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Characterization of functional, noncovalently assembled zinc finger nucleases

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ABSTRACT

Zinc finger nuclease (ZFN) is a chimeric restriction enzyme made of a C_2H_2-type zinc finger protein (ZFP) and the *Fok*I nuclease domain (F_N). ZFN technology has been considered as a powerful tool for genome editing. Here, we report a new type of ZFN system based on the coiled-coil interaction used as a noncovalent assembler. Like conventional ZFNs, noncovalently assembled ZFNs (ncZFNs) structurally have two domains, a ZFP and a F_N. Each domain carries one of antiparallel heterodimeric leucine zippers, respectively, to form an ncZFN through leucine zipper assembly. The characterization of ncZFNs revealed that they behave as fully functional sequence-specific endonucleases, comparable to those of conventional ZFNs. Interestingly, some ncZFNs displayed augmented off-target cleavage, possibly by degenerate DNA binding of the ZFP domain of ncZFNs based on our data. We postulate that DNA cleavage of ncZFN(s) seems to be more sensitive to the ZFP binding to lesser-optimal sites. Facile design of ncZFNs through the mix-and-assemble approach could be applicable to other DNA binding proteins for evaluation of sequence-specificity. In addition, our work establishes that the coiled-coil interaction could be used as the peptide-based noncovalent assembler for the formation of a noncovalently-linked functional multi-domain protein.

1. Introduction

*Fok*I restriction enzyme is composed of two separable domains: a sequence-specific DNA recognition domain (F_R) at the N terminus, and a DNA cleavage domain (F_N) at the C terminus (Fig. S1A) [1]. This modular structure of *Fok*I has made it possible to create a chimeric nuclease with novel sequence specificity by replacing the F_R domain of *Fok*I with other DNA binding domain (DBD) (Fig. S1B) [2]. This approach has successfully created novel sequence-specific endonucleases with various DBDs [3–8].

ZFN is largely based on the modular structure of *Fok*I. ZFN is composed of a C_2H_2-type zinc finger protein (ZFP) domain and the F_N domain of *Fok*I, and it has been utilized as a tailor-made, rare-cutter restriction enzyme [9]. In the advance of genome editing technology, zinc finger nuclease (ZFN) has been one of the leading and most investigated gene targeting methods [9]. The sequence-specific gene targeting using ZFNs was first described by Bibikova et al. [10]. Since then, a number of works have been reported, notably the gene targeting against X-linked severe combined immune deficiency (SCID) by Urnov et al. [11]. There have been several new designing approaches such as the single-chain F_N domain dimer [12–14] and the photocaged F_N domain [15]. In addition, the heterodimerization of the mutant F_N domains has been successfully used for avoiding the homodimerization of ZFNs to reduce off-target cleavage [16–18].

The coiled-coil is a versatile structural motif consisting of two or more α-helices [19]. In nature, it is used for a vast variety of biologically important interactions. The coiled-coil has been also utilized as a building block to produce polypeptide polyhedral [20,21], new biomaterials such as hydrogels [22,23], and assembling fragmented enzyme [24,25]. As the simplest way to make a dimer complex, the coiled-coil dimer-typically, leucine zippers-is particularly an attractive model system for the study of protein assembly and design [26,27]. It can be used to effectively guide polypeptide chains for assembly in preconceived ways.

In this study, we reasoned that the coiled-coil interaction can be devised as a 'noncovalent assembler'. We designed a new type of ZFN system by noncovalently associating a ZFP and a F_N through the leucine zipper assembly (Fig. 1). Noncovalently assembled ZFNs (ncZFNs) were successfully created through noncovalently combining two separately expressed, folded protein domains. Characterization of ncZFNs showed high sequence-specific DNA cleavages, comparable to those by conventional ZFN counterparts.

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2. Materials and methods

2.1. DNA construction

Expression vectors, pZFP-F_N, pZFP-CZ and pNZ-F_N (Fig. S2), were constructed with the pET28a vector (Novagen). The genes of ZFPs and the antiparallel heterodimeric leucine zipper pair (NZ and CZ) were synthesized by assembling oligonucleotides. To construct a vector for expression of ZFP-CZ fusion protein, pZFP-CZ, a ZFP gene was cloned into the modified pET28a at *Nde*I/*Age*I sites, and the CZ gene was then cloned at *Age*I/*Xho*I sites. pNZ-F_N vector was constructed by cloning the NZ gene and the PCR-amplified F_N gene into the modified pET28a at *Nde*I/*Spe*I and *Age*I/*Xho*I sites, respectively. To construct pZFP-F_N that expresses a conventional ZFN, we first constructed pZif268-F_N made by inserting the *Nco*I-*Xho*I DNA fragment of the Zif268-ZFN gene from the Zif268-ZFN expression vector (gift from Professor Jin Soo Kim at Seoul National University) into pET28a. For other conventional ZFP-ZFNs, an appropriate ZFP gene was inserted into pZif268-F_N vector to replace the Zif268 gene at *BamH*I/*Age*I sites. For the construction of mammalian expression vectors, PCR-amplified DNAs of natural or synthetic Zif268-ZFPs from pZFP-F_N vectors were digested with *Nhe*I/*Mlu*I. The *Nhe*I-*Mlu*I DNA fragment was then cloned into pIRES vector at *Nhe*I/*Mlu*I sites.

Construction of substrate DNAs for DNA cleavage assay was performed by inserting an annealed oligonucleotides (Bioneer Corporation) into pUC19 plasmid at *BamH*I/*Hind*III sites. DNA substrates with different spacer lengths were cloned by the same manner.

2.2. In vitro protein synthesis

Expression of all genes in vector constructs based on the pET28a was controlled by T7 promoter. For *in vitro* protein expression, $T_NT^{®}$T7 Quick Coupled Transcription/Translation System (Promega), T_NT in short, was used. All plasmid DNAs were purified using the plasmid cleanup kit (Qiagen) to remove RNases before the T_NT reaction. Proteins were produced by following the manufacturer's manual (Promega).

2.3. DNA cleavage assay

For sequence-specific DNA cleavage assay, proteins produced from pZFP-CZ and pNZ-F_N by the T_NT reaction were pre-incubated for 1 h at 22 °C for facilitating self-assembly of antiparallel heterodimeric leucine zippers. The ratio of reaction products from pZFP-CZ and pNZ-F_N was optimized to 1:4 for reproducible and efficient reaction condition after titration (Fig. 2). Typically, a reaction volume was 20 μl containing 2 μl of the 10× reaction buffer (50 mM potassium acetate, 20 mM Tris–acetate, pH7.9, 10 mM magnesium acetate, 1 mM DTT), 500 ng of a *Sca*I-linearized plasmid DNA substrate, and 5 μl of the preincubated ncZFN mix (the T_NT reactions of pZFP-CZ and pNZ-F_N) as 1:4 ratio. The reaction was incubated for 16 h at 22 °C. Similarly, sequence-specific DNA cleavage of conventional ZFNs was also performed by using 2 μl of the T_NT reaction and incubated for 16 h at 22 °C. After completing the DNA cleavage reaction, 10 μg of RNase A was added into reactions and incubated for 30 min at 22 °C. Then, 10 μg of protease K was added

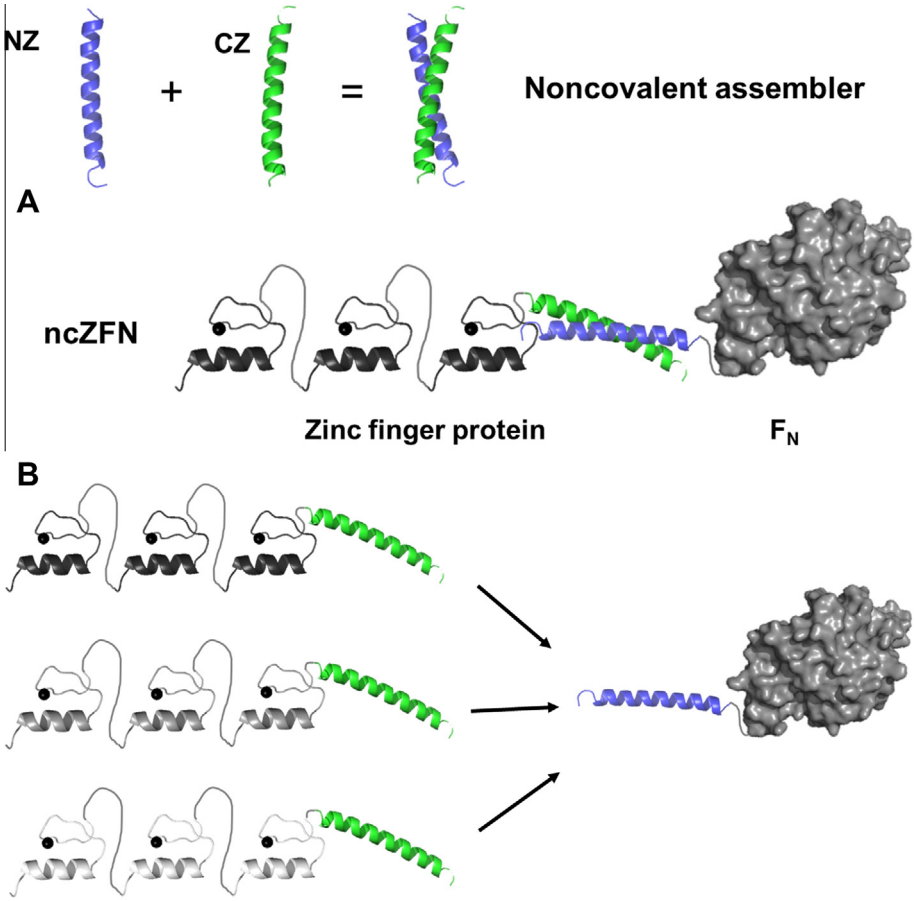

Fig. 1. Strategy for noncovalently assembled zinc finger nuclease. (A) *Fok*I nuclease domain (F_N) and zinc finger protein are fused to heterodimeric antiparallel leucine zipper pair (NZ and CZ), respectively. The noncovalently assembled ZFN (ncZFN) can be formed through the leucine zipper interaction. (B) Schematic illustration of mix-and-assemble approach for ncZFN formation using the noncovalent assembler. Diverse zinc finger proteins fused to the CZ were used to form ncZFNs simply by mixing one with the NZ-F_N.

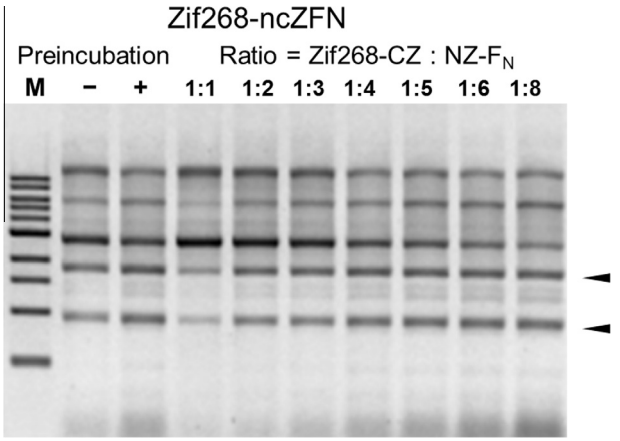

Fig. 2. Optimization of DNA cleavage condition for ncZFN. Ratio of two *in vitro* translation products, Zif268-CZ and NZ-F_N, was varied to find an optimal condition for efficient cleavage activity. When the two translation products were preincubated for 1 h at 22 °C without DNA substrates before the cleavage reaction for facilitating formation of antiparallel heterodimeric leucine zipper (preincubation, +), it resulted in improved cleavage activity. Numbers indicate the volumes (μl) of Zif268-CZ and NZ-F_N used for reactions. The arrows indicate sequence-specific DNA cleavage products, 0.9 kb and 1.8 kb.

to the reactions and incubated for 2 h at 55 °C. Reactions were subjected to electrophoresis and stained with ethidium bromide. DNAs were visualized under UV light.

3. Results

3.1. Design and construction of noncovalently assembled ZFNs

In this study, we designed and constructed noncovalently assembled ZFN (ncZFN). Conventional ZFNs have two covalently linked functional domains, a ZFP and a F_N (Fig. S1). As an alternative approach to combining the two domains, we surmised that the coiled-coil interaction could be used to form a stable complex of the two domains with a sequence-specific endonuclease activity. To create ncZFNs, we employed the well-characterized antiparallel heterodimeric leucine zipper pair as a noncovalent assembler [28,29] that previously used for specifically stabilizing the assembly of protein fragments [24,25]. We fused one of these heterodimeric leucine zippers (hereinafter referred to as NZ) to the N terminus of the F_N and the other leucine zipper (CZ) to the C terminus of the ZFP, respectively (Fig. 1A). These leucine zippers present in two fusion proteins (ZFP-CZ and NZ-F_N) can self-assemble each other. Accordingly, the two functional domains are brought to close proximity by the leucine zippers and resulted in a pseudo-multidomain protein behaving as a single entity.

To demonstrate facile construction of various ncZFNs possessing distinct sequence-specificities, we chose four different ZFPs as a DBD domain. Firstly, Zif268, one of well-characterized and naturally-occurring ZFPs, has three fingers and has been used as a frame to design novel sequence-specific DNA binding proteins to recognize desired target DNA sequences [30,31]. Other three ZFPs-denoted as ZifNRE (binding to nuclear receptor element sequence), ZifP53 (binding to p53 binding sequence), and ZifTAB (binding to TATA-box sequence), respectively-were derived from Zif268 through the rigorous sequential selection method [32]. These engineered ZFPs recognize entirely different DNA sequences from that of the wild-type Zif268 (Table 1).

Mixing ZFP-CZ and NZ-F_N together can generate an ncZFN assembly through the coiled-coil interaction between the two leucine zippers, NZ and CZ. This mix-and-assemble approach promptly generated four assemblies of ncZFNs by simply choosing a corresponding ZFP-CZ (Fig. 1B). To compare specificity and efficiency of DNA cleavage by ncZFNs, conventional ZFNs with the same four ZFPs were also constructed.

3.2. Characterization of sequence-specific DNA cleavage activity of ncZFNs

It is known that ZFNs work in pairs for efficient double-stranded DNA (dsDNA) cleavage that requires dimerization of two F_N domains [33]. Thus, DNA substrates were designed to have a 6-bp spacer between two oppositely-oriented, corresponding ZFP binding sequences in the polylinker region of pUC19 plasmid. Consequently, sequence-specific DNA cleavage of 2.7-kb *Sca*I-linearized DNA substrates by ncZFNs or ZFNs would result in two DNA fragments, 0.9 kb and 1.8 kb.

The proteins were synthesized *in vitro* by the coupled transcription/translation (T_NT) reaction as described in Materials and methods. As illustrated in Fig. 1, the F_N and the Zif268 domains were expressed as fusion proteins with either NZ or CZ, respectively. Zif268-ncZFN is formed from Zif268-CZ and NZ- F_N through the coiled-coil interaction between two leucine zippers. To give sufficient flexibility for both facile dimerization of the antiparallel leucine zippers and enzymatic activity of ncZFNs, short linkers were introduced between two fusing domains. The GGSGSGSS linker was chosen between ZFP and CZ, while the 11-aa linker containing GGSGTSGGGGS was chosen for NZ-F_N after testing various linkers (Table S1 and Fig. S3).

DNA cleavage reaction was carried out with the mixture of two fusion protein products from the *in vitro* protein synthesis. As the first demonstration of an ncZFN, Zif268-CZ was mixed with NZ-F_N to form Zif268-ncZFN. As shown in Fig. 2, Zif268-ncZFN cleaved the *Sca*I-linearized Zif268 DNA substrate into two 0.9 kb and 1.8 kb DNA fragments, as an indicative of dsDNA cleavages occurring at target sites of the DNA substrate. This result clearly showed that Zif268-ncZFN behaves as a sequence-specific endonuclease like conventional Zif268-ZFN does.

The optimal condition for sequence-specific DNA cleavage by ncZFNs was principally obtained by adjusting amounts of ZFP-CZ and NZ-F_N. The 1:4 ratio of ZFP-CZ and NZ-F_N showed the most effective sequence-specific DNA cleavage (Fig. 2). It is noteworthy that the preincubation of the two fusion proteins for facilitating assembly before DNA cleavage reaction noticeably increased sequence-specific DNA cleavage by Zif268-ncZFN (Fig. 2, preincubation + and −), which manifests that the stable formation of ncZFNs is important for efficient DNA cleavage. The standard reaction condition described in more detail in Materials and methods provided reliable reproducibility for DNA cleavage specificity and efficiency.

DNA cleavage activity of other ncZFNs containing artificially-selected, engineered ZFPs was also examined. Consistent with our prediction, these three ncZFNs clearly cleaved their cognate DNA substrates in a sequence-specific manner although there were variations in DNA cleavage efficiency (Fig. 3). As shown in Fig. S4, sequence-specific DNA cleavage of conventional ZFN counterparts was also examined and confirmed for comparison.

Since ncZFN has a different structural flexibility in the domain arrangement from that of conventional ZFN, we investigated the requirement of spacing between two recognition sites of an ncZFN for DNA cleavage. DNA substrates with various lengths of spacers were constructed and analyzed (Table S2). Unlike ZFN that needs a spacer between two recognition sites longer than 3-bp for efficient DNA cleavage, surprisingly ncZFNs can cleave all DNA substrates including even without a spacer (Fig. S5). We speculated that the more flexible domain organization of ncZFN due to the noncovalent assembly and flexible linkers may explain no apparent spacing-dependency of ncZFN.

Table 1
Zinc finger proteins used in this study. Amino acid sequences in the DNA recognition helix and cognate sequences are listed below.

ZFPs	Recognition Sequence (3′ → 5′)	Finger 1[a]	Finger 2	Finger 3
Zif268	GCG GGT GCG	RSDELTR	RSDHLTT	RSDERKR
ZifNRE	ACT TGG GAA	QSHDLDK	DSSKLSR	RLDNRTA
ZifP53	TGT ACA GGG	MSHHLKE	QRGTLTR	RLHHRLL
ZifTAB	AAA ATA TCG	QKTNLDT	QQASLNA	TLHTRTR
synZif268	GCG GGT GCG	RSDDLTR	RSDHLTT	RSDDLVR
synZ1-Zif268	GCG GGT GCG	RSDDLTR	RSDHLTT	RSDERKR
synZ3-Zif268	GCG GGT GCG	RSDELTR	RSDHLTT	RSDDLVR

[a] Sequences represent amino acids in the recognition helix (−1 to +6).

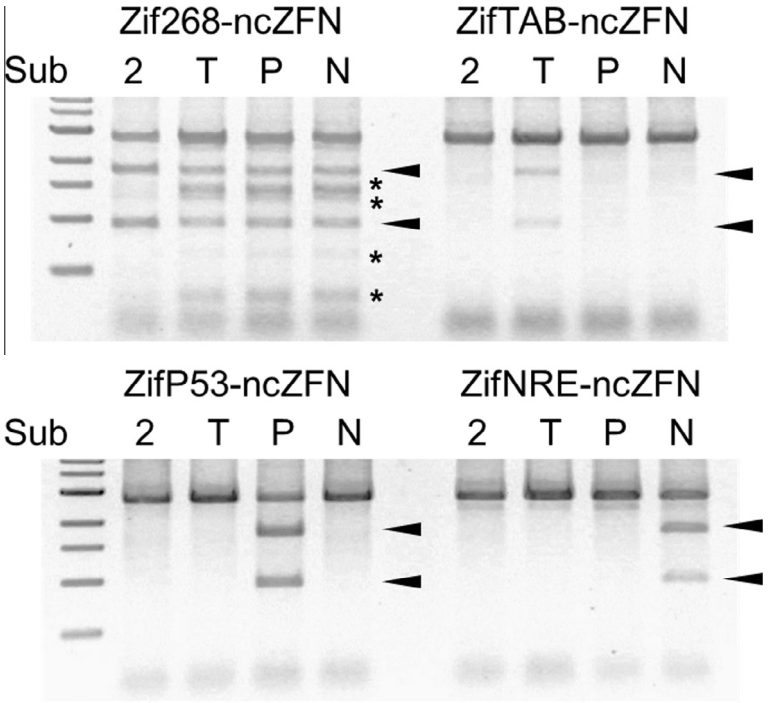

Fig. 3. Sequence-specific DNA cleavage of ncZFNs. DNA cleavage experiments with four ncZFNs containing different ZFP domains, Zif268, ZifTAB, ZifP53 and ZifNRE, were carried out with four different DNA substrates-Zif268(2) ZifTAB(T), ZifP53(P) and ZifNRE(N). The result showed that all ncZFNs sequence-specifically cleaved their cognate DNA substrates (arrows). However, only Zif268-ncZFN also exhibited considerable off-target cleavages (*) with noncognate DNA substrates.

3.3. Augmented off-target cleavage observed in Zif268-ncZFN in vitro resulted from degenerate bindings of ZFP domain

Cleavage of noncognate DNA substrates by ncZFNs revealed an intriguing result. For cognate DNA substrates, all ncZFN and conventional ZFNs faithfully cleave their DNA cognate substrate in a sequence-specific manner (Figs. 3 and S4). Likewise, ncZFNs and ZFNs with the exception of Zif268-ncZFN showed almost no DNA cleavages for noncognate DNA substrates (Figs. 3 and S4). However, Zif268-ncZFN had a significant level of off-target cleavage with noncognate DNA substrates (Fig. 3, Zif268-ncZFN with T, P and N). This observation is contrasting to the DNA cleavage results from Zif268-ZFN with noncognate DNA substrates (Fig. S4, Zif268-ZFN with T, P and N). To explain this observed difference, we postulated that degenerate DNA binding of Zif268 domain may be responsible since naturally-occurring Zif268 is biologically less strictly selected for cognate target site(s) than artificially created ZFPs. It is known that Zif268 regulates multiple genes by recognizing different target sequences embedded in promoter regions [34]. In contrast, artificially-selected ZFPs derived from Zif268 underwent very strict and stringent screening processes for more exclusive sequence-specific binding ability to their target sequences than the wild-type Zif268.

To probe the source of the augmented off-target cleavage, we firstly carried out the competition assay using short cognate and noncognate dsDNAs. In the DNA cleavage reactions of Zif268-ncZFN with the ZifP53 DNA substrate (*i.e.* noncognate DNA substrate), we added 34-bp competitor DNAs that contain either Zif268-binding (cognate) sequences or ZifP53-binding (noncognate) sequences. This competition experiment showed that off-target cleavage by Zif268-ncZFN with the ZifP53 DNA substrate was more reduced in the presence of the cognate competitor than the noncognate competitor (Fig. S6). This result indicated that this off-target cleavage produced by Zif268-ncZFN was likely caused by bindings of the Zif268 domain to lesser-optimal recognition sequences.

To further clarify the cause of augmented off-target DNA cleavage by Zif268-ncZFN, we constructed artificial Zif268-ZFPs that recognize the same sequence instead of the wild-type Zif268. Currently, zinc fingers (ZFs) database for triplet DNAs are predominantly selected by the rigorous phage display method [32]. In the synthetic Zif268 (synZif268), the ZF1 and ZF3 (the first and third ZFs) of the wild-type (naturally-occurring) Zif268 were replaced with ZF modules from the ZF selection of the Barbas group (Table 1) [35]. The ZF2 of Zif268 was not changed because an alternative choice of the ZF for the 'TGG' triplet is not available. Additionally,

either of the ZF1 and the ZF3 of the wild-type Zif268 was also individually replaced with synthetic ZFs (referred to as synZ1-Zif268 and synZ3-Zif268, respectively). All three synthetic Zif268s were then served as a DBD in both ncZFN and conventional ZFN constructs, which were examined their sequence-specific DNA cleavage and off-target cleavage with cognate or noncognate DNA substrate.

All synthetic Zif268-ZFNs mimicking the natural Zif268-ZFN cleaved their cognate DNA substrate sequence-specifically and efficiently without considerable off-target cleavage (Fig. 4A). However, natural and synthetic Zif268-ncZFNs have a significant difference in regard to off-target cleavage with noncognate DNA substrate. Both synZif268-ncZFN and synZ3-Zif268-ncZFN showed almost none or little amounts of off-target cleavage with the noncognate DNA substrate (Fig. 4B, ZifP53(s)) while synZ1-Zif268 exhibited significant level of off-target cleavage with the noncognate DNA substrate (Fig. 4B) like the wild-type (natural) Zif268 counterpart. This result implicates that the ZF3 of Zif268 is possibly responsible for off-target cleavage products made by Zif268-ncZFN.

Taken together, we concluded that off-target cleavage by Zif268-ncZFN *in vitro* is probably resulted from the degenerate binding of the wild-type Zif268 domain. In the ncZFN system, it may be possible that DNA cleavage at the lesser-optimal sites recognized by the wild-type Zif268 domain is increased so as to become more visibly detectable. Why is the Zif268-ncZFN or maybe other ncZFNs more sensitive to degenerate bindings by the ZFP domain than that of the conventional Zif268-ZFN counterpart? One possible explanation, we suggest, is that the structural flexibility of ncZFN may contribute to increased cleavages at the lesser binding sites. Additionally, plasticity of DNA cleavage activity on the spacing between two target sequences by ncZFNs (Fig. S4) may also support our account.

3.4. Correlation between off-target cleavage activity of Zif268-ncZFN in vitro and cytotoxicity of Zif268-ZFN in vivo

Next, we were intrigued if such a high level of off-target cleavages from Zif268-ncZFN *in vitro* may reflect the off-target cleavage activity of Zif268-ZFN inside cells. To examine the difference in off-target cleavage activity between the natural and synthetic Zif268-ZFNs at chromosomal DNA level (Fig. 4A), we measured their cytotoxicity by quantifying DNA double-strand breaks (DSBs) in human HEK293 cells using visualization of 53BP1-foci by immunofluorescence microscopy to elucidate DNA damages [16]. In accordance with our observation on the augmented off-target cleavage activity of Zif268-ncZFN *in vitro*, Zif268-ZFN (41.6 ± 4.2%) showed higher cytotoxicity than that of synZif268-ZFN (31.6 ± 4.4%) (Fig. S7). Thus, this higher cytotoxicity of Zif268-ZFNs *in vivo* seems to

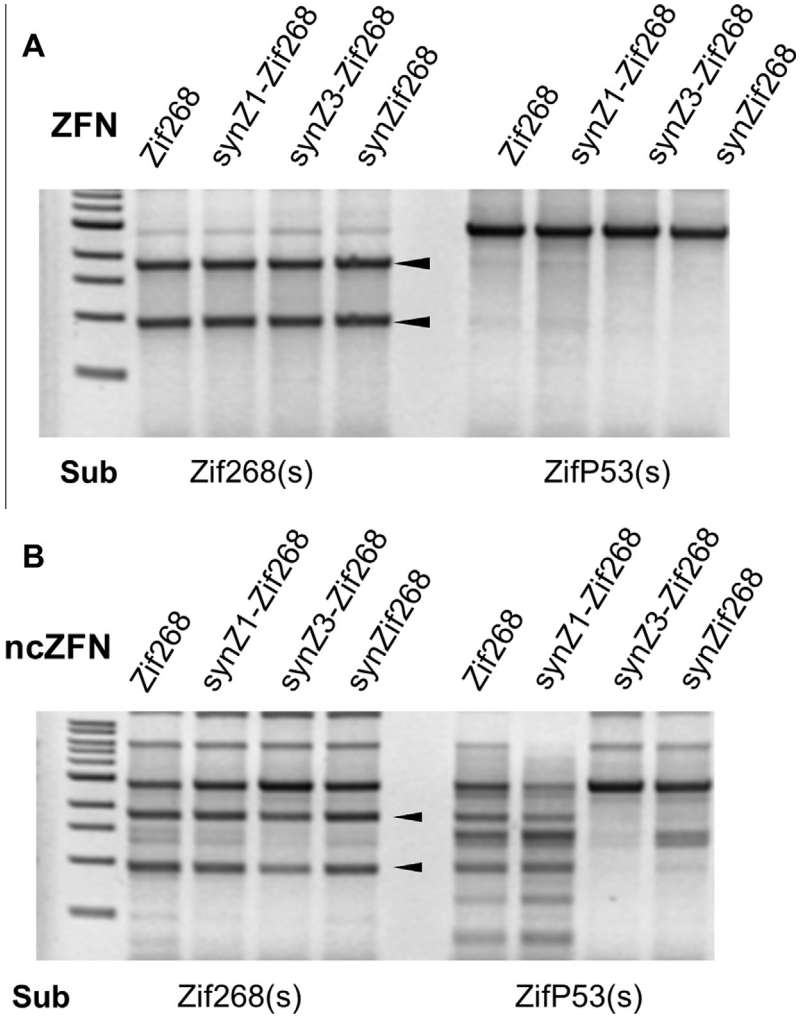

Fig. 4. Comparison of the wild-type Zif268 and synthetic Zif268 ZFPs in ZFN constructs (A) and ncZFN constructs (B). The wild-type and synthetic Zif268s (synZif268, synZ1-Zif268 and synZ3-Zif268) that recognize the same sequence were examined for DNA cleavage activity. DNA cleavage with a cognate DNA substrate (Zif268(s)) and a noncognate DNA substrate (ZifP53(s)) suggested that off-target cleavage of the Zif268-ncZFN was resulted from degenerate binding of the Zif268 domain, more specifically its ZF3.

reflect off-target cleavage activity observed *in vitro* by Zif268-ncZFNs when it compared with those of synZif268-ZFN *in vivo* and synZif268-ncZFN *in vitro*.

4. Discussion

In this study, we demonstrated the new type of ZFN system that uses the coiled-coil motif as a noncovalent assembler. In ncZFN, the noncovalent assembly of two separately expressed proteins works like a single functional entity with two domains. Characterization of ncZFNs revealed that they behave as fully active endonucleases with high level of sequence-specificity. One noticeable observation was unusually the augmented off-target cleavage on noncognate DNA substrates by Zif268-ncZFNs, whereas other ncZFNs with artificially-selected engineered ZFPs showed significant sequence-selective DNA cleavage activities with cognate and noncognate DNA substrates.

By using the competition assay, we first demonstrated that augmented off-target cleavage on noncognate DNA substrates by Zif268-ncZFN may be stemmed from degenerate bindings of the Zif268 domain. Off-target cleavage by Zif268-ncZFN was more drastically reduced by the cognate competitor DNA (Fig. S6). Next, we used synthetic Zif268s that have artificially-selected, engineered ZF module(s) that recognize the same target sequence with that of the wild-type Zif268. In comparison with the wild-type Zif268-ncZFN, synthetic Zif268-ncZFNs showed higher sequence-specificity and reduced off-target cleavage (Fig. 4B). This result further evidenced that weak sequence-discrimination capability of the wild-type Zif268 is likely the basis of the augmented off-target cleavage by Zif268-ncZFN. Finally, cytotoxicity difference between the wild-type Zif268-ZFN and synZif268-ZFNs clearly correlated with off-target cleavage activity difference by the natural Zif268-ncZFN and synthetic Zif268-ncZFNs *in vitro* (Fig. S7). All these results indicate that the intrinsic degenerate DNA binding of the wild-type Zif268 is possibly responsible for the observed off-target cleavage by Zif268-ncZFN on noncognate DNA substrates *in vitro*.

Zif268, a naturally-occurring ZFP, has biologically less strictly selected target sites than artificially engineered ZFPs do. Many DBDs including C_2H_2–type ZFPs, which are commonly a part of transcription factors, do show degeneracy of their recognition sites *in vitro* as well as *in vivo* [36]. Accordingly, Zif268 binds to multiple promoter regions of target genes, which contain different recognition sequences, and thus modulation of gene expression is often controlled by degree of affinities of Zif268 for different target sequences located within various promoters [34]. Thus, Zif268-ncZFN could produce more promiscuous DNA cleavages than other ncZFNs that contain strictly selected, engineered ZFPs. This distinctive feature of ncZFN(s) *in vitro* may offer important information for DNA binding nature of ZFPs used in conventional ZFNs. Can ncZFNs expose off-target cleavage activity by augmenting DNA cleavages at lesser-optimal sites, which is not clearly detected in ZFN counterparts? Further studies on ncZFN are certainly required to provide sufficient supports to generalize our postulation.

The ncZFN system could be one of good examples to demonstrate creation of new multidomain proteins from existing domains through the coiled-coil interaction, *i.e.*, noncovalent assembler. Using our strategy, we think that the noncovalent assembler approach can be applied to the design of new *Fok*I-based chimeric enzymes with other DBDs. Moreover, it would be interesting if the noncovalent assembler approach can create multidomain-like protein structures or even multienzyme complexes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.140.

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