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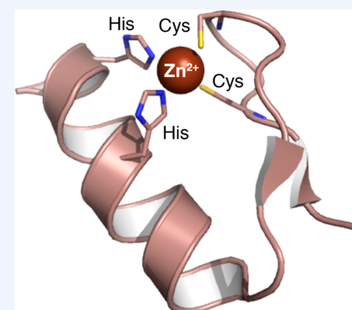
Synthetic Zinc Finger Proteins: The Advent of Targeted Gene Regulation and Genome Modification Technologies

Charles A. Gersbach,[†,§] Thomas Gaj,[‡,§] and Carlos F. Barbas, III*[,‡]

[†]Department of Biomedical Engineering, Duke University, Durham, North Carolina 27708, United States

[‡]The Skaggs Institute for Chemical Biology and the Departments of Chemistry and Cell and Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, United States

CONSPECTUS: The understanding of gene regulation and the structure and function of the human genome increased dramatically at the end of the 20th century. Yet the technologies for manipulating the genome have been slower to develop. For instance, the field of gene therapy has been focused on correcting genetic diseases and augmenting tissue repair for more than 40 years. However, with the exception of a few very low efficiency approaches, conventional genetic engineering methods have only been able to add auxiliary genes to cells. This has been a substantial obstacle to the clinical success of gene therapies and has also led to severe unintended consequences in several cases. Therefore, technologies that facilitate the precise modification of cellular genomes have diverse and significant implications in many facets of research and are essential for translating the products of the Genomic Revolution into tangible benefits for medicine and biotechnology. To address this need, in the 1990s, we embarked on a mission to develop technologies for engineering protein−DNA interactions with the aim of creating custom tools capable of targeting any DNA sequence. Our goal has been to allow researchers to reach into genomes to specifically regulate, knock out, or replace any gene. To realize these goals, we initially focused on understanding and manipulating zinc finger proteins. In particular, we sought to create a simple and straightforward method that enables unspecialized laboratories to engineer custom DNA-modifying proteins using only defined modular components, a web-based utility, and standard recombinant DNA technology. Two significant challenges we faced were (i) the development of zinc finger domains that target sequences not recognized by naturally occurring zinc finger proteins and (ii) determining how individual zinc finger domains could be tethered together as polydactyl proteins to recognize unique locations within complex genomes. We and others have since used this modular assembly method to engineer artificial proteins and enzymes that activate, repress, or create defined changes to user-specified genes in human cells, plants, and other organisms. We have also engineered novel methods for externally controlling protein activity and delivery, as well as developed new strategies for the directed evolution of protein and enzyme function. This Account summarizes our work in these areas and highlights independent studies that have successfully used the modular assembly approach to create proteins with novel function. We also discuss emerging alternative methods for genomic targeting, including transcription activator-like effectors (TALEs) and CRISPR/Cas systems, and how they complement the synthetic zinc finger protein technology.



1. INTRODUCTION

A new phase of the Genomic Revolution is beginning. In the first phase, the genomes of dozens of animals and many more plant, bacteria, and viral species were sequenced. In the second phase, functional genomics, genome-wide association studies, and fundamental molecular biology efforts led to substantial annotation of the tens of thousands of genes and other noncoding regulatory elements within these genomes. Now, a primary challenge to scientists, engineers, and clinicians is to convert this wealth of information into benefits for society. To address this challenge, it is necessary not only to understand the components of naturally occurring genomes, but also to easily, precisely, and robustly manipulate genome structure to effect functional changes. In the mid-1990s, our laboratory and others began to describe strategies for the creation of synthetic DNA-binding zinc finger proteins. We showed that zinc finger proteins could be engineered to bind to a wide range of DNA sequences to activate, repress, cut, and paste genes as well as modulate the epigenetic state of targeted loci (Figure 1). Importantly, these methods did not require any specialized expertise beyond standard recombinant DNA techniques. In this Account, we review fundamental aspects concerning the development of modular assembly of zinc finger proteins and highlight numerous examples of their successful implementation by our laboratory and others.

2. ZINC FINGER PROTEINS: STRUCTURE, FUNCTION, AND VERSATILITY

Zinc finger proteins comprise the most common class of DNA-binding proteins across all of biology. In 1991, the first crystal structure of a zinc finger protein, Zif268, bound to its DNA target was published.[1] This study revealed that each zinc finger domain consists of approximately 30 amino acids in a $\beta\beta\alpha$

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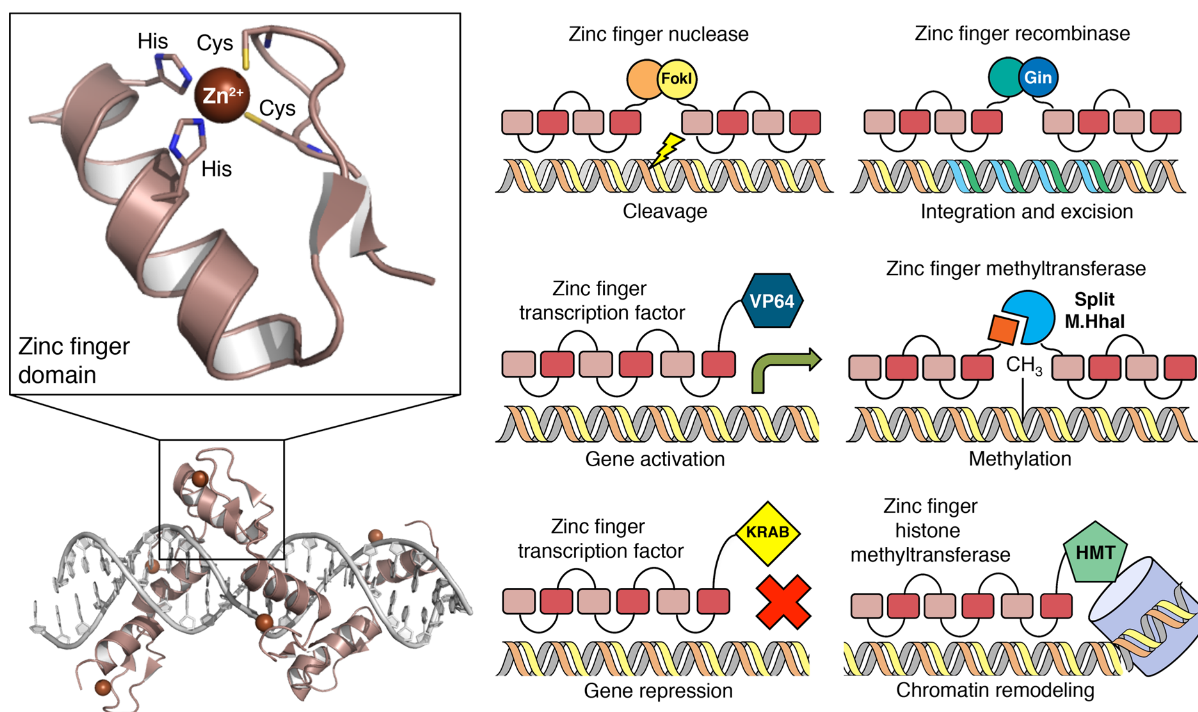

Figure 1. Structure and applications of zinc finger proteins. (Left) The designed six-finger zinc finger protein, Aart (light brown), in complex with target DNA (gray) (PDB ID: 2I13). The inset shows a single zinc finger domain. The side-chains of the conserved Cys and His residues that coordinate with a Zn ion (red sphere) are shown as sticks. (Right) Cartoon illustrating the applications of zinc finger technology.

configuration, with the DNA-binding residues of each zinc finger localized within a short contiguous stretch of residues, designated positions −1, 3, and 6, on the surface of the zinc finger α-helix (Figure 1). The side-chains of these residues interact with the major groove of DNA to make specific contacts, typically with three nucleotides. This landmark structural study also suggested that individual zinc finger domains each recognize three base pairs (bp) independently, and that modifying zinc finger specificity should only require altering the identity of the −1, 3, and 6 positions of a given domain. The modular recognition of serial zinc finger domains to consecutive three bp targets led to the hypothesis that individual domains could be interchangeable and that exchanging domains would confer new binding specificities to the whole protein, allowing for targeting of unique sequences.

By 1991, we had established phage display as a method for selecting highly specific recombinant monoclonal antibodies from large libraries.[2] Inspired by the parallels of molecular target recognition by antibody−antigen and zinc finger protein−DNA pairs, we developed an approach to express libraries of zinc finger proteins on the surface of phage in which the DNA-binding residues of a central zinc finger protein were randomized (Figure 2).[3] With this method we attempted to answer two key issues with respect to molecular recognition of DNA by zinc fingers. First, we sought to determine whether only changes at positions −1, 3, and 6 were sufficient to select for new zinc finger domains that bound various triplets with high specificity. Second, we attempted to ascertain whether zinc finger domains could be selected for each of the 64 possible 5′-NNN-3′ triplets, and if a universal system for DNA targeting based on preselected domains could be established. To address these questions, we constructed and selected phage display libraries wherein all residues (i.e., −1, 1, 2, 3, 4, 5, and 6) within the zinc finger α-helix were randomized. By selecting for phage

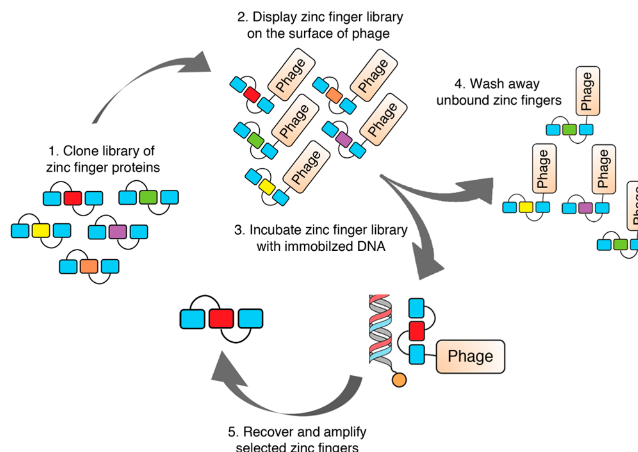

Figure 2. Phage-display selection of zinc finger proteins. Highly diverse three-finger zinc finger libraries were generated by randomization of the α-helical residues (−1, 1, 2, 3, 5, and 6) of the central zinc finger. These zinc finger libraries were then displayed on the surface of phage and incubated with biotinylated hairpin DNA targets. Phage-display libraries were subjected to stringent selection pressure to ensure sequence specificity. Phages that bound to single biotinylated DNA targets were recovered and amplified, and the selection process was repeated.

using labeled oligonucleotides containing specific target sites, new synthetic zinc finger domains of defined three bp specificity were isolated.[3,4] Our selections quickly revealed that zinc finger domains could be selected for not only 5′-GNN-3′ triplets, which are overrepresented in naturally occurring zinc finger target sites, but also 5′-ANN-3′ and 5′-CNN-3′ triplets. In subsequent studies, synthetic zinc finger domains that recognized all of the 16 possible 5′-GNN-3′,[5,6] 5′-ANN-3′,[7] and 5′-CNN-3′[8] triplets, as well as several 5′-TNN-3′

sequences[8] were developed through selection as well as design (Figure 3). Surprisingly, many of our engineered domains bound their intended triplets with specificity that even exceeded the corresponding naturally occurring zinc finger domain, as certain optimized zinc fingers discriminated between targets containing single base mismatches by greater than 100-fold in the context of three-finger proteins (Figure 4). Subsequent crystallographic studies of Aart,[9] a designed six-finger zinc finger protein, revealed that the selected residues at positions −1, 3, and 6 do indeed play key roles in mediating DNA specificity, but that positions 2, 4, and 5 are also important for enforcing target specificity by excluding recognition of other types of sequences (Figure 5), a general finding supported by the diversity observed at these positions among our selected zinc finger domains (Figure 3).

First position	Second position: T	Second position: C	Second position: A	Second position: G	Third position
T	TTT -	TCT - RLRDIQF	TAT - ARGNLRT	TGT -	T
T	TTC -	TCC - RSDERKR	TAC - SRGNLKS	TGC - APKALGW	C
T	TTA -	TCA - RSDHLTT	TAA - QASNLIS	TGA - QAGHLAS	A
T	TTG - RKDALRG	TCG - RLRALDR	TAG - REDNLHT	TGG - RSDHLTT	G
C	CTT - TTGALTE	CCT - TKNSLTE	CAT - TSGNLTE	CGT - SRRTCRA	T
C	CTC - QRHHLVE	CCC - SKKHLAE	CAC - SKKALTE	CGC - HTGHLLE	C
C	CTA - QNSTLTE	CCA - TSHSLTE	CAA - QSGNLTE	CGA - QSGHLTE	A
C	CTG - RNDALTE	CCG - RNDTLTE	CAG - RADNLTE	CGG - RSDKLTE	G
A	ATT - HKNALQN	ACT - THLDLIR	AAT - TTGNLTV	AGT - HRTTLTN	T
A	ATC - RRSACRR	ACC - DKKDLTR	AAC - DSGNLRV	AGC - ERSHLRE	C
A	ATA - QKSSLIA	ACA - SPADLTR	AAA - QRANLRA	AGA - QLAHLRA	A
A	ATG - RRDELNV	ACG - RTDTLRD	AAG - RKDNLKN	AGG - RSDHLTN	G
G	GTT - TSGSLVR	GCT - TSGELVR	GAT - TSGNLVR	GGT - TSGHLVR	T
G	GTC - DPGALVR	GCC - DCRDLAR	GAC - DPGNLVR	GGC - DPGHLVR	C
G	GTA - QSSSLVR	GCA - QSGDLRR	GAA - QSSNLVR	GGA - QRAHLER	A
G	GTG - RSDELVR	GCG - RSDDLVR	GAG - RSDNLVR	GGG - RSDKLVR	G

Figure 3. Summary of the selected zinc finger domains used for modular assembly. The α-helical residues (−1, 1, 2, 3, 5, and 6) for each zinc finger are shown. Positions −1, 3, and 6 are underlined.

3. POLYDACTYL ZINC FINGER PROTEINS

Another critical question for the development of zinc finger technology concerned the length of DNA that could be specifically targeted by these proteins. Because the haploid human genome consists of three billion DNA bp, proteins such as Zif268, which bind nine bp of DNA, are predicted to recognize ∼12 000 distinct sites, making single site recognition virtually impossible. Since we desired to create proteins capable of recognizing a single address within a genome, we sought a means to develop zinc finger proteins capable of binding specific DNA sequences of >15 bp. This, however, would require the use of novel polydactyl proteins not typically found in nature. Molecular modeling of a six-finger zinc finger protein based on Zif268 led us to determine that the canonical linker peptides TGEKP or TGQKP would be ideal candidates for assembling polydactyl proteins.[10] While it was generally assumed that these linkers would be insufficient for polydactyl zinc fingers to accommodate the helicity of DNA, numerous studies have since supported the use of this linker strategy. Thus, while initially very controversial,[11] this approach has now facilitated the development of other zinc finger selection strategies and is commonly used in both commercial (CompoZr, Sigma-Aldrich) and clinical applications of zinc finger proteins.

Having created a lexicon of zinc finger domains and a means of connecting them to achieve genome-wide levels of

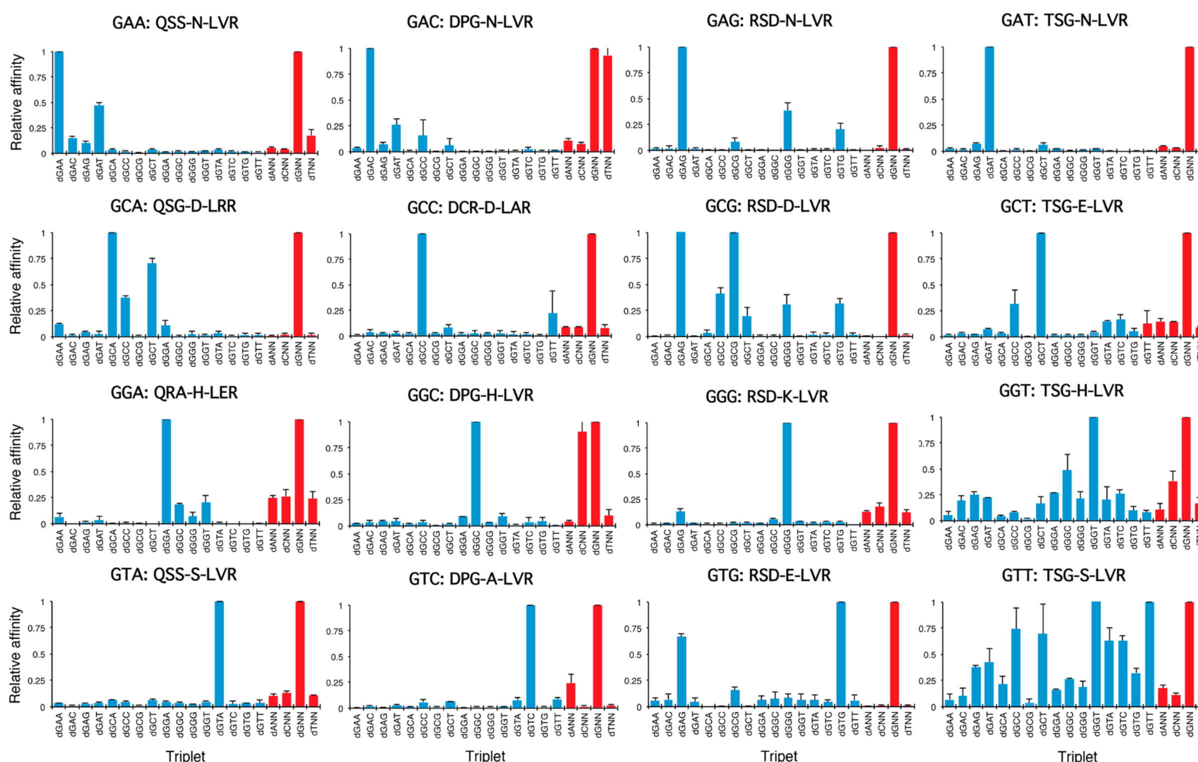

Figure 4. Specificity profiles of the zinc finger domains selected or designed to recognize each of the 16 possible 5′-GNN-3′ triplets. Blue bars represent binding to all 16 possible 5′-GNN-3′ triplets. Red bars represent binding to pools of 5′-GNN-3′, 5′-ANN-3′, 5′-CNN-3′, and 5′-TNN-3′ triplets. Data previously published in refs 5 and 12.

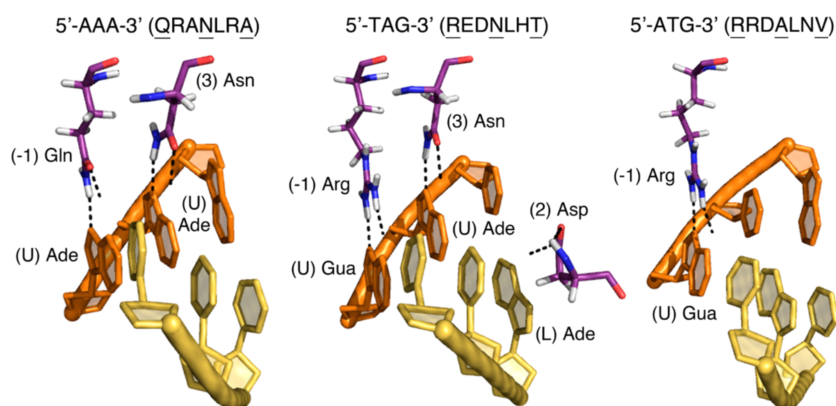

Figure 5. Contacts between the recognition helices of Aart, a designed six-finger zinc finger protein, and target DNA. The α-helical residues that specifically interact with DNA are shown as purple sticks. All residues are numbered according to their α-helical position (−1, 3, or 6). DNA is shown as orange and yellow sticks. The indicated DNA triplet and the α-helical residues specific for that target are indicated above each structure.

specificity, an ever-growing number of zinc finger-based applications became possible. The inherent modularity of our customization strategy also allowed us to develop the first web server, Zinc Finger Tools, for automated zinc finger protein design.[12] Now, with advances in standardized recombinant DNA technologies and custom DNA synthesis, novel zinc finger proteins can be rapidly prepared for a variety of purposes.[13] Below we highlight three applications that have been facilitated by our approach to zinc finger construction.

4. TRANSCRIPTION FACTORS

One of the first applications of synthetic zinc finger technology was the creation of artificial modulators of gene expression. In contrast to conventional genetic engineering methods, which induce gene expression via addition of transgenes to cells, zinc finger proteins allow for regulation of endogenous genes in their natural chromosomal locations. In 1998, we created the first synthetic activators and repressors of a human gene by fusing engineered polydactyl zinc finger proteins to a tetrameric repeat derived from the herpes simplex VP16 activation domain (VP64) and the Krüppel-associated box (KRAB) repression domain, respectively.[14] We subsequently showed that these proteins could specifically up- and down-regulate the endogenous *epidermal growth factor receptors* 2 and 3 (*ERBB2* and *ERBB3*) genes in human cells, and that these proteins could discriminate between their highly homologous target sites, even when the targeted sequences differed by only 3 of 18 bp.[15] Our *ERBB3* transcription factor was subsequently used to define the role of ErbB3 in breast cancer.[16]

Having demonstrated the ability to make synthetic repressors targeted to specific genes, we next sought to inhibit the transcription and replication of HIV-1. We engineered zinc finger repressors that were able to specifically down-regulate the HIV promoter and decrease HIV replication in primary cells up to 100-fold.[17,18] To further demonstrate the therapeutic potential of zinc finger technology, we designed synthetic zinc finger transcriptional activators targeted to the promoter of the γ-globin gene, which can be activated as a therapeutic approach to sickle cell disease and β-thalassemia.[19] We showed that synthetic activators targeted to this promoter up-regulated γ-globin expression in human cell lines[19] and activated the silent γ-globin gene in primary human hematopoietic stem cells[20] and in vivo in a transgenic mouse model.[21] These results established a promising blueprint for gene therapy of sickle cell disease and β-thalassemia in which targeted gene activation in a patient's own cells could compensate for the genetic disorder.

We have also used zinc finger technology to discover new genes associated with important cellular phenotypes, such as cancer progression and drug resistance. We created a library of 8.4×10^7 unique six-finger zinc finger variants[13,22] designed to recognize random 18 bp sequences through combinatorial assembly of individual zinc finger domains. When fused to the VP64 activation domain and delivered to human cells by retrovirus, we found that each protein could find one or more targets in the genome and regulate adjacent genes. By selecting for cells in which specific cell surface markers were up-regulated, we isolated zinc finger proteins that targeted the promoters of those gene products.[22,23] We later used a similar approach to identify genes that controlled tumor progression[24,25] and transcription factors that conferred drug resistance and increased migration and invasion in drug-sensitive cancer cells, thereby providing valuable insight into the mechanisms governing tumor progression. We have also used alternative selection schemes to recover activators of the γ-globin gene from libraries of zinc finger transcription factors in human cells.[26]

Because the delivery of multiple zinc finger transcription factors to one cell for activation of multiple genes may be challenging, we engineered bispecific zinc finger transcription factors with two independent and modular DNA binding domains.[27] Expression of this single transcription factor led to activation of both target promoters in human cells, providing researchers with a tool capable of yielding insight into the dynamics of complex signaling pathways. Furthermore, to explore the possibility of modulating gene expression by manipulating the epigenetic state of a gene, we and others have fused DNA methyltransferase domains to synthetic zinc finger proteins to direct methylation to specific DNA sequences. Numerous biological processes, including organism development, gene imprinting, X-chromosome inactivation, and carcinogenesis are known to be associated with gene silencing via DNA methylation. However, one major challenge associated with this technology has been off-target methylation. To address this, we engineered a split *Hha*I DNA methyltransferase in which the N- and C-termini of this enzyme were attached to two separate synthetic zinc finger proteins.[28] Recent studies by other groups have similarly used modularly assembled zinc finger proteins to direct DNA methylation to endogenous gene

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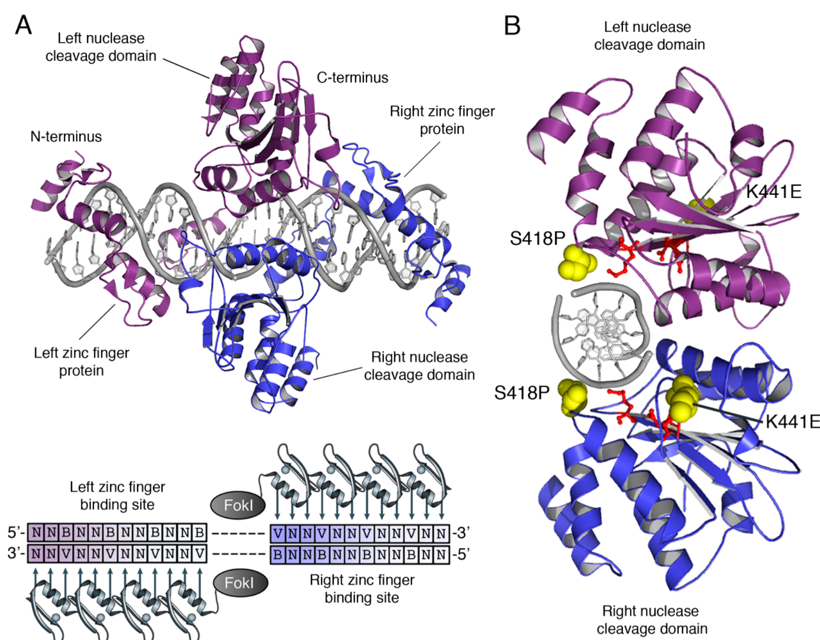

Figure 6. Zinc finger nuclease (ZFN) structure. (A) (Top) Three-dimensional model of the ZFN dimer (purple and blue) in complex with DNA (gray) (PDB IDs: 1FOK and 2I13, respectively). (Bottom) Cartoon of the ZFN dimer bound to DNA. (B) Model of the FokI cleavage domain dimer (purple and blue) in complex with DNA (PDB ID: 2FOK). Sharkey mutations (S418P and K441E) are shown as yellow spheres. The catalytic amino acids Asp 450, Asp 467, and Lys 469 are shown as red sticks.

promoters[29,30] or to demethylate promoters to activate gene expression.[31,32]

In many cases, external control over the magnitude and timing of gene activation or repression is desirable. To achieve this, we developed chemically inducible transcription factors by fusing zinc finger proteins to steroid receptors that are activated only in the presence of complementary small molecule ligand.[33] We later expanded on this work and showed that ligand-inducible expression of endogenous human genes could be achieved.[34] We have also developed an inducible expression system that responds only to light by linking synthetic zinc finger proteins and transcriptional activation domains to plant proteins that dimerize in response to blue light illumination.[35]

Finally, to expand upon current methods for genetically modifying plants for agricultural benefits, we demonstrated regulation of transgenes in tobacco plants and endogenous genes in *Arabidopsis* with modularly assembled zinc finger transcription factors.[36−38] We later used synthetic zinc finger repressors to suppress genes that inhibit plant pathogens.[39]

5. NUCLEASES

Although approaches for gene targeting based on homologous recombination are established for certain systems, until recently there has been no general technology that worked robustly in diverse species and cell types, including human cells. In 1994, Jasin and colleagues discovered that induction of a double-strand DNA break by the endonuclease I-SceI stimulated homologous recombination by several orders of magnitude.[40] Synthetic zinc finger proteins have since been fused to the cleavage domain of the FokI restriction endonuclease for targeting of nuclease activity to user-defined sites, enabling both site-specific integration or gene modification through homologous recombination and gene knockout via nonhomologous end joining (Figure 6A).[41] These zinc finger nucleases (ZFNs) have now been widely used for genome editing in many species and cell types for basic science, biotechnology, and medical applications, such as targeted disruption of the *CCR5* gene for HIV-1 therapy, as proposed in 1997.[10] We[42,43] and others[44,45] have comprehensively reviewed the development and application of ZFN technology elsewhere. Here, we focus on how modular assembly of custom zinc finger proteins has enabled ZFN construction, as well as other contributions by our laboratory for enhancing this technology.

A vast number of ZFNs have been constructed by modular assembly and shown to mediate efficient genome editing of endogenous genes. Among these are several of the first ZFN pairs to stimulate gene targeting in *Xenopus laevis* oocytes[41] and *Drosophila*,[46] as well as foundational work that derived important parameters for ZFN-mediated homologous recombination.[47] We have also created ZFNs targeted to the mouse ROSA26 locus for the creation of isogenic transgenic cell lines with consistent levels of transgene expression.[48] Yet despite the widespread success of ZFN-mediated gene targeting, one limitation of this technology has been the efficiency with which these enzymes induce modifications. To address this, we created a high-throughput directed evolution strategy for identifying activating mutations in the FokI cleavage domain.[49] Using this approach, we discovered mutations that increased ZFN activity by >15-fold in bacterial assays and 3- to 6-fold in mammalian cell-based assays (Figure 6B). This enhanced FokI variant, dubbed Sharkey, has now been used in numerous studies to broadly enhance nuclease activity.[48,50]

The efficiency of ZFN-mediated genome editing is largely dependent on the ability of ZFNs to enter the cell nucleus, where they can access genomic DNA and induce targeted modifications. To date, most studies have delivered ZFN-encoding genes via plasmid DNA or viral vectors. These approaches, however, are subject to numerous limitations, including toxicity from plasmid transfection or electroporation, viral vector immunogenicity, and the potential for vector integration into the genome. We therefore explored the possibility of delivering ZFNs directly into cells as proteins.

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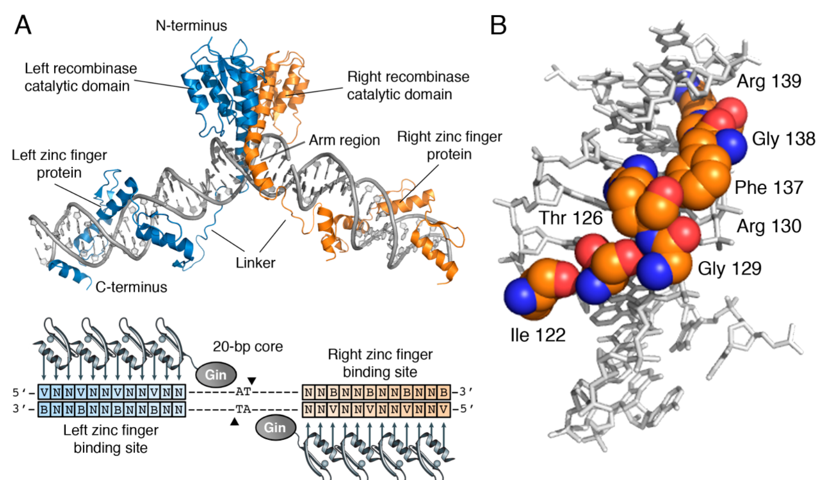

Figure 7. Zinc finger recombinase (ZFR) structure. (A) (Top) Three-dimensional model of the ZFR dimer (blue and orange) in complex with DNA (gray), adapted from Gaj et al.[60] (PDB IDs: 1GDT and 2I13, respectively). (Bottom) Cartoon of the ZFR dimer bound to DNA. (B) Residues that confer recombinase catalytic specificity and subject to reprogramming for recognition of new sequences are shown as spheres. Carbon, oxygen and nitrogen atoms are colored orange, red and blue, respectively. DNA is shown as gray sticks.

Indeed, we discovered that purified ZFN proteins have the innate capacity to cross cell membranes and mediate highly efficient gene knockout in human cells.[50] Because ZFN proteins delivered directly into cells were degraded shortly after internalization, this approach led to fewer off-target effects than expressing ZFNs from plasmid DNA.

6. RECOMBINASES

In recent years, ZFNs have been used for diverse applications in many areas of research. However, off-target cleavage by ZFNs and subsequent activation of the DNA damage response pathway has repeatedly led to cellular toxicity after treatment. To address these concerns, our laboratory and others have investigated the possibility of engineering new classes of enzymes that autonomously catalyze targeted DNA recombination in the absence of double-strand DNA breaks and thus do not depend on endogenous DNA repair mechanisms. Our initial attempts focused on fusing zinc finger proteins to the HIV viral integrase for directed retroviral integration into the genome.[51] Other groups have fused our modularly assembled zinc finger proteins to transposases for site-specific integration.[52,53] However, in each of these cases, fusion of the catalytic domain to the zinc finger did not abrogate nonspecific integration, as targeted integration was less frequent than unwanted random integration events.

To build enzymes that only catalyze targeted integration or excision, we investigated the fusion of modularly assembled zinc finger proteins to the serine recombinases (Figure 7A).[54] Within this family of enzymes, the catalytic domain promotes DNA recombination, while the DNA-binding domain mediates target specificity, as both domains are structurally and functionally distinct. This modularity allows for replacement of the native DNA-binding domain with engineered zinc finger proteins. We have shown that these zinc finger recombinases (ZFRs) are able to efficiently excise genes[55] and integrate plasmid DNA into the human genome with >98% specificity.[56,57] These studies also confirmed that the ZFR catalytic domain maintained sequence specificity for its natural target site.[55,56] Because this constraint limits the capacity of ZFRs to be re-engineered to recognize new sequences, we developed a powerful system for the directed evolution of recombinases that led to the discovery of enzyme variants with a >1000-fold increase in activity against new target sites.[55,58] However, these ZFRs demonstrated relaxed target specificity, an undesirable feature for applications that require precise genetic engineering. We therefore pursued a structure-guided approach for reprogramming serine recombinase specificity by randomizing only those residues predicted to contact DNA (Figure 7B).[59] Using this strategy, the catalytic specificities of two distinct recombinases, Gin and Tn3, were reprogrammed. We expanded on this approach and developed a catalog of redesigned Gin recombinases capable of recognizing a wide variety of possible sequences and showed that ZFRs assembled from these re-engineered domains recombined user-defined targets with high specificity.[60] Ongoing work in this area includes the design of ZFRs with extended[61] and improved[62] targeting capabilities.

7. ALTERNATIVE ZINC FINGER ASSEMBLY STRATEGIES

Alternative strategies for engineering DNA-binding domains have emerged that complement the modular assembly of zinc finger proteins. In contrast to the synthetic domains isolated by our laboratory using phage display, several studies have used naturally occurring zinc finger domains as modular components.[63,64] Other studies have used modules of prevalidated two-finger domains, rather than the individual zinc finger domains used in our method.[65,66] In contrast to modular assembly, strategies that select for new zinc finger proteins from large libraries have been well documented to produce functional proteins.[67,68] Because this strategy is very labor-intensive, it has served as the basis for a hybrid modular assembly approach in which the domains of the selected proteins can be recombined to target new sequences.[69] These varied methods of engineering new proteins provide a spectrum of balance between simplicity, effort, and success rates. Therefore, each individual investigator needs to decide which approach is most suitable for a particular study and laboratory. However, the successes of modular assembly covered in this Account, combined with a recent study[70] providing guidelines for engineering highly active zinc finger nucleases by modular

assembly, clearly document the efficacy and simplicity of this approach.

8. ALTERNATE DNA-BINDING DOMAINS

Recently, the DNA recognition code for transcription activator-like effectors (TALEs) was solved.[71,72] TALEs are naturally occurring proteins from plant pathogenic bacteria, which contain DNA-binding domains composed of a series of 34 amino acid repeat domains that each recognize a single bp of DNA through two hypervariable residues. We and others have incorporated engineered TALEs into synthetic transcription factors,[73−77] nucleases,[74] recombinases,[78] and epigenetic modifiers.[32,79,80] Numerous reports have suggested that engineering new TALEs may be easier and more effective than constructing active zinc finger proteins,[81] and that TALE nucleases (TALENs) may not cause the toxic effects associated with some ZFNs.[82] This technology is still in its infancy, and much work remains to be done to comprehensively characterize the advantages of TALEs and zinc finger proteins. However, some fundamental differences between these two proteins indicate that the best choice may largely be decided on a case-by-case basis. For example, TALEs are more than three times larger than zinc finger proteins. Consequently, a single adeno-associated viral (AAV) vector cannot be packaged with two TALEN monomers due to vector size restriction, hindering potential gene therapy applications. TALEs also do not have the overall cationic charge presumably responsible for the innate cell-penetrating properties of zinc finger proteins.[50] As a result, it may be significantly more challenging to design cell-penetrating TALE-based proteins, although our laboratory has recently shown that cell-permeability can be artificially introduced into TALENs via bioconjugation of cell-penetrating peptides.[83]

In addition, CRISPR/Cas systems have recently emerged as an alternative to zinc finger and TALE-based DNA targeting platforms. In 2012, Doudna, Charpentier, and colleagues demonstrated that the Cas9 protein facilitates sequence-specific cleavage of pathogenic DNA via complementary CRISPR RNA, and that CRISPR/Cas systems can be retargeted to cleave virtually any DNA sequence simply by redesigning the CRISPR RNA template.[84] The ease with which this system can be implemented has led to a plethora of studies demonstrating the applicability of CRISPR/Cas for genome engineering.[85,86] The Cas9 protein complexed with CRISPR RNA has since been repurposed for gene regulation, as we and others have shown that a cleavage-incompetent mutant of the Cas9 protein can be fused to transcriptional activation and repression domains for RNA-guided control of gene expression.[87−90] Much like TALEs, this system is still in its infancy, and many questions remain related to the specificity as well as the overall robustness of these Cas9-based tools. However, we expect that having multiple options for engineering DNA-binding proteins will provide greater opportunity for success in leveraging these technologies. Importantly, the speed at which these new systems have progressed would likely not have been possible without the fundamental advances made with the zinc finger technology.

9. CONCLUSIONS

The full impact of the fundamental advances generated by the modular assembly approach to engineering zinc finger proteins has only begun to be realized. Our understanding of the interactions between zinc finger proteins and DNA is continuously improving, applications for their use are rapidly evolving, and emerging complementary genome engineering technologies are providing new opportunities to capitalize on 20 years of research in this field. These methods will continue to catalyze progress in the targeted manipulation and regulation of genomic and epigenetic structure, and translate the products of the Genomic Revolution into advances in science, medicine, and biotechnology.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: carlos@scripps.edu. Deceased. Please see the Dedication for additional contact information.

Author Contributions

[§]C.A.G. and T.G. contributed equally to this work.

Notes

The authors declare no competing financial interest.

Biographies

Charles A. Gersbach is an Assistant Professor in the Department of Biomedical Engineering and Institute for Genome Science and Policy at Duke University. He received his Ph.D. in Biomedical Engineering jointly from the Georgia Institute of Technology and Emory University. He completed a postdoctoral fellowship with Carlos F. Barbas, III at The Scripps Research Institute. His lab is focused on applying molecular engineering to gene therapy, regenerative medicine, and synthetic biology.

Thomas Gaj received his Ph.D. in Chemistry from The Scripps Research Institute working with Carlos F. Barbas, III. He is now a postdoctoral research associate in the Department of Chemical and Biomolecular Engineering at the University of California, Berkeley. His research interests include biomolecular engineering, synthetic biology, and gene therapy.

Carlos F. Barbas, III received his Ph.D. in Chemistry with Chi-Huey Wong at Texas A&M University. Following postdoctoral studies with Richard Lerner, he began his academic career as Assistant Professor in 1991. He is now Kellogg Professor of Chemistry and Cell and Molecular Biology at The Scripps Research Institute. His research is focused at the interface of organic chemistry, biology, and medicine and focuses on the development of organocatalysis, protein engineering, and novel therapeutic approaches to human disease.

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■ DEDICATION

Dedicated to the memory of Carlos F. Barbas, III, a close friend and colleague to many, and a pioneer of research at the interface of medicine, chemistry, and biology. Address

correspondence to the co-authors at charles.gersbach@duke.edu and gaj@berkeley.edu.

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