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Adding Fingers to an Engineered Zinc Finger Nuclease Can Reduce Activity

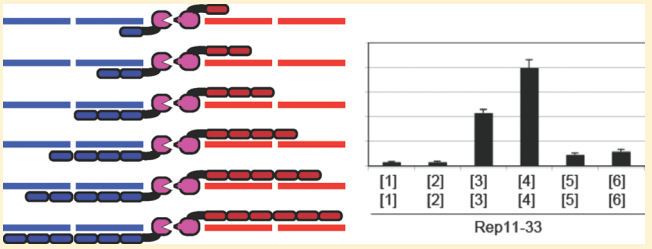
Yuka Shimizu,[†] Cem Şöllü,[‡] Joshua F. Meckler,[†] Alice Adriaenssens,[†] Artem Zykovich,[†] Toni Cathomen,[*,‡] and David J. Segal[*,†]

[†]Genome Center and Department of Biochemistry and Molecular Medicine, University of California, Davis, California 95616, United States

[‡]Department of Experimental Hematology, Hannover Medical School, D-30625 Hannover, Germany

Supporting Information

ABSTRACT: Zinc finger nucleases (ZFNs) have been used to direct precise modifications of the genetic information in living cells at high efficiency. An important consideration in the design of ZFNs is the number of zinc fingers that are required for efficient and specific cleavage. We examined dimeric ZFNs composed of [1]+[1], [2]+[2], [3]+[3], [4]+[4], [5]+[5], and [6]+[6] zinc fingers, targeting 6, 12, 18, 24, 30, and 36 bp, respectively. We found that [1]+[1] and [2]+[2] fingers supported neither in vitro cleavage nor single-strand annealing in a cell-based recombination assay. An optimal ZFN activity was observed for [3]+[3] and [4]+[4] fingers. Surprisingly, [5]+[5] and [6]+[6] fingers exhibited significantly reduced activity. While the extra fingers were not found to dramatically increase toxicity, directly inhibit recombination, or perturb the ZFN target site, we demonstrate the ability of subsets of three fingers in six-finger arrays to bind independently to regions of the target site, possibly explaining the decrease in activity. These results have important implications for the design of new ZFNs, as they show that in some cases an excess of fingers may actually negatively affect the performance of engineered multifinger proteins. Maximal ZFN activity will require an optimization of both DNA binding affinity and specificity.



ZFNs consist of tandem arrays of engineered zinc finger proteins fused to a monomer of the dimeric nuclease FokI. When two such proteins bind to adjacent target sites, an active FokI dimer is formed and creates a double-strand break (DSB) in the DNA. In cells, the DSB is rapidly targeted for repair by cellular factors using either nonhomologous end joining (NHEJ) or homologous recombination (HR) pathways. This approach has been used to create targeted gene deletions both in cultured cells, including human embryonic and induced pluripotent stem cells, and in whole organisms such as fruit flies, nematodes, zebrafish, and rats and to significantly improve the frequency of homologous recombination in gene therapy and genome engineering applications.[1,2]

An important consideration in the design of ZFNs is the number of zinc fingers (ZFs) that are required for efficient and specific cleavage. The ability to recognize 15−18 bp of DNA should be sufficient to specify a unique locus in a complex genome such as the human genome. In principle, six fingers should provide such specificity because each zinc finger recognizes approximately 3−4 bp of DNA.[3] Although no in vivo binding studies (i.e., chromatin immunoprecipitation followed by sequencing, ChIP-Seq[4]) have examined this proposition directly, some artificial transcription factors containing six engineered ZFs have been shown to regulate a single gene in human cells, suggesting high specificity.[5] ZFNs consisting of two monomers of three ZFs each ([3]+[3] fingers) can recognize two 9 bp zinc finger binding sites separated by several nucleotides that allow the FokI cleavage domains to dimerize and cleave (Figure 1A). The exact spacing between the two binding sites is dependent on the length and composition of the linker between the terminal zinc finger and the FokI domain, and the most commonly used linkers restrict the tolerated variation in spacer length to ∼1 bp.[6,7] Therefore, ZFNs consisting of [3]+[3] fingers might be expected to be active only on unique target sites in the human genome. A recent study of a ZFN composed of [3]+[3] fingers at 141 potential off-target sites reported very weak but dose-dependent ZFN activity at ≤1% of examined sites.[8] Other similar studies using ZFNs composed of four to six fingers per monomer have reported similarly limited off-target events.[9−11] However, the host organisms and composition of fingers were different, precluding a direct comparison of the effect of finger number on off-target events. A cytological analysis found ZFNs composed of [4]+[4], [6]+[6], and [3]+[3] fingers produced decreasing amounts of off-target cleavage events.[12] However, the fingers

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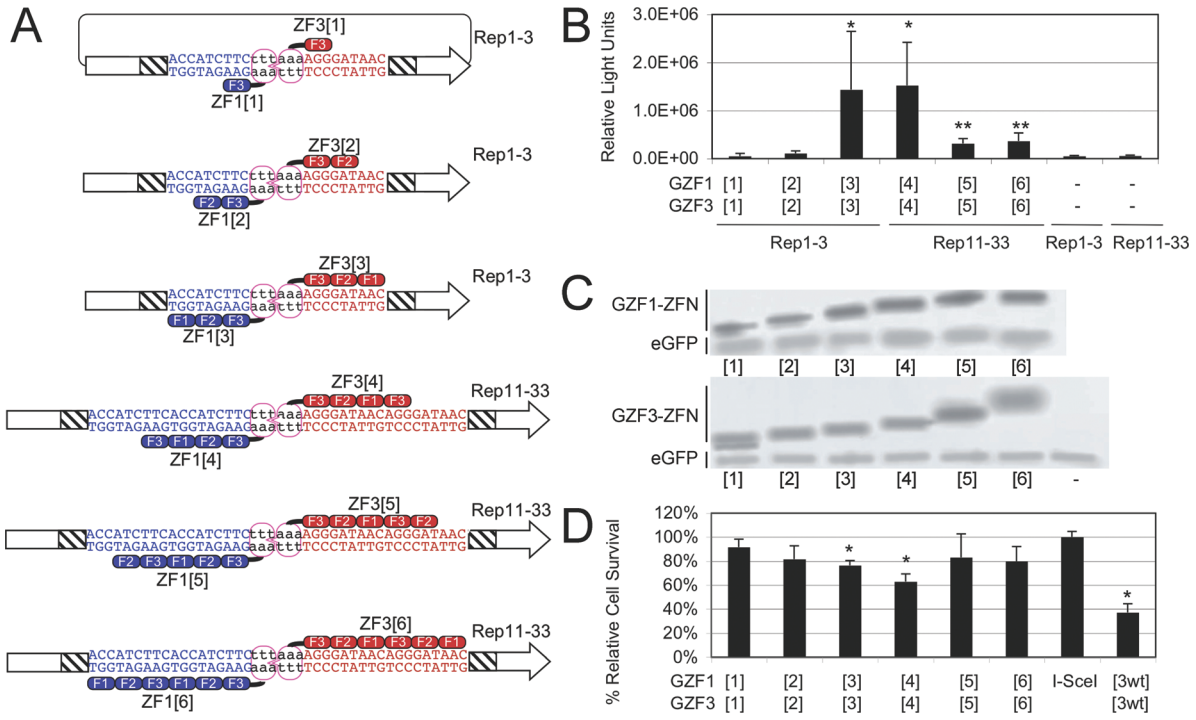

Figure 1. Effects of finger number on ZFN activity in cells. (A) Schematic of the experimental setup. A systematic series of multifinger heterodimeric ZFNs and the various target DNA sites were studied. DNA-binding domains that mediate binding to the left (blue) and right (red) target subsite juxtapose the obligate heterodimeric FokI cleavage domains (purple ovals). The target sites were located between two fragments of a luciferase reporter (boxed arrows), which shared a region of homology (hatched region). The elements are not drawn to scale. A double-strand break at the target site in cells restores an active luciferase gene by SSA recombination. (B) ZFN-stimulated SSA recombination assay. Luciferase activity induced by ZFN-mediated cleavage is shown as the combined results of six independent experiments. The significance of the measurements over no ZFN controls (indicated with −) was determined using a paired, one-tailed *t* test (*$p < 0.02$; **$p < 0.002$). (C) ZFN expression levels. HEK293T cells were cotransfected with ZFN expression vectors and pEGFP, and cell lysates were probed with antibodies against the HA tag and EGFP. A dash indicates transfection with pEGFP only. (D) Quantitative cytotoxicity assay. HEK293T cells were cotransfected with pEGFP and ZFN expression vectors as indicated below the graph. The columns represent the average fraction of EGFP-positive cells at day 5 as compared to the fraction 30 h after transfection and are shown relative to transfection with an expression vector encoding the endonuclease I-SceI. A statistically significant decrease in cell survival compared to that of non-toxic I-SceI was determined using one-tailed *t* test with unequal variance (*$p < 0.002$). [3wt] denotes expression of GZF1[3] and GZF3[3], which contain wild-type FokI domains and have been previously shown to be cytotoxic.[14]

differed not only in number but also in composition and recognition sequences, thus confounding a direct comparison between them.

The intention of this study was to systematically investigate the relationship between the number of ZFs and ZFN activity. As one conceptual hypothesis, if the [3]+[3] finger were active, the [4]+[4] version would be expected to possess both greater affinity and greater specificity and thus be even more active. Using this reasoning, the [6]+[6] version would be expected to have a combined recognition capability of 36 bp (or at least more specificity than the shorter zinc finger arrays) and be the most active. Here, a series of ZFNs consisting of one to six fingers per monomer were tested for activity in a plasmid-based single-strand annealing (SSA) recombination assay and an in vitro cleavage assay. We found that ZFNs containing any monomer with one or two ZFs were rarely active in any combination, while ZFNs containing monomers with three or four ZFs were active in all combinations. Surprisingly, ZFNs containing [5]+[5] or [6]+[6] fingers showed significantly reduced activity in cells and failed to cleave target sites in vitro. This study therefore identifies a potential limitation to the use of arrays with more than four fingers for ZFN applications.

MATERIALS AND METHODS

Expression and Reporter Plasmids. ZFN expression vectors pPGK.GZF1-N and pPGK.GZF3-N carrying the obligate heterodimer modifications RR and DD, respectively, have been described previously.[13,14] Additional fingers were digested with XhoI and AgeI and cloned between the XhoI and XmaI sites of these vectors to preserve canonical TGEKP linkers between all ZFs. Construction of SSA luciferase reporter plasmid pSSA Rep 3-1 has been described previously.[14] Construction of additional SSA reporters was performed similarly, using long oligonucleotides to introduce the target sequences described.

SSA Assay. In 24-well plates, HEK293T cells at 80% confluency in DMEM supplemented with 10% fetal calf serum were cotransfected with 200 ng of each ZFN monomer expression plasmid and 25 ng of SSA reporter plasmid using Lipofectamine 2000 (Invitrogen). Luciferase activity was determined 48 h post-transfection using BrightGlo reagents (Promega) in an Analyst microplate luminometer (Molecular Devices).

Cell Survival Assay. The assay was basically performed as described previously.[15] Briefly, HEK293T cells in 12-well plates were transfected with 1000 ng of the respective ZFN expression

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plasmid and 100 ng of pEGFP-C1 (Stratagene) and assessed by flow cytometry (FACSCalibur, BD Biosciences) after 2 and 5 days. For the calculation of cell survival, the ratio of the number of eGFP-positive cells at day 2 versus day 5 was determined and normalized to the number of cells transfected with an I-SceI control expression vector.

In Vitro Cleavage Assay. ZFNs were expressed in vitro using the TNT SP6 Quick Coupled Transcription/Translation System according to the manufacturer's instructions (Promega).[16] The target DNA fragment was generated by polymerase chain reaction with Pfu polymerase (Stratagene) using plasmid pSSA Rep 3-1 or pSSA Rep 33-11 as the template and primers 5′-GCTGTTTCTGAGGAGCCTTC and 5′-CCCTTCTTGGCCTTTATGAG (Eurofins MWG Operon, Ebersberg, Germany) to amplify an 800 bp fragment. A target site for restriction enzyme EcoRI was located next to the ZFN target site and used as a positive control. For in vitro cleavage, 1 μL of each TNT lysate containing one ZFN subunit was mixed with 200 ng of the DNA template, 1 μg of BSA, and NEBuffer 4 [50 mM potassium acetate, 20 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, and 1 mM dithiothreitol (New England Biolabs)] in a total volume of 10 μL. After incubation at 37 °C for 90 min, the reaction was analyzed on a 1% agarose gel.

Western Blot. HEK293T cells in six-well plates were transfected via polyethyleneimine (PEI)-mediated transfection. The transfection mix contained 800 ng of ZFN expression plasmid, 200 ng of pEGFP-N1 (Clontech), 5 μg of pUC118, and PEI (0.1 g/L, pH 5.5) in 150 mM NaCl. Cells were harvested after 30 h and lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% deoxycholate), and 50 μg of lysate was separated via sodium dodecyl sulfate−polyacrylamide gel electrophoresis. After the transfer to polyvinylidene difluoride membranes, ZFN and EGFP expression was detected simultaneously with antibodies directed against the HA tag [NB600-363 (Novus Biologicals)] and EGFP [MAB3580 (Millipore)] and visualized by infrared imaging after incubation with secondary antibodies conjugated with either IR-Dyes 680 or 800CW (LI-COR Biosciences).

Electromobility Shift Assay (EMSA). The LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL) was used according to the manufacturer's protocol. Complementary pairs of 5′-biotin-labeled forward and 5′-poly-T (to improve cross-linking to the membrane) reverse oligonucleotides were annealed to obtain double-stranded target DNAs. The sequences of all oligonucleotide targets are provided in the Supporting Information. Binding reactions were performed for 1 h at room temperature (22 °C) in zinc buffer A (ZBA) [100 mM Tris base, 90 mM KCl, 1 mM $MgCl_2$, and 90 μm $ZnCl_2$ (pH 7.5)] containing 150 mM KCl, 5 mM DTT, 10% glycerol, 0.1 mg/mL BSA, 0.05% NP-40, 25−55 pM target DNA, and purified ZFPs at concentrations of 0.025−1000 nM. Gel electrophoresis was performed on a 10% native polyacrylamide gel in 0.5× TBE buffer at 4 °C. After being blotted on a Biodyne B nylon membrane (Pierce) for 1 h at 100 V and 4 °C, the DNA was cross-linked by a UV cross-linker (Stratagene) for 4 min. Equilibrium binding constants (K_D) were calculated from protein titration experiments imaged on X-ray film. The reported values represent the results of at least two experiments, with a standard deviation of ±50%.

Binding Site Specificity Assay (Bind-n-Seq). Bind-n-Seq was performed essentially as described previously.[17] Briefly, the coding regions for the DBDs were subcloned into pMAL-c2X (New England Biolabs), expressed in *Escherichia coli* BL21(DE3) cells (Invitrogen), and purified over amylose resin in ZBA and 10 mM maltose. The maltose was removed by overnight dialysis. Bar-coded 93-mer double-stranded oligonucleotide targets containing Illumina primer binding sites and a 21-nucleotide random region were incubated with proteins at various concentrations and under various salt conditions. Bound complexes were enriched by six washing steps over amylose resin. Eluted DNA was sequenced on an Illumina Genome Analyzer. The motifs shown for GZF1[3] and GZF3[3] are based on approximately 100000 reads that were enriched 12- and 7-fold over a non-selected background, respectively.

RESULTS

Number of Zinc Finger Domains and Activity in Cells. To examine the relationship between the number of ZFs and ZFN activity in a systematic manner, we constructed a series of proteins based on the three-finger proteins GZF1 and GZF3, which have been extensively characterized as a ZFN pair.[13,14] ZFN pairs were constructed containing only one finger 3 of each protein (GZF1[1] and GZF3[1], with the number of ZFs given in brackets), fingers 2 and 3 of each protein (GZF1[2] and GZF3[2]), and all three fingers (GZF1[3] and GZF3[3]) (Figure 1A). To create proteins of four, five, and six fingers, we generated ZFN pairs containing the three fingers of GZF1 or GZF3 and an additional finger 3 of each protein (GZF1[4] and GZF3[4]), additional fingers 2 and 3 of each protein (GZF1[5] and GZF3[5]), and an addition of all three fingers (GZF1[6] and GZF3[6]) (Figure 1A). The resulting six-finger proteins were therefore tandem dimers of the two three-finger arrays. All ZFNs contained FokI cleavage domains that carried the obligate heterodimer modification RR or DD.[14] These variants restrict activity to only heterodimers, reducing cytotoxicity due to off-target DSBs.

A plasmid-based single-strand annealing (SSA) reporter assay in HEK293T cells was performed to assess the activity of the ZFN pairs (Figure 1A).[14] Briefly, a gene encoding luciferase was divided into two segments containing an 870 bp region of homology, separated by a stop codon and a ZFN target site. A ZFN-induced double-strand break (DSB) between the segments stimulates SSA homologous recombination, resulting in an active luciferase gene. Luciferase activity should therefore be proportional to ZFN activity. GZF1[3] and GZF3[3] were previously shown to have optimal activity in this assay on a heterodimeric target site consisting of 9 bp binding sites for each monomer in an inverted orientation separated by a 6 bp spacer [Rep1-3 (Figure 1A)].[18] To accommodate the ZFN pairs containing four to six fingers, we constructed two tandem 9 bp binding sites for each monomer in an inverted orientation separated by a 6 bp spacer [Rep11-33 (Figure 1A)]. GZF1−GZF3 ZFN heterodimers consisting of [1]+[1] and [2]+[2] fingers did not stimulate SSA recombination, while [3]+[3] and [4]+[4] fingers stimulated robust recombination (Figure 1B). Although the protein expression levels were comparable (Figure 1C), [5]+[5] and [6]+[6] fingers produced ∼4-fold less recombination than [3]+[3] and [4]+[4] fingers.

Some ZFNs have been shown to be cytotoxic, presumably because of numerous cleavages at off-target sites.[13,14,19−21] Cytotoxicity may manifest as an apparent reduction in the level of recombination in the SSA assay. To investigate if the reduced SSA activity was due to cytotoxicity, cell survival frequency was examined using a ZFN-associated toxicity assay.[14,22] HEK293T cells were cotransfected with plasmids expressing the series of ZFNs and a green fluorescent marker protein. Cell survival

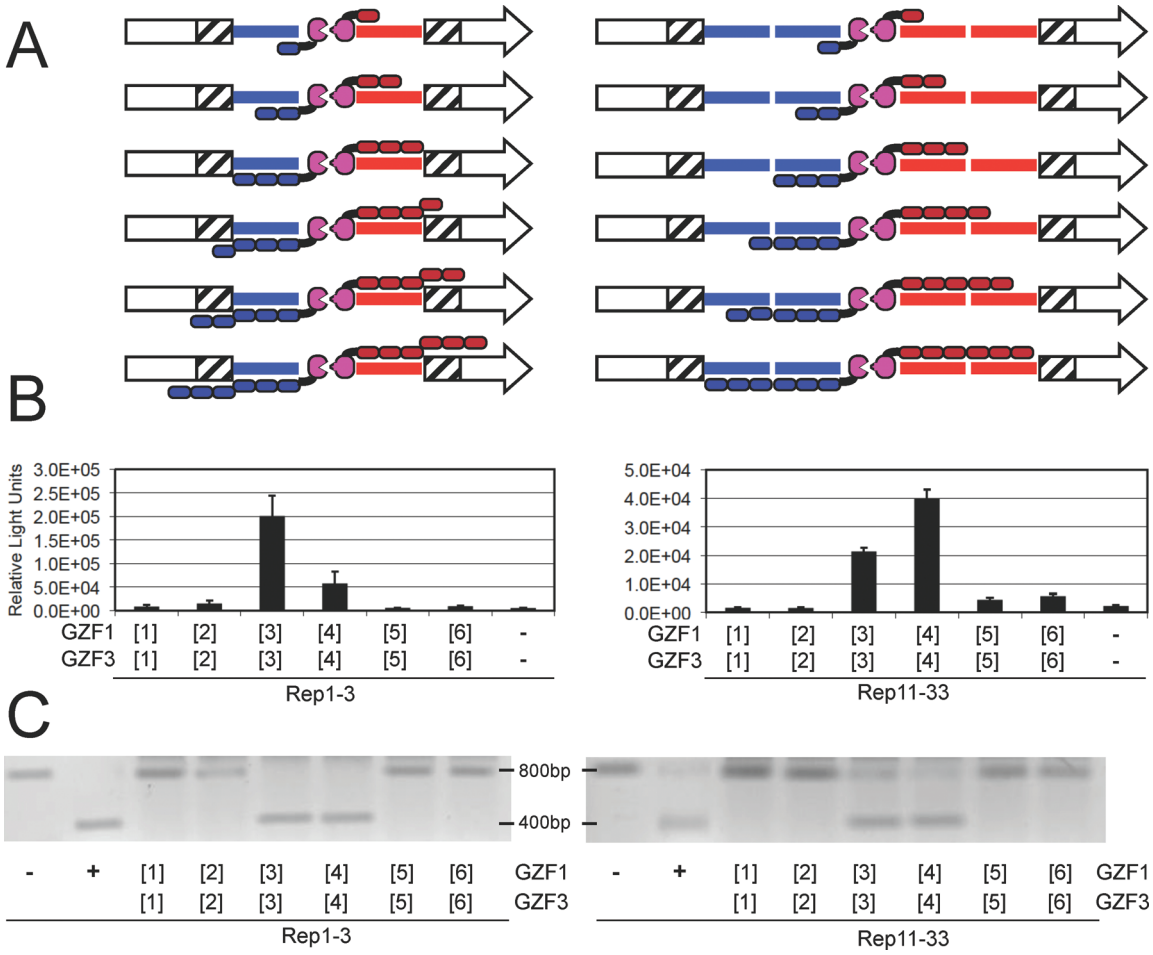

Figure 2. Effect of target site composition on ZFN activity. (A) Cartoon of the experiments in which the full series of ZFNs were assayed on either target Rep1-3 (left) or target Rep11-33 (right). Components are labeled as in Figure 1. (B) ZFN activity in cells. The graph shows luciferase activity upon ZFN-mediated SSA using Rep1-3 (left) or Rep11-33 (right). (C) In vitro cleavage activity. Activity of the ZFN pairs in an in vitro cleavage assay using ~800 bp DNA containing the ZFN target site of Rep1-3 (left) or Rep11-33 (right). Cleavage produces two bands of ~400 bp. Lane −, DNA with no ZFN; lane +, DNA digested by EcoRI, which cleaves just 3′ to the ZFN target site.

frequency was determined by calculating the fraction of green fluorescent cells at day 5 as compared to 30 h post-transfection. We observed that cells expressing the highly active ZFNs [3]+[3] and [4]+[4] had similar or even slightly reduced viability compared to those expressing ZFNs [5]+[5] or [6]+[6] (Figure 1D). Overexpression of ZFN pairs [1]+[1] and [2]+[2] did not considerably affect cell viability.

To further investigate the relationship between the number of fingers and ZFN activity, we examined the effect of target site composition using the full series of ZFNs on either target Rep1-3 or Rep11-33 (Figure 2A). ZFN pairs with one to three fingers should be accommodated on the 9 bp monomer binding sites of Rep1-3. However, ZFNs with four to six fingers would not have binding sites for more than the three C-terminal fingers. In principle, these experiments could reveal an effect of unbound fingers (e.g., whether the level of recombination is reduced if not all fingers have a cognate binding site). In contrast, all ZFN pairs should be accommodated on the 18 bp monomer binding sites of Rep11-33. As in our previous SSA experiment, we observed for both target Rep1-3 and Rep11-33 that GZF1−GZF3 heterodimers consisting of [1]+[1] and [2]+[2] fingers did not stimulate SSA recombination (Figure 2B). On Rep1-3, [3]+[3] fingers appeared to be more active than [4]+[4] fingers, while on Rep11-33, the reverse was observed. In general, the activity of [3]+[3] and [4]+[4] fingers seemed somewhat greater on Rep1-3 than on Rep11-33. As observed previously, ZFNs [5]+[5] and [6]+[6] produced dramatically less recombination than ZFNs [3]+[3] and [4]+[4].

Number of Zinc Finger Domains and Activity in Vitro. Because others have reported highly active ZFNs with six-finger arrays,[10,12,23] the reduced activity of GZF1−GZF3 [5]+[5] and [6]+[6] heterodimers was unexpected. To investigate if the reduced activity could be due to the inhibition of a cellular process (e.g., inhibition of the recombination mechanism), we examined ZFN activities using an in vitro cleavage assay.[16] A segment containing the ZFN target site of Rep1-3 or Rep11-33 was amplified and exposed to in vitro translated ZFNs. Conversion of the original DNA to a lower-molecular weight fragment should provide a direct measure of cleavage activity without potentially confounding cellular processes, such as recombination or cell viability. The observed in vitro cleavage pattern was nearly identical to the results obtained by the SSA assay (Figure 2C). In agreement with the SSA data, ZFN pairs [3]+[3] and [4]+[4] were able to cleave targets Rep1-3 and

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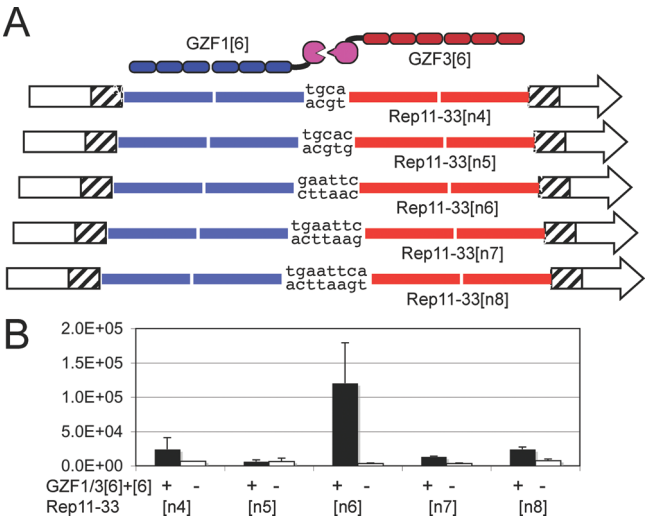

Figure 3. Effect of target site spacer length on ZFN [6]+[6] activity. (A) Cartoon of the experiments in which ZFN [6]+[6] was assayed on a series of Rep11-33 variants having 4−8 bp between zinc finger binding sites. (B) Luciferase activity upon ZFN-induced SSA recombination of the Rep11-33 series in the presence (+) or absence (−) of ZFN.

Rep11-33 in vitro, while ZFNs [1]+[1], [2]+[2], [5]+[5], and [6]+[6] did not reveal detectable activity.

The Six-Finger ZFN Maintains the Expected Target Site Spacing. ZFs distort the B-form structure of the DNA double helix upon binding,[24] and it has been suggested that longer arrays of ZFs may create greater strain in the protein−DNA complex.[25,26] We have previously shown, using the same ZFN architecture and SSA assay used here, that GZF1[3] and GZF3[3] heterodimers display a narrow peak of activity when their binding sites are separated by 6 bp.[18] Spacers of 5 or 7 bp resulted in a loss of SSA activity. To investigate if the reduced activity of GZF1−GZF3 [5]+[5] and [6]+[6] heterodimers might be due to increased DNA distortions that change the spacing requirements of the target site, ZFN [6]+[6] was tested on a series of Rep11-33 variants in which the spacing between the 18 bp binding sites varied from 4 to 8 bp (Figure 3A). We observed a narrow peak of recombination activity when the binding sites were separated by 6 bp (Figure 3B), consistent with the results previously obtained with ZFN [3]+[3].

Reduced ZFN Activity Can Be Rescued by Using Fewer Fingers on One Side. Because ZFN monomers with three or four fingers were active, we explored if the combination of an active monomer with an inactive monomer would rescue the activity of the resulting ZFN heterodimer. Combinations of GZF1[6] with the full series of GZF3 monomers (Figure 4A, left) and GZF3[6] with the full series of GZF1 monomers (Figure 4A, right) were examined using the SSA assay (Figure 4B) and the in vitro cleavage assay (Figure 4C). The results of the two assays were highly consistent. We observed that combined expression of the GZF1−GZF3 [6]+[3] or [6]+[4] heterodimer improved the recombination frequency as well as the cleavage activity compared to those of [6]+[5] and [6]+[6] heterodimers. ZFN [6]+[2] showed weak but observable activity, in contrast to inactive ZFN [2]+[2] (Figures 1 and 2). Similarly, expression of GZF1−GZF3 [3]+[6] and [4]+[6] heterodimers showed improved activity in the cellular recombination and the in vitro cleavage assay compared to those of [5]+[6] and [6]+[6] heterodimers. Although the rescue was only partial, a 2−5-fold increase in activity over that of ZFN [6]+[6] compared to a 7−20-fold increase in activity of ZFNs [3]+[3] and [4]+[4] over that of ZFN [6]+[6] (Figures 1 and 2), these results demonstrated that the six-finger monomers used in this study had the capacity to be functional.

The Number of Zinc Finger Domains Does Not Always Correlate with Binding Affinity and Specificity. Several possible models could explain the observations in this study, including the six-finger proteins binding to additional sites compared to the three-finger versions (i.e., reduced specificity), the six-finger proteins binding their targets less well (i.e., reduced affinity), the six-finger proteins binding their specific sites using alternative binding modes (i.e., destructive binding modes), or the six-finger proteins binding with such high affinity that the DNA is distorted and the target site is rendered resistant to nuclease activity (i.e., destructive high affinity). An EMSA was used to investigate the binding affinity and specificity of the three- and six-finger proteins. The affinity of GZF1[3] was similar to that of the well-characterized Zif268 protein for their respective 9 bp targets (Table 1, $K_d^{specific}$). The affinity of GZF3[3] was 30-fold lower. The addition of three fingers to GZF3[3] increased its affinity more than 80-fold. Unexpectedly, the addition of three fingers to GZF1[3] slightly decreased its affinity by ∼2-fold. As one measure of specificity, binding of the six-finger proteins to targets containing only 9 bp of the 18 bp sites reduced their affinity only ∼2-fold (Table 1, $K_d^{half\text{-}site}$, and Figure S-1 of the Supporting Information). As another measure of specificity, each protein was assayed for nonspecific binding the reciprocal 18 bp target [e.g., GZF1[6] on the target for GZF3[6] (Table 1, $K_d^{nonspecific}$)]. In most cases, the level of binding to the nonspecific target was below the measurable range of our assay, which had a 9000-fold dynamic range. This corresponded to a ratio of nonspecific to specific binding of >1700. An important exception was GZF3[3], which had a specificity ratio of only 2.8. The low specificity of this protein was confirmed by target site selection analysis using the Bind-n-Seq method,[17] which revealed that GZF3[3] recognizes only 4−5 bp of the intended 9 bp binding site (Figure 5). The low specificity of GZF3[3] in these assays is consistent with its known toxic activity as a homodimeric ZFN in vivo.[13,14] The results of these analyses therefore indicate that the additional fingers dramatically improved both the affinity and specificity of GZF3 while unexpectedly having a negligible or modestly impairing effect on the affinity and specificity of GZF1.

■ DISCUSSION

The goal of this study was to systematically investigate the relationship between the number of ZFs and ZFN activity. We examined ZFN heterodimers containing one to six ZFs using a plasmid-based single-strand annealing (SSA) recombination assay and an in vitro cleavage assay. The in vitro data were in good correlation with the results obtained in cells, indicating that the phenomena we observed reflect the ability of the ZFN to cleave its target DNA rather than the effect of the ZFN on cellular processes, such as recombination or cell viability. In agreement with this hypothesis, a direct test of ZFN-induced cytotoxicity showed a weak inverse correlation between cell viability and ZFN activity.

We found that ZFNs composed of [1]+[1] and [2]+[2] fingers did not stimulate SSA recombination or cleave their targets in vitro. It is likely that these monomers lacked sufficient affinity to bind individually to their targets. Although the FokI dimerization interface could have allowed GZF1[2] and GZF3[2] to bind cooperatively, approximating a four-finger

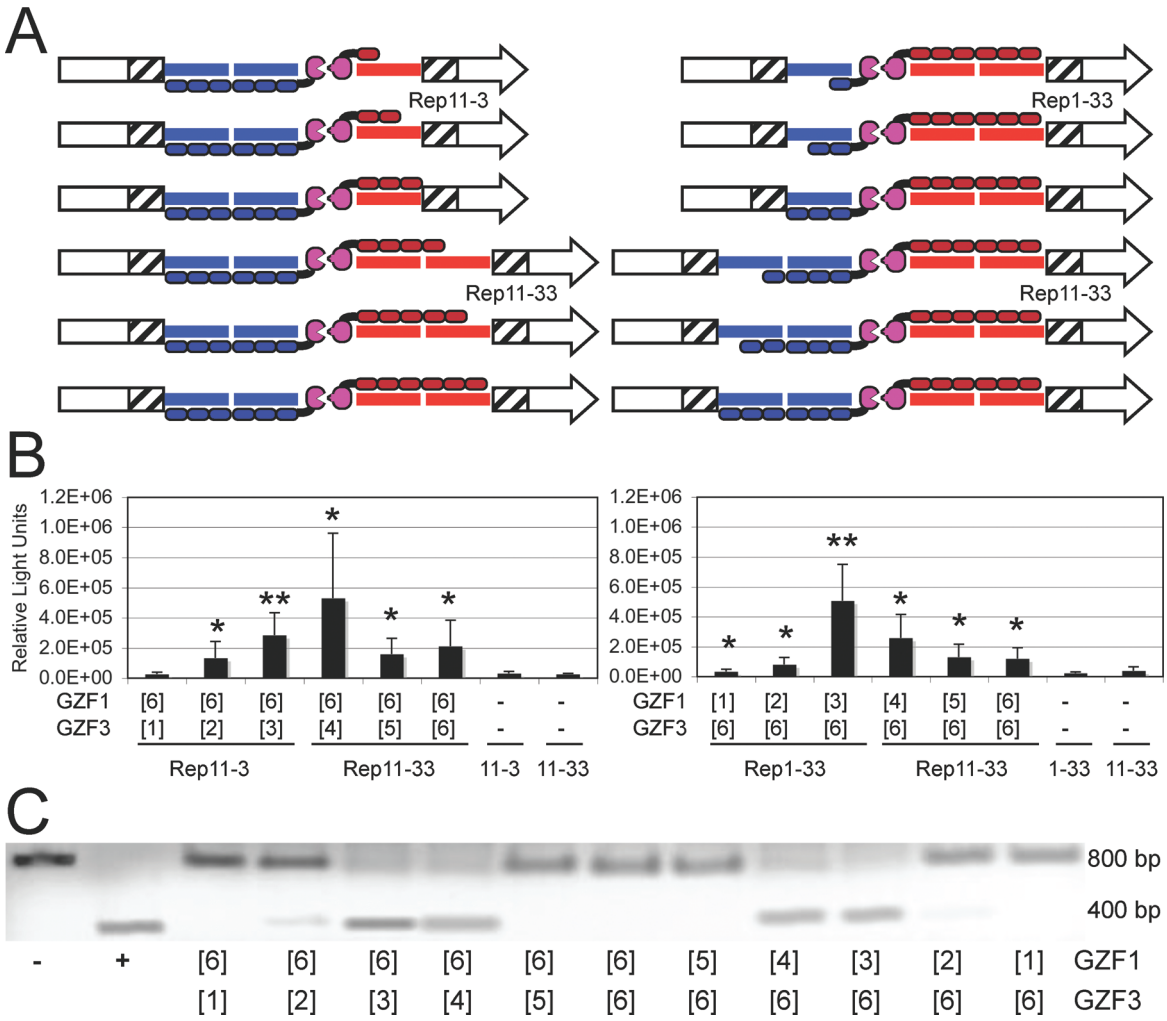

Figure 4. Rescue of ZFN[6] activity by shorter arrays. (A) Cartoon of the experiments in which full series of ZFN monomers were assayed with either GZF1[6] (left) or GZF3[6] (right). (B) Luciferase activity upon ZFN-induced SSA recombination. Activity of the ZFN pairs in the SSA assay is shown as the combined results of five independent experiments. The significance of the measurements over no ZFN controls (−) was determined using a paired, one-tailed *t* test (*$p < 0.05$; **$p < 0.01$). (C) In vitro cleavage activity of ZFN. Activity of the ZFN pairs in an in vitro cleavage assay using ~800 bp DNA containing the ZFN target site of Rep11-33. Cleavage produces two bands of ~400 bp. Lane −, DNA with no ZFN; lane +, DNA digested by EcoRI, which cleaves just 3′ to the ZFN target site.

Table 1. Affinities and Specificities of Three- and Six-Finger Proteins

	$K_d^{specific}$ (nM)	$K_d^{half\text{-}site}$ (nM)	$K_d^{half\text{-}site}/K_d^{specific}$	$K_d^{nonspecific}$ (nM)	$K_d^{nonspecific}/K_d^{specific}$
GZF1[3]	0.12			>450	>3846
GZF1[6]	0.26	0.62	2.4	>450	>1744
GZF3[3]	4.0			11	2.8
GZF3[6]	<0.05	0.085	1.7	>120	>2400
Zif268[3]	0.34				

protein, this effect was not observed. The affinity of the wild-type FokI dimerization interface is estimated to be ~100 nM,[27] and the use of a modified obligate heterodimerization interface in this study may have further reduced this affinity.[14] The [6]+[2] and [2]+[6] configurations displayed modest activity, suggesting a more stable partner could partially compensate for the weaknesses of the shorter arrays.

ZFNs composed of [3]+[3] and [4]+[4] fingers were active in all assays, including assays in which the target site contained binding sites for only three of the four fingers. On Rep1-3, ZFN [4]+[4] appeared to have reduced activity compared to that of ZFN [3]+[3] in the SSA assay, particularly when compared to the increased activity of ZFN [4]+[4] compared to that of ZFN [3]+[3] on Rep11-33 in which binding sites were present for all four fingers. This suggests that binding of the terminal fourth finger to the target site was beneficial but not essential under the experimental conditions used.

An unexpected observation was that ZFNs composed of [5]+[5] or [6]+[6] ZFs showed significantly reduced activity compared to those of [3]+[3] and [4]+[4] fingers, while others have reported the use of five- and six-finger monomers in highly active ZFNs.[10,12,23,28] One obvious possible explanation for the lower ZFN activity might be a reduced level of DNA binding, which could be explained by either (i) context-dependent negative effects upon addition of additional fingers[29] or (ii) the use of the canonical "TGEKP" linker in six-finger arrays that may

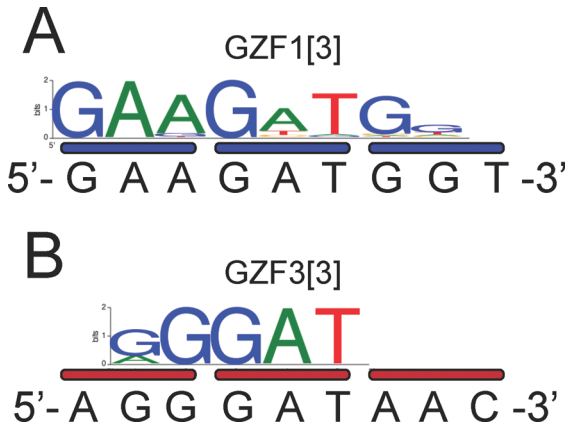

Figure 5. Binding specificity determined by the Bind-n-Seq target site selection assay. Binding motifs are shown for (A) GZF1[3] and (B) GZF3[3], with intended binding sites shown below.

be too short for optimal binding, hence resulting in deformation of the DNA and making binding of longer arrays energetically less favorable.[26,30] However, in contrast to a weakened binding model, we observed that fusion of three additional fingers to GZF3[3] improved the affinity and specificity of the protein more than 100-fold. This result is in agreement with observations made by Guo et al.,[28] who reported an increase in affinity when comparing related three-finger (K_d = 35 nM), four-finger (K_d = 11 nM), five-finger (K_d = 4.2 nM), and six-finger (K_d = 0.85 nM) arrays. Together, these data demonstrate that low-affinity binders can be rescued by the addition of further low-affinity ZFs and that linker modifications are not strictly required to generate high-affinity ZF arrays.

A second potential explanation for the decreased activity of six-finger ZFNs could be increased toxicity. However, in this study, the extra fingers were not found to dramatically affect ZFN-associated toxicity, directly inhibit recombination, perturb the optimal configuration on the ZFN target site, or substantially reduce binding affinity or specificity. Interestingly, Guo et al.[28] also reported a 2-fold decrease in activity when comparing a high-affinity six-finger ZFN with lower-affinity four- and five-finger ZFNs, suggesting that high affinity does not necessarily translate into high activity. Cornu et al.[15] showed that minor modifications to the DNA-binding domain of ZFN EB0, which reduced its affinity ~3-fold but increased its specificity ~14-fold, resulted in a ZFN with improved activity and significantly reduced toxicity. This suggests that the number of fingers in a ZF array is not important, but rather the balance between affinity and specificity.

One difference between this study and several others is that both six-finger monomers were actually tandem dimers of three-finger monomers. GZF1[6] and GZF3[6] were found to bind their half-sites with only 2-fold weaker affinity than for the 18 bp sites. In principle, the independent binding of subsets of fingers could affect ZFN activity by several mechanisms, including additional nonproductive binding modes at the target site (suggested in Figure S-2 of the Supporting Information), or a reduced concentration of protein at the target site due to increased binding at off-target sites. The ability of subsets of fingers in multizinc finger proteins to bind independently has been well established for other natural and engineered DNA binding proteins. For instance, the ability of the human 11-finger protein CTCF to employ different combinations of zinc fingers to bind diverged promoter sequences was discovered more than a decade ago.[31] The first engineered six-finger protein, C7-C7, was reported to have an affinity for a constituent 9 bp three-finger site that was only 10-fold weaker than the affinity for the 18 bp six-finger site.[3] The first six-finger protein engineered to an endogenous site, E2C, was reported to have an affinity for a constituent 9 bp site that was only 2−3-fold weaker than for the 18 bp site.[32] Yant et al.[33] invoked alternative binding modes of the E2C protein at its target site as a mechanism to explain the increased number of transposition events in a mutated E2C target site (9 of 18 bp) compared to a perfect target site (18 bp) in the presence of an E2C-Sleeping Beauty chimeric transposase. Therefore, these studies support a model in which subsets of fingers in a multidomain zinc finger protein can bind with substantial affinity, resulting in unexpected binding behaviors that can adversely affect activity.

Such effects are likely to be observed only in cases for which multifinger proteins contain subsets of fingers that can bind independently with high affinity. Three-finger proteins composed of fingers recognizing only 5′-GNN-3′ sites, such as GZF1 (5′-GAA GAT GGT-3′), have been reported to generally bind their target sites better than those containing fingers recognizing 5′-ANN-3′, 5′-CNN-3′, and 5′-TNN-3′ sites,[34] such as GZF3 (5′-AGG GAT AAC-3′). The small number of high-affinity 5′-GNN-3′ fingers in the [6]+[6] ZFN Chk2-ZFN1-MA (target site 5′-ACC CGG GTT CCC CTC GGG-3′)[12] might be anticipated to diminish the potential for independently binding subsets of fingers, possibly providing an explanation for the high activity of that [6]+[6] ZFN compared to those in our study. Similarly, we found that when proteins with a low probability of independently binding subsets (i.e., three and four fingers) were combined with proteins with a high probability (i.e., the five- and six-finger proteins used here), a partial rescue of ZFN activity was observed. It is possible that this rescue was dependent on the particular composition of fingers and conditions used here.

This study and the studies mentioned above identify an important potential limitation to the use of multifinger proteins. They demonstrate that long arrays of zinc fingers have the potential to display complex binding behaviors and that subsets of fingers within longer arrays can mediate efficient binding to DNA. Hence, more zinc fingers are not always better, and in some cases, an excess of fingers may actually negatively affect the performance of engineered multifinger proteins. The success of any particular array may depend less on the number of fingers and more on the composition of the fingers. Maximal ZFN activity requires an optimization of both affinity and specificity. These results therefore have important implications for the design of new ZFNs, artificial transcription factors, and other zinc finger applications.

■ ASSOCIATED CONTENT

Supporting Information. EMSA results and oligonucleotide sequences (Figure S-1) and diagram of potential nonproductive binding modes (Figure S-2). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*D.J.S.: telephone, (530) 754-9134; fax, (530) 754-9658; e-mail, djsegal@ucdavis.edu. T.C.: telephone, +49 511 532 5170; fax, +49 511 532 5121; e-mail, cathomen.toni@mh-hannover.de.

Author Contributions

Y.S. and C.Ş. contributed equally to this work.

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■ ABBREVIATIONS

DSB, double-strand break; HR, homologous recombination; NHEJ, nonhomologous end joining; SSA, single-strand annealing; ZFNs, zinc finger nucleases.

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