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Synthetic zinc finger peptides: old and novel applications

Nicoletta Corbi, Valentina Libri, Annalisa Onori, and Claudio Passananti

Abstract: In the last decade, the efforts in clarifying the interaction between zinc finger proteins and DNA targets strongly stimulated the creativity of scientists in the field of protein engineering. In particular, the versatility and the modularity of zinc finger (ZF) motives make these domains optimal building blocks for generating artificial zinc finger peptides (ZFPs). ZFPs can act as transcription modulators potentially able to control the expression of any desired gene, when fused to an appropriate effector domain. Artificial ZFPs open the possibility to re-program the expression of specific genes at will and can represent a powerful tool in basic science, biotechnology and gene therapy. In this review we will focus on old, novel and possible future applications of artificial ZFPs.

Key words: synthetic zinc finger, recognition code, artificial transcription factor, chromatin modification, gene therapy.

Résumé : Au cours de la dernière décennie, les efforts visant à clarifier les interactions entre les protéines à doigts de zinc et leurs cibles sur l'ADN ont fortement stimulé la créativité des scientifiques œuvrant dans le domaine de l'ingénierie des protéines. La versatilité et la modularité des motifs à doigts de zinc (ZF) font de ces domaines en particulier des matériaux optimaux afin de générer des peptides à doigts de zinc artificiels (ZFP). Les ZFP peuvent agir comme modulateurs transcriptionnels, potentiellement capable de contrôler l'expression de n'importe quel gène cible, lorsque fusionnés à un domaine effecteur approprié. Les ZFP artificiels offrent la possibilité de reprogrammer l'expression de gènes spécifiques si besoin est, constituant des outils puissants en science fondamentale, biotechnologie et thérapie génique. Dans cette revue, nous nous concentrerons sur les applications anciennes, nouvelles et potentielles des ZFP.

Mots clés : doigts de zinc synthétiques, code de reconnaissance, facteurs de transcription artificiels. Modification de la chromatine, thérapie génique.

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Introduction

Despite the fact that the sequence of the whole genome of different organisms is now available, the mechanisms by which a particular cell type coordinates gene regulation remain largely unknown. The complex process of eukaryotic gene expression is the result of the integration of partially overlapping events, including the assembly of general transcription factors, the recruitment of the transcription machinery to the promoter, the use of cofactor complexes involved in activation or repression, and chromatin remodeling (Hampsey 1998; Cremer and Cremer 2001; Levine and Tjian 2003).

Transcription regulation is mainly achieved through the action of proteins known as transcription factors, which are commonly organized in two separate modules: a DNA-binding domain that can target specific DNA sequences, and an effector domain that regulates transcription. Transcription factors have a complex relationship with the chromatin template; chromatin-modifying factors can act as crucial determinants of the transcriptional status of a given gene (Reik et al. 2002).

In the large class of DNA-binding molecules, the Cys_2His_2 (C_2H_2) zinc-finger (ZF) domain, with 4500 examples identified to date, is the most abundant DNA-binding motif in the human genome (Venter et al. 2001). The C_2H_2 ZF domain is a compact motif of 28–30 amino acids, folded into a compact globular module. The domain consists of an α helix that contains two invariant histidine residues, which are coordinated through a zinc atom to two cysteines of a single β turn. The X-ray crystal structures of the three ZF domains of the transcription factor Zif268 bound to its DNA target site reveal

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that an individual finger domain essentially binds to three base pairs of a double-stranded DNA sequence, with specific contacts through the amino-terminal part of the α helix (Pavletich and Pabo 1991) (Fig. 1). Changes in these crucial residues alter the DNA-binding specificity of a ZF, and this makes it possible to adapt ZF so that it recognizes novel DNA sequences. In fact, a "code" that relates the amino acids of a single ZF to its associated DNA target has been produced (Jamieson et al. 1994; Choo and Klug 1994*a*, 1994*b*). Using and expanding this code, several research groups have engineered and (or) selected peptides containing ZF domains that can recognize and bind to specific DNA sequences. When these DNA-binding peptides are fused to an appropriate effector domain, they can act as artificial transcription modulators (Kang and Kim 2000; Beerli et al. 2000*a*), making these synthetic ZF peptides (ZFPs) a powerful tool for genome analysis and control (Pabo et al. 2001; Urnov et al. 2002; Urnov and Rebar 2002; Jamieson et al. 2003).

Zinc-finger recognition code

The physical interaction between the α helix of the C_2H_2 ZF domain and the major groove indicates that contact between particular amino acids and specific DNA bases follow a simple set of rules that can be combined to form a stereochemical recognition code. Before the Zif268 crystal structure was established, a large database of natural ZF domains and mutagenesis experiments were analyzed, revealing a number of specific amino acid-base contact (Nardelli et al. 1991, 1992; Desjarlais and Berg 1992*a*, 1992*b*). The establishment of the Zif268 protein – DNA complex at 2.1-Å resolution deepened the understanding of how C_2H_2 proteins contact DNA (Pavletich and Pabo 1991). The three Zif268 fingers wrap around the DNA in an antiparallel fashion, and each ZF has a similar relation to the DNA. In each ZF, amino acids in positions –1, +3, and +6 of the α helix make contact with three adjacent bases (the 3-bp subsite) on one strand of the DNA duplex, in a one-to-one interaction between amino acids and bases (Fig. 2B). These α-helix amino-acid positions are responsible for contacting the 3′, middle, and 5′ nucleotides of the 3-bp subsite. Contiguous ZFs recognize adjacent but independent subsites. Dejarlais and Berg (1992*b*) combined the correlations between specific amino acids and bases in a matrix. When they performed binding studies on variants of the central ZF of the Sp1 transcription factor, they observed that the amino acid discrimination among the different bases is rarely absolute, thus introducing the concept of degeneracy of the code. It is now clear that, in addition to the primary contact residues (–1, +3, and +6 in the α helix), flanking amino residues may also play a role in base specificity (Choo and Isalan 2000; Pabo et al. 2001), making the relation of one amino acid to one nucleotide too simplistic. In particular, as shown in Fig. 2B, a fourth base, located in the antisense strand relative to the 3-bp subsite, is recognized by residue +2 of the α helix. Rebar and Pabo (1994) and Jamieson et al. (1994), using the phage-display system, created a library of Zif268 variants by randomizing the amino-acid positions of the first ZF (–1, +2, +3, +6) and selected them against the Zif268 target with a single modified subsite triplet. Choo and Klug (1994*b*), taking into account the importance of context, performed more extensive randomization on the central ZF α helix (–1, +1, +2, +3, +5, +6, +8) of Zif268. Many research groups contributed to the stereochemical recognition code table (Fig. 2A) (Choo and Klug 1995, 1997; Wolfe et al. 2000*a*; Lee et al. 2003*b*), and many ZFPs have been constructed on the basis of this recognition code (Desjarlais and Berg 1993; Choo et al. 1994; Corbi et al. 2000; McNamara and Ford 2000; Libri et al. 2004). Our research group published two papers in which ZFPs were designed to bind to arbitrary DNA sequences (Corbi et al. 1997, 1998). As proof of specificity, the ZFPs selected strictly related target DNA sequences among randomized ones in a SELEX assay. These results validate some of the rules derived from the code. Sera and Uranga (2002) proposed a rapid approach, based on a nondegenerate recognition code, to develop new ZFPs with satisfactory binding properties in a high-throughput manner. This method can be used to manipulate gene expression by screening multiple ZFP transcription factors (ZFP-TFs) for multiple sites in a given promoter, even in the absence of information on the chromatin structure.

Recently, a novel selection approach was developed in yeast and mammalian cells. This approach is based on functional screening of multifingered ZFPs, in which each finger had already been defined to bind a specific DNA site. Using this protocol, ZFPs randomly selected from combinatorial ZFP libraries can exert their functions on predetermined genes (Blancafort et al. 2003). Alternatively, ZFPs can be selected on the basis of the phenotype, with no predetermined genes

Fig. 1. Interactions between the Zif268 middle finger and its DNA target. Modular recognition between the central Zif268 finger and its triplet subsite DNA target. Essentially, the individual finger domain binds three base pairs of double-stranded DNA sequence (sense strand) with specific contacts through the amino-terminal part of the α helix in positions –1, +3, and +6. The amino acid in position +2 (indicated in grey), usually an aspartate or a serine, makes an auxiliary contact on the antisense strand.

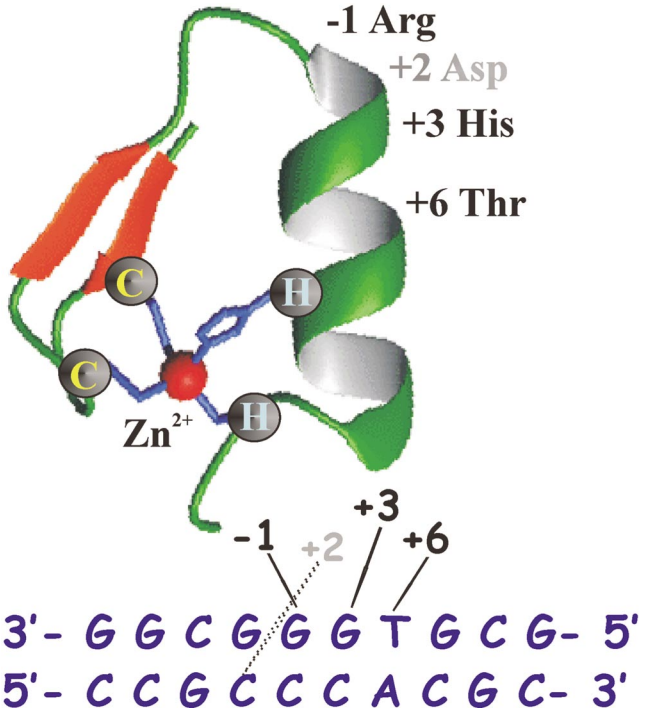
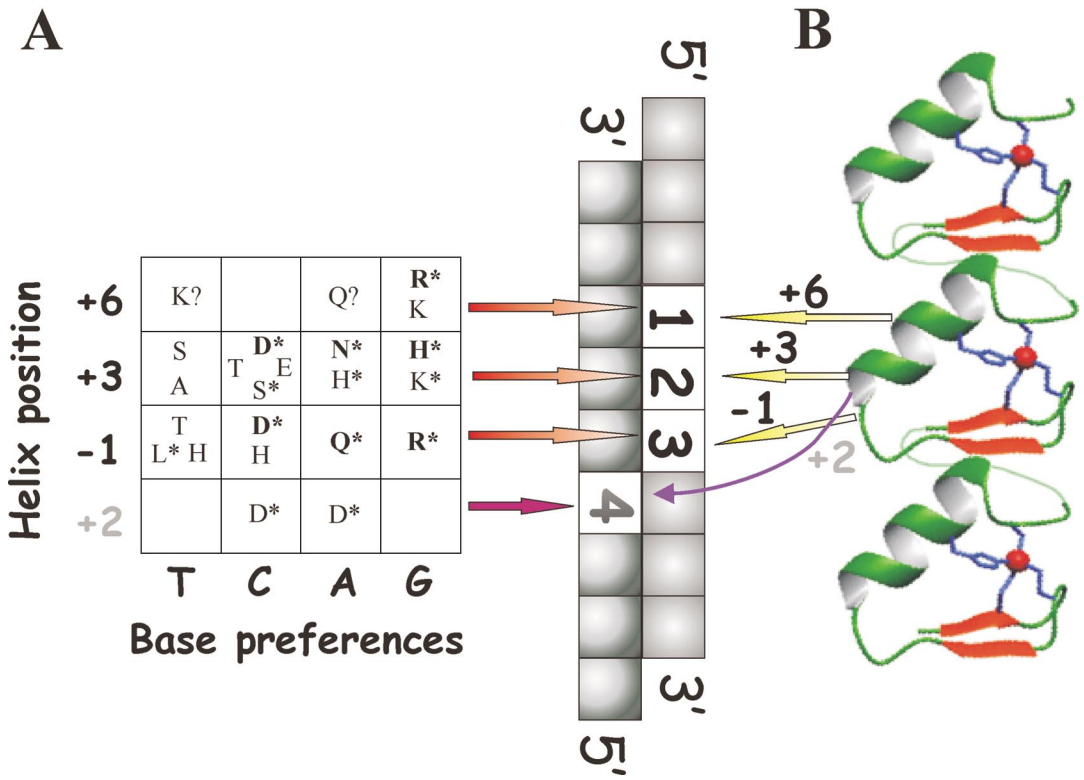


Fig. 2. Recognition code. (A) Amino acids at the crucial positions in the α helix finger (–1, +2, +3, +6) are listed in a matrix, relating to the four bases at each position of a DNA subsite. Amino-acid residues that arise recurrently from phage display selections are in bold, and asterisks indicate interactions observed in structural studies. Some correspondences are still uncertain (indicated by the curly arrow) or poorly defined (left blank). (B) Schematic diagram of contacts between the middle finger of a prototype three-ZFP and its DNA-binding subsite. Contact between the finger and the antisense DNA strand is indicated by the curved arrow.



(Park et al. 2003). In this case, the random genome perturbation obtained using artificial ZFP-TFs can provide a novel functional genomic tool for annotating and classifying genes in a chosen organism (Lee et al. 2003*a*).

Zinc-finger domains selected and assembled as building blocks

Single ZF domains linked together can generate novel ZFPs with the desired DNA-binding specificity. This structural and functional ZF modularity enables the use of different construction methods for the selection of novel ZFPs: the parallel selection, the sequential selection, and the bipartite selection (Choo and Isalan 2000; Segal and Barbas 2000; Beerli and Barbas 2002). The parallel selection approach combines three individual ZFPs previously selected by phage display. Specifically, two Zif268 fixed fingers (anchor fingers), the first and the third, guarantee correct positioning on the target site, and the middle ZF is randomized in crucial positions (Choo and Klug 1995; Segal et al. 1999; Dreier et al. 2001). The selected monomers provide prefabricated building blocks that can be assembled (Segal 2002; Liu et al. 2002; Segal et al. 2003*a*). This strategy is based on the assumption that each ZF is a functional independent unit. Pabo's group addressed the matter of functional dependence of neighboring fingers by developing the sequential selection strategy (Greisman and Pabo 1997). The sequential approach takes into account context-dependent interactions between fingers and subsites by serially selecting fingers, adding one ZF at a time. Thus, a new three-ZFP is created in which each finger is selected from multiple ZF libraries and optimized in relation to the finger next to it. This selection, even though it is quite laborious, means that any sequence desired can be targeted, which is particularly useful in cases where there is no flexibility in target choice. Bipartite selection is a variation of the two preceding methods that is designed to overcome incompatibilities between adjacent fingers and to significantly shorten ZFP construction time. Selection is carried out in parallel from two premade "half" libraries, in which one-and-one-half fingers of the three-finger Zif268 are randomized. After the selections have been made, the two ZF portions are assembled to form a novel three-ZFP (Isalan et al. 2001).

Other strategies for selecting ZFPs rely on yeast one-hybrid and bacterial two-hybrid systems (Bartsevich and Juliano 2000; Joung et al. 2000). These systems have two useful features: the selection is performed in the context of living cells, and the best proteins can be obtained in a one-step selection process.

Efforts in synthetic ZFP design have also focused on the assembly of polydactyl ZFPs (ZFPs with more than three fingers), in an attempt to increase binding specificity and to approach the uniqueness of the target sequence in the genome (Imanishi et al. 2001; Beerli and Barbas 2002). The increased number of ZFs and, consequently, the increased length of the target sequence should, in principle, enhance ZFP binding specificity. Statistically, for a target sequence to be unique in

the human genome, it must be at least 16 bp long. Therefore, the specificity, in terms of binding to a unique locus in the genome, should be ensured by using polydactyl ZFPs containing six fingers capable of targeting an 18-bp DNA sequence. Interestingly, polydactyl ZFPs containing six to nine ZFs do not always exhibit the expected increased binding affinity. This could be caused by unfavorable cooperation between certain fingers when binding long target sequences (Ansari 2003) or by an intrinsic ZFP rigidity that could force too much unfolding of the DNA duplex (Uil et al. 2003). For instance, computer graphics modeling with the Zif268–DNA complex has suggested the possibility of connecting two three-ZFPs with the conserved five-residue linker TGEKP, which joins many naturally occurring multifinger domains (Liu et al. 1997; Kamiuchi et al. 1998). To decrease multi-fingered protein rigidity, alternative construction approaches have been proposed, including the use of noncanonical linkers. In one study, two three-ZFP units were linked by flexible linkers that were four or seven residues longer than canonical linkers, spanning gaps of up to 1 and 2 bp, respectively. The resulting six-ZFP bound more than 6000-fold tighter than its three-ZFP constituent parts (Kim and Pabo 1998). In another study, a structured linker, made up of the TFIIIA finger 4 and a nonsequence-specific ZF (capable of spanning up to 10 bp of nonbound DNA), has been used to link two three-finger units. The resulting molecules bound with high affinity and specificity (Moore et al. 2001). The investigation of linker contribution to recognition site selectivity is currently a widely discussed topic (Nomura and Sugiura 2003). Notably, the results obtained suggest that varying the length of the linkers can contribute to expansion of the recognition code of ZFPs.

Yet another approach in polydactyl ZFP construction involves the use of dimerization domains to bring together two separate three ZFPs. ZFPs, fused either to the Gal4 dimerization domain or to a leucine zipper, can dimerize and bind to a longer DNA target (Pomerantz et al. 1998; Wolfe et al. 2000*b*). One recent study (Wolfe et al. 2003) reports on the 1.5-Å resolution cocrystal structure of the Zif268-GCN4 homodimer bound to DNA. This dimeric ZF molecule was developed by attaching the two monomers (each oconsisting in fingers 2 and 3 of Zif268) using the leucine zipper of GCN4. Dimeric complexes can also form when ZFPs are fused to steroid hormone receptor ligand-binding domains (LBDs) (Beerli et al. 2000*b*).

Regulation of gene expression by artificial ZFPs: effectiveness

To alter gene expression in vivo, several strategies can be used. Methods such as homologous recombination, antisense reagent, and RNA interference can only downregulate or knock down a target gene. ZFPs, as well as other types of sequence-specific DNA binding agents, including triplex-forming oligonucletides and synthetic polyamides, have the potential to perturb, both up and down, the level of expression of the target gene. There are several methods by which artificial DNA-binding agents can modulate gene transcription, including blocking the path of RNA polymerase II by targeting a site internal to the transcribed region of a gene, blocking or promoting transcription by competing with endogenous factors for specific binding sites in vivo within the regulative region of a gene, and repressing or activating gene transcription by fusing a proper effector domain (ED) to the DNA-binding agents (Uil et al. 2003).

Thanks to their plasticity and versatility, ZFPs have many successful applications (Pabo et al. 2001; Beerli and Barbas 2002; Urnov and Rebar 2002). In fact, ZFP-ED can mimic the functional modularity of natural transcription factors better than the other classes of DNA-binding agents. To make an artificial transcription factor act like a natural one, many aspects need to be empirically tested, including the accessibility of the factor to the target site in the endogenous chromatin context, the position of the target site with respect to the target promoter, and the nature of the fused effector–regulatory domain (Yaghmai and Cutting 2002; Levine and Tjian 2003). When an artificial transcription factor binds to its DNA target and exerts the regulatory effect on transcription, its activity must be specific and must deliver the desired level of gene expression in a given cell type, especially if it is for therapeutic use in humans. These requirements can be achieved with an inducible-expression system, based on a small-molecule ligand, that can trigger a pathway to activate a dormant transcription factor that contains a signal-responsive element. Barbas' group used a tetracycline/doxycicline-dependent expression vector to regulate the expression of a ZFP to obtain inducible regulation of the endogenous erb-2 gene (Beerli et al. 2000*a*). This strategy is based on the delivery of two genes: one encoding the ZFP under the control of a regulatable promoter that contains the tetracycline response element (TRE), and the other encoding the regulatory protein (tetracycline-controlled transactivator) (Gossen and Bujard 1992; Gossen et al. 1995). Delivery of multiple vectors can create difficulties, particularly for gene therapy. As an alternative approach, Barbas' group proposed the use of fusion proteins between designed ZFPs and the eukaryotic nuclear hormone steroid receptor LBDs for the inducible control of gene expression (Beerli et al. 2000*b*). Steroid receptors bind to DNA as dimers and recognize typically palindromic sequences. To be able to also target nonpalindromic sequences, they engineered a single-chain steroid hormone receptor LBD by rearranging, intramolecularly, two serially connected LBDs fused to a six ZFP. Another strategy is the use of a rapamycin-based dimerizer system to regulate the expression of a specific gene. In this case, administration of the drug brings together the ZPF and the ED, because they each bind to separate halves of the small-molecular drug (Pollock et al. 2002) (Fig. 3).

Regulation of gene expression by artificial ZFPs: old and novel applications

One of the most promising applications of designed ZFPs is in generating artificial transcription factors by fusing engineered ZFPs to natural and (or) engineered EDs, as shown in Fig. 3. Several different EDs have been fused in artificial ZFPs. For transcriptional activation, these include VP16 (one or multimerized copies) from the herpes simplex virus, the Gal-4 activation domain from yeast GAL4 transcription factor, p65 from the cellular transcription factor NF-κB. For transcriptional repression, these include KRAB (Kruppel-associated box) from natural ZFP TFs and Sid (Sin3A inter-

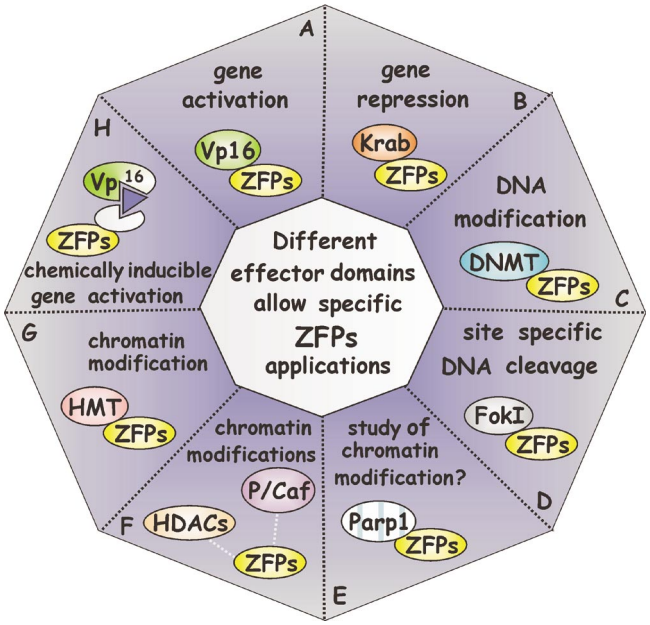
action domain). Notably, a ZFP that does not contain an activation or repression domain can function as transcription repressor when it competes with endogenous transcription factors to bind to DNA sequences. Moreover, by binding to RNA or proteins, ZFPs can exert effects by employing other mechanisms.

Choo et al. (1994) were the first to realize a functional artificial ZFP. They engineered a three-ZFP able to bind specifically to the unique nine-bp region of the BCR–ABL fusion oncogene, generated by chromosomal translocation in acute lymphoblastic leukemia. In transfected cell lines, the ZFP that was able to target the BCR–ABL sequence led to BCR–ABL mRNA reduction. Our research group engineered several synthetic three-ZFP TFs on the basis of the recognition code. One of these artificial genes, named Jazz, was designed to target the promoter region of human and mouse utrophin, a dystrophin-related gene, and to upregulate the expression level of utrophin in Duchenne's muscular dystrophy (DMD) (Corbi et al. 2000). The upregulation of utrophin gene expression, a recognized treatment for Duchenne's muscular dystrophy (Tinsley et al. 1996), can be achieved by artificial ZFP TFs, and provide a valid alternative to pharmacological therapy. ZFPs can also provide a model for screening compounds and for drug-target validation. Another artificial ZFP engineered in our laboratory, named Blues and currently under study, targets fibroblast growth factor 4 (FGF-4, K-fgf). FGF-4 was originally identified as an oncogene, whose proto-oncogene expression is restricted to early stages of embryogenesis (Yuan et al. 1995). Our aim is to generate a ZFP-based model to study the biological role of FGF-4 during development and tumorigenesis (Libri et al. 2004). Interestingly, in a recent study, Bartsevich and colleagues (2003) constructed ZFP TFs that target Oct-4 gene (also called Oct-3), a major regulator of embryonic stem cells, whose expression overlaps the FGF-4 expression pattern. These artificial ZFP TFs can regulate the transcription of Oct-4, affecting the expression of downstream genes and thus regulating embryonic stem cell differentiation.

Bartsevich and Juliano (2000) used artificial ZFP TFs able to regulate the transcription of the MDR1 multidrug resistance gene in the chromosomal context. Barbas' group used six-finger Sp1-based ZFPs able to target and regulate the endogenous erB-2 and erB-3 proto-oncogenes (Beerli et al. 1998, 2000*a*; Segal et al. 1999), which are frequently overexpressed in human tumors. Significantly, regulation of the two genes was highly specific, because ZFP TFs can distinguish the genes from each other, even though their binding sites share 15 of 18 nucleotides. Moreover, this research group was able to induce expression of the artificial ZFP TFs, which is particularly useful in gene therapy protocols. In another study, Zhang et al. (2000) activated, in transfected cell lines, the endogenous erythropoietin gene using a series of ZFP TFs that were able to gain access to their recognition sequences within the chromatin infrastructure.

Similarly, Liu et al. (2001) used a panel of ZFP TFs to target the endogenous locus of vascular endothelial growth factor (VEGF-A), a specific inducer of new blood vessel growth that is involved in a variety of medical conditions. They showed that combinations of three-ZFP TFs targeted to distinct adjacent chromosomal sites functioned synergistically on VEGF-A activation. Moreover, when the behavior of ZFP TFs targeted to accessible regions (identified by DNAase I hypersensitivity assay) was compared with that of ZFP TFs targeted to inaccessible regions, a clear difference was found, underlining once again the importance of chromatin accessibility.

Fig. 3. Different effector domains allow specific ZFPs applications. An engineered ZFP can be fused to different functional domains for different applications. (A) A ZFP fused to a transcriptional activation domain, such as the VP16 domain from herpes simplex virus. (B) A ZFP fused to a transcriptional repression domain, such as the KRAB domain from KOX protein. (C) A ZFP fused to a DNA methyltransferase (DNMT) domain for DNA modification. (D) A ZFP fused to a DNA nuclease domain from a restriction enzyme, such as FokI, for site-specific DNA cleavage. (E) A proper ZFP fused to the poly(ADP-ribosyl)ation domain of one of the PARP enzymes can be used to study specific chromatin modifications. (F) A ZFP can be directly fused to enzymes that modify chromatin, such as histone deacetylase proteins (HDACs), or histone acetylases, such as P/CAF, to locally perturb chromatin organization. (G) A ZFP fused to a histone methyltransferase (HMT) domain for chromatin modification. (H) A complete ZFP artificial transcription factor that assembles only in presence of a small-molecule drug.



Peroxisome proliferator activated receptor-γ (PPARγ) (Ren et al. 2002) is an interesting example of knockdown expression. PPARγ is a nuclear hormonal receptor and exists as two isoforms, γ1 and γ2. When a ZFP TF repressed the expression of both forms, adipogenesis was restored selectively after PPARγ2 but not PPARγ1 reintroduction. The use of this ZFP TF made it possible to identify the role of each isoform in adipogenesis. In addition, it could be functional for the design of specific drugs able to affect only the desired isoform (Jamieson et al. 2003). Customized ZFP TFs were able to inhibit transcription of viral genes, and therefore viral replication. Papworth and colleagues (2003) designed ZFPs that targeted the viral immediate-early promoter of HSV-1, and that were able to reduce the viral titer by about 90%. Similarly, they repressed the HIV-1 LTR promoter and inhibited HIV-1

replication (Reynolds et al. 2003). A recent paper (Segal et al. 2004) suggested a potential ZFP strategy for treating viral infections, in particular HIV infections. Falke and coworkers (2003) reported on an artificial ZFP TF that could stimulate the expression of the endogenous proapoptotic gene bax, one of the p53 targets. The p53-gene product is inactivated in more than 50% of cancers. p53-negative cells fail to induce bax and the apoptotic pathway, which seems to make them more refractory to chemotherapic agents. Therefore, Falke and colleagues proposed the induction of the bax gene by ZFPs as a valuable tool in cancer chemotherapy, by diminishing the survival of p53-deficient tumor cells. A recent study (Jouvenot et al. 2003) demonstrated the ability of a ZFP TF to reactivate the transcriptionally silent IGF2 and H19 alleles, overriding the imprinted transcriptional status and highlighting the potential of synthetic transcription factors in the treatment of epigenetic lesions. Tan and coworkers (2003) realized a six-ZFP TF repressor that was able to abolish the function of the cell-cycle checkpoint kinase CHK2 in different cell types.A microarray analysis demonstrated that CHK2 was the only gene repressed by the ZFP TF in the genome. This remarkable specificity is promising for both target validation and therapeutic interventions in vivo. All these data, obtained in cell lines expressing ZFP TFs, either by transfection (stable or inducible) or infection, indicate stable long-term target gene regulation. This is a fundamental starting point for the effective use of ZFP TFs in a whole-organism model. In fact, the expression of a ZFP TF (delivered by adeno-vectors) in a mouse ear led to the induction of the VEGF-A, which stimulates blood vessel growth. These data demonstrated, for the first time, the feasibility of regulating, in vivo, crucial biological processes such as angiogenesis (Rebar et al. 2002). Interestingly, the induced activation of the VEGF-A gene led to the expression of its naturally occurring splicing variants, which are required for correct angiogenesis. Because it is easier to deliver a single ZFP TF than to multiple splice variants using cDNA treatments, complex biological processes that require the coordination of multiple gene expressions can be in this way regulated with a single intervention.

In the field of large-scale three-ZFP engineering, Kim's group proposed the GeneGrip method. This method is based on the screening of natural" ZFs from the human genome that can be combined to generate modular ZFPs (Bae et al. 2003). Because they evade immune surveillance, these ZFPs may be optimal for the development of gene therapy protocols.

In addition to the transcriptional modulators discussed above, EDs can be fused to ZFPs to generate proteins with novel functions (Fig. 3). For example, ZFPs fused to DNA-restriction nuclease can recognize and cut specific DNA sequences at will. One class of engineered nucleases consists of the nonspecific-DNA cleavage domain of Fok1 (a type IIS restriction endonuclease) fused to selected ZFPs (Chandrasegaran and Smith 1999; Bibikova et al. 2001). To test such chimeric nucleases for their ability to find and cleave their target sites in living cells, both engineered DNA substrates and nucleases were injected into frog oocyte nuclei. The injected enzymes made site-specific double-strand breaks in the targets, even after the assembly of DNA into the chromatin. In addition, this cleavage activated the target molecules for efficient homologous recombination. This property, combined with the DNA-recognition specificity conferred by artificial ZFPs, looks promising for inducing targeted recombination in a variety of organisms, and may be particularly useful for human gene therapy (Porteus and Baltimore 2003).

ZFPs linked to a minimal histone methyltransferase (HMT)-catalytic domain can affect the local methylation of histone H3 lysine 9 (H3K9), and consequently repress the expression of a target gene through chromatin modification (Snowden et al. 2002). Methylated histones are involved in heterochromatic repression, promoter regulation, and propagation of a repressed state via DNA methylation (Kouzarides 2002). In particular, it has been postulated that the methylation of H3K9 acts as a signal for the recruitment of heterochromatin protein 1 (HP1), which is thought to be involved in spreading the methylation signal away from the original methylated H3K9/HP1 binding site. Accordingly, Sangamo Biosciences demonstrated the ability of a ZFP-HMT to generate an H3K9 histone methylation signal specifically at the promoter targeted, verifying that this methylation signal is not restricted to the immediate vicinity of the ZFP-binding site (Snowden et al. 2002).

It has been suggested that ZFPs fused to C5-cytosine DNA methyltransferases (DNMT) is a potential tool for promoter gene silencing through a DNA-methylation-mediated cellular response (McNamara et al. 2002). In particular, it has been reported that a ZFP fused to the DNA methyltransferase M.HpaII binds to and methylates the target site specifically, up to a separation of 40 bp between the ZF and the methyltransferase subsite. Interestingly, the chimeric ZFP-DNMT has a much higher preference for binding to its target site than its component peptides (ZFP and ED) alone. These targeted enzymes, however, are shown to retain some non-targeted activity particularly evident at elevated protein concentration.

Chromatin-modifying activities, such as histone deacetetylases (HDAC) (Minucci et al. 2001), which are often associated to hematological disorders, have also been linked to ZFPs (Jamieson et al. 2003) (Fig. 3).

The list of EDs that can be coupled to ZFPs is expected to increase, as will consequent applications. A possible candidate is the enzymatic domain of poly(ADP-ribose) polymerase-I (Parp-1), which is thought to play a fundamental role in the control of cell-type DNA-methylation patterns (Zardo et al. 2003) (Fig. 3).

In general, covalent modifications of histones (acetylation, methylation) and other epigenetic modifications (DNA methylation) can be obtained, in principle, by fusing a specific ED to ZFPs. These artificial molecules may shed light on fundamental cellular molecular mechanisms, focusing specifically on the connection between aberrant epigenetic modifications and multistep processes, such as carcinogenesis. Finally, we want to mention other hot fields for ZFPs applications. In agricultural biotechnology, designed ZFP TFs can be used to alter the expression of target genes to producehealthier or more nutritious crop plants. Genes related to disease and resistance could be turned on, and genes related to pathologic status or the production of antinutritive proteins could be turned off (Stege et al. 2002; Sanchez et al. 2002; Segal et al. 2003*b*; Frommer and Beachy 2003). ZFPs can also bind to RNA in a specific manner, which can be used to develop antiviral agents and correct defects of RNA metabo-

lism associated with a variety of diseases (Corbi et al. 1998; Friesen and Darby 2001; Lee et al. 2003*b*).

Conclusions and perspectives

The human genome project identified more than 30 000 genes (Venter et al. 2001). Now the challenge is to systemically characterize the function of novel genes. An aberrant transcription profile is often a marker for a specific disease. In the future, so-called "transcription therapy" by artificial ZFP TFs may be able to correct the abnormalities in gene expression and consequently reverse the disease process (Pandolfi 2001).

Currently, the delivery of artificial transcription modulators into organisms and the ability to maintain the desired expression level of target genes are challenges to be overcome (Verma and Somia 1997; Somia and Verma 2000; Falke and Juliano 2003). Thanks to artificial ZFPs, we may someday be able to dissect cell functions and make gene therapy a reality. To meet this challenge, contributions from all branches of biology, from geneticists to virologists to immunologists, are needed.

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