmentation. Alternatively, the transgene could be inserted downstream of any desired endogenous promoter for regulated expression.

## POSSIBLE PROBLEMS/RISKS

### Zinc Finger Specificity and Toxicity

The most significant immediate concern for the ZFN technology is poor specificity of some zinc finger proteins. Currently, we have the capability to target many, though perhaps not all, DNA sequences. Among the sequences we can target with the existing lexicon of optimized ZF modules, some assembled proteins bind better than others. Occasionally proteins are constructed that do not seem to have sufficient affinity for detectable binding. However, the greater problem is specificity; that is, does the protein bind a unique site or several related sites? This is a particularly significant problem for ZFN. If an artificial transcription factor binds 100 off-target sites in addition to its intended target, it is statistically unlikely to bind near one of the 24,000 gene promoters in the 3,000,000,000 bp genome and thus should display little or no off-target activity. If a ZFN binds 100 off-target sites, the result could be 100 additional DSBs, leading to apoptosis, random mutations, or unintended genomic rearrangements. Such results may be difficult to detect. We currently do not have any experimentally validated methods for *predicting* specificity, nor has any study yet reported *measuring* the *in vitro* specificity of their ZFN by target site selection assays (i.e., CAST, SELEX, etc…). No genomewide chromatin binding analysis (i.e., ChIP-chip, ChIP-seq, etc…) has been reported for any engineered zinc finger protein. However, cytotoxicity has been reported in several ZFN studies, suggesting that "off-target" DSBs and their undesired consequences are indeed occurring [33, 39].

One practical approach to obtain high rates of HR was to design several ZFN monomers and hope that at least one pair would not be toxic. The fact that some ZFNs were found to be non-toxic [12, 33] strongly suggests that DNA binding specificity plays a critical role in determining toxicity. The recently solved structure of the engineered 6-finger protein *Aart* provides insights into some potential limitations of the "modular assembly" approach [40]. *Aart* was designed to bind the A-rich sequence 5′-ATG-TAG-AGA-AAA-ACC-AGG-3′ [18]. However, target site selection studies found the protein preferred the sequence 5′-ATG-(G/T)AG-(A/G)GA-AAA-GCC-CNN-3′ (differences underlined) [41]. The crystal structure of *Aart* bound to DNA revealed that the short amino acid side chains that were designed to recognize the 5'-A nucleotides in the target site were in fact too far from the DNA to influence specificity. Thus, although many of the ZF modules performed well in the context in which they

were originally optimized, their high-specificity was not always maintained in the context of a new multi-finger protein. Similar "context-dependant effects", often unpredictable, are the likely cause of the observed variability in ZFN activity and specificity.

Early attempts to limit toxicity involved reducing ZFN expression levels [27, 32]. However, that approach does not directly address the specificity problem, and it has become clear that high levels of nuclease expression are required to obtain the very high rates of HR. Recently, two concurrent publications described a generally applicable method to reduce ZFN toxicity based on redesigning the ZFN dimerization interface (Fig. **3**) [39, 42]. Wild type FokI acts as a homodimer, and the dimerization interface of the two cleavage domains is symmetric. However, ZFNs typically target a heterodimer site, in which the sequence bound by the left monomer is different than that bound by the right monomer. An unfortunate consequence of the symmetric FokI interface is that homodimers consisting of two left or two right monomers can also bind and cleave DNA. By redesigning the interface to be asymmetric, only heterodimers were active and toxicity due to DSBs at homodimer sites was greatly reduced. Lower toxicity allowed a higher expression level of the ZFN, resulting in the desired high rates of HR (>10%).

At least three methods have also been described for improving the binding specificity of assembled multi-finger ZF proteins [43-45]. Essentially, libraries of ZF proteins containing different fingers, or different amino acids within the fingers, are selected in a bacterial one-hybrid or two-hybrid system. These protein optimization methods hold tremendous promise for overcoming context-dependant effects to produce ZFN of high affinity and specificity. Unfortunately, these methods are highly technical and laborious, and will likely only be performed in specialized laboratories. However, it also seems possible that a careful analysis of re-optimized proteins will reveal general principles that can used to design highly-specific proteins using methods as accessible as modular assembly.

## Undesired Competing Reactions

Even if a ZFN had perfect specificity and all DSBs occurred only at the intended target site, undesired competing reactions in the cell could complicate the desired outcome. For example, if the intention was to correct a mutation in an exon by homologous recombination, there is some likelihood that the ZFN-induced DSB will instead be repaired by mutagenic NHEJ. In some experiments, NHEJ events were observed to outnumber HR events [27, 34]. Such a mutagenized site would likely be resistant to further ZFN activity. The result would therefore be to introduce an additional mutation in some population of the cells rather than correcting the original allele. A second type of competing reaction is the random integration of expression or repair plasmids. Two recent studies found that non-specific integration was not dependant on ZFN activity, but nonetheless occurred at frequencies that were about 10% of the observed HR frequencies (as high as 5% integration in one study) [34, 36]. In addition to the potential risk of insertional mutagenesis, long-term and heritable ZFN expression resulting from integration of the ZFN expression vector could increase the probability of off-target NHEJ-mediated mutagenesis. Non-specific integration may in part require NHEJ activity [46]. Therefore, transient inhibition of the NHEJ pathway, for example by silencing the gene for Ku70 using RNAi, may be useful to reduce both undesired NHEJ mutagenesis and non-specific integration [46, 47].

## Chromatin Inaccessibility

Earlier work with zinc finger transcription factors elegantly demonstrated that zinc finger proteins require "accessible" target sites on chromosomes [48, 49]. Sites occupied by nucleosomes or other endogenous binding proteins will compete with or preclude ZFN binding. Since existing methods to measure accessibility (i.e., DNaseI hypersensitivity, micrococcal nuclease digestion) are difficult and may be uninformative, it is often easier to design several ZFN to different regions near the desired recombination site. Anecdotally, it is not uncommon to examine 5-8 ZFN in order to

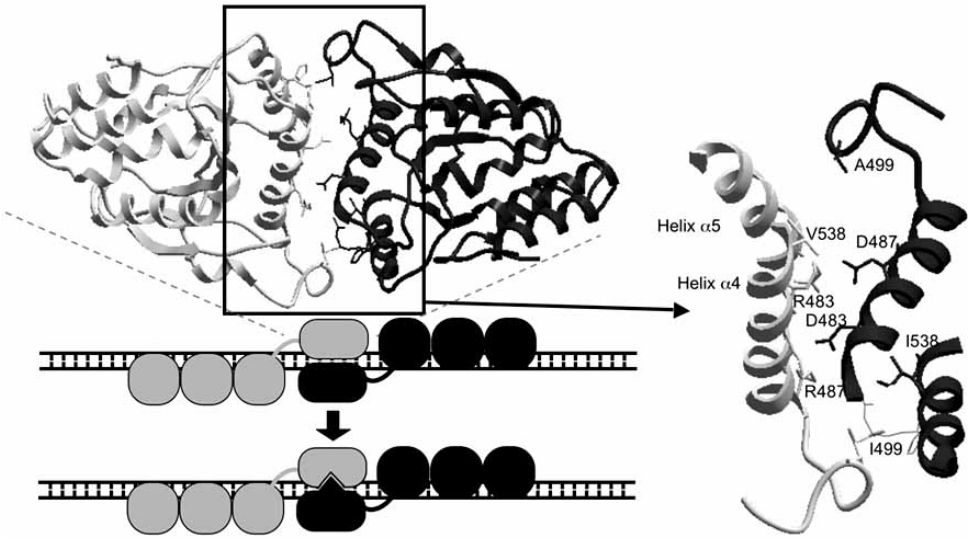


**Fig. (3).** Reengineering the dimerization interface of a ZFN. Structure-based redesign was applied to create nucleases that are obligate heterodimers. These next-generation ZFNs display less off-target effects, reducing toxicity and improving recombination frequencies.

achieve productive recombination. Some of this is likely due to chromatin inaccessibility; some may be due to poor zinc finger affinity or specificity, or other unknown problems. Some sites may be untargetable. Targeting transcribed genes will likely improve the probability to finding an accessible site. The transient use of histone methylase and deacetylase inhibitors might provide temporary accessibility. Better predictive or observational methods for chromatin accessibility would lead to improved ZFN activity.

## DELIVERY

Delivery is a significant limitation for all potential gene therapies. Delivery of ZFNs will likely limit their use *in vivo*, and will initially restrict the use of these methods to diseases in which some clinical benefit can be derived without 100% cell targeting (ie: not cancer). The plethora of viral and non-viral delivery methods developed by the gene therapy community can be applied for ZFNs [1, 2]. However, unlike a typical gene therapy transgene, ZFN-mediated gene editing requires only transient expression of the nuclease. Once recombination occurs, continued nuclease expression or enzymatic activity is undesirable. Therefore, methods designed to silence expression of the nuclease after treatment might be warranted.

Zinc finger-based artificial transcription factors (ATFs) have been in development longer than nucleases and have consequently progressed further towards pre-clinical and clinical gene therapy studies. In cell culture, the most common methods for delivery of ATFs are transient transfection of plasmid DNA or retroviral vectors (as one example, [50]). Delivery into animal models has been accomplished by adenoviral and adeno-associated virus (AAV) vectors [51, 52]. An interesting non-viral approach has been the delivery of ATFs not as genes but as proteins, by fusing them with cell penetrating peptides (CPP) such as residues 47-57 of the HIV TAT protein, or a 9-mer of arginine [53]. Another successful non-viral method has been intramuscular (IM) injection of an ATF expression plasmid [54]. In particular, IM injection of an ATF designed to downregulate the VEGF gene was recently shown to be effective in the treatment of peripheral arterial disease and peripheral neuropathy in diabetic mice and rats [55, 56]. Sangamo is currently entering this ATF into a Phase II clinical trial (www.sangamo.com).

ZFNs have been tested in whole animals and plants including *Drosophila, C. elegans*, tobacco, and *Arabidopsis* [13, 29-31]. For these experiments, the ZFNs were introduced into the germline of the organisms by standard methods, such as P-element insertion into *Drosophila* embryos, injection of plasmid DNA into young nematodes, or *Agrobacterium*-mediated transfer into plants. Clearly, germline approaches would not be appropriate for gene therapy, and most experiments involving ZFNs in human cells have been performed in cell cultures, using standard transfection methods for delivery. In principle, all of the methods used for ATFs could be used for ZFN delivery. A particularly exciting method was described recently involving the use of integrase-defective lentiviral vectors (IDLV) [34]. Lentiviral vectors are capable of highly efficient transduction of both dividing and non-dividing cells. IDLV have the additional feature that viral integration into the host genome is eliminated, thus greatly reducing the potential for insertional mutagenesis or transgene silencing. Transgenes are instead expressed transiently from extrachomosomal circular forms of reverse-transcribed lentiviral DNA. Using ZFNs expressed from these vectors, the Sangamo and Naldini groups were able to achieve HR at a frequency of 5% in human embryonic stem cells, and up to 50% in other cell lines.

## CONCLUSIONS

ZFN-mediated gene editing has demonstrated fantastic potential. Several endogenous loci have been modified at efficiencies >10%. Spurned on by these achievements, ZFNs are likely to have tremendous impact in the areas of gene therapy, functional genomics, transgenics and model organisms. However, it is likely that the best results over the next 5-10 years will be obtained by those laboratories that have the capability to perform ZFN re-optimization. It is hoped eventually that general principles for highly-active, highly-specific ZFN can be defined. Enhanced ZFN design methods, together with improved methods for addressing chromatin accessibility and ZFN delivery, will help advance this promising area of medicinal chemistry.

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