



# The past, present, and future of artificial zinc finger proteins: design strategies and chemical and biological applications

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Received: 18 October 2022 / Accepted: 25 January 2023 / Published online: 7 February 2023  
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## Abstract

Zinc finger proteins are abundant in the human proteome and are responsible for a variety of functions. The domains that constitute zinc finger proteins are compact spherical structures, each comprising approximately 30 amino acid residues, but they also have precise molecular factor functions: zinc binding and DNA recognition. Due to the biological importance of zinc finger proteins and their unique structural and functional properties, many artificial zinc finger proteins have been created and are expected to improve their functions and biological applications. In this study, we review previous studies on the redesign and application of artificial zinc finger proteins, focusing on the experimental results obtained by our research group. In addition, we systematically review various design strategies used to construct artificial zinc finger proteins and discuss in detail their potential biological applications, including gene editing. This review will provide relevant information to researchers involved or interested in the field of artificial zinc finger proteins as a potential new treatment for various diseases.

**Keywords** Zinc finger · Redesign · Metalloprotein · Zinc protein · Artificial protein · Gene editing

## Introduction

Zinc fingers (ZFs) are representative DNA-binding motifs found in many transcription factors. Genome analysis of human and mouse genomes has revealed that ZFs are present in 3–10% of protein-coding genes [1, 2]. ZFs are known to be involved in various biological functions such as development, differentiation, and suppression of tumors [2]. Thus, due to the recognized biological importance of ZFs, their structure and function has been extensively studied from various perspectives.

Generally, ZF domain comprises 20–30 amino acid residues. ZFs are classified according to the number and type of amino acids involved in zinc coordination, and typical examples include Cys<sub>2</sub>His<sub>2</sub>, Cys<sub>2</sub>HisCys, Cys<sub>4</sub> ribbon, Cys<sub>4</sub> GATA family, Cys<sub>6</sub>, Cys<sub>8</sub>, Cys<sub>3</sub>HisCys<sub>4</sub> ring finger, and His<sub>2</sub>Cys<sub>2</sub> [3]. Among these, the Cys<sub>2</sub>His<sub>2</sub>-type ZF is a representative classical motif whose structure and function have been most intensively studied (Fig. 1) [4]. Focusing on the domain structure, two Cys residues and two His residues form a tetrahedral coordination bond with Zn(II). This induces a secondary structure of an inverted parallel  $\beta$ -sheet at the amino group terminus and an  $\alpha$ -helix (DNA recognition helix) at the carboxyl group terminus, which further folds to form a compact globular structure (Fig. 1A). Furthermore, in numerous ZFs, several single finger domains are linked by specific linkers to form tandem structures (Fig. 1B). Regarding DNA recognition, Cys<sub>2</sub>His<sub>2</sub>-type ZFs are characterized by the following features: (1) one finger domain recognizes three to four bases, (2) the tandem structure allows selective binding to contiguous DNA sequences, and (3) unlike restriction enzymes, they can bind to DNA in monomeric units and recognize asymmetric sequences (Fig. 1B). Thus, ZFs are promising templates for the redesign and creation of functional artificial proteins because

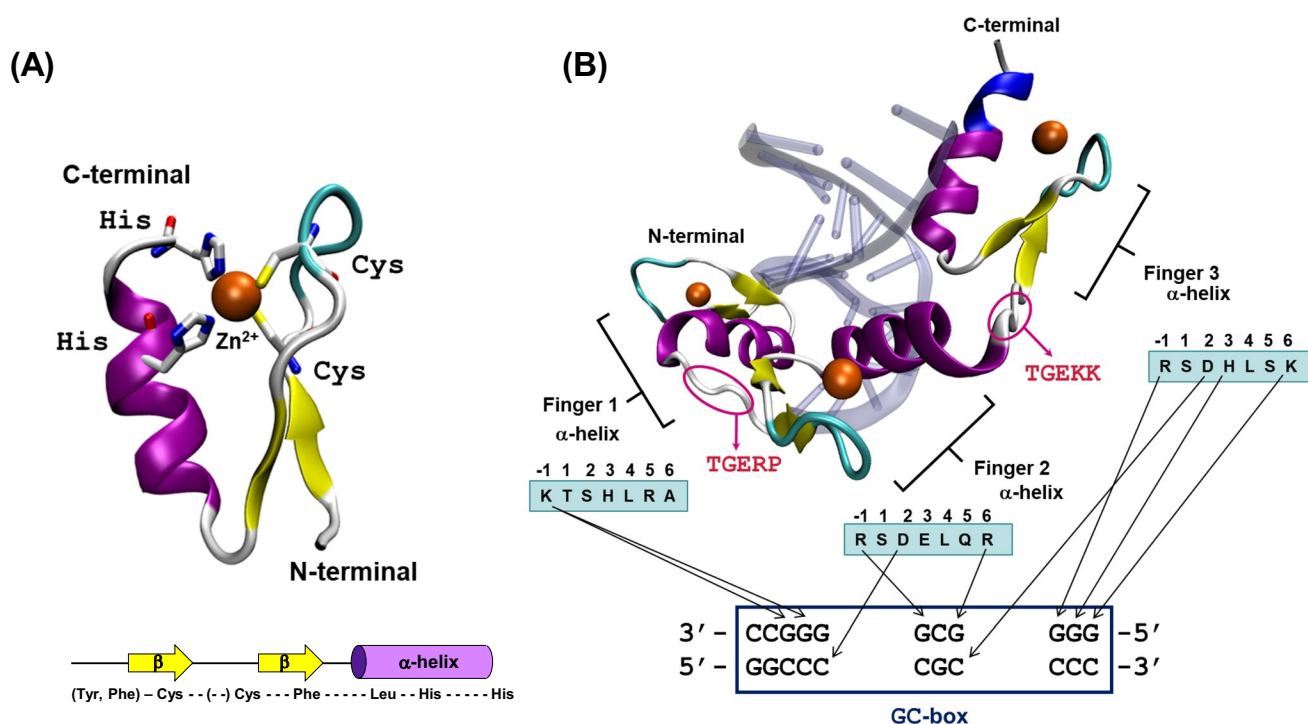
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**Fig. 1** **A** Schematic representation of a typical  $C_2H_2$ -type zinc finger motif with the bba fold (upper) and consensus sequence of  $C_2H_2$ -type zinc finger motif “(Tyr, Phe)-x-Cys-x<sub>2,4</sub>-Cys-x<sub>3</sub>-Phe-x<sub>5</sub>-Leu-x<sub>2</sub>-His-

x<sub>3,5</sub>-His (x: nonconserved amino acids)” [ref. 10 and 41] (lower). **B** X-ray structure of the Zif268-DNA complex (PDB: 1ZAA)

of their unique structure and precise molecular recognition capabilities (metal coordination binding and DNA recognition). Against this background, numerous research groups, including ours, have been working on the creation of functional artificial proteins using ZFs as templates and using various approaches from different angles. Design approaches have primarily attempted to target the secondary structural sites ( $\alpha$ -helix and  $\beta$ -sheet sites), zinc coordination sites, and linker sites that constitute the domain.

Recently, there has been revived interest in the metalloprotein aspect of ZF from a protein design perspective. This is likely because the numerous natural metalloproteins with their unique structures are now widely recognized as useful for the creation of artificial proteins. One-third of human natural proteins belong to metalloproteins, which are known to cooperate with metal ions to play structural and functional roles in maintaining biological activities [1, 5–14]. These metalloproteins have been optimized by natural evolution to perform complex structural formation and functional regulation that cannot be achieved by either protein or metal ion alone. Recently, significant advances in protein design and engineering have made it possible to create artificial metalloproteins with novel functions by redesigning natural proteins as templates or by constructing entirely new amino acid sequences from computer calculations [7–9]. As mentioned

above, various coordination patterns of ZFs make their metal coordination sites interesting targets for redesigning artificial proteins.

In this review, we will discuss the creation and application of artificial proteins that are redesigned from natural ZFs and their application to gene editing and other recent biological tools, including our own research findings.

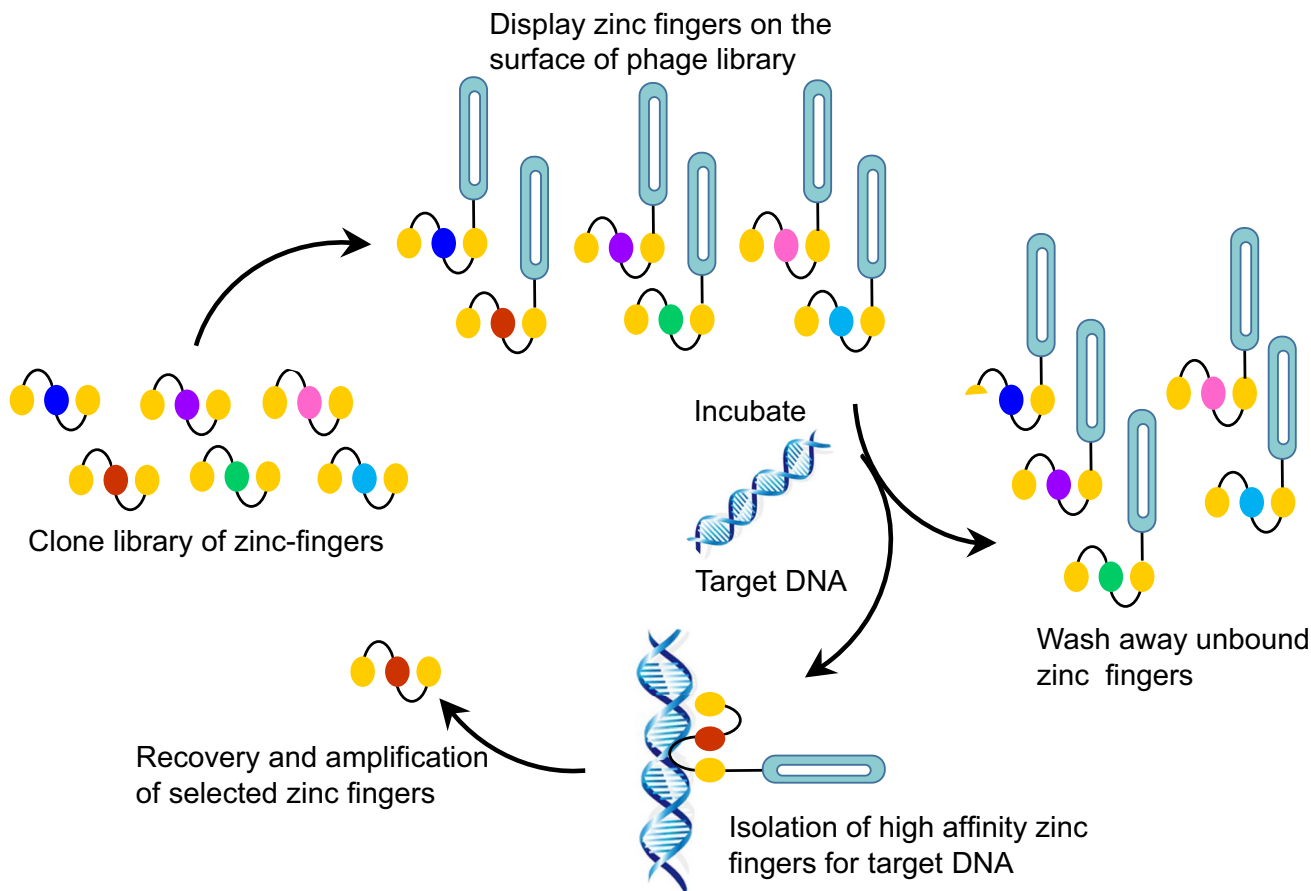
### Modification of DNA recognition ability of ZFs targeting secondary structures within the domain

The binding of ZFs to DNA is generally achieved through the interaction of the  $\alpha$ -helix region with the major groove of DNA. In the  $Cys_2His_2$ -type ZF, amino acid residues -1, 2, 3, and 6 at the N-terminus of the  $\alpha$ -helix interact with DNA bases to selectively recognize specific DNA sequences (Fig. 1B) [5, 15]. The mutation of amino acid residues directly involved in DNA sequence recognition is considered an effective approach for modifying the DNA recognition ability of wild-type ZFs. The most effective method using the phage display method, in which a ZF library is constructed by randomizing amino acid residues involved in DNA recognition and selecting ZFs that can bind to the

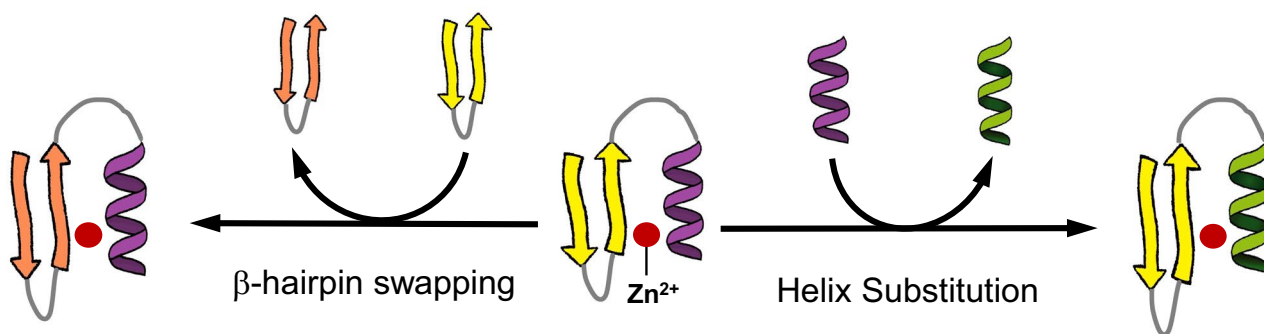
target DNA sequence [5, 16, 17]. Barbas et al. successfully constructed a ZF domain library using a phage display capable of accommodating approximately 75% of the 64 sequences that combine all triplet sequences, thereby enabling customization of the artificial ZFs that can accommodate recognition of most DNA sequences (Fig. 2) [18–20].

In contrast, the so-called domain swapping method, in which secondary structural units are recombined among

target proteins, is one of the effective methods for protein redesign [21, 22]. We attempted to modify the DNA recognition ability of ZF proteins using the domain swap method, in which secondary structural units such as  $\alpha$ -helix and  $\beta$ -hairpin of ZFs are swapped with those of other ZFs (Fig. 3). First, artificial ZFs were created by replacing the  $\alpha$ -helix parts of ZFs of two different origins [23]. A chimeric ZF, Sp1HM, was produced by replacing the  $\alpha$ -helix



**Fig. 2** Schematic diagram of the selection of zinc finger proteins by the directed evolution method using phage display libraries



**Fig. 3** Modification of DNA binding properties of zinc finger proteins by swapping the  $\alpha$ -helix and  $\beta$ -hairpin regions

region of the human transcription factor-derived Sp1 ZF with the  $\alpha$ -helix region of the *Drosophila*-derived transcription factor CF2-II ZF (Fig. 3). The Sp1 ZF binds to the GC sequence; however, in the case of the CF2-II ZF, it binds selectively to the AT sequence. The secondary structure of Sp1HM was examined using circular dichroism spectra. The results showed that Sp1HM exhibited almost the same spectrum as wild-type Sp1, indicating that a similar structure to that of wild-type Sp1 is expected to be retained after the swapping. The DNA binding of Sp1HM was also primarily involved in folding, as observed using a gel shift assay. The results showed that Sp1HM bound to DNA containing AT sequences with very high affinity ( $K_d = 1.3$  nM), but hardly bound to DNA containing GC sequences.

Similarly, the effect of exchanging the  $\beta$ -hairpin site of two different ZFs on DNA-binding capacity was also examined (Fig. 3). As described above, the  $\alpha$ -helix portion is involved in the DNA recognition of ZFs by directly binding to the major DNA groove. In contrast, the  $\beta$ -hairpin portion is thought to be primarily involved in folding but not directly involved in binding to DNA. Therefore, the  $\beta$ -hairpin portion has not received much attention in the redesign of ZFs with respect to functional modification of DNA binding. However, even if the  $\beta$ -hairpin does not directly interact with DNA, perturbation of the folded structure by domain exchange may alter the DNA-binding ability. Therefore, we created mutants in which the  $\beta$ -hairpin portion of the Sp1 ZF was alternately replaced with the GLI ZF and investigated the role of the  $\beta$ -hairpin portion of the ZF in DNA binding (Fig. 3) [24]. Interestingly, in Sp1(zf23)BG, a mutant of Sp1 with the  $\beta$ -hairpin portion of GLI, binding to the GC box was stronger than binding to wild-type Sp1. In contrast, the GLI mutant GLI(zf45)BS, which contained the  $\beta$ -hairpin portion of Sp1, completely lost its binding affinity to DNA. Thus, it is clear that the  $\beta$ -hairpin portion of the ZF domain affects the DNA-binding ability. In summary, we found that the DNA-binding affinity and sequence selectivity can be changed by redesigning ZFs using the domain swap method, indicating the possibility of applying this method to the design of functional artificial ZFs.

## Redesign of linker site-modified ZFs

ZFs are typically connected in a tandem manner using a conserved linker consisting of the five amino acids “TGEK(R)P” with two to three finger domains, thereby allowing sequence-selective binding to contiguous DNA sequences. Generally, a ZF must recognize and bind to more than 18 bases to locate a specific site in the genome sequence.

We attempted to extend the DNA base recognition region of the ZF by tandemly binding multiple finger domains via a linker [25, 26]. Two or three wild-type Sp1ZFs were linked

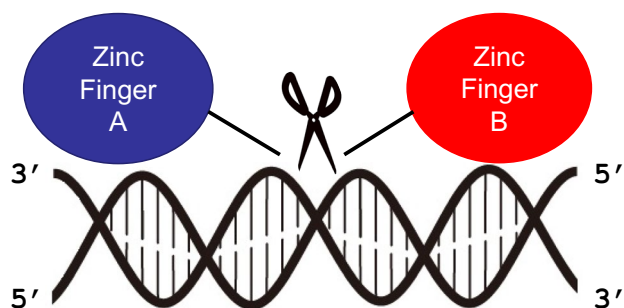
using the conserved linker “TGEKP” to create multi-ZFs, Sp1ZF6 and Sp1ZF9, containing six and nine ZF domains, respectively. As mentioned above, Sp1ZF3 binds to nine base pairs enriched in GC bases, known as GC boxes. The DNA-binding behaviors of Sp1ZF6 and Sp1ZF9 were examined using a gel shift assay, which confirmed that Sp1ZF6 and Sp1ZF9 selectively bind to 18 and 27 base pairs, corresponding to 2 and 3 GC boxes, respectively. We also constructed the longest artificial ZF, Sp1ZF15, to evaluate the potential for multiple linkage of ZF domains [27]. The results revealed that Sp1ZF15 regulates the number of finger domains active for DNA binding in response to the length and sequence of the target DNA. Thus, the creation of multi-fingers by combining multiple ZF domains by a conserved linker can facilitate binding to longer DNA sequences, indicating that this redesign approach effectively extends the contiguous DNA recognition region of the ZFs.

Next, artificial ZFs capable of recognizing discontinuous DNA sequences were redesigned by connecting Sp1ZF3 to each other with a longer and more flexible glycine linker [28, 29]. Sp1ZF6(Gly)7 and Sp1ZF6(Gly)10 have linkers consisting of 7 and 10 glycine residues, respectively, and their binding behavior to discontinuous DNA sequences was examined. The results showed that these 6-ZFs could bind to two target sequences, “2GC(10),” which are 10 base pairs apart, corresponding to one helical turn of DNA. Furthermore, the 6-ZFs induced a curvature between the two target DNA sequences by binding to DNA, and the magnitude of the distortion was dependent on the linker length and was larger for Sp1ZF6(Gly)7 than that for Sp1ZF6(Gly)10. Since conformational changes in DNA are important in the process of transcription, artificial ZFs that can bend DNA created by this redesign method could be applied to artificial transcription manipulation in the cell.

We further investigated the use of oligoarginine as a linker [30]. Arginine is positively charged and has a highly sterically hindered side chain. Sp1ZF6(Arg)8 was created by linking two Sp1ZF3 with eight consecutive arginine-containing sequences (GRRRRRRRRRRRQ) as a linker, and its DNA-binding ability was compared with that of Sp1ZF6(Gly)10, which has the same number of amino acid residues and a flexible linker. The binding affinities of these ZFs to DNA from contiguous (2GC(0)) and non-contiguous (2GC(10)) target sequences were examined. As a result, Sp1ZF6(Gly)10 showed similar affinity to both target sequences. In contrast, Sp1ZF6(Arg)8 showed the same affinity for 2GC(10) as Sp1ZF6(Gly)10, but its affinity for 2GC(0) was reduced more than 20-fold from that of 2GC(10). Possible reasons for the reduced binding of Sp1ZF6(Arg)8 to 2GC(0) include electrostatic and physical repulsion within the linker and inhibition of binding to the GC box sequence due to nonspecific binding of the arginine linker to DNA. In addition, Sp1ZF6(EAAAR)<sub>4</sub>, which has a

linker that forms an  $\alpha$ -helix structure, showed binding preference for the discontinuous recognition sites in the same phase, 2GC(10) [31]. Our results suggest that the degree of DNA binding, selectivity, and affinity can be controlled by appropriately redesigning the structure of the linker part.

We then created an artificial nuclease molecule Sp1(P1G)GLI by attaching a functional linker, the cerium-binding peptide sequence P1 (DKDGDGYISAAE), to two different ZFs, Sp1(zf23) and GLI(zf45) (Fig. 4) [32]. In general, exonuclease-type artificial restriction enzymes are often created by joining the DNA-binding site and the cleavage site in parallel, and this type cleaves DNA sequences outside the DNA-binding site. In contrast, Sp1(P1G)GLI is considered an endonuclease-like artificial restriction enzyme that can cut inside the target DNA sequence by introducing a P1 linker between two ZFs. DNA cleavage experiments were



**Fig. 4** Schematic diagram of an endonuclease-type artificial enzyme obtained by linking two ZFs with a functional linker

performed by adding tetravalent cerium ions to Sp1(P1G)GLI. We found that Sp1(P1G)GLI selectively cleaves the DNA region between the DNA-binding sites of two ZFs by binding of cerium ions to the P1 linker moiety.

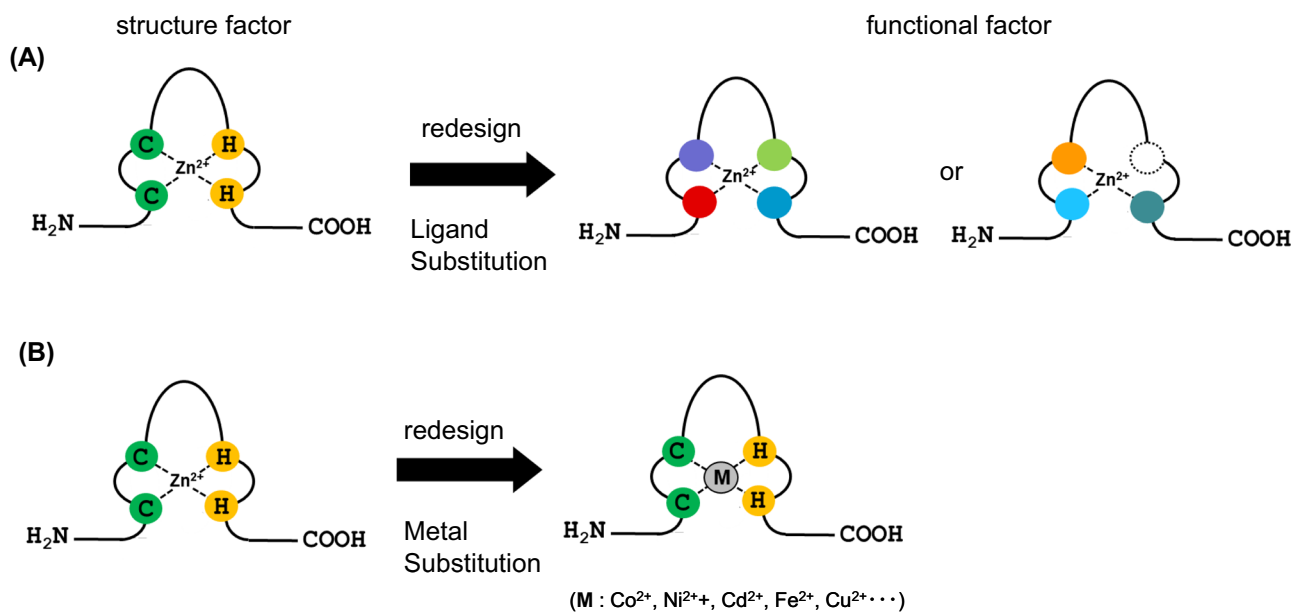
Thus, by appropriately designing the linker portion, it is possible to create artificial ZFs with various new functions, such as DNA cleavage ability and extension of DNA-binding regions, which are expected to be applied to artificial gene regulation.

## Redesign of ZF to target Zn(II) coordination sites

Thermodynamic and structural analyses have revealed that Zn(II) is an essential structural factor for ZF but not a functional factor, because it cannot catalyze biological reactions such as enzymes [10–14]. With regard to the creation of artificial ZFs focusing on the zinc coordination site, two main approaches have been attempted to redesign the zinc (II) coordination site: mutation of amino acid residues involved in the coordination properties and direct replacement of zinc (II) with exogenous metal ions. (Fig. 5).

### The amino acid pattern of naturally occurring zinc finger proteins

We studied the possibility of introducing a new function to ZF by redesigning the amino acid ligand of the Zn(II) coordination site, giving the structural factor of Zn(II) a new



**Fig. 5** Schematic of the redesign approach for the Zn(II)-binding site: **A** substitution of coordinating amino acids for non-coordinating amino acids, **B** substitution of Zn(II) for exogenous metal ions

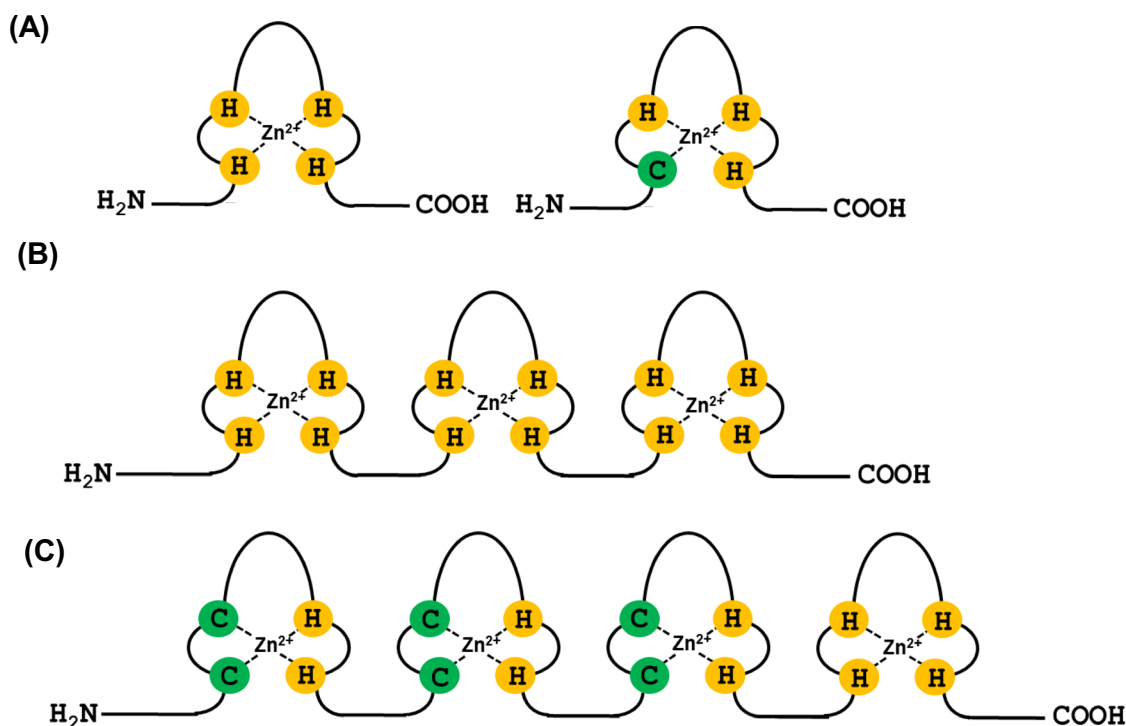
role as a functional factor. Andreini et al. classified approximately 3000 zinc proteins encoded by the human genome by function and zinc ion-binding pattern [1]. The results show that about one-third of human zinc proteins belong to the functional category of transcription factors. In addition, zinc proteins were classified according to the amino acid pattern of their zinc ion-binding sites. The results showed that the Cys<sub>4</sub> type was the most abundant, followed by the Cys<sub>2</sub>His<sub>2</sub> tetraordinated type, with these two types accounting for approximately 70% of the total coordination pattern.

Interestingly, the CysHis<sub>3</sub> and His<sub>4</sub> type ligand patterns are absent in human zinc proteins. One possible reason for the absence of these ligand patterns in nature is that zinc proteins with these sequences may have been eliminated during evolution because of some inconvenience to the organism. In contrast, we hypothesized that introducing these unnatural ligand patterns into natural ZFs might confer new functions not observed in existing ZFs.

### Substitution of amino acid residues ligated to Zn(II)

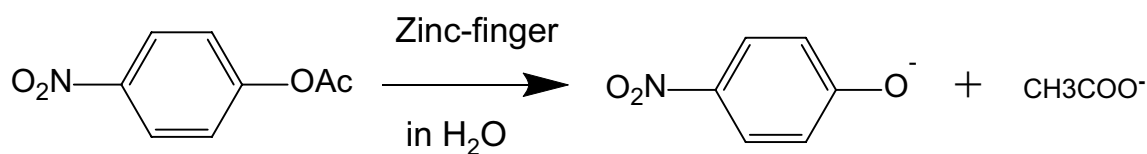
We mutated two Cys residues involved in the Zn(II) coordination site to His using Sp1 ZF to create artificial ZFs of CysHis<sub>3</sub> and His<sub>4</sub> types and evaluated their structure and function (Fig. 6A) [33–35]. Circular dichroism and nuclear

magnetic resonance spectroscopic analysis revealed that the His<sub>4</sub> mutants induce the same typical ββα structure as the wild type upon binding to Zn(II). H4Sp1, consisting of three His<sub>4</sub>-type ZF domains with all Cys residues of Sp1 ZF mutated to His residues (Fig. 6B), was created and its DNA-binding behavior was examined using gel shift assay and DNase I footprinting. The results showed that H4Sp1 can sequence-selectively bind to the GC box, the DNA-binding site of wild-type Sp1 ZF. In general, Zn(II) complexes with high Lewis acidity show high hydrolytic activity. The coordination of cysteine residues to zinc(II) reduces the Lewis acidity of zinc(II) due to its high electron-donating property, thereby reducing hydrolytic reactivity. In this regard, the Lewis acidity of Zn(II) in CysHis<sub>3</sub>-type and His<sub>4</sub>-type ZFs was considered to be higher than that of natural-type ZFs, because more His residues are coordinated to Zn(II) than Cys residues, and their hydrolytic activity was further investigated [34, 35]. 4-Nitrophenyl acetate (4-NP), a commonly used substrate, was selected for the hydrolysis study (Scheme 1). The results showed that natural ZF does not promote hydrolysis of ester bonds at all, while both CysHis<sub>3</sub>- and His<sub>4</sub>-type ZF hydrolyze ester bonds efficiently (Fig. 7). These mutants were also found to be able to hydrolyze phosphate bonds, which is generally considered difficult.

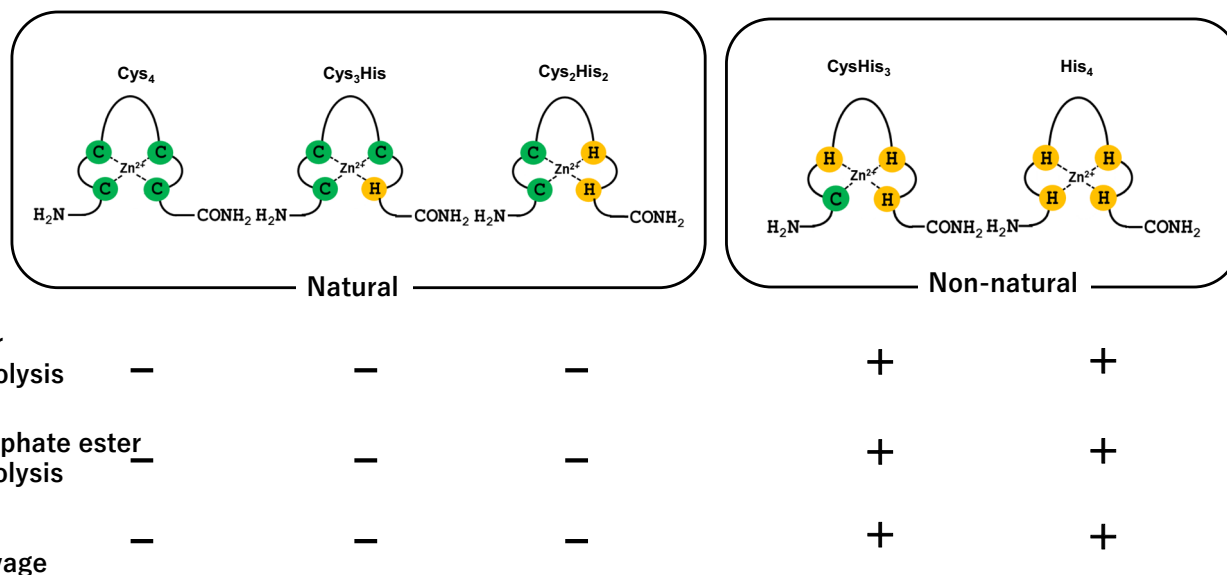


**Fig. 6** Artificial ZF proteins with unnatural Zn(II) coordination sites. **A** Artificial ZF domains of H<sub>4</sub> and CH<sub>3</sub> types; **B** Structure of H<sub>4</sub>-type artificial ZF protein H4Sp1 by redesigning wild-type Sp1. **C** Struc-

ture of a non-*Fok* I-type ZF nuclease with a His<sub>4</sub>-type domain added to the C-terminal side of the wild-type Sp1 ZF



**Scheme 1.** Hydrolysis reaction by artificial ZFs created by ligand substitution



**Fig. 7** Comparison of ester hydrolysis and DNA cleavage ability of natural and non-natural forms of ZF protein

Next, we examined the DNA cleavage potential of H4-type ZFs. Interestingly, H4Sp1 was able to specifically cleave the DNA duplex at the GC box site of plasmid DNA (pUC19GC) under physiological conditions [34]. We also created an artificial restriction endonuclease with a His<sub>4</sub>-type domain added to the C-terminal side of wild-type Sp1 ZF (called non-*Fok* I type ZF) nuclease (Fig. 6C) [36–38]. We found that this artificial restriction enzyme exhibited higher sequence selectivity and DNA double-strand cleavage activity. These results indicate that zinc ions, which originally existed as structural factors in their natural forms, can be converted into functional factors capable of catalytic functions by modifying the coordination site of zinc ions. Furthermore, from a biological point of view, this result may be one possible reason why zinc-binding sequences of the CysHis<sub>3</sub> and His<sub>4</sub> types were eliminated from the human protein component during natural evolution. These results indicate that Zn(II), which originally plays the role of a structure factor in its natural form, can be converted into a catalytic function factor by redesigning its coordination site.

As an example of another ligand substitution method, several groups, including ours, have created ZF mutants in which one of the amino acids in the Cys<sub>2</sub>His<sub>2</sub> coordination

site is replaced by an uncoordinated amino acid (Gly or Ala), such as the CCHH to CCHG or CCHA type (C: Cys, H: His, G: Gly, A: Ala), and studied their structure and hydrolytic enzyme-like function [39–41], considering that most zinc proteins with a tridentate zinc (II) coordination structure belong to hydrolytic enzymes [1, 2]. We constructed a series of ZF mutants in which one of the four zinc-coordinated amino acid residues of finger 2 of Sp1 ZF was replaced with non-coordinating Gly or Ala residues. The results show that the tridentate ZF mutants form a compact domain structure similar to the wild type in the presence of Zn(II), provided that the interaction between the first His ligand in the  $\alpha$ -helical region and the hydrophobic core is conserved [10, 39, 41]. In addition, these mutants have hydrolytic activity for 4-NP, which decreases in the order CCHA > CGHH > GCHH > CCHG > CCAH > CCGH [40]. Presumably, the loss of one coordinating amino acid allows one water molecule to bind to the vacant coordination site, thereby conferring hydrolytic activity to the ZF mutants against 4-NP. Kinetic studies have also revealed that the hydrolysis reaction is influenced by the electron-donating ability of the protein ligand and the coordination environment. These studies are another example of how redesigning the coordination site

of Zn(II) could add a new functional factor to the structural factor Zn(II).

We also created CXHH-type ZFs ( $X = C, H, D, E$ ) in which the second Cys in the CCHH-type coordination site was replaced with other coordinating amino acid residues, His, Asp, and Glu, and examined the effects of ligand substitution on both Zn(II) binding and DNA binding [42, 43]. Thus, by substituting ligands that coordinate to Zn(II), artificial ZFs with various DNA-binding affinities in response to Zn(II) concentrations can be redesigned.

Thus, the ligand redesign method is capable of adjusting the intrinsic function of natural proteins or introducing completely new functions, indicating that it is one of the most promising methods for the creation of artificial functional ZFs using natural ones as templates.

### Replacement of Zn(II) with various exogenous metal ions

Another approach to redesign the coordination site of Zn(II) is to replace Zn(II) in ZF with another exogenous metal ion (Fig. 5B). Given that Zn(II) is important for the structure formation and functional expression of ZF, its function can be modulated by replacement with exogenous metals or other metal complexes and new functions could be introduced more directly into ZF. In general, Zn(II) is known to bind tightly to various intracellular proteins, and the intracellular concentration of free Zn(II) maintains homeostasis in the range of  $10^{-9}$  to  $10^{-11}$  M [44, 45]. The ZF motif has been designed during evolution to bind Zn(II) with high selectivity, and its dissociation constant is low relative to the concentration of free Zn(II). In contrast, the ZF motif can also bind other metal ions *in vivo* [46, 47]. Moreover, substitution of Zn(II) with exogenous metal ions is of biological interest, because it can induce toxic effects (including carcinogenic effects) specific to certain metal ions [48, 49]. There have been extensive studies on metal substitutions in ZF and their interactions with metal complexes [50–56]. In addition, it is crucial to evaluate the genotoxic effects of metal substitution of Zn(II) in ZFs from both biological and toxicological perspectives. It has been reported that metal ions such as Co(II) [12, 14, 57–59], Ni(II) [58, 60–66], Cd(II) [14, 67–70], Pb(II) [71–73], Cu(I) [12, 74], Fe(II) [75–77], Au(I) [78–84], and Ag(I) [85] can interact with various ZFs. The effects of these metal substitutions on the structure and chemistry of ZFs are diverse depending on the metal ion.

Co(II), like Zn(II), exhibits intermediate Lewis acidity and thus high affinity for oxygen, nitrogen, and sulfur donors, because of which it is widely used to study the coordination environment and determine the stability constants of zinc proteins by replacing the spectroscopically inert Zn(II) with Co(II) as a spectroscopic probe [12, 14, 57–59].

Ni(II) is also frequently used as a spectroscopic probe in the study of zinc proteins. In ZF, Ni(II), along with Co(II), is often used to study their metal-binding properties. Instead of using Ni(II) as a probe, we created nickel-substituted ZFs (nickel finger) in which all three Zn(II) are replaced by Ni(II) in wild-type Sp1 ZFs and studied their structure and DNA-binding ability [62]. Interestingly, the substitution of Zn(II) for Ni(II) in Sp1 ZF did not result in a significant difference in the mode of interaction with DNA, but the preference for DNA sequences was significantly altered from that of the wild type. This is an example of how Ni(II) substitution alters the DNA sequence-specific recognition ability of ZF. Perhaps, the change in DNA-binding sequence selectivity in the nickel finger is due to perturbations to the domain structure caused by Ni(II) coordination, which would be expected to have a slightly different structure than that of the wild type. Furthermore, Ni(II) substitution significantly alters the folding structure and properties of ZF, previously reported. For example, in XPA ZF, the Ni(II) substitutions resulted in the formation of a square planar complex, which is different from the tetrahedral structure of the original Zn(II) complex [61]. Furthermore, ZF in Zn(II) coordination is significantly resistant to air oxidation, whereas the presence of Ni(II) ions promotes its oxidation [61]. This change in oxidative reactivity is thought to be due to the disruption of the ZF domain structure by Ni(II) substitution, which in turn promotes oxidation of the Cys residues. Additionally, hydrolysis of peptide bonds has been reported as an interesting property of Ni(II)-substituted ZFs. In general, Ni(II) is known to bind to -TESHHK- amino acid sequences to form square planar complexes and catalyze the hydrolysis of peptide bonds between Gln and Ser residues [64–66]. This amino acid sequence is abundant in the ZF domain, where Ni(II) selectively hydrolyzes peptide bonds in the ZF [63]. From a biological perspective, the oxidative and peptide hydrolysis properties of nickel-substituted ZF may further increase the toxicity of nickel *in vivo*.

Recently, complex formation of ZFs with precious metals such as gold, platinum, and silver ions and their metal complexes, and subsequent binding to DNA and RNA has been reported [78–85]. Gold ion complexes strongly interact with cysteine residues on ZFs, and the binding of Gold ion has been shown to alter the folding structure of ZFs. Therefore, gold complexes may form “gold fingers” by targeting the Zn(II) coordination sites of ZF *in vivo*. Similarly, the interaction of platinum complexes such as cisplatin with ZFs has been studied, and cases of Zn(II) release and loss of secondary structure have been reported. Silver ions are being actively researched for practical applications in the fields of antibacterial, anti-inflammatory, and anti-cancer properties, and the utilization of silver nanoparticles (AgNPs) is increasing in line with this research. Because of the global popularity



of COVID-19, many personal items are now coated with AgNPs, and as a result, our exposure to AgNPs and Ag ions is increasing more than ever. Against this background, a recent study on the interaction between Ag(I) and ZF reported interesting results in which Zn(II) instead of Ag(I) directly forms a silver cluster structure within the ZF domain, resulting in loss of structure and function. Thus, silver ions may not necessarily be harmless metal ions for living organisms either.

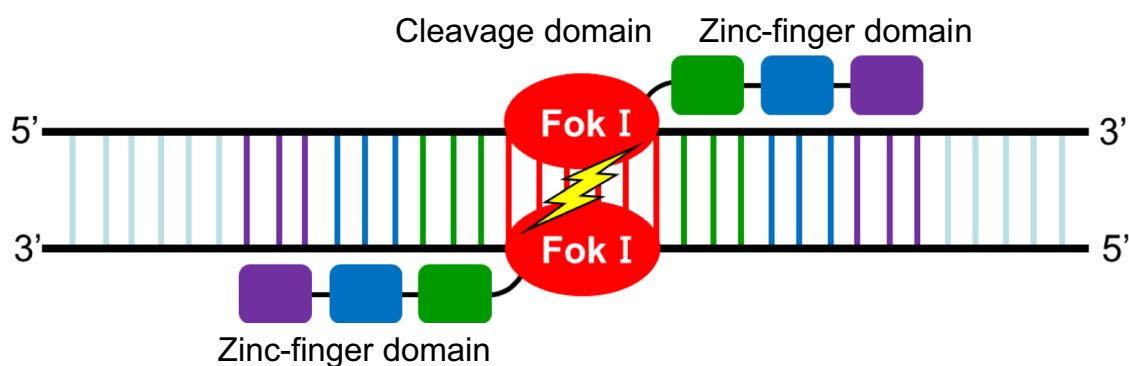
Recently, Michel et al. focused on tristetraprolin protein (TTP), an RNA-binding protein of the CCCH family of ZFs, and are actively engaged in research on the exchange of TTP with exogenous metal ions, the design of metal complexes that stabilize or destabilize TTP, and the possibility of developing new anti-inflammatory and anti-cancer drugs by modulating TTP activity with exogenous molecules [56, 57, 69, 70, 74, 77, 82, 86]. Iron-substituted TTPs achieve nanomolar affinity and retain the ability to recognize homologous RNA sequences compared to Zn(II)-containing sites [77]. While classical ZFs, such as TFIIIA, lose structure and function upon iron substitution, this study shows interesting results in that non-classical ZF TTPs maintain structure and function upon Fe(II)/Fe(III) substitution.

In summary, when ZF is substituted with exogenous metal ions, its structure and function are often lost, and there are only a few cases where metal substitution has successfully introduced new functions into ZF itself. However, extending the study of metal substitution to non-classical ZFs such as TTP, or even combining it with methods such as computational chemistry, could lead to further studies of interactions with exogenous metal ions. This is highly promising and could lead to the creation of artificial metal-substituted ZFs with novel functions.

## Biological applications of designer ZF proteins

Finally, we describe the use of artificial ZF technology for biological applications. The representative biological application of artificial ZFs is genome editing. Genome editing is a technology that uses site-specific nucleases to modify target genes as desired. The major site-specific nucleases are ZF nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9. These technologies have a much wider range of applications compared to conventional genetic engineering and gene therapy and are being intensively studied [87–92]. Here, we will introduce ZFNs, which have been at the center of genome editing research since the early stages of its development.

The above-mentioned phage display system allows for the production of finger motifs that specifically recognize any three bases; by combining these motifs, artificial ZFs that can bind to any base sequence can be created. Artificial ZFNs have been designed by fusing the DNA cleavage domain of the restriction enzyme *Fok I* to ZFs (Fig. 8). Some attempts have been made to “correct” and normalize mutated chromosomes using ZFNs for practical use in gene therapy [87, 93, 94]. Sangamo successfully altered DNA sequences by inducing homologous recombination between chromosomes and extrachromosomal DNA at specific sites of DNA double-strand breaks on chromosomes by ZFNs. In fact, genome editing using a ZF designed to target a mutation site on the interleukin-2 receptor (IL2R) gene that causes X-linked severe combined immunodeficiency, an incurable human disease, resulted in a high frequency of DNA homologous recombination (15–20%) in mutant human cells. Thus, genetically repaired human cells were obtained without selection conditions, and the mutant gene



**Fig. 8** Schematic diagram of the structure of ZF nuclease (ZFN) and the mechanism by which it induces double-stranded breaks (DSB) in its targets. Target sites for gene editing are defined by “left” and “right” zinc finger units with 3–6 ZF domains arranged in tan-

dem. The ZF sites are bound to the nuclease domain of the restriction enzyme *Fok I*. When the left and right ZFs recognize the target sequence, the *Fok I* nuclease dimerizes and cleaves the DNA along the spacer sequence between the two ZFP recognition sites

was successfully recombined into the normal sequence. Furthermore, in 2018, a phase I trial of genome editing therapy for hemophilia was initiated under the leadership of Sangamo (NCT02695160). ZFNs were employed as the genome editing tool, with liver-directed AAV6 vectors carrying two ZFNs and an F9 donor template.

Since then, numerous studies using ZFN-based genome editing have been conducted [93, 95, 96], and highly versatile genome editing system is now commercially available. Although CRISPR/Cas9 is currently the mainstream method for genome editing, because of the convenience in designing and preparing guide RNA [97], there are still difficulties in packaging the large Cas9 gene into virus vectors to deliver into cells. In contrast, it is much easier to package the ZFN genes in the virus vectors than Cas9, because the size of ZFNs recognize a specific 18 bp DNA is approximately 20 kDa and that of the *FokI* nuclease domain is around 20 kDa.

Furthermore, ZF, TALE, and engineered RNA-binding proteins have been used to alter the levels of mutated mitochondrial DNA and mitochondrial RNA in mammals [98, 99]. For example, it has become clear that mitochondrial diseases caused by defects in mitochondrial DNA are behind numerous intractable pediatric diseases. Typical mitochondrial diseases, such as Leigh's syndrome and NARP (mental retardation due to ataxia), are intractable chronic progressive diseases with a wide range of symptoms including mental and motor delays and respiratory disorders due to brain abnormalities caused by impaired energy production in the central nervous system. ZFN-based approaches have been tried for these diseases, and it has been shown that it is possible in principle to modify mitochondrial DNA in a sequence-selective manner [98].

Recently, there have also been attempts to visualize dynamic phenomena in cells using artificial ZFNs. Stasevich et al. have successfully developed a genome visualization technique based on a ZF DNA-binding protein with a repetitive epitope tag and signal amplification by epitope-specific intracellular antibodies [100]. This demonstrated that the dynamics of non-repetitive genomic loci can be imaged by designing and using ZF probes.

ZF-based molecular tools, which still have high sequence selectivity and diverse functions, are expected to be applied for gene analysis and editing and as novel gene therapy.

## Outlook

In this review, we outline the creation of functional ZFNs using several redesign techniques. Various designer ZFNs have been created by targeting characteristic structural sites such as secondary structural units in the ZF domain, Zn(II) coordination sites, and linker sites used for tandem structure formation, resulting in unique structures and functions.

Although these studies have been conducted for a long time, we believe that there are still numerous challenges in the study of artificial protein design using ZFNs and that this is a research field that is promising for further development. In the future, redesigned artificial ZFNs will play an increasingly important role in advancing research in various fields, including protein engineering, medicine, and pharmacology. Research on new functions of ZFNs themselves, such as interactions between ZFNs and other biomolecules, is also expected to advance, leading to the development of new artificial ZFNs that will enable the construction of complex artificial biological systems and the control of more sophisticated biological functions. In the future, we expect that artificial ZFNs could provide entirely new treatments for intractable diseases such as cancer, genetic diseases, AIDS, and Alzheimer's disease.

**Data availability** The authors confirm that the data of this study are available within the article.

## Declarations

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose. No funding was received for conducting this study.

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