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Chimeric Restriction Enzymes: What Is Next?

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Abstract

Chimeric restriction enzymes are a novel class of engineered nucleases in which the non-specific DNA cleavage domain of *FokI* (a type IIS restriction endonuclease) is fused to other DNA-binding motifs. The latter include the three common eukaryotic DNA-binding motifs, namely the helix-turn-helix motif, the zinc finger motif and the basic helix-loop-helix protein containing a leucine zipper motif. Such chimeric nucleases have been shown to make specific cuts *in vitro* very close to the expected recognition sequences. The most important chimeric nucleases are those based on zinc finger DNA-binding proteins because of their modular structure. Recently, one such chimeric nuclease, Zif-QQR-F_N was shown to find and cleave its target *in vivo*. This was tested by microinjection of DNA substrates and the enzyme into frog oocytes (Carroll *et al.*, 1999). The injected enzyme made site-specific double-strand breaks in the targets even after assembly of the DNA into chromatin. In addition, this cleavage activated the target molecules for efficient homologous recombination. Since the recognition specificity of zinc fingers can be manipulated experimentally, chimeric nucleases could be engineered so as to target a specific site within a genome. The availability of such engineered chimeric restriction enzymes should make it feasible to do genome engineering, also commonly referred to as gene therapy.

Keywords

Chimeric restriction endonuclease; *Flavobacterium okeanokoites*; Gene therapy; Genome engineering; Hybrid restrictions enzymes; Recognition and cleavage domains

Introduction

Each human cell contains an estimated 3 billion base pairs within its genome. Figure 1 shows a schematic diagram of a genome engineering thought experiment. The objective here is to excise the mutant gene or the segment of the gene that contains a mutation (shown in red) from the genome and replace it with a normal allele or the segment of the gene that does not contain a mutation (shown in green). Two obvious questions come to mind:

1. do we have the necessary molecular tools to carry out such an experiment? and
2. if we had the necessary tools, could we successfully complete the experiment without actually killing the cell?

Of course, one could also do the converse experiment, i. e., replace a normal gene within the genome with a mutant allele. If this experiment were performed in germ line cells, one could produce transgenic animals.

A decade ago when we initiated our research on chimeric restriction enzymes, it was quite clear that the tools necessary to do the genome engineering experiment were not available. Since most type II restriction enzymes typically recognize short palindromic DNA sites that are four to eight base pairs in length, they are not useful for this task (Chandrasegaran, 2000). For example, restriction enzymes that recognize 6 base pairs result in cuts as often as every 4096 bases. Even rare cutters (enzymes that recognize 8 base pair long sequences) cut DNA once every 65536 bases on average. So far, only a limited number of restriction enzymes that recognize sequences longer than 6 base pairs have been identified. We need enzymes that recognize sequences of 16 – 18 bp in length to make unique double-strand breaks (DSB) within the genome. Therefore, a long-term goal in the field of restriction-modification enzymes has been to generate novel restriction endonucleases with longer recognition sites by mutating or engineering existing enzymes.

Before restriction enzymes can cleave DNA, they must bind to the correct DNA sequence. Thus, they have a dual function, namely DNA recognition and DNA cleavage. In the case of type II enzymes, these functions overlap each other. Several methods including the bacteriophage P22 challenge-phage system have been applied for the selection of mutations that alter sequence-specificity in restriction-modification enzymes (Fisher *et al.*, 1995). However, attempts to generate new specificities, particularly longer recognition sites, by genetic manipulation of the existing type II enzymes have not been successful. This may be simply due to the fact that multiple mutations are needed before a change in specificity can be achieved. Alternatively, since the DNA recognition and catalytic functions overlap each other in type II enzymes, it is possible that attempts to change amino acid residues responsible for the sequence-specificity may also affect the catalytic activity. Changes in the DNA-binding domain may alter the geometry of catalytic sites; this is likely accompanied by a drop in cleavage activity over several orders of magnitude. The type II enzymes simply may not be suitable subjects for changing sequence specificity.

Our laboratory took an alternative approach to address this problem. There are numerous bacterial enzymes that recognize an asymmetric sequence and cleave a short distance from that sequence (Szybalski *et al.*, 1991). These are termed type IIS enzymes, where 's' stands for shifted cleavage. These enzymes do not recognize any specific sequence at the site cut. For example, *FokI* restriction endonuclease recognizes the non-palindromic pentadeoxyribonucleotide 5'-GGATG-3':5'-CATCC-3' in duplex DNA and cleaves 9/13 nucleotides downstream of the recognition site (Sugisaki and Kanazawa, 1981). This property implies the presence of two separate protein domains within *FokI*: one for sequence-specific recognition of DNA and the other for the endonuclease activity. Once the DNA-binding domain is anchored at the recognition site, a signal is transmitted to the

endonuclease domain, probably, through allosteric interactions, and cleavage occurs. We reasoned that the type IIS enzymes probably are the ideal candidates for changing sequence specificities because one may be able to swap the recognition domain of these enzymes with other naturally occurring DNA-binding proteins which recognize longer sequences. Therefore, we undertook a detailed study of the *FokI* restriction-modification system from *Flavobacterium okeanokoites*.

Functional Domains in *FokI* Restriction Endonuclease

Several laboratories including ours have independently cloned the *FokI* restriction-modification system from *Flavobacterium okeanokoites* (Kita *et al.*, 1989; Looney *et al.*, 1989; Wu and Chandrasegaran, 1989: unpublished work). Our studies on proteolytic fragments of *FokI* endonuclease revealed an N-terminal DNA-binding domain and a C-terminal domain with non-specific DNA-cleavage activity (Li *et al.*, 1992; Li and Chandrasegaran, 1993). Waugh and Sauer (1993, 1994) showed that single amino acid substitutions uncouple the DNA-binding and scission activities of *FokI* endonuclease. The DNA-binding mode of *FokI* endonuclease has been analyzed by DNA footprinting (Li *et al.*, 1993; Yonezawa and Sugiura, 1994). These studies show a lack of protection at the cleavage site. Recently, the crystal structures of the native *FokI* and *FokI* bound to DNA were reported by Wah *et al.* (1997, 1998). The structures confirm the modular nature of *FokI* endonuclease. It appears that the cleavage domain is sequestered in a piggyback fashion by the recognition domain. This is consistent with the DNA footprinting analysis. Thus, the crystal structure is in complete agreement with the model derived from rigorous biochemical studies.

Based on our work on *FokI* endonuclease, we postulated that the type IIS endonucleases probably evolved by random fusions of the DNA-binding domains to non-specific endonucleases. Over time, these fusions were further refined into sequence-specific type IIS restriction enzymes by acquiring allosteric interactions between the recognition domain and the catalytic domain (Kim and Chandrasegaran, 1994). It appears that the modular architecture of type IIS enzymes is much more common in nature than previously thought. I-*TevI* and PI-*SceI*, group I intron-encoded homing endonucleases, appear to have a similar bipartite structure (Derbyshire *et al.*, 1997; Duan *et al.*, 1997; Grindl *et al.*, 1998). Studies on proteolytic fragments of I-*TevI* suggest that unlike *FokI*, in which the recognition domain is at the amino-terminus and the cleavage domain is at the carboxyl-terminal third of the molecule, the homing endonuclease appears to be an enzyme with an N-terminal catalytic domain and C-terminal DNA-binding domain connected by a flexible linker (Derbyshire *et al.*, 1997). The crystal structure of PI-*SceI* has revealed an N-terminal protein splicing domain and a C-terminal endonucleolytic domain (Duan *et al.*, 1997). Biochemical studies have shown that the PI-*SceI* protein splicing domain is also responsible for specific DNA binding (Grindl *et al.*, 1998). Recent studies suggest that similar multimodular endonuclease fusions may be much more prevalent (Yang *et al.*, 1999). R2 retrotransposon endonuclease, *Drosophila* P1 transposase and the bacterial RecBCD enzyme involved in recombination may fall into this category of modular architecture (Figure 2).

Chimeric Restriction Enzymes

The modular nature of *FokI* endonuclease suggested that it might be feasible to construct chimeric restriction enzymes with novel sequence-specificities by linking other DNA-binding proteins to the cleavage domain of *FokI* endonuclease. This indeed proved to be the case. We reported the construction of the first 'chimeric' restriction endonuclease by linking the *Drosophila* Ubx homeodomain to the cleavage domain of *FokI* (Kim and Chandrasegaran, 1994). We then reported the creation of novel site-specific endonucleases by linking two different threezinc- finger proteins to the *FokI* cleavage domain (Kim *et al.*, 1996). This work has been confirmed by another article that reported the construction of a similar three-zinc-finger restriction enzyme with a slightly different DNA sequence specificity (Huang *et al.*, 1996). Recently, we reported the creation of a novel site-specific endonuclease by linking the N-terminal 147 amino acids of the yeast Gal4 protein to the cleavage domain of *FokI* (Kim *et al.*, 1998). Thus, we have shown that the three common eukaryotic DNA-binding motifs, namely the helix-turn-helix motif, the zinc finger motif and the basic helix-loop-helix protein containing a leucine zipper motif, can be converted into novel site-specific endonucleases by fusing them to the *FokI* cleavage domain. Such engineered chimeric nucleases have been shown to make specific cuts *in vitro* very close to the expected recognition sequences. Other literature reports include the construction of Z-DNA conformation specific endonuclease (Kim *et al.*, 1997) and the use of *FokI* fusions to study the recruitment of Sp1 to the β -globin promoter with an *in vivo* method called protein position identification with a nuclease tail (PIN*POINT) (Lee *et al.*, 1998).

Of these chimeric nucleases, the most important are those based on zinc-finger DNA-binding motifs. Because of their modular nature, the zinc finger proteins offer an attractive framework for designing chimeric restriction enzymes with tailor-made sequence-specificities. The Cys₂-His₂ zinc finger proteins are a class of DNA-binding proteins that contain sequences of the form (Tyr, Phe)-Xaa-Cys-Xaa₂₋₄-Cys-Xaa₃-Phe-Xaa₅-Leu-Xaa₂-His-Xaa₃₋₅-His, usually in tandem arrays. Xaa represents an unspecified amino acid. Each of these sequences binds a zinc(II) ion to form the structural domain termed a zinc finger. These proteins bind to DNA by inserting an α -helix into the major groove of the double helix. The crystallographic structures of zinc finger proteins bound to a cognate oligonucleotide have revealed that each finger interacts with a base pair triplet within the DNA substrates (Pavletich and Pabo, 1991). Each finger, because of variations of certain key amino acids from one zinc finger to the next, makes its own unique contribution to DNA-binding affinity and specificity. The zinc fingers, because they appear to bind as independent modules, can be linked together in a peptide designed to bind a predetermined DNA site. Although more recent studies suggest that there might be a synergistic interaction between adjacent zinc fingers (Isalen *et al.*, 1997) and that the zinc finger-DNA recognition is more complex than originally perceived, it still appears that zinc finger motifs will provide an excellent framework for designing DNA-binding proteins with a variety of new sequence-specificities. In theory, one can design a zinc finger for each of the 64 possible triplet codons, and, using a combination of these fingers, one could design a protein for sequence-specific recognition of any segment of DNA. Studies to understand the rules relating to zinc finger sequences/DNA-binding preferences and redesigning of DNA-binding

specificities of zinc finger proteins are well underway (Berg, 1995). An alternative approach to the design of zinc finger proteins with new specificities involves the selection of desirable mutants from a library of randomized fingers displayed on phages (Greisman and Pabo, 1997; Isalan *et al.*, 1997).

The ability to design or select zinc fingers with desired specificity implies that DNA-binding proteins containing zinc fingers will be made to order. Therefore, we reasoned that one could design 'artificial' nucleases that will cut DNA at any preferred site by making fusions of zinc finger proteins to the cleavage domain of *FokI* endonuclease. We have been successful in engineering several novel chimeric restriction enzymes (Zif-F_N) by fusing three-zincfinger proteins to the cleavage domain of *FokI* (Kim *et al.*, 1996; 1997). We have shown that the fusion of the *FokI* cleavage domain to the zinc finger motif does not change the sequence specificity of the zinc finger protein and does not change its binding affinity significantly (Smith *et al.*, 1999).

While several research groups have contributed to the study of zinc finger proteins, much of the elegant work originated from the laboratories of Carl Pabo, Jeremy Berg, and Aaron Klug. Although we were the earliest laboratory to fuse another functional moiety (*FokI* cleavage domain) to zinc finger proteins, our long-term focus and efforts have remained on the development of chimeric restriction enzymes. Other functional domains, like activator domains and repressor domains, can also be fused to the designed zinc finger motifs to form hybrid proteins that act as transcription activators and transcription repressors within cells (Kim *et al.*, 1997; Beerli *et al.*, 1998). Recently, Tim Bestor's group has shown that cytosine methylation can be targeted to pre-determined sequences by attaching CpG-specific DNA methyltransferase to Zif 268 three-zinc-finger proteins (Xu and Bestor, 1997). It appears that important applications in medicine and biological research arising from this work are almost certain to follow. Furthermore, a commercial firm called Sangamo Bio- Sciences, Inc. was established in 1995 by Ed Lanphier (President and CEO); the first of its kind, the company's sole focus is to harness the utility of the zinc finger platform technology by attaching other functional moieties to the zinc finger proteins (Figure 3).

Stimulation of Homologous Recombination Through Targeted Cleavage by a Chimeric Nuclease

The modular structure of *FokI* endonuclease and zinc finger proteins has made it possible to create artificial nucleases (Figure 4) that will cut DNA at a pre-determined site. This approach opens the way to generate many new restriction enzymes with tailor-made sequence-specificities desirable for various applications. Now that it appears one can design chimeric nucleases, we need to address the second question of the genome engineering thought experiment (Figure 1).

How might these chimeric restriction enzymes be used in genome engineering? One approach would be to recruit the pre-existing cellular machinery to achieve the goal. Cells of many different organisms use recombination to repair DNA damage, especially double-strand breaks (DSB). An unrepaired DSB in a cellular chromosome would be lethal. Carroll and coworkers (1996) have shown that recombination in frog oocytes proceeds by

exonuclease resection and the annealing of complementary strands. This is called single-strand annealing; and this same mechanism is thought to operate in mammalian cells. One could utilize this process in gene targeting experiments. The essence of this approach is to target the chromosomal DNA for sequence-specific cleavage at or near the site where one wants the recombination to occur. Exonuclease would then resect the DNA at that chromosomal site, generating single-strand tails. Recombination would proceed by a single-strand annealing mechanism with the exogenous DNA that is present in the cell. The homologous foreign DNA would be incorporated at this chromosomal site. If the chromosomal target site is not cleaved, then it would not be accessible for exonucleolytic resection. In this case, the exogenous DNA will be degraded while the target remains unchanged. Thus, making a targeted DSB would greatly stimulate homologous recombination between the exogenous DNA and a chromosomal sequence. Such experiments have been performed using group I intron-encoded homing endonucleases in yeast, cultured mammalian and plant cells (Carroll *et al.*, 1996). Although the target sites for the homing endonucleases range from 15 to 40 bp length, they exhibit a broad range of sequence-specificities and cutting frequencies because of their binding to degenerate sites. In addition, their sequence-specificities are not amenable to easy manipulation. As a result they have limited use in genome engineering.

Recently, the chimeric nuclease Zif-QQR-F_N (Kim *et al.*, 1997) was shown to find and cleave its target *in vivo* (Carroll *et al.*, 1999). This was tested by microinjection of DNA substrates and the enzyme into frog oocytes. *In vitro* cleavage experiments indicated that at the enzyme concentrations used in microinjection experiments Zif-QQRF_N predominantly cuts only one strand of DNA at its recognition site. Double-strand cleavage required an inverted repeat of the 9-bp target (Figure 5). When the appropriate sites were placed in the recombination substrate, this DNA was cleaved in frog oocytes by the injected enzyme and homologous recombination ensued (Carroll *et al.*, 1999). Recent microinjection experiments have shown that greater than 90% of the substrate was cleaved *in vivo*; almost all cleaved substrates underwent homologous recombination.

The ability of the chimeric nucleases to distinguish the chosen target site from all the other sequences in the genome is critical in these gene targeting experiments. Any sequence shorter than 16-bp would be present in multiple copies in the human genome. The three-zincfinger protein, Zif-QQR-F_N, makes predominantly only a single-strand cut beside each 9-bp binding site (Kim *et al.*, 1997). Since two copies of the inverted site are required to produce a DSB, the enzyme effectively has an 18-bp recognition site. One can envision that two such enzymes with different sequence-specificities could cleave in tandem to produce a DSB when their binding sites are appropriately placed and oriented with respect to each other (Figure 5). Nicking by the individual enzymes at non-homologous sites is likely not to be a problem, since singlestrand breaks are readily repaired by the DNA ligase to become resistant to recombination.

Because the focus of this minireview is chimeric nucleases, we have not discussed here alternative technologies. These include the elegant triple-helix approach that originated from Peter Dervan's laboratory as well as other work from several laboratories on ribozymes and

antisense methods that specifically target messenger RNA to control gene expression at the translation level within cells.

What Is Next for Chimeric Restriction Enzymes?

Our immediate focus is on three important issues that need to be addressed for the utility of chimeric nucleases as tools for gene targeting. First, our goal is to refine and improve the properties of the chimeric restriction enzymes. Previous studies (Waugh and Sauer, 1993; Kim *et al.*, 1994) suggested that the cleavage domain activity is regulated through the protein-protein interaction with the recognition domain. The crystal structure of *FokI* bound to DNA confirms this (Wah *et al.*, 1997, 1998). The cleavage domain is sequestered in a piggyback fashion by the recognition domain. This interaction is absent in our current constructs of chimeric nucleases. As a result, Zif-F_N does not behave like the naturally occurring restriction endonucleases. There is a low level of non-specific cleavage activity associated with the chimeric nucleases. Experiments are underway to refine Zif-F_N (Figure 4) into an enzyme that communicates between the DNA-binding domain and the catalytic domain. The protein-protein interaction of the DNA-recognition domain and the cleavage domain of *FokI* that prevents cleavage except when bound to the target site needs to be incorporated in the new constructs of the hybrid endonucleases. One approach to achieving the protein-protein interaction would be to include more of the linker region of *FokI* that is responsible for the interaction when the new hybrids are constructed. The availability of the crystal structures of native *FokI* and *FokI* bound to its cognate site may help in the rational redesign of the chimeric nucleases. Computer modeling should greatly aid this process. Such a redesign and refinement of chimeric restriction enzymes could help to regulate their non-specific nuclease activity.

Second is the issue of increasing the sequence-specificity of the chimeric restriction enzymes. One approach would be to design polydactyl proteins with additional zinc fingers. This has been shown to lead to greater affinity (Kim and Pabo, 1998; Beerli *et al.*, 1998). However, the specificity of the proteins remains to be examined in detail. Addition of a longer flexible linker between two sets of three-zinc-fingers appears to help in the binding of the protein to nearly two turns of the double helix. To produce DSBs with such an enzyme, one might have to engineer the dimerization of the cleavage domain (Bitinaite *et al.*, 1998). Preliminary analysis of the mechanism of cleavage by chimeric restriction enzymes suggests that dimerization of *FokI* nuclease domain promotes DSB. An alternative approach that targets two chimeric nucleases with different sequence specificities to adjacent sites is discussed above (Figure 4). In this case, the orientation and spacing between the recognition sites appear to be critical for the efficient production of DSB.

Third, Dana Carroll's laboratory (in a collaborative effort with our group) by using model substrates has shown that chimeric nucleases stimulate homologous recombination through targeted cleavage in frog oocytes (Carroll *et al.*, 1999). This observation needs to be followed up by a direct experiment that shows induction of homologous recombination at a chromosomal site in a human cell line. This would entail the following steps:

1. Identification of a target site within the gene of interest,

2. designing or selecting the zinc finger proteins that recognize this target site,
3. conversion of the engineered zinc finger proteins into chimeric nucleases,
4. delivery of the chimeric nucleases and the normal gene directly into the nucleus either by microinjection or through viral vectors that carry the genes coding for the chimeric nucleases to induce cleavage at the target site and stimulate homologous recombination, and
5. monitoring for homologous recombination at the target site.

Importantly, we have to show that recombination fails to occur at non-homologous sites due to residual cleavage by the chimeric nuclease. The proof-of-principle experiment should be followed by an application to correct a genetic defect in a transgenic animal model.

As a practical matter, since no counterpart methylases are available for the chimeric nucleases, increased production of these enzymes is often lethal to cells. During the overproduction and purification steps, much of the chimeric nuclease is lost as inclusion bodies or through degradation by the cellular proteases. As a consequence, recovery of soluble chimeric nuclease from the crude extract is difficult and the yield is quite low. The chimeric restriction enzymes are also not very stable; they lose their activity even when stored at -80°C in glycerol. Some effort must be expended to improve the vectors for cloning and overproduction of the enzymes. Also, the necessary conditions to make these chimeric nucleases stable for long term storage need to be determined.

Use of Chimeric Nucleases in Gene Therapy

The introduction of a defined chromosomal break at a unique site is known to induce homologous recombination in a large fraction of cells in a population. The challenge, so far unfulfilled, is to develop a general means for inducing a DSB uniquely at a given locus in the genome. It appears that chimeric nucleases may meet this challenge. If this proves to be true, then the only remaining obstacle for gene therapy will be efficient delivery of the chimeric nucleases to cells in patients to stimulate correction of the defect through homologous recombination.

Gene therapy provides a new paradigm for treating human disease by correcting the causative genetic defect. The implementation of this novel concept into clinical practice and therapeutic reality has proved to be extremely difficult and the progress has been slow (Friedmann, 1998). Many of the difficulties associated with gene therapy are likely to be overcome if one could insert the corrected version of the mutation at the precise location of the genetic defect within the genome (Figure 1). Current gene therapy vectors lack the requisite sequence specificity necessary for the targeted correction of the defective site within the genome. Incorporation of chimeric nucleases into the gene therapy viral vectors may provide the requisite target specificity.

Future Outlook

There is an excited anticipation to make 'artificial' restriction enzymes that will recognize and cleave a particular sequence within a genome. The generation of many novel enzymes

with tailor-made sequence-specificities that are desirable for various applications especially in diagnostics and therapeutics appears feasible. Ultimately, we might be able to target specific genes for cleavage within human cells. Several areas of research appear to be converging to make gene therapy a reality. These include the recent isolation and identification of human stem cells that are fundamental building blocks of human tissues (Shamblott *et al.*, 1998; Thomson *et al.*, 1998). Also, the complete nucleotide sequence of the human genome will be known as early as the dawn of the next century. The availability of chimeric nucleases, a new type of molecular scissors that target a specific site within the human genome, will likely contribute and greatly aid the feasibility of genome engineering, and in particular, *ex vivo* gene therapy using stem cells. Ethical issues aside, it is not unreasonable to expect that in a decade or two, all the technical problems associated with gene delivery will be overcome; and gene therapy will be routinely used in clinical practice, signifying a paradigm shift in the treatment of human disease. Only time will tell if chimeric nucleases will contribute to these goals and fulfill their great promise.

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References

- Beerli RR, Segal DJ, Dreier B, Barbas CF. Toward controlling gene expression at will: Specific regulation of the *erbB-2/HER-2* promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proc. Natl. Acad. Sci. USA*. 1998; 95:14628–14633. [PubMed: 9843940]
- Berg JM. Zinc finger domains: From predictions to design. *Accounts of Chemical Research*. 1995; 28:14–19.
- Bitinaite J, Wah DA, Aggarwal AK, Schildkraut I. *FokI* dimerization is required for cleavage. *Proc. Natl. Acad. Sci. USA*. 1998; 95:10570–10575. [PubMed: 9724744]
- Carroll D. Homologous genetic recombination in *Xenopus*: Mechanisms and implications for gene manipulation. *Progress in Nucleic Acid Research and Molecular Biology*. 1996; 54:101–125. [PubMed: 8768073]
- Carroll D, Segal DJ, Trautman JK, Smith J, Kim Y-G, Chandrasegaran S. Stimulation of homologous recombination through targeted cleavage by a chimeric nuclease. *Proc. Natl. Acad. Sci. USA*. 1999 in press.
- Chandrasegaran, S. *Encyclopedia of Life Sciences*. McMillan Reference Ltd.; 2000. Restriction Enzymes Article Reference code: 973. in press.
- Derbyshire V, Kowalski JC, Dansereau JT, Hauer CR, Belfort M. Two-domain structure of the td intron-encoded endonuclease *I-TevI* correlates with the two-domain configuration of the homing site. *J. Mol. Biol.* 1997; 266:494–506. [PubMed: 9048944]
- Duan X, Gimble FS, Quijcho FA. Crystal structures of *PI-SceI*, a homing endonuclease with protein splicing activity. *Cell*. 1997; 89:555–564. [PubMed: 9160747]
- Fisher EW, Yang M-T, Jeng S-t, Gardner JF, Gumpert RI. Selection of mutations altering specificity in restriction-modification enzymes using the bacteriophage P22 challenge-phage system. *Gene*. 1995; 157:119–121. [PubMed: 7607471]
- Friedmann, T. *The Development of Human Gene Therapy*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1998.
- Greisman HA, Pabo CO. A general strategy for selecting high-affinity zinc finger proteins for diverse target sites. *Science*. 1997; 275:657–661. [PubMed: 9005850]

- Grindl W, Wende W, Pingoud V, Pingoud A. The protein splicing domain of the homing endonuclease PI-SceI is responsible for specific DNA-binding. *Nucleic Acids Res.* 1998; 26:1857–1862. [PubMed: 9518476]
- Huang B, Schaeffer CJ, Li Q, Tsai M-W. Splase: A new class IIS zinc finger restriction endonuclease with specificity for Sp1 binding sites. *J. Protein Chemistry.* 1996; 15:481–489.
- Isalan M, Klug A, Choo Y. Comprehensive DNA recognition through concerted interactions from adjacent zinc fingers. *Biochemistry.* 1998; 37:12026–12033. [PubMed: 9724513]
- Kim J-S, Pabo CO. Getting a handhold on DNA: Design of poly-zinc finger proteins with femtomolar dissociation constants. *Proc. Natl. Acad. Sci. USA.* 1998; 95:2812–2817. [PubMed: 9501172]
- Kim J-S, Kim J, Cepek KL, Sharp PA, Pabo CO. Design of TATA box binding protein/zinc finger fusions for targeted regulation of gene expression. *Proc. Natl. Acad. Sci. USA.* 1997; 94:3616–3620. [PubMed: 9108026]
- Kim Y-G, Cha J, Chandrasegaran S. Hybrid restriction enzymes: zinc finger fusion to the *FokI* cleavage domain. *Proc. Natl. Acad. Sci. USA.* 1996; 93:1156–1160. [PubMed: 8577732]
- Kim Y-G, Shi Y, Berg JM, Chandrasegaran S. Site-specific cleavage of RNA/DNA hybrids by zinc finger/*FokI* cleavage domain fusions. *Gene.* 1997; 203:43–49. [PubMed: 9426005]
- Kim Y-G, Chandrasegaran S. Chimeric restriction endonuclease. *Proc. Natl. Acad. Sci. USA.* 1994; 91:883–887. [PubMed: 7905633]
- Kim Y-G, Kim PS, Herbert A, Rich A. Construction of a Z-DNA-specific restriction endonuclease. *Proc. Natl. Acad. Sci. USA.* 1997; 94:12875–12879. [PubMed: 9371768]
- Kim Y-G, Li L, Chandrasegaran S. Insertion and deletion mutants of *FokI* restriction endonuclease. *J. Biol. Chem.* 1994; 269:31978–31982. [PubMed: 7989374]
- Kim Y-G, Smith J, Durgesha M, Chandrasegaran S. Chimeric restriction enzyme: GAL4 fusion to *FokI* cleavage domain. *Biol. Chem.* 1998; 379:489–495. [PubMed: 9628342]
- Kita K, Kotani H, Sugisaki H, Takanami M. The *FokI* restriction-modification system: Organization and nucleotide sequences of the restriction-modification system. *J. Biol. Chem.* 1989; 264:5751–5756. [PubMed: 2784436]
- Lee J-S, Lee C-H, Chung JH. Studying the recruitment of Sp1 to the β -globin promoter with an *in vivo* method: Protein position identification with nuclease tail (PIN*POINT). *Proc. Natl. Acad. Sci. USA.* 1998; 95:969–974. [PubMed: 9448269]
- Li L, Wu LP, Clark R, Chandrasegaran S. C-terminal deletion mutants of *FokI* restriction endonuclease. *Gene.* 1993; 133:79–84. [PubMed: 8224897]
- Li L, Chandrasegaran S. Alteration of the cleavage distance of *FokI* restriction endonuclease by insertion mutagenesis. *Proc. Natl. Acad. Sci. USA.* 1993; 90:2764–2768. [PubMed: 8464886]
- Li L, Wu LP, Chandrasegaran S. Functional domains in *FokI* restriction endonuclease. *Proc. Natl. Acad. Sci. USA.* 1992; 89:4275–4279. [PubMed: 1584761]
- Looney MC, Moran LS, Jack WE, Feehery GR, Benner JS, Slatko BE, Wilson GG. Nucleotide sequence of the *FokI* restriction-modification system: separate strand specificity domain in the methyltransferase. *Gene.* 1989; 80:209–216. [PubMed: 2583511]
- Pavletich NP, Pabo CO. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. *Science.* 1991; 252:809–817. [PubMed: 2028256]
- Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, Blumenthal PD, Huggins GR, Gearhart JD. Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl. Acad. Sci. USA.* 1998; 95:13726–13731. [PubMed: 9811868]
- Smith J, Berg JM, Chandrasegaran S. A detailed study of the substrate specificity of a chimeric restriction enzyme. *Nucleic Acids Research.* 1999; 27:674–681. [PubMed: 9862996]
- Sugisaki H, Kanazawa S. New restriction endonucleases from *Flavobacterium okeanokoites* (*FokI*) and *Micrococcus luteus* (*MluI*). *Gene.* 1981; 16:73–78. [PubMed: 6282705]
- Szybalski W, Kim SC, Hasan N, Podhajski AJ. Class-IIS restriction enzymes – a review. *Gene.* 1991; 100:13–26. [PubMed: 2055464]
- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science.* 1998; 282:1145–1147. [PubMed: 9804556]

- Wah DA, Bitinaite J, Schildkraut I, Aggarwal AK. Structure of *FokI* has implications for DNA cleavage. *Proc. Natl. Acad. Sci. USA*. 1998; 95:10564–10569. [PubMed: 9724743]
- Wah DA, Hirsch JA, Dormer LF, Schildkraut I, Aggarwal AK. Structure of the multimodular endonuclease *FokI* bound to DNA. *Nature*. 1997; 399:97–100. [PubMed: 9214510]
- Waugh DS, Sauer RT. A novel class of *FokI* restriction endonuclease mutants that cleave hemimethylated substrates. *J. Biol. Chem*. 1994; 269:369–379. [PubMed: 8276820]
- Waugh DS, Sauer RT. Single amino acid substitutions uncouple the DNA-binding and strand scission activities of *FokI* endonuclease. *Proc. Natl. Acad. Sci*. 1993; 90:9596–9600. [PubMed: 8415747]
- Xu G-L, Bestor T. Cytosine methylation targeted to pre-determined sequences. *Nature Genetics*. 1997; 17:376–378. [PubMed: 9398832]
- Yang J, Malik HS, Eickbush TH. Identification of the endonuclease domain encoded by R2 and other sitespecific non-long terminal repeat retrotransposable element. *Proc. Natl. Acad. Sci. USA*. 1999; 96:7847–7852. [PubMed: 10393910]
- Yonezawa A, Sugiura Y. DNA-binding mode of class-IIIS restriction endonuclease *FokI* revealed by DNA foot-printing analysis. *Biochem. Biophys. Acta*. 1994; 1219:369–379. [PubMed: 7918634]

Figure 1. A Genome Engineering Thought Experiment

The objective here is to replace the mutant gene (red) with the normal allele (green). If the converse experiment, where the normal gene is replaced with a mutant version is performed in germ line cells, then one can produce transgenic animals.

Multimodular Enzymes

Figure 2. A Schematic Representation of Multimodular Enzymes

FokI, a type IIS restriction endonuclease; *I-TevI*, a homing endonuclease; and *R2*, a retrotransposon endonuclease.

Figure 3. Zinc Finger Protein Platform Technology

Additional functional domains like the *FokI* cleavage domain, activator domain, repressor domain and methylases can be fused to the designed zinc finger motifs to form chimeric proteins that act as chimeric nucleases, transcriptional activators, transcriptional repressors and targeted methyltransferases, respectively.

Figure 4. A Computer-Generated Model of the Chimeric Nuclease Bound to DNA
Zif- QNK (red) is fused to the *FokI* nuclease domain (orange) through the (Gly₄Ser)₃ linker (broken white line). The DNA is shown in yellow and zinc atoms are shown in white. The construction of Zif- QNK-F_N is described elsewhere (Smith *et al.*, 1999).

Figure 5. A Chimeric Nuclease Binding to Its Cognate Site

(A) Zif-QQR-F_N binding to a 9 bp site. (B) Mode of the Zif-QQR-F_N binding to an inverted repeat to produce a DSB. (C) Zif-QQR-F_N and Zif-QNK-F_N chimeric nucleases with different sequence-specificities bind to their cognate site in tandem to produce a DSB. The Zif-QQR-F_N binding site is 5'-GGG GAA GAA-3' (shown in red) and that of Zif-QNK-F_N is 5'-GGG GCG GAA-3' (shown in blue), respectively. The binding sites are also indicated by red and blue arrows, respectively.