Nucleic Acids Research Advance Access published December 1, 2011

Nucleic Acids Research, 2011, 1–16
doi:10.1093/nar/gkr1112

A novel zinc-finger nuclease platform with a sequence-specific cleavage module

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Received April 7, 2011; Revised November 4, 2011; Accepted November 6, 2011

ABSTRACT

Zinc-finger nucleases (ZFNs) typically consist of three to four zinc fingers (ZFs) and the non-specific DNA-cleavage domain of the restriction endonuclease FokI. In this configuration, the ZFs constitute the binding module and the FokI domain the cleavage module. Whereas new binding modules, e.g. TALE sequences, have been considered as alternatives to ZFs, no efforts have been undertaken so far to replace the catalytic domain of FokI as the cleavage module in ZFNs. Here, we have fused a three ZF array to the restriction endonuclease PvuII to generate an alternative ZFN. While PvuII adds an extra element of specificity when combined with ZFs, ZF-PvuII constructs must be designed such that only PvuII sites with adjacent ZF-binding sites are cleaved. To achieve this, we introduced amino acid substitutions into PvuII that alter K_m and k_{cat} and increase fidelity. The optimized ZF-PvuII fusion constructs cleave DNA at addressed sites with a >1000-fold preference over unaddressed PvuII sites *in vitro* as well as *in cellula*. In contrast to the 'analogous' ZF-FokI nucleases, neither excess of enzyme over substrate nor prolonged incubation times induced unaddressed cleavage *in vitro*. These results present the ZF-PvuII platform as a valid alternative to conventional ZFNs.

INTRODUCTION

Specific targeting and modification of defined DNA sequences within the human genome is a major challenge for gene therapy (1–3). By using highly specific and programmable nucleases (4), such as zinc-finger nucleases (ZFNs) (5–9), homing endonucleases (10–12), chemical nucleases (13,14) or TALE nucleases (15–17), specific DNA double-strand breaks (DSBs) can be introduced next to or within defective genes to trigger the cellular DSB repair (18) required for DSB-mediated gene surgery. Repair via non-homologous end joining (NHEJ) often results in small insertions or deletions and can be used for the creation of specific gene knockouts. If a donor DNA containing the correct DNA sequence is provided *in trans*, repair via homologous recombination can result in the reconstitution of the defective gene (19).

In this context, ZFNs have been successfully used for genetic modifications within different organisms, tissues and cells (20–31) including human pluripotent stem cells (32–35), culminating in the initiation of at least three clinical trials for a ZFN-mediated treatment of glioblastoma and HIV infections so far (36). 'Classical' ZFNs (ZF-FokI, Figure 1A) are chimeric endonucleases consisting of Cys_2His_2 zinc-finger (ZF) DNA-binding modules (37) and the non-specific catalytic domain of the Type IIS restriction endonuclease FokI (38). Two ZF-FokI monomers have to bind to their respective ZF DNA-binding sites on opposite strands in an inverted orientation, usually separated by 5–7 nt, in order to form a catalytically active dimer of two FokI-cleavage modules that catalyze double-strand cleavage (39). Because the FokI-cleavage domain itself has no further sequence specificity, the ZFN target site is determined solely by the specific ZF DNA interactions. Since the first description of ZFNs in 1996 (40), several efforts have been made to optimize the ZFNs in terms of increased specificity *in vivo*: (i) a steadily growing library of ZF-binding modules in three or four-finger arrays that have been optimized by e.g. OPEN (oligomerized pool engineering) (41) or CoDA (context-dependent assembly) (42) to target 9 or 12 bp DNA sequences in a highly specific manner; (ii) optimization of the amino acid linker connecting the ZF and the FokI-cleavage domain (43); (iii) the generation of variants of the FokI-cleavage domain resulting in DNA binding by an obligate heterodimer (24,44–46) and enhanced activity (47,48); and (iv) controlling the ZFN

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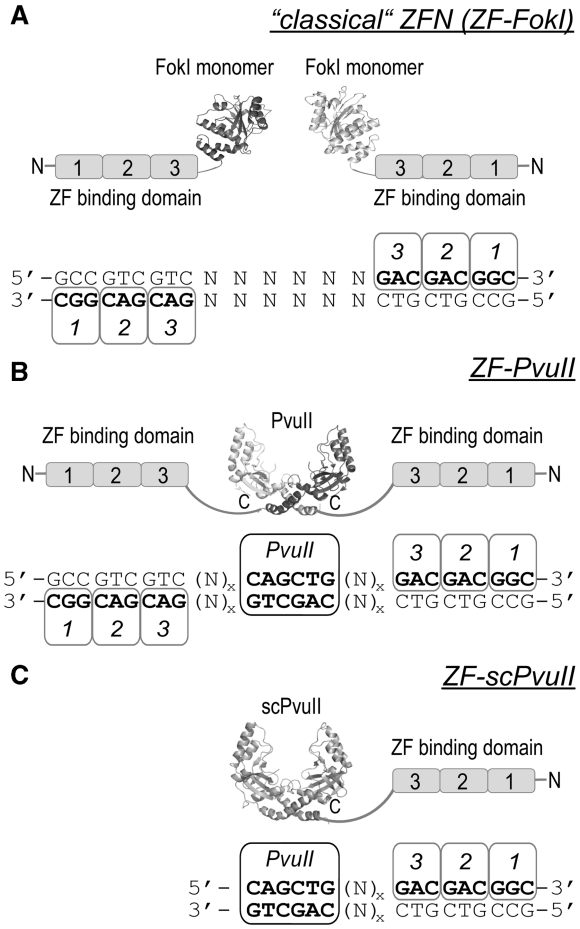

Figure 1. Strategies for developing ZF-PvuII fusion enzymes compared to the 'classical' ZFN design. (**A**) Scheme of a 'classical' ZFN (ZF-FokI) with two ZFN monomers, each consisting of a ZF DNA-binding domain fused to the N-terminal end of the unspecific cleavage domain of FokI (2FOK). Upon dimerization of the two ZFN monomers at the DNA target sequence 'Z–Z', cleavage within the intervening DNA sequence of random 6 bp will be catalyzed by the interacting FokI-cleavage domains. Recombinant ZF-FokI nucleases were purified by affinity chromatography using their N-terminal Strep-tag. (**B**) Scheme of a ZF-PvuII homodimer (a homodimer fusion approach), in which the ZF DNA-binding domain (N-1-2-3-C) is fused to the N-terminal end of each subunit of the PvuII homodimer (1PVU). The tripartite DNA target sequence 'Z–P–Z' is addressed by this ZF-PvuII fusion enzyme. (**C**) Scheme of ZF-scPvuII (a monomer fusion approach), in which the ZF-binding module is fused to the N-terminal end of the single-chain variant of PvuII (3KSK). This ZF-scPvuII fusion enzyme addresses the bipartite target sequence 'P–Z'. Purification of recombinant ZF-PvuII and ZF-scPvuII was performed by tandem affinity purification using the N-terminal Strep- and the C-terminal His-tag of both types of fusion enzymes.

expression level (49) and optimize cleavage conditions, respectively (50). In addition to the ZF-directed cleavage at the desired DNA target site, it has been reported that ZF-FokI nucleases also show unwanted cleavage at so called 'off-target' sites *in vivo*, which is associated with ZFN-mediated toxicity (6). Although is has been shown that the increased specificity of the designer ZFN directly results in a greatly reduced genotoxicity (51–53), the optimized ZF-FokI nucleases so far still exhibit some residual off-target site cleavage (23,54), as recently investigated by two genome-wide studies of ZFN-mediated cleavage specificity (55,56). By improved DNA sequencing technologies, a thorough investigation of all off- and on-target site modifications within the human genome that are induced by a ZFN-mediated treatment might clarify in the near future, if an application of optimized designer ZFN will be eventually available for the treatment of human genetic diseases and viral infections with an acceptable risk/benefit ratio.

ZF DNA-binding domains have been fused to domains with different functions, resulting in chimeric proteins such as a ZF DNA methyltransferase (57), a ZF recombinase/integrase (58–60) or a ZF transposase (61). However, with only one singular exception, in which the monomeric and also non-specific staphylococcal nuclease has been fused to ZF (62), the 'classical' ZFNs always contain the non-specific catalytic domain of FokI and its engineered variants as cleavage module.

In order to improve the specificity of ZFNs, we have developed a novel ZFN with the sequence-specific Type IIP restriction endonuclease PvuII as the cleavage module (ZF-PvuII) and characterized the targeted DNA cleavage *in vitro* and in cells. The final ZF-PvuII fusion construct, consisting of an optimized three-finger ZF DNA-binding protein and a partially inactivated homodimeric PvuII variant, catalyzes DNA cleavage only when the 6 bp PvuII recognition site is addressed by two adjacent 9 bp ZF-binding sites (in both directions of the PvuII site on opposite strands and in an inverted orientation). Cleavage at ZF-binding sites without an adjacent PvuII site or unaddressed singular PvuII sites does not occur. For the evaluation of the ZF-PvuII as a novel ZFN platform, we also characterized the cleavage specificity of the corresponding 'classical' ZFNs (ZF-FokI) containing the unspecific nuclease domain of FokI and its obligate heterodimeric variants *in vitro*. By comparing the *in vitro* specificities of ZF-FokI and ZF-PvuII, we demonstrate that PvuII can substitute for the non-specific FokI DNA-cleavage domain in ZFNs. In principle, as with conventional ZFNs, the ZF-PvuII specificity can be further increased by using heterodimeric versions of PvuII (63). Since we further show that ZF-PvuII is able to cleave the addressed target site on an episomal substrate *in cellula*, we suggest that the ZF-PvuII nuclease platform indeed can be used for gene modification of endogenous targets *in vivo*.

MATERIALS AND METHODS

Design and construction of ZF-PvuII fusion proteins and the corresponding classical ZFNs containing the FokI-cleavage domain

For the construction of a chimeric ZF-PvuII fusion protein, the three-finger ZF DNA-binding domain OZ039 (ZF) (41), which had been optimized for specifically binding the DNA sequence 5′-GACGACGGC-3′, was fused to the N-terminal end(s) of the high-fidelity variant T46G [Zhenyu Zhu, New England Biolabs (64)] of the homodimeric Type II restriction endonuclease PvuII

(PvuII) or the single-chain variant of PvuII (scPvuII) (65) via an 18 amino acid linker (LRSSVIPNR GVTKQLVKG) based on the FokI-linker sequence. Therefore, the ZF gene and the gene coding for PvuII/scPvuII, which contains the coding sequence for a C-terminal His_6-tag, were connected by the coding sequence of the linker and cloned in the expression vector pQE30 (Qiagen), which additionally contains the coding sequence for an N-terminal Strep-tag. For the corresponding classical ZFNs (ZF-FokI), the ZF gene and the gene coding for the unspecific cleavage domain of FokI were connected via a short linker sequence coding for a four amino acid inter-domain linker (LRGS) (43) and cloned into the vector pQE30, resulting in the expression of an N-terminal Strep-tagged ZFN according to the classical ZFN setup. Mutations within the gene coding for *pvuII* or the *fokI* cleavage domain were introduced by a PCR-based site-directed mutagenesis method (66). The genetic integrity of all fusion constructs was confirmed by DNA sequencing over the entire coding region.

Protein expression and purification

The expression vectors, containing the genes for the ZF-PvuII fusion constructs were introduced into the *Escherichia coli* strain XL10-Gold (Stratagene), which previously had been transformed with the pLGM plasmid containing the coding sequence for the PvuII DNA methyltransferase. The expression vectors for the classical ZFNs containing the FokI-cleavage domain were introduced into the *E. coli* strain XL1-Blue (Stratagene). Cultures were grown at 37°C to OD_{600} ~0.7 in the presence of 100 µM $ZnCl_2$ and protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 16 h of induction at 20°C, cells were harvested by centrifugation, resuspended in 30 mM K-phosphate pH 7.4, 1 M NaCl, 20 mM imidazol, 100 µM $ZnCl_2$, 0.01% w/v Lubrol, 1 mM phenyl methane sulfonyl fluoride (PMSF) (for ZF-PvuII) or in 100 mM Tris–HCl pH 8.0, 1 M NaCl, 1 mM PMSF (for ZF-FokI) and lysed by sonication. Cell debris was removed by centrifugation (>17 000 *g*) for 30 min at 4°C. The recombinant His- and Strep-tagged ZF-PvuII fusion proteins were purified by tandem affinity chromatography over (i) Ni-NTA agarose (Qiagen) using 30 mM K-phosphate pH 7.4, 300 mM NaCl, 200 mM imidazol, 0.01% w/v Lubrol and (ii) Strep-Tactin Sepharose (IBA) using 100 mM Tris–HCl pH 8.0, 300 mM NaCl, 2.5 mM Desthiobiotin. The recombinant Strep-tagged ZF-FokI variants and the ZF-protein itself were purified by affinity chromatography over Strep-Tactin Sepharose as described above, only. Fractions containing pure protein were dialyzed overnight at 4°C against 20 mM K-phosphate pH 7.4, 150 mM KCl, 10 µM $ZnCl_2$, 50% v/v glycerol (ZF-PvuII/ZF) or 20 mM Tris–HCl pH 7.9, 50 mM NaCl, 200 µM dithiothreitol (DTT), 100 µM $ZnCl_2$, 50% v/v glycerol (ZF-FokI) and stored at −20°C. The protein concentration was determined by measuring the absorbance at 280 nm [the molar extinction coefficient (ε) was determined according to Pace *et al.* (67)] and the protein purification was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis.

Electrophoretic mobility shift assay

The DNA-binding affinities of the ZF-PvuII fusion constructs and their individual modules were monitored by electrophoretic mobility shift assay (EMSA) using [α-^{32}P]dATP-labeled PCR products (Table 1). The binding assay of the PvuII variants was performed similarly as previously described (68) using 1 nM labeled DNA and protein concentrations from 10 to 500 nM in the presence of 10 mM $CaCl_2$. For the ZF protein and the ZF-PvuII fusion constructs, 1 nM labeled DNA and 1–100 nM protein were incubated in 10 mM Tris–HCl pH 7.2, 50 mM NaCl, 1 mM DTT, 0.1 mg/ml bovine serum albumin (BSA), 1 µM $ZnCl_2$, 10 µg/ml poly(dI-dC) for 1 h at 23°C in a volume of 10 µl. Complex formation was analyzed by electrophoresis on 6% polyacrylamide gels in 20 mM Tris–acetate pH 8.5, and visualized using an InstantImager system (Packard). The radioactive bands were quantitated using the InstantImager software. The respective binding affinities were determined from the fraction of DNA bound by a non-linear regression analysis.

Competitive DNA-cleavage assay

To analyze targeted DNA cleavage by the ZF-PvuII fusion proteins, a DNA-cleavage assay with an unaddressed substrate (containing a single PvuII site) and an addressed substrate (containing a PvuII site with one or two adjacent ZF-binding sites in one or both directions of the PvuII site, respectively) was carried out in competition. Therefore, radioactively labeled substrates were generated by PCR using [α-^{32}P]dATP. The target sites of the various substrates used are given in Table 1. To analyze the preference for addressed over unaddressed cleavage, 20 nM unaddressed substrate and 20 nM addressed substrate were incubated with 18 nM of the respective ZF-PvuII fusion enzyme in 10 mM Tris–HCl pH 7.2, 50 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA, 1 µM $ZnCl_2$, 0.8 mM $MgCl_2$ at 37°C (for PvuII G46 an enzyme concentration of 0.9 nM and for scPvuII G46—with G46 in both domains—and for PvuII G46/F94 an enzyme concentration of 9 nM was used in the competition assay). The reaction progress was analyzed after defined time intervals (up to 24 h) by electrophoresis on 6% polyacrylamide gels; unaddressed and addressed substrate and product bands were visualized with an InstantImager system (Packard), quantified by using the InstantImager software and analyzed by non-linear regression analysis. The cleavage preference was calculated as the ratio of the activity for the addressed substrate over the one for the unaddressed substrate.

Plasmid cleavage assay

To characterize the targeted cleavage of larger substrates by the different ZF-PvuII fusion enzymes, different substrate plasmids (4878 bp) were generated, each containing four PvuII sites (4xP) and one specific 'addressed cassette' (e.g. Z12P12Z), which contains either the tripartite target

Table 1. Compilation of the sequences of various substrates used for the characterization of the activity and specificity of the ZF-PvuII fusion proteins

PCR substrates	Target sequences
P	5′-TGGATACCAACTGTAGATCCT**CAGCTG**TCACTTGACATCGCATCGTGC-3′
P4Z	5′-**CAGCTG**TCACGACGACGGC-3′
P8Z	5′-**CAGCTG**TCACTTGAGACGACGGC-3′
P12Z	5′-**CAGCTG**TCACTTGACATCGACGACGGC-3′
Z4P4Z	5′-GCCGTCGTCTCCT**CAGCTG**TCACGACGACGGC-3′
Z8P8Z	5′-GCCGTCGTCTAGATCCT**CAGCTG**TCACTTGAGACGACGGC-3′
Z12P12Z	5′-GCCGTCGTCACTGTAGATCCT**CAGCTG**TCACTTGACATCGACGACGGC-3′
Plasmid substrates (*in vitro*)	**Addressed target sequences**
4xP-P	5′-TGGATACCAACTGTAGATCCT**CAGCTG**TCACTTGACATCGCATCGTGC-3′
4xP-P12Z	5′-TGGATACCAACTGTAGATCCT**CAGCTG**TCACTTGACATCGACGACGGC-3′
4xP-Z12P12Z	5′-GCCGTCGTCACTGTAGATCCT**CAGCTG**TCACTTGACATCGACGACGGC-3′
4xP-Z30Z	5′-GCCGTCGTCACTGTAGATCCTTCCGTATCACTTGACATCGACGACGGC-3′
4xP-Z6Z	5′-GCCGTCGTCTACAGTGACGACGGC-3′
4xP-Z	5′-GACGACGGC-3′
Plasmid substrates (*in cellula*)	**Target sequences**
Z12P12Z	5′-GCCGTCGTCACTGTAGATCCT**CAGCTG**TCACTTGACATCGACGACGGC-3′
21P21	5′-GTACTCCTGACTGTAGATCCT**CAGCTG**TCACTTGACATCCAGCAGCGG-3′

P denotes a PCR fragment with a singular PvuII site; P4Z, e.g. denotes a PCR fragment with a PvuII site in a 4 bp distance to a ZF-binding site; and Z4P4Z, e.g. denotes a PCR fragment with a PvuII site flanked on both sides by a ZF-binding site in a distance of 4 bp. A similar nomenclature was used for the plasmid substrates for the *in vitro* cleavage assays, which in addition to the addressed site contained four unaddressed PvuII sites (4xP), as well as for the plasmid substrates for the cellular cleavage assays. PvuII sites are in bold, ZF-binding sites underlined.

sequence Z12P12Z (or 4xP-Z12P12Z, respectively), or the bipartite target sequence P12Z (or 4xP-P12Z, respectively), or two ZF-binding sites in 30 bp distance in an inverted orientation without a PvuII site in between (4xP-Z30Z), or another unaddressed PvuII site as control (4xP-P). Further substrate plasmids containing two ZF-binding sites in 6 bp distance in an inverted orientation (4xP-Z6Z), or just a single ZF-binding site (4xP-Z) were used as substrates for the ZF-FokI variants, as well as the substrate plasmid 4xP-P as unspecific target plasmid (see Table 1 for a detailed description of the target sites). For the analysis of the addressed cleavage with near-stoichiometric concentrations of substrate and enzyme, 20 nM of target plasmid was incubated with 18 nM of 'active' enzyme [i.e. 18 nM of ZF-PvuII homodimer or 36 nM of ZF-FokI monomer(s)] in 10 mM Tris–HCl pH 7.2, 50 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA, 1 μM $ZnCl_2$, 0.8 mM $MgCl_2$ (for the assay with ZF-PvuII) or 20 mM Tris–HCl pH 8.5, 50 mM NaCl, 1 mM DTT, 5 μg/ml BSA, 1 μM $ZnCl_2$, 0.8 mM $MgCl_2$ (for the assay with ZF-FokI) at 37°C. The reaction progress was analyzed after defined time intervals (up to 24 h) by agarose gel electrophoresis; gels were stained with ethidium bromide, and the fluorescence of the substrate and different product bands was visualized with a BioDocAnalyze system (Biometra). For the investigation of targeted cleavage with excess of enzyme, 20 nM target plasmid was incubated with 100 nM of 'active' enzyme [i.e. 100 nM of ZF-PvuII homodimer or 200 nM of ZF-FokI monomer(s)] and cleavage was analyzed as described. For a further characterization of linear products produced, a subsequent cleavage with HindIII (FastDigest® HindIII, Fermentas) for 1 h at 37°C was carried out in some cases.

Cellular plasmid cleavage assay

HEK293T cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen). Cells were seeded in 6-well plates at a density of 300 000 cells/well. After 18 h, cells were transfected using polyethylenimin (PEI) as described before (17) with 1 μg of addressed (Z12P12Z) or unaddressed (21P21) target plasmid [target sequences cloned into the plasmid pRK5.mCherry (17); see Table 1], respectively, and 4 μg of ZF-PvuII G46/A83/F94 or PvuII G46/A83/F94 encoding expression plasmids [the respective genes had been cloned in the plasmid pRK5 (43)]. Cells were harvested 4 days after transfection, total DNA was extracted using QIAamp DNA Mini Kit (Qiagen) and 500 ng subjected to incomplete digestion with 20 U of PvuII-HF (New England BioLabs). The region encompassing the PvuII-binding site on the target plasmid was amplified by PCR using 10 ng of total DNA as a template, along with 0.5 μM of each primer (#1535 5′-ATCCACGCTGTT TTGACCTC and #1536 5′- ACATGAACTGAGGGGA CAGG), 200 μM dNTPs, and 1 U of Phire Hot Start DNA Polymerase (Fisher Scientific) in 1× reaction buffer for 25 cycles. Amplicons were purified with QIAquick PCR Purification Kit (Qiagen) and then digested with either PvuII-HF or the mismatch-sensitive T7 endonuclease I (T7E1; NEB). For the T7E1 assay, DNA was denatured at 95°C for 5 min, slowly cooled down to room temperature to allow for formation of

heteroduplex DNA, treated with 5 U of T7E1 for 20 min at 37°C and fragments were separated on a 2% agarose gel.

Immunoblotting

Immunoblotting was performed as previously described (43). Briefly, 300 000 HEK293T cells per 6-well were transfected with 4 μg of ZF-PvuII G46/A83/F94 or PvuII G46/A83/F94 encoding plasmids, 500 ng of pEGFP-N1 (Clontech), and 500 ng of pUC118 using the PEI transfection method. The cells were harvested after 30 h, and 50 μg of lysate was separated by SDS–PAGE. After transfer to polyvinyliden difluoride membranes, HA-tagged ZF-PvuII G46/A83/F94 or PvuII G46/A83/F94, respectively, and enhanced green fluorescent protein (EGFP) were 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).

RESULTS

Design of chimeric ZF-PvuII fusion proteins

For the generation of a novel and highly specific chimeric nuclease, a three-finger ZF DNA-binding protein as specific DNA-binding module and the sequence-specific restriction endonuclease PvuII as DNA-cleavage module were fused via a short linker. The resulting ZF-PvuII fusion enzyme is directed to the specific ZF DNA-binding sites via the ZF-binding module, and DNA cleavage should occur only if a PvuII recognition site is in close proximity. A main requirement for using ZF-PvuII constructs for gene targeting applications is that unaddressed PvuII sites without adjacent ZF sites and, vice versa, ZF sites without an adjacent PvuII site should not be cleaved by the chimeric enzyme. As a binding module, we used a well-characterized ZF protein with a high DNA-binding specificity that was optimized by *in vivo* engineering methods for binding its specific target sequence 5′-GACGACGGC-3′ (41). The cleavage module PvuII is a homodimeric Type IIP restriction endonuclease that recognizes and cleaves the double-stranded DNA sequence 5′-CAG↓CTG-3′ in the presence of Mg^{2+}. In terms of structure and function, it is one of the best-studied restriction enzymes (69–72). In this fusion approach, we used 'high-fidelity' PvuII variants that contained the amino acid substitution T46G, which is known to effectively reduce the observed 'star' activity of the enzyme [Zhenyu Zhu, New England Biolabs (64)]. To further improve the specificity of the chimeric nuclease, additional amino acid substitutions that lower the activity of PvuII, were introduced (Supplementary Figure S1). Amino acid substitutions were chosen, which had been shown to lower the affinity of PvuII to DNA (His83→A) or affect the catalytic process (Tyr94→F). The H83A variant binds a PvuII substrate more weakly than wild-type (wt) PvuII, because His83 makes a specific phosphate contact to the PvuII recognition site (68). The Y94F variant has at least a 10-fold lower catalytic activity than wt PvuII, because it exhibits an altered Mg^{2+} binding, which disrupts the cooperativity in DNA cleavage (73). We figured that DNA binding of the ZF-binding module in the ZF-PvuII constructs would position the active site of the PvuII-cleavage module firmly (than with PvuII H83A) and precisely (than with PvuII Y94F) on the composite binding site. This would make DNA cleavage at addressed PvuII sites absolutely dependent on ZF binding to the DNA, and at the same time prevent cleavage of unaddressed PvuII sites.

Over all, we pursued two particular strategies: (i) A homodimeric ZF-PvuII fusion approach (Figure 1B), where the ZF was fused to each of the two identical subunits of the PvuII homodimer. The homodimeric ZF-PvuII construct should recognize the target sequence 'Zinc-finger site–PvuII site–Zinc-finger site' (Z–P–Z), a tripartite recognition sequence of 24 bp (ZF ≡ 9 bp + PvuII ≡ 6 bp + ZF ≡ 9 bp). (ii) A monomeric ZF-scPvuII fusion approach (Figure 1C), where the ZF was fused to the monomeric single-chain variant of PvuII (scPvuII) (65), which should target the bipartite 'PvuII site–Zinc-finger' site (P–Z) with a total length of 15 bp (ZF ≡ 9 bp + PvuII ≡ 6 bp). All ZF-PvuII fusion enzymes and the ZF protein itself could be expressed in *E. coli* and purified by affinity chromatography; no degradation or instability was observed (Supplementary Figure S2).

Affinity of ZF-PvuII variants for the addressed target site

Gel shift experiments with the fusion enzymes and their individual components were performed to get an estimate of their affinities towards their respective target sites. The ZF protein itself was found to bind with an apparent dissociation constant of $K_{d(app)}$ ~1 nM to the ZF-binding site (Supplementary Figure S3), whereas PvuII G46 had an affinity of $K_{d(app)}$ ~100 nM to the PvuII recognition site. The affinity of the ZF-PvuII variants to their respective target sites turned out [$K_{d(app)}$ ~10 nM; Supplementary Figure S4] to be intermediate between that of the PvuII G46 enzyme and of the ZF protein alone.

Analysis of the *in vitro* cleavage preference for addressed target sites by the ZF-PvuII variants

The activity and specificity of the chimeric ZF-PvuII fusion enzymes were tested using an addressed substrate, which contained the bi- or tripartite target sequences (P–Z or Z–P–Z), and an unaddressed substrate with a singular PvuII site (P). The cleavage of both substrates by the different ZF-PvuII variants was carried out in competition and with a (near) equimolar stoichiometry, to quantify the cleavage preference for the addressed target site over the unaddressed site. The results of all competitive DNA-cleavage assays are summarized in Table 2. As expected, the PvuII variants without an additional ZF fusion cleaved the addressed and unaddressed substrates with similar rates (Supplementary Figure S5). The comparison of the absolute activities shows that PvuII G46/F94 is ~32 times less active than PvuII G46 and that the

Table 2. Cleavage preference of the various ZF-PvuII fusion enzymes for their addressed target sites

Enzymes	Substrates		Absolute activity [nM substrate/(min × nM enzyme)]		Cleavage preference (addressed/unaddressed)
	Addressed	Unaddressed	Addressed	Unaddressed	
PvuII G46	Z4P4Z	P	2.76 ± 0.25	1.98 ± 0.12	1.4
	Z8P8Z	P	2.30 ± 0.18	1.71 ± 0.03	1.3
	Z12P12Z	P	1.66 ± 0.52	1.53 ± 0.26	1.1
ZF-PvuII G46	Z4P4Z	P	0.0419 ± 0.0024	0.0077 ± 0.0003	5.4
	Z8P8Z	P	0.0603 ± 0.0023	0.0137 ± 0.0009	4.4
	Z12P12Z	P	0.0283 ± 0.0005	0.0027 ± 0.0001	10.4
	–	P	–	0.0166 ± 0.0004	–
scPvuII G46	P4Z	P	0.167 ± 0.013	0.127 ± 0.007	1.3
	P8Z	P	0.164 ± 0.015	0.155 ± 0.007	1.1
	P12Z	P	0.091 ± 0.011	0.098 ± 0.002	0.9
ZF-scPvuII G46	P4Z	P	0.166 ± 0.010	0.0419 ± 0.0066	4.0
	P8Z	P	0.209 ± 0.009	0.0728 ± 0.0072	2.9
	P12Z	P	0.115 ± 0.012	0.0262 ± 0.0047	4.4
PvuII G46/F94	Z12P12Z	P	0.0519 ± 0.0034	0.0498 ± 0.0011	1.0
ZF-PvuII G46/F94	Z12P12Z	P	0.0072 ± 0.0002	<3.9E-06[a]	>1800
	P12Z	P	0.0058 ± 0.0001	<3.9E-06[a]	>1400
	–	P	–	<3.9E-06[a]	–
PvuII G46/A83/F94	Z12P12Z	P	0.0033 ± 0.0002	0.0035 ± 0.0001	0.9
ZF-PvuII G46/A83/F94	Z12P12Z	P	0.0009 ± 0.0001	<3.9E-06[a]	>230
	P12Z	P	1.9E-05 ± 4.2E-06	<3.9E-06[a]	~5.0
	–	P	–	<3.9E-06[a]	–

The absolute activities (±standard deviation) of the ZF-PvuII fusion enzymes and their individual cleavage modules (PvuII variants) for addressed and unaddressed target site cleavage were determined by a cleavage assay using the indicated addressed and unaddressed substrates in competition in a near equimolar stoichiometry (20 nM addressed substrate/20 nM unaddressed substrate/18 nM enzyme). Cleavage preference is given as the ratio of the activities determined for the addressed and the unaddressed substrate, respectively.
[a]The observed cleavage activity was beyond the detection limit of the assay, i.e. the activity was <0.5% cleavage in 24 h.

PvuII G46/A83/F94 variant, shows a ~16 times reduced cleavage activity compared to PvuII G46/F94.

Several addressed substrates with 4, 8 and 12 bp between the ZF and PvuII target sites were tested to determine, which distances in the DNA target are optimal for the relative positioning of the binding and cleavage modules in the ZF-PvuII fusion constructs. In the case of ZF-PvuII G46, the highest cleavage preference for the addressed substrate was observed for the target sequence with a 12 bp spacer. For Z12P12Z a cleavage preference of 10.4 was determined (Figure 2A), as compared to values of 5.4 and 4.0 for the addressed substrates Z4P4Z and Z8P8Z, respectively. Since the ZF-PvuII G46 and ZF-scPvuII G46 fusion enzyme showed the same order for the cleavage preferences (Z12P > Z4P > Z8P), we assume that the geometry of the composite DNA-binding site of both enzyme variants is similar, being complementary to the geometry of the DNA target sites with a 12 bp spacer between the recognition sites for the ZF module and the PvuII module (Figure 2A and B; Supplementary Figures S6 and S7). Thus, the addressed substrates with the 12 bp spacer were used for further investigating addressed cleavage.

By comparing the activity of the PvuII variants with and without a ZF module cleaving the unaddressed substrate, it is obvious that fusing the ZF-binding module to the N-terminus of PvuII lowers the cleavage activity by approximately two orders of magnitude (see column 3 in Table 2). If a ZF module is attached to only one subunit as in the monomeric ZF-scPvuII fusion, the cleavage activity is only lowered by a factor of ~10. Thus, the ZF fusion seems to interfere with the binding and/or catalysis of PvuII presumably due to steric reasons, but interference with the conformational dynamics of the enzyme cannot be excluded. Table 2 also shows that ZF-PvuII G46 has an approximately two times higher cleavage preference for the tripartite target sequence Z12P12Z than ZF-scPvuII G46 for the corresponding bipartite target sequence P12Z, which is mainly due to a relatively low cleavage activity towards the unaddressed substrate in the competition assay (Figure 2A and B; Table 2). The homodimeric ZF-PvuII G46 fusion enzyme exhibits a higher cleavage preference combined with a higher sequence specificity for the tripartite target in comparison with the monomeric ZF-scPvuII G46 construct addressing the bipartite target. We therefore focused on the homodimeric ZF-PvuII approach for investigating the effect on the cleavage preference by lowering the activity of PvuII through additional amino acid substitutions, in order to make the effect of the ZF-binding module in the context in the ZF-PvuII fusion construct more dominant.

Introducing the substitution Y94F into the PvuII-cleavage module to decrease its activity indeed resulted in a significant increase in the cleavage preference. ZF-PvuII G46/F94 cleaves the addressed target Z12P12Z with a >1800-times higher preference over the unaddressed PvuII site (Figure 2C and Table 2). Moreover, no cleavage of the unaddressed substrate could be detected within 24 h. When adding the H83A amino acid substitution, which affects the K_m of PvuII, the same high cleavage preference for the addressed tripartite target sequence with no detectable cleavage of the unaddressed

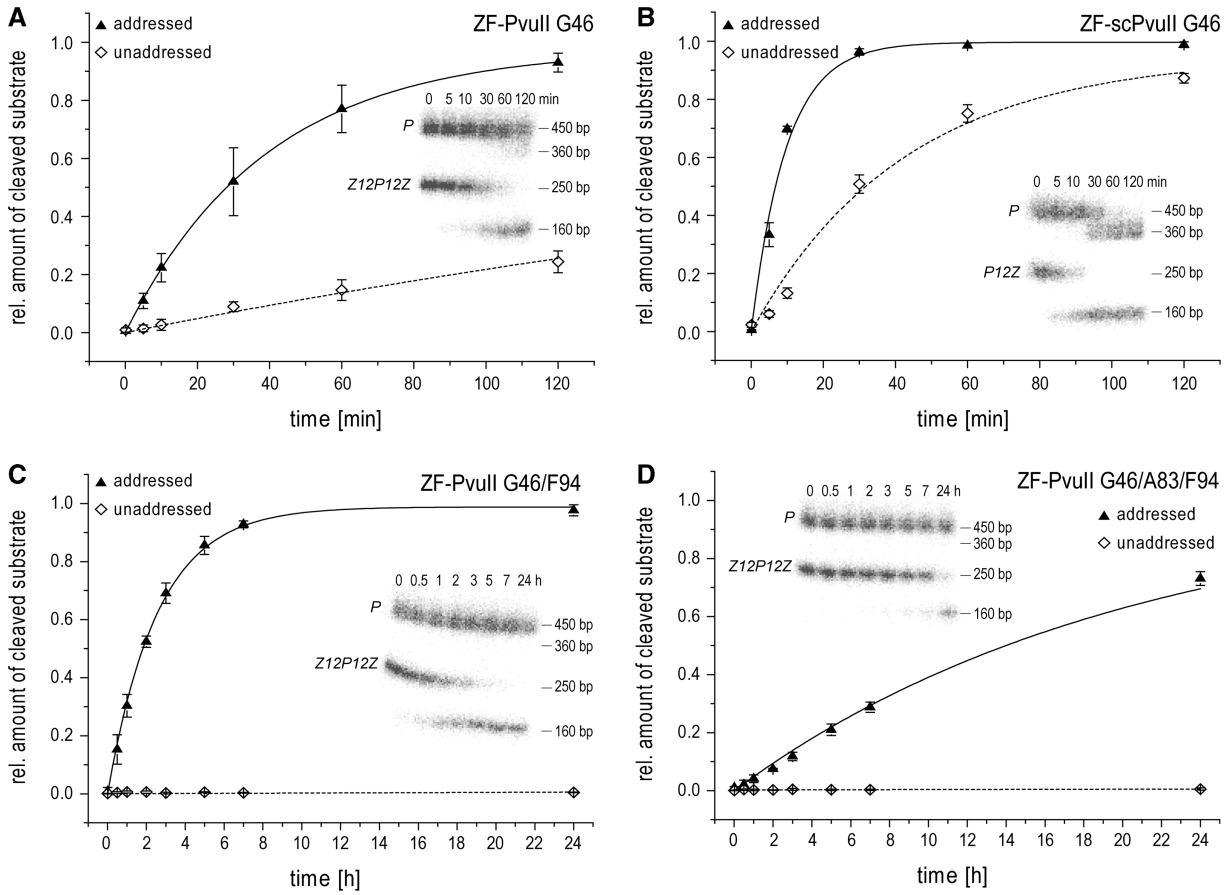

Figure 2. Kinetic analysis of the ZF-PvuII fusion enzymes cleaving an addressed substrate [black triangle; 250 bp PCR product containing either the tripartite (Z12P12Z) or bipartite (P12Z) target sites] and an unaddressed substrate [open diamond; 450 bp PCR product containing a single PvuII site (P)] in competition in a near equimolar stoichiometry (20 nM addressed substrate/20 nM unaddressed substrate/18 nM enzyme). The gel electrophoretic analysis of samples withdrawn from the incubation mixture at defined time intervals is shown as an insert of the activity versus time profile. (**A**) Preferential DNA cleavage (addressed versus unaddressed) by ZF-PvuII G46 (Z12P12Z versus P). (**B**) Preferential cleavage by ZF-scPvuII G46 (P12Z versus P). (**C**) Preferential DNA cleavage by ZF-PvuII G46/F94 (Z12P12Z versus P). (**D**) Preferential DNA cleavage by ZF-PvuII G46/A83/F94 (Z12P12Z versus P).

target was observed. Since the absolute activity of ZF-PvuII G46/A83/F94 was further decreased with the H83A substitution, a cleavage preference of only >230 could be calculated (Figure 2D and Table 2). Due to the detection limit of the competitive cleavage assay only a lower limit can be given for the cleavage preference. The 'real' cleavage preference of ZF-PvuII G46/A83/F94 might be even more pronounced than for ZF-PvuII G46/F94. It is also noteworthy that ZF-PvuII G46/F94 cleaves the bipartite target sequence P12Z only with a slightly reduced cleavage preference of >1400 compared to the tripartite target Z12P12Z (cleavage preference >1800), whereas ZF-PvuII G46/A83/F94 shows almost no cleavage at the addressed bipartite target (cleavage preference of ~5.0) (Supplementary Figures S8 and S9). This means, that even when only one ZF-binding module is specifically bound to the DNA (P12Z), the cleavage module PvuII G46/F94 is still able to bind and cleave the adjacent PvuII site, whereas PvuII G46/A83/F94 cannot cleave the DNA properly in this case.

In order to prove the specificity of the fusion constructs, a cleavage assay was performed using only the unaddressed target as substrate without a ZF-binding site containing target in competition. Whereas ZF-PvuII G46 shows a residual activity for cleaving a singular PvuII site, no unaddressed cleavage was observed with the constructs ZF-PvuII G46/F94 and ZF-PvuII G46/A83/F94 (Supplementary Figures S6, S8 and S9). Although our results show that a pre-incubation step of the ZF-PvuII variants in the absence of Mg^{2+} in order to pre-form the specific binding complex before starting the cleavage by addition of Mg^{2+} is not essential for specifically targeting the addressed target site, it can further increase the respective cleavage preferences. For example, ZF-PvuII G46/F94, which has a cleavage preference of >1800 for the Z12P12Z site over a non-specific site (Table 2) has a cleavage preference of >5000 after pre-incubation in the absence of Mg^{2+} (Supplementary Table S1).

Preferential cleavage of addressed target sites in a larger sequence context by the ZF-PvuII variants *in vitro*

To analyze the targeted cleavage by the ZF-PvuII fusion constructs in the context of a larger DNA substrate, a 4878 bp plasmid with four unaddressed PvuII sites and

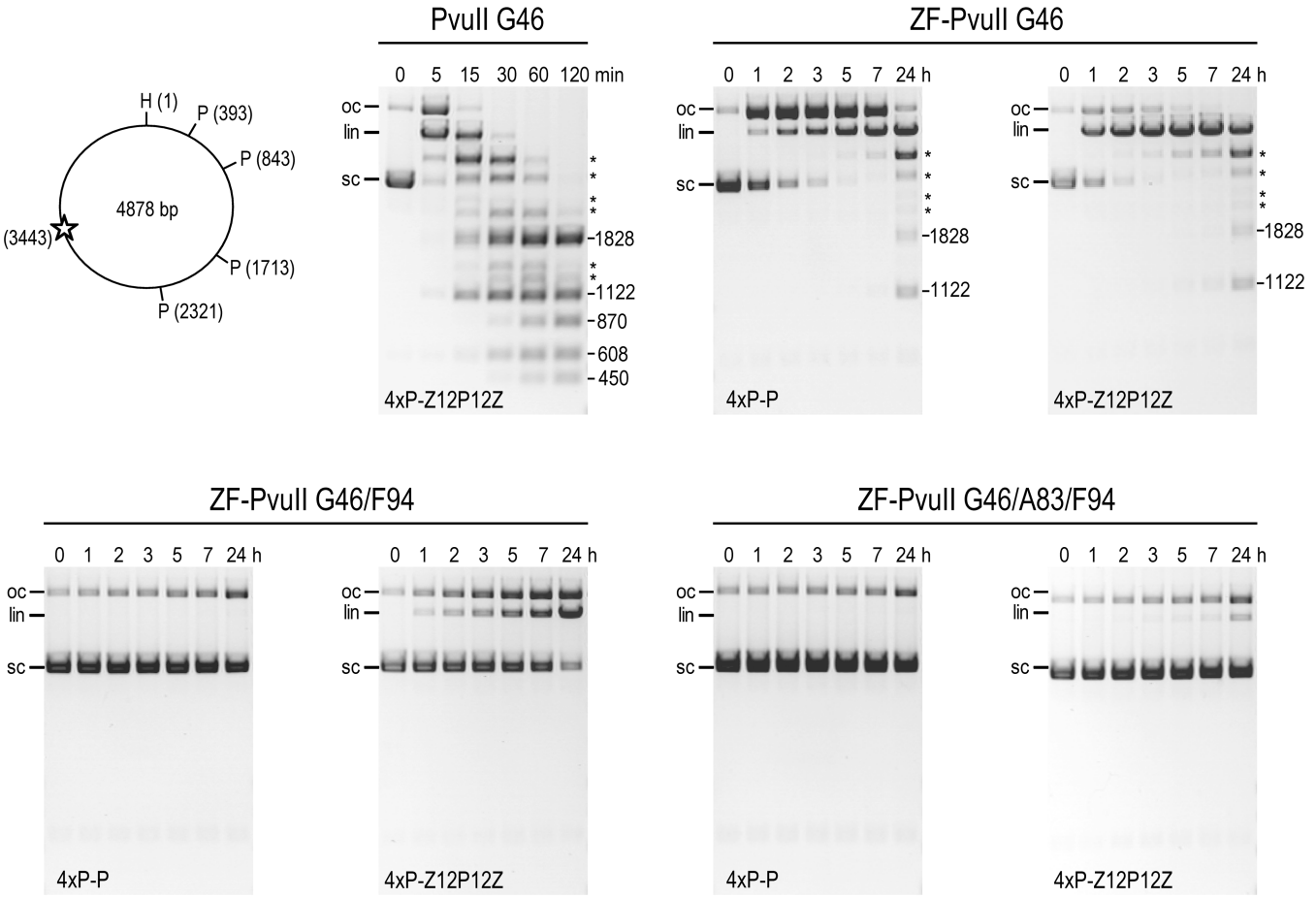

Figure 3. Addressed cleavage of a 4878 bp plasmid substrate by the ZF-PvuII fusion enzymes using a near equimolar stoichiometry (20 nM plasmid/18 nM ZF-PvuII homodimer). The plasmid contains four unaddressed PvuII sites (P) and an 'addressed cassette' region (indicated by an open star) with either the 'addressed tripartite target sequence' (4xP-Z12P12Z) or another unaddressed PvuII site (P) as control (4xP-P). A unique HindIII site (H) is also included in the plasmid sequence for further analysis of the addressed DNA cleavage (Figure 4). The gel electrophoretic analyses of the cleavage products obtained after defined times of incubation of the plasmids harboring 4xP-Z12P12Z or 4xP-P with PvuII G46, ZF-PvuII G46, ZF-PvuII G46/F94 or ZF-PvuII G46/A83/F94 are shown, respectively. The supercoiled, open circular and linear forms of the plasmid are denoted by sc, oc and lin. The final cleavage products are indicated by their respective lengths (1828, 1122, 870, 608 and 450 bp); intermediate cleavage products are marked by an asterisks. It should be noted that the ZF-PvuII G46/F94 and the ZF-PvuII G46/A83/F94 fusion proteins cleave the plasmid only at the addressed sequence (Z12P12Z).

one addressed PvuII site (Z12P12Z) was used (Figure 3). Cleavage of the supercoiled plasmid with PvuII G46 results in five cleavage products, as expected. By using the ZF-PvuII fusion enzymes in near-stoichiometric amounts, the ZF-PvuII G46 variant showed the formation of the linear plasmid as the main product, indicating preferred cleavage at the addressed PvuII site. However, several further cleavage products occurred within 24 h, which indicates that upon long-term incubation cleavage took place also at unaddressed PvuII sites, also seen using a control plasmid that contains five unaddressed PvuII sites only (4xP-P). In contrast, the more specific fusion enzymes ZF-PvuII G46/F94 and ZF-PvuII G46/A83/F94 did not cleave the control plasmid 4xP-P within 24 h, but both cleaved the plasmid containing the addressed tripartite target site (4xP-Z12P12Z) only once, namely at the addressed site (c.f. Figure 4).

To test the limitations of this highly specific targeted cleavage, plasmid cleavage assays were performed using a 5-fold excess of enzyme (Figure 4). For ZF-PvuII G46/F94, rapid linearization of the supercoiled plasmid containing the tripartite target sequence was observed. However, this variant also showed some further cleavage at unaddressed sites after 24 h under the conditions chosen. In the reaction using ZF-PvuII G46/F94 and the control plasmid 4xP-P, the occurrence of the linearized plasmid confirmed the DNA double-strand cleavage of the ZF fusion enzyme at unaddressed PvuII sites, albeit at an excess of enzyme. Interestingly, the main product in this reaction was the open-circle plasmid, i.e. a single nick in only one DNA strand was introduced that converted the supercoiled plasmid into the open-circle form. This nicking activity could be explained by the nature of the Y94F amino acid substitution within PvuII, which is known to interfere with the cooperative interaction of the two catalytic centers in the PvuII homodimer, resulting in an increased formation of nicked DNA substrates (73). If ZF-PvuII G46/A83/F94, which contains the

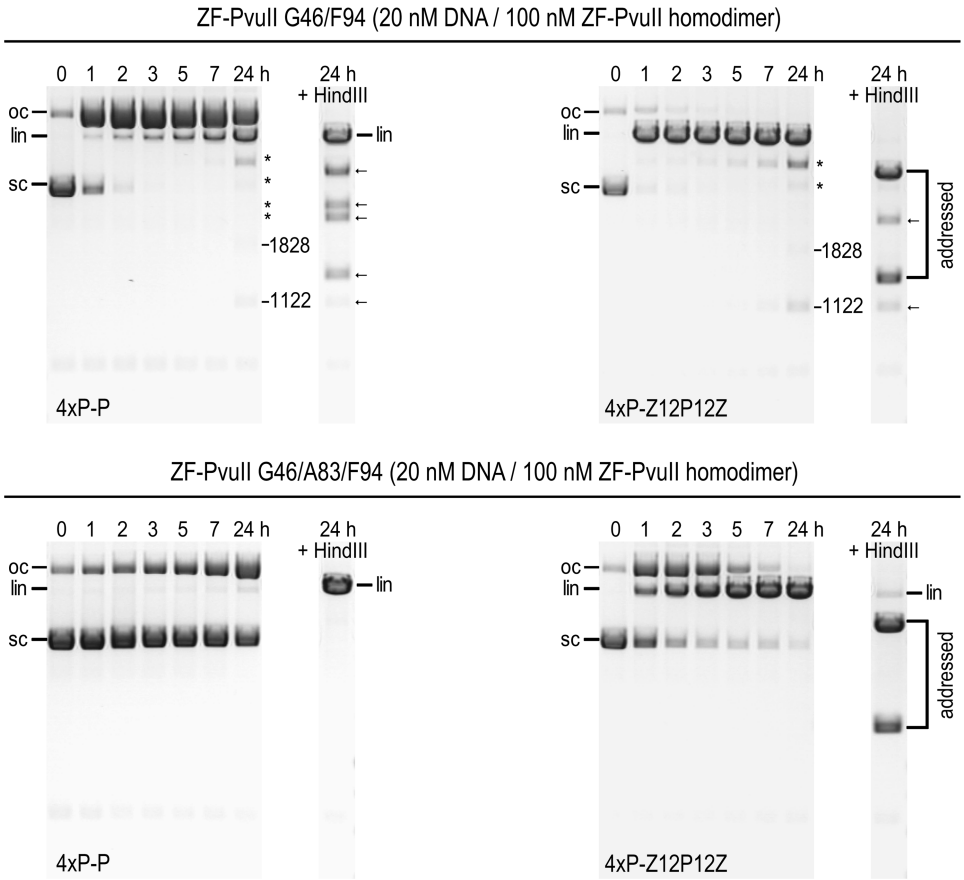

Figure 4. Addressed cleavage of a 4878 bp plasmid substrate by the ZF-PvuII fusion enzymes using an excess of enzyme (20 nM plasmid/100 nM ZF-PvuII homodimer). The gel electrophoretic analyses of the cleavage products of the plasmids 4xP-P and 4xP-Z12P12Z produced by ZF-PvuII G46/F94 or ZF-PvuII G46/A83/F94, respectively, are shown. The supercoiled, open circular and linear forms of the plasmid are denoted by sc, oc and lin. The final cleavage products are indicated by their respective lengths (1828 and 1122 bp); intermediate cleavage products are marked by an asterisks. After 24 h of cleavage by the fusion enzyme, a secondary cleavage with HindIII was performed to characterize the linear cleavage product. Only cleavage at the addressed PvuII site and the HindIII site, results in formation of two cleavage products, 3442 and 1436 bp in length. The occurrence of these two products, which are marked by a bracket and denoted with 'addressed', demonstrates that the ZF-PvuII fusion enzyme before cleavage by HindIII had cleaved the plasmid only at the addressed PvuII target site. Further cleavage products that are occurring after the subsequent HindIII cleavage and document previous cleavage events at unaddressed PvuII sites by the ZF-PvuII fusion enzymes, are marked by an arrow.

additional H83A substitution in the cleavage module, is used under these conditions, the complete linearization of the substrate plasmid containing the tripartite target sequence (4xP-Z12P12Z) can be observed without the appearance of any further 'unspecific' cleavage products. Subsequent cleavage with HindIII, which cleaves at a singular site in the plasmid, proved that only specific cleavage at the tripartite target sequence had occurred. In contrast to ZF-PvuII G46/F94, hardly any linearization of the control plasmid 4xP-P by the ZF-PvuII G46/A83/F94 fusion enzyme could be observed (<1.5% linear plasmid within 24 h). Furthermore, there was also only a moderate increase in open-circle plasmids detectable. It is noteworthy that the substrate plasmid 4xP-Z30Z, which contains two ZF-binding sites in 30 bp distance of each other without a PvuII site in between, was also not cleaved by ZF-PvuII G46/A83/F94 (Supplementary Figures S10 and S11). These results demonstrate that the introduction of a highly specific DNA DSB is only catalyzed, if the tripartite target sequence with two ZF-binding sites in an inverted orientation and a central PvuII site is present.

Analysis of the *in vitro* cleavage specificity of the 'analogous' ZF-FokI variants

To appreciate the observed cleavage specificity of the ZF-PvuII constructs, several 'analogous' ZF-FokI nucleases, consisting of the same ZF DNA-binding domain and the homodimeric wt FokI-cleavage domain or its obligate heterodimeric variants (EA + KV, ELD + KKR) (24,45,47) (Supplementary Table S2), respectively, were produced (Supplementary Figure S2) and tested under comparable conditions as the ZF-PvuII for addressed cleavage *in vitro* (Figure 5, Supplementary Figures S12–S18). In a near equimolar stoichiometry, the ZF-FokI wt or the heterodimeric nuclease pairs ZF-FokI EA + KV and ZF-FokI ELD + KKR specifically cleaved the substrate plasmid containing the specific target

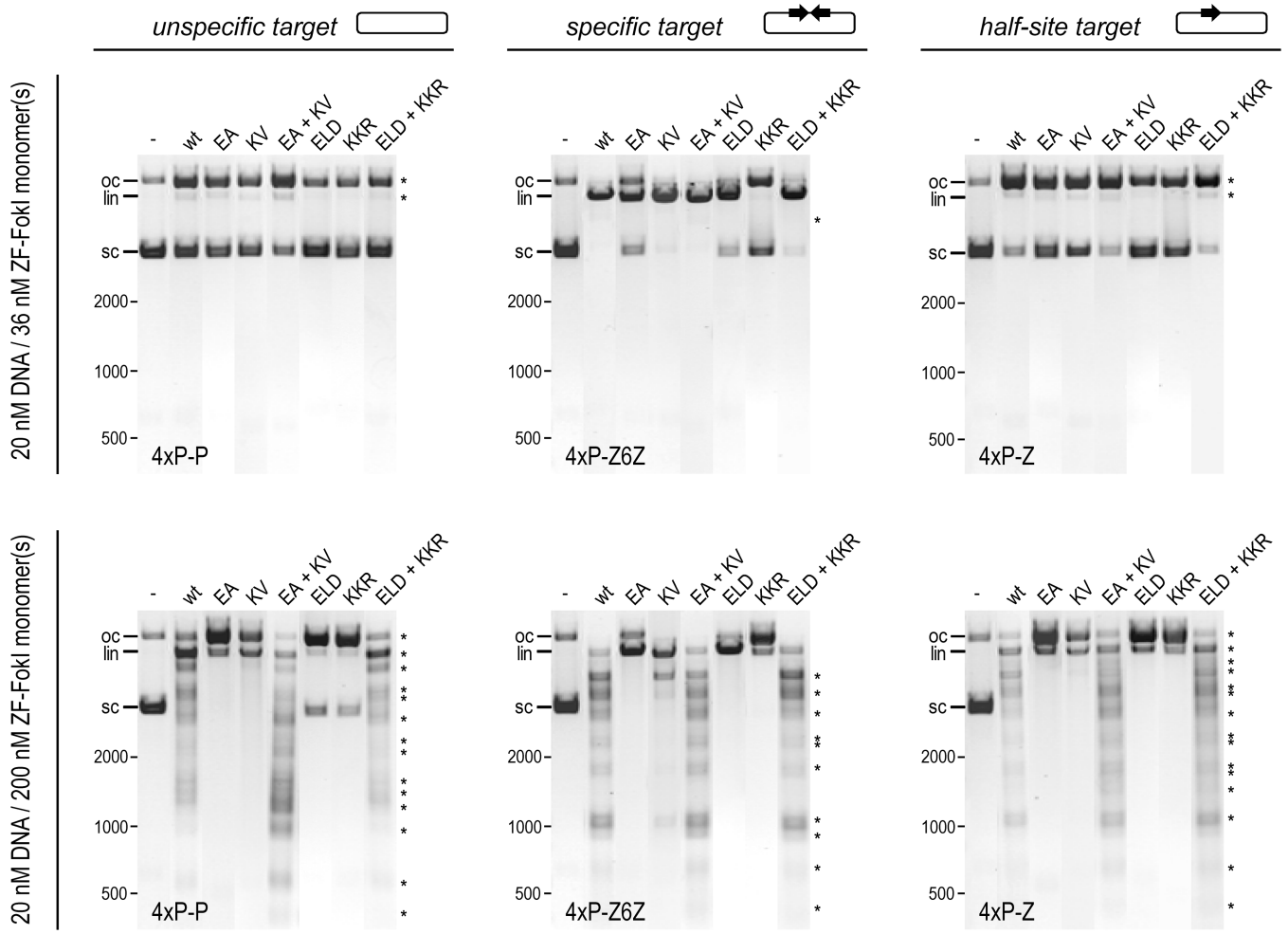

Figure 5. Influence of mutations in the FokI-cleavage domain (ZF-FokI EA, ZF-FokI KV, ZF-FokI ELD, ZF-FokI KKR) on the ZF-FokI-cleavage specificity of 'classical' ZFNs. Cleavage of a 4878 bp plasmid substrate was performed for 24 h, using a near equimolar stoichiometry of DNA substrate and ZFN dimers [20 nM plasmid/36 nM ZF-FokI monomer(s); top panel] and an excess of enzyme [20 nM plasmid/200 nM ZF-FokI monomer(s); bottom panel], respectively. ZF-FokI variants with either the homodimeric wt FokI-cleavage domain, or the obligate heterodimeric FokI-cleavage domains EA, KV, ELD, KKR, each individually tested, or the obligate heterodimeric FokI-cleavage domain pairs EA + KV and ELD + KKR in combination were investigated. A summary of the gel electrophoretic analyses of the cleavage products obtained after 24 h of incubation of the plasmids 4xP-P (unspecific target), 4xP-Z6Z (specific target) and 4xP-Z (half-site target) with the different ZF-FokI variants is shown. The supercoiled, open circular and linear forms of the plasmid are denoted by sc, oc and lin. Further cleavage products that result from cleavage at unspecific target sites are marked by an asterisks. (The detailed kinetic analyses of DNA cleavage by all ZF-FokI variants are shown in Supplementary Figures S12–S18.)

site (4xP-Z6Z), but also some linearization or nicking of the substrate plasmids containing only one (4xP-Z) or no ZF-binding site at all (4xP-P) occurred after 24 h. By increasing the ZF-FokI concentration to a 5-fold excess of 'active' ZF-FokI over DNA, a considerable amount of unspecific cleavage even of the unspecific substrate plasmid (4xP-P) was observed by all investigated ZF-FokI nucleases (wt, EA + KV, ELD + KKR), indicated by multiple unwanted cleavage products and a DNA smear. Furthermore, the obligate heterodimeric ZF-FokI variants (EA, KV, ELD, KKR), individually tested, also showed some, but to a lesser extent, specific and unspecific cleavage, indicating that a homodimerization of the obligate heterodimeric variants is not fully inhibited *in vitro*.

Dependence of the cleavage specificity of ZF-FokI and ZF-PvuII on the salt concentration

Insufficient binding specificity of the ZF domains seems to be a major concern for ZF-FokI mediated off-target site cleavage. Thus, DNA-cleavage assays using a 5-fold excess of 'active' ZFN (ZF-FokI or ZF-PvuII) were also performed with the addition of increasing salt concentrations, in order to decrease the amount of unspecific protein/DNA-interactions by increasing the ionic strength (Figure 6, Supplementary Figure S19). For ZF-FokI, an increase of salt concentration positively correlates with its cleavage specificity. As shown in detail in Figure 5, at a salt concentration of 50 mM unspecific cleavage of the ZF-FokI variants is quite pronounced. By adjusting the total salt concentration to >150 mM, ZF-FokI

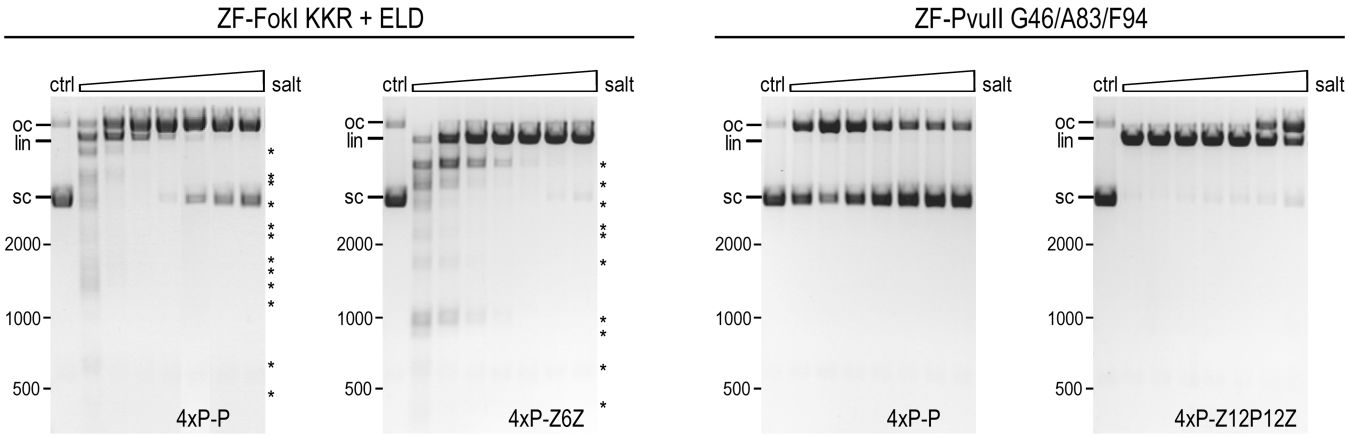

Figure 6. Influence of the salt concentration on the addressed cleavage of a 4878 bp plasmid substrate by the obligate heterodimeric ZFN pair ZF-FokI ELD + KKR in combination (20 nM DNA/100 nM ZF-FokI ELD monomer/100 nM ZF-FokI KKR monomer; left panel) and by the ZF-PvuII G46/A83/F94 fusion enzyme (20 nM DNA/100 ZF-PvuII G46/A83/F94 homodimer; right panel), respectively. The salt concentration of 50 mM NaCl was complemented with KCl to give total salt concentrations of 50, 75, 100, 125, 150, 175 and 200 mM salt. The gel electrophoretic analyses of the cleavage products of 4xP-P (unspecific target), 4xP-Z6Z (specific target for ZFN) and 4xP-Z12P12Z (specific target for ZF-PvuII) cleaved by ZF-FokI ELD + KKR or ZF-PvuII G46/A83/F94 after 24 h of incubation are shown. The supercoiled, open circular and linear forms of the plasmid are denoted by sc, oc and lin. Further cleavage products that result from cleavage at unspecific target sites are marked by an asterisks. (The corresponding gel electrophoretic analyses of the products obtained after 1 h of incubation are shown in Supplementary Figure S19.)

ELD + KKR specifically catalyzes a DSB at its addressed target site (Z6Z). The unspecific substrate plasmid (4xP-P) is only nicked after 24 h; and a small amount of linearized product is produced. In contrast, the cleavage preference of ZF-PvuII G46/A83/F94 for cleaving the tripartite target sequence Z12P12Z apparently is not affected by variation of the ionic strength. Especially for salt concentrations of >150 mM, no unaddressed off-target site cleavage of the substrate plasmid 4xP-P by ZF-PvuII G46/A83/F94 (with only little formation of nicked product) was observed within 24 h. These data show the ZF-PvuII variant to have an improved on- versus off-target ratio *in vitro* compared to ZF-FokI, even under an increased ionic strength.

ZF-PvuII-mediated cleavage in human cells

Based on the *in vitro* findings, we designed target plasmids that contain either an addressed Z12P12Z or unaddressed 21P21 target site (Figure 7A). Even though both ZF-PvuII G46/A83/F94 and PvuII G46/A83/F94 were expressed in human cells, ZF-PvuII seemed more stable than PvuII (Figure 7B). To test ZF-PvuII G46/A83/F94 or PvuII G46/A83/F94 for their ability to cleave the target site *in cellula*, HEK293T cells were co-transfected with the respective expression vectors and either target plasmid. Nuclease-induced DSBs are frequently repaired by the error-prone NHEJ pathway, leading to insertions/deletions (indels) at the target site and/or disruption of the central PvuII site (Figure 7A). PCR amplicons encompassing the central PvuII site were generated from extracted plasmids and either digested with the mismatch-sensitive T7 endonuclease I (T7E1; Figure 7C) or PvuII (Figure 7D). Cleavage of the PCR fragments at the addressed target site Z12P12Z by T7E1 was only detected in samples transfected with ZF-PvuII G46/A83/F94 but not in samples with the unaddressed target site 21P21 or the equivalent samples transfected with PvuII G46/A83/F94 (Figure 7C). In agreement with these results, partial resistance to PvuII cleavage was only observed for PCR amplicons generated from cells transfected with the Z12P12Z target plasmid and the ZF-PvuII G46/A83/F94 expression vector (Figure 7D). These results show that ZF-PvuII can be expressed in human cells and that the enzyme specifically cleaves addressed target sites.

DISCUSSION

The ZFN technology represents a big step forward towards applying precision genome surgery in human gene therapy. Nevertheless, two recent studies performing a genome-wide screen for potential ZFN off-target sites revealed that even optimized ZF-FokI nucleases show detectable unspecific cleavage at multiple off-target sites (54–56). Since the off-target sites found are similar in sequence to the intended on-target site (>65% identity to the on-target site), one major concern for the lack in precision of the ZF-FokI nucleases is the insufficient binding specificity of the ZF domains. If binding of two ZFN subunits occur to near cognate ZF sites in correct spacing and orientation, a double-strand cleavage will likely be catalyzed by the unspecific FokI nuclease domains. In order to avoid such unspecific DNA cleavage, we have developed a novel ZFN platform that, in addition to the specificity provided by the ZF, comprises the sequence specificity of a Type IIP restriction enzyme. A main requirement for such a fusion enzyme is that the ZF-binding module is the major determinant for binding of the fusion enzyme to the ZF recognition sites and that DNA cleavage is only initiated when a cognate target site for the restriction enzyme is in defined proximity to the ZF-binding site. On the other hand, nuclease activity at unaddressed recognition sites must be

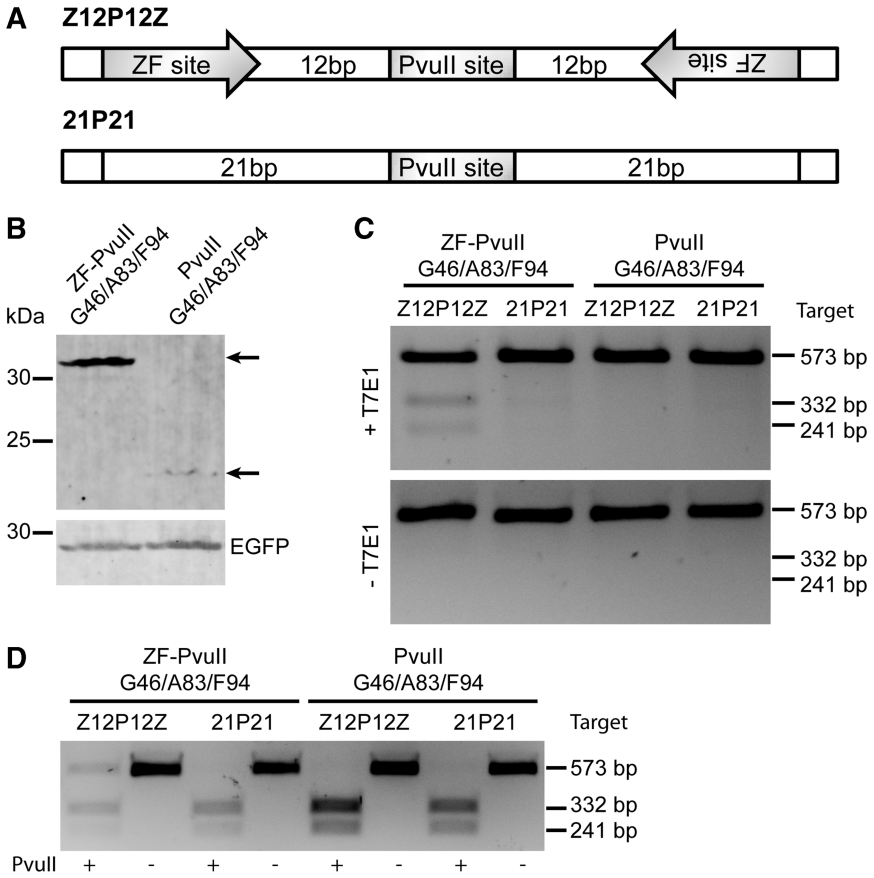

Figure 7. ZF-PvuII G46/A83/F94 mediated DNA cleavage in mammalian cells. (**A**) Schematic of the ZF-PvuII G46/A83/F94 target sites. The addressed Z12P12Z target site harbors an inverted repeat of ZF-binding sites separated by 12-bp spacer sequences that flank a central PvuII site. The unaddressed target site 21P21 is structured identically but lacks the ZF-binding sites. (**B**) Expression levels of ZF-PvuII G46/A83/F94 and PvuII G46/A83/F94. Cell lysates of transfected HEK293T cells were probed with antibodies against HA-tag or EGFP. (**C** and **D**) Cleavage activity *in cellula*. Cleavage of target plasmids in transfected HEK293T cells was assessed by detecting nuclease-induced mutations due to imperfect repair of DNA DSBs by NHEJ. PCR fragments encompassing the target site were either subjected to digestion with the mismatch-sensitive T7 endonuclease 1 (C) or PvuII (D).

suppressed. For this purpose, we have coupled an optimized three finger ZF DNA-binding domain to the N-terminal end(s) of the well-characterized homodimeric restriction enzyme PvuII, which catalyzes a DNA DSB at the palindromic 6 bp recognition site 5′-CAG↓CTG-3′ in a highly accurate manner. Besides the homodimeric PvuII we have also used a single-chain variant of PvuII, which allowed us to investigate the effect of either one or two ZF domain fusions on addressed DNA cleavage. It turned out that the homodimeric ZF-PvuII fusion enzyme has a higher cleavage preference for the tripartite target sequence (Z– P–Z) than the ZF-scPvuII fusion enzyme for cleaving the bipartite target sequence (P–Z), mainly due to a better suppression of cleavage at unaddressed PvuII sites.

By introducing additional amino acid substitutions within the cleavage module that had been described to interfere with binding or catalysis and thus lowering the activity of PvuII, the specificity of the fusion enzyme could be significantly increased. Binding experiments show that the ZF-PvuII fusion enzymes have a high affinity to the tripartite target site [$K_{d(app)}$ ~10 nM], which is largely independent of the contributions of the PvuII-cleavage module and thus is mainly determined by the specific DNA interaction of the ZF-binding modules. By analyzing the cleavage specificity in the activity assays it is apparent that the final fusion enzyme ZF-PvuII G46/A83/F94 (containing the high-fidelity mutation G46, the mutation A83, which mainly affects binding, and the mutation F94, which mainly affects catalysis) has a pronounced cleavage preference for the tripartite target sequence with an optimum distance of 12 bp between the PvuII and the ZF-binding sites. Whereas the tripartite DNA target sequence Z12P12Z is accurately cleaved by the fusion enzyme, neither DNA cleavage at unaddressed PvuII sites nor at a site with two inverted ZF-binding sites without a central PvuII recognition site was detectable after 24 h, even under stoichiometric conditions of enzyme and substrate.

It is also noteworthy that ZF-PvuII G46/A83/F94, in contrast to ZF-PvuII G46/F94, shows a considerably reduced cleavage activity at the bipartite target sequence P12Z. The explanation that the ZF-PvuII fusion enzyme forms monomers in solution that dimerize upon binding at

the tripartite target site is rather unlikely, since PvuII itself forms stable dimers in solution and the extended subunit–subunit interface of PvuII should not be affected by the ZF fusion or by the additional substitutions. Thus, it is more likely that the cleavage module PvuII G46/F94 is still able to bind and cleave the PvuII target site, when only one ZF has bound to the DNA. In contrast, the cleavage module PvuII G46/A83/F94 has a more reduced binding affinity and needs both ZF-binding modules to bind to the two inverted ZF-binding sites to initiate cleavage at the central PvuII site.

For a detailed evaluation of the ZF-PvuII-cleavage specificities, the corresponding ZF-FokI nucleases were produced and tested for their *in vitro* cleavage specificities under similar long-term cleavage conditions. In contrast to the clear cleavage preference of ZF-PvuII G46/A83/F94 for its specific target site, we observed considerable amount of unspecific DNA cleavage by the ZF-FokI nucleases, when using a 5-fold excess of enzyme over substrate DNA. Since the substrate plasmids contain no obvious off-target site besides the specifically introduced one, the ZF-FokI-cleavage pattern of multiple defined cleavage fragments combined with some DNA smear can be explained by: (i) one ZF-FokI monomer binds to a near cognate ZF site and the bound FokI-cleavage domain interacts with the FokI-cleavage domain of another monomer recruited from solution, resulting in a defined DSB; and (ii) two FokI-cleavage domains interact in solution and stimulate unspecific DSBs, indicated by the observed DNA smear. At a near equimolar stoichiometry, the ZF-FokI variants cleave the specific target site (Z6Z) as expected, but incubation with the unspecific substrate plasmid (4xP-P) or the plasmid containing the half-target site (4xP-Z) resulted also in formation of open circle and some linearized product. Accumulation of nicked product can result from an unstable complex; a ZF-FokI monomer bound to a near cognate ZF site might interact with another FokI-cleavage domain recruited from solution, forming a stable complex capable of introducing a nick, but which dissociates before the second nick resulting in a DSB has occurred. Similar observations have been already reported for the native FokI restriction endonuclease (39,74,75). Unexpectedly, the observed cleavage patterns of ZF-FokI were largely independent of the variant of the FokI-cleavage domain used. The homodimeric ZF-FokI with the FokI wt cleavage domain showed the same *in vitro* cleavage pattern as the combination of the obligate heterodimeric variants ZF-FokI EA + KV or ELD + KKR. It is further noteworthy that, even though to a lesser extent than wt, the individual subunits of ZF-FokI are EA, KV, ELD and KKR, respectively, were able to nick or cleave specific and unspecific substrate plasmids. This means that under low salt conditions the oppositely charged interface impairs but does not completely block the ability of the individual subunits ZF-FokI EA, ZF-FokI KV, ZF-FokI ELD and ZF-FokI KKR to form homodimers *in vitro*. On the other hand, Gabriel *et al.* (55) showed that with the use of obligate heterodimeric ZF-FokI variants cleavage of homodimeric off-target sites is not observed *in cellula*.

Since the unspecific cleavage of the ZF-FokI variants mainly relies on the unspecific binding of the ZF domains, we increased the ZF-FokI specificity *in vitro* by increasing the ionic strength in order to shift the ZF DNA-binding equilibrium towards binding the specific target site. Upon increasing the salt concentration to more physiological conditions (~150 mM NaCl/KCl), ZF-FokI specifically cleaves the target site Z6Z, but still exhibited nicking and some cleavage activity on the unspecific substrate plasmid. Compared to the considerable amount of unspecific cleavage obtained under low salt conditions (50 mM NaCl), these results might reflect the *in vivo* cleavage conditions more properly. However, in their *in vitro* selection method to screen for potential ZF-FokI nucleases off-target site, Pattanayak *et al.* (56) found a good correlation between the observed *in vitro* off-target sites under similar low salt conditions and the off-target sites that are cleaved *in vivo*. From these results we conclude that the ZF-FokI nucleases do not exhibit such a high amount of off-site cleavage *in vivo* as we observed at low ionic strength *in vitro*, which is in agreement with the current view of ZF-FokI nuclease off-target cleavage *in vivo*. But our data show that the specificity of ZF-FokI nucleases is determined by the ZF-binding specificity, and if a ZF-FokI monomer binds to a near cognate ZF site once, interaction with another ZF-FokI monomer can always cause formation of a nick or a DSB (76), which can be the case for ZFNs containing the wt as well as the obligate heterodimeric FokI-cleavage domains. Besides the prominent off-target sites that have a high similarity to the intended on-target site, such rare and more random DNA-cleavage events are likely to occur as well and might not be detectable by the current methods for detecting ZFN off-target activity. Furthermore, we could show that unspecific cleavage by ZF-FokI is strongly dependent on the enzyme concentration. The more ZF-FokI nucleases are present, the more cleavage at unspecific sites does occur, an observation which supports the current considerations for ZFN treatment *in vivo*, namely to apply the lowest amount of ZFNs possible (54).

In contrast, the ZF-PvuII nuclease does not fully rely on the ZF-binding specificity only because another level of specificity was added. If the ZF-binding domain binds to a near cognate ZF site, cleavage will only occur if an additional PvuII site is in close proximity. Whereas ZF-FokI catalyzed unspecific cleavage under low salt conditions *in vitro*, no cleavage of the unaddressed plasmid was catalyzed by ZF-PvuII under the same conditions. By increasing the ionic strength towards more physiological salt concentrations, ZF-PvuII G46/A83/F94 still preferentially cleaved the addressed target site of two inverted ZF-binding sites with a central PvuII site, and no cleavage or nicking of the unaddressed substrate was observed within 24 h, as seen for the ZF-FokI variants. Importantly, the results for ZF-PvuII could be confirmed by a cellular cleavage assay in HEK293T cells, as demonstrated by the detection of insertions/deletions (indels) or the partial disruption of the addressed PvuII site based on NHEJ after double-strand cleavage.

Compared to ZF-FokI nucleases, another type of off-target cleavage by ZF-PvuII may be possible, namely

cleavage at unaddressed PvuII sites. But crippling of the activity of PvuII in the fusion construct by introducing the G46/A83/F94 mutations clearly prevented cleavage at unaddressed PvuII sites. The approach to weaken the DNA-binding and/or catalytic properties of an 'effector' enzyme might also be transferred to other fusion proteins for highly specific genome modifications, in order to make a tethered binding module more dominant.

Similar to the classical ZF-FokI nucleases, the ZF-binding modules used within the ZF-PvuII fusion can be replaced by ZF domains with other DNA-binding specificities, resulting in designer fusion enzymes that can address different target sites consisting of a central PvuII site flanked by two ZF-binding sites. In principle, as with conventional ZFNs, the specificity could be further increased by using heterodimeric versions of ZF-PvuII. Heterodimeric versions could be produced also with a catalytically defective PvuII subunit that would promote targeted nicking, which is believed to decrease the incidence of NHEJ (77). Considering the frequencies for possible and unique ZF-binding sites by the current ZF engineering platforms (29) and allowing only a minor tolerance in the distance of the 12 bp spacer sequence within the tripartite ZF-PvuII target site (Z–P–Z) of ± 1 bp or ± 2 bp, respectively, we calculate that approximately every third or nearly every single PvuII site within a complex genome (occurring statistically once every ~4000 bp) can be addressed in principle by two adjacent ZF target sites in the proper orientation. Besides the ZF-based DNA-binding domains, other binding domains, e.g. based on homing endonucleases or designer TALEs, could be coupled to the PvuII-cleavage module for the creation of novel, customizable designer nucleases, as recently shown in a proof-of-concept approach by fusing PvuII to a catalytically inactive I-SceI variant (78). In addition, ZF-PvuII fusion enzymes could be combined with other functionalities that regulate the cleavage activity of PvuII, such as photocaged (79) or photoswitchable (80) variants of PvuII. Altogether, further studies including gene targeting of endogenous target sites and toxicity assays will be carried out to validate the ZF-PvuII platform as a valid alternative to conventional ZF-FokI nucleases.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–19 and Supplementary Tables 1 and 2.

ACKNOWLEDGEMENTS

We thank Peter Friedhoff, Mert Yanik and Marika Midon for fruitful discussions, Birgit Reinhardt and Nikolas Martin for assistance and Ines Fonfara for plasmids.

FUNDING

This work was supported by the Justus-Liebig University Giessen (program Justus to B.S.); the DFG-RFBR program 'International Research Training Groups' (GRK 1384 and 08-04-91974 to A.P. and W.W.); and the European Commission's Seventh Framework Programme (PERSIST–222878 to T.C.). Funding for open access charge: DFG (Deutsche Forschungsgemeinschaft) and the Justus-Liebig University Giessen.

Conflict of interest statement. None declared.

REFERENCES

1. Mackay,J.P. and Segal,D.J. (2010) *Engineered Zinc Finger Proteins*. Humana Press, New York.
2. Wong,G.K. and Chiu,A.T. (2011) Gene therapy, gene targeting and induced pluripotent stem cells: applications in monogenic disease treatment. *Biotechnol. Adv.*, **29**, 1–10.
3. Pingoud,A. and Silva,G.H. (2007) Precision genome surgery. *Nat. Biotechnol.*, **25**, 743–744.
4. Pingoud,A. and Wende,W. (2011) Generation of novel nucleases with extended specificity by rational and combinatorial strategies. *ChemBioChem*, **12**, 1495–1500.
5. Davis,D. and Stokoe,D. (2010) Zinc finger nucleases as tools to understand and treat human diseases. *BMC Med.*, **8**, 42.
6. Handel,E.M. and Cathomen,T. (2011) Zinc-finger nuclease based genome surgery: it's all about specificity. *Curr. Gene Ther.*, **11**, 28–37.
7. Carroll,D. (2011) Zinc-finger nucleases: a panoramic view. *Curr. Gene Ther.*, **11**, 2–10.
8. Rahman,S.H., Maeder,M.L., Joung,J.K. and Cathomen,T. (2011) Zinc-finger nucleases for somatic gene therapy: the next frontier. *Hum. Gene Ther.*, **22**, 925–933.
9. Carroll,D. (2011) Genome engineering with zinc-finger nucleases. *Genetics*, **188**, 773–782.
10. Silva,G., Poirot,L., Galetto,R., Smith,J., Montoya,G., Duchateau,P. and Paques,F. (2011) Meganucleases and other tools for targeted genome engineering: perspectives and challenges for gene therapy. *Curr. Gene Ther.*, **11**, 11–27.
11. Grizot,S., Smith,J., Daboussi,F., Prieto,J., Redondo,P., Merino,N., Villate,M., Thomas,S., Lemaire,L., Montoya,G. *et al.* (2009) Efficient targeting of a SCID gene by an engineered single-chain homing endonuclease. *Nucleic Acids Res.*, **37**, 5405–5419.
12. Takeuchi,R., Lambert,A.R., Mak,A.N., Jacoby,K., Dickson,R.J., Gloor,G.B., Scharenberg,A.M., Edgell,D.R. and Stoddard,B.L. (2011) Tapping natural reservoirs of homing endonucleases for targeted gene modification. *Proc. Natl Acad. Sci. USA*, **108**, 13077–13082.
13. Eisenschmidt,K., Lanio,T., Simoncsits,A., Jeltsch,A., Pingoud,V., Wende,W. and Pingoud,A. (2005) Developing a programmed restriction endonuclease for highly specific DNA cleavage. *Nucleic Acids Res.*, **33**, 7039–7047.
14. Tsai,T.L., Shieh,D.B., Yeh,C.S., Tzeng,Y., Htet,K., Chuang,K.S., Hwu,J.R. and Su,W.C. (2010) The down regulation of target genes by photo activated DNA nanoscissors. *Biomaterials*, **31**, 6545–6554.
15. Miller,J.C., Tan,S., Qiao,G., Barlow,K.A., Wang,J., Xia,D.F., Meng,X., Paschon,D.E., Leung,E., Hinkley,S.J. *et al.* (2011) A TALE nuclease architecture for efficient genome editing. *Nat. Biotechnol.*, **29**, 143–148.
16. Li,T., Huang,S., Zhao,X., Wright,D.A., Carpenter,S., Spalding,M.H., Weeks,D.P. and Yang,B. (2011) Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. *Nucleic Acids Res.*, **39**, 6315–6325.
17. Mussolino,C., Morbitzer,R., Lutge,F., Dannemann,N., Lahaye,T. and Cathomen,T. (2011) A novel TALE nuclease scaffold enables

high genome editing activity in combination with low toxicity. *Nucleic Acids Res.*, doi: 10.1093/nar/gkr1597.
18. Jackson,S.P. and Bartek,J. (2009) The DNA-damage response in human biology and disease. *Nature*, **461**, 1071–1078.
19. Rouet,P., Smih,F. and Jasin,M. (1994) Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc. Natl Acad. Sci. USA*, **91**, 6064–6068.
20. Cui,X., Ji,D., Fisher,D.A., Wu,Y., Briner,D.M. and Weinstein,E.J. (2011) Targeted integration in rat and mouse embryos with zinc-finger nucleases. *Nat. Biotechnol.*, **29**, 64–67.
21. DeKelver,R.C., Choi,V.M., Moehle,E.A., Paschon,D.E., Hockemeyer,D., Meijsing,S.H., Sancak,Y., Cui,X., Steine,E.J., Miller,J.C. *et al.* (2010) Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. *Genome Res.*, **20**, 1133–1142.
22. Doyon,Y., McCammon,J.M., Miller,J.C., Faraji,F., Ngo,C., Katibah,G.E., Amora,R., Hocking,T.D., Zhang,L., Rebar,E.J. *et al.* (2008) Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat. Biotechnol.*, **26**, 702–708.
23. Perez,E.E., Wang,J., Miller,J.C., Jouvenot,Y., Kim,K.A., Liu,O., Wang,N., Lee,G., Bartsevich,V.V., Lee,Y.L. *et al.* (2008) Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat. Biotechnol.*, **26**, 808–816.
24. Sollu,C., Pars,K., Cornu,T.I., Thibodeau-Beganny,S., Maeder,M.L., Joung,J.K., Heilbronn,R. and Cathomen,T. (2010) Autonomous zinc-finger nuclease pairs for targeted chromosomal deletion. *Nucleic Acids Res.*, **38**, 8269–8276.
25. Urnov,F.D., Miller,J.C., Lee,Y.L., Beausejour,C.M., Rock,J.M., Augustus,S., Jamieson,A.C., Porteus,M.H., Gregory,P.D. and Holmes,M.C. (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*, **435**, 646–651.
26. Weinthal,D., Tovkach,A., Zeevi,V. and Tzfira,T. (2010) Genome editing in plant cells by zinc finger nucleases. *Trends Plant Sci.*, **15**, 308–321.
27. Bozas,A., Beumer,K.J., Trautman,J.K. and Carroll,D. (2009) Genetic analysis of zinc-finger nuclease-induced gene targeting in Drosophila. *Genetics*, **182**, 641–651.
28. Lombardo,A., Genovese,P., Beausejour,C.M., Colleoni,S., Lee,Y.L., Kim,K.A., Ando,D., Urnov,F.D., Galli,C., Gregory,P.D. *et al.* (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat. Biotechnol.*, **25**, 1298–1306.
29. Shukla,V.K., Doyon,Y., Miller,J.C., DeKelver,R.C., Moehle,E.A., Worden,S.E., Mitchell,J.C., Arnold,N.L., Gopalan,S., Meng,X. *et al.* (2009) Precise genome modification in the crop species Zea mays using zinc-finger nucleases. *Nature*, **459**, 437–441.
30. Young,J.J., Cherone,J.M., Doyon,Y., Ankoudinova,I., Faraji,F.M., Lee,A.H., Ngo,C., Guschin,D.Y., Paschon,D.E., Miller,J.C. *et al.* (2011) Efficient targeted gene disruption in the soma and germ line of the frog Xenopus tropicalis using engineered zinc-finger nucleases. *Proc. Natl Acad. Sci. USA*, **108**, 7052–7057.
31. Herrmann,F., Garriga-Canut,M., Baumstark,R., Fajardo-Sanchez,E., Cotterell,J., Minoche,A., Himmelbauer,H. and Isalan,M. (2011) p53 Gene repair with zinc finger nucleases optimised by yeast 1-hybrid and validated by Solexa sequencing. *PLoS One*, **6**, e20913.
32. Zou,J., Maeder,M.L., Mali,P., Pruett-Miller,S.M., Thibodeau-Beganny,S., Chou,B.K., Chen,G., Ye,Z., Park,I.H., Daley,G.Q. *et al.* (2009) Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell Stem Cell*, **5**, 97–110.
33. Hockemeyer,D., Soldner,F., Beard,C., Gao,Q., Mitalipova,M., DeKelver,R.C., Katibah,G.E., Amora,R., Boydston,E.A., Zeitler,B. *et al.* (2009) Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat. Biotechnol.*, **27**, 851–857.
34. Collin,J. and Lako,M. (2011) Concise review: putting a finger on stem cell biology: zinc finger nuclease-driven targeted genetic editing in human pluripotent stem cells. *Stem Cells*, **29**, 1021–1033.
35. Lei,Y., Lee,C.L., Joo,K.I., Zarzar,J., Liu,Y., Dai,B., Fox,V. and Wang,P. (2011) Gene editing of human embryonic stem cells via an engineered baculoviral vector carrying zinc-finger nucleases. *Mol. Ther.*, **19**, 942–950.
36. Urnov,F.D., Rebar,E.J., Holmes,M.C., Zhang,H.S. and Gregory,P.D. (2010) Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.*, **11**, 636–646.
37. Klug,A. (2010) The discovery of zinc fingers and their applications in gene regulation and genome manipulation. *Annu. Rev. Biochem.*, **79**, 213–231.
38. Wah,D.A., Bitinaite,J., Schildkraut,I. and Aggarwal,A.K. (1998) Structure of FokI has implications for DNA cleavage. *Proc. Natl Acad. Sci. USA*, **95**, 10564–10569.
39. Bitinaite,J., Wah,D.A., Aggarwal,A.K. and Schildkraut,I. (1998) FokI dimerization is required for DNA cleavage. *Proc. Natl Acad. Sci. USA*, **95**, 10570–10575.
40. Kim,Y.G., Cha,J. and Chandrasegaran,S. (1996) Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc. Natl Acad. Sci. USA*, **93**, 1156–1160.
41. Maeder,M.L., Thibodeau-Beganny,S., Osiak,A., Wright,D.A., Anthony,R.M., Eichtinger,M., Jiang,T., Foley,J.E., Winfrey,R.J., Townsend,J.A. *et al.* (2008) Rapid "open-source" engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol. Cells*, **31**, 294–301.
42. Sander,J.D., Dahlborg,E.J., Goodwin,M.J., Cade,L., Zhang,F., Cifuentes,D., Curtin,S.J., Blackburn,J.S., Thibodeau-Beganny,S., Qi,Y. *et al.* (2011) Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat. Methods*, **8**, 67–69.
43. Handel,E.M., Alwin,S. and Cathomen,T. (2009) Expanding or restricting the target site repertoire of zinc-finger nucleases: the inter-domain linker as a major determinant of target site selectivity. *Mol. Ther.*, **17**, 104–111.
44. Ramalingam,S., Kandavelou,K., Rajenderan,R. and Chandrasegaran,S. (2011) Creating designed zinc-finger nucleases with minimal cytotoxicity. *J. Mol. Biol.*, **405**, 630–641.
45. Szczepek,M., Brondani,V., Buchel,J., Serrano,L., Segal,D.J. and Cathomen,T. (2007) Structure-based redesign of the dimerization interface reduces the toxicity of zinc-finger nucleases. *Nat. Biotechnol.*, **25**, 786–793.
46. Miller,J.C., Holmes,M.C., Wang,J., Guschin,D.Y., Lee,Y.L., Rupniewski,I., Beausejour,C.M., Waite,A.J., Wang,N.S., Kim,K.A. *et al.* (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat. Biotechnol.*, **25**, 778–785.
47. Doyon,Y., Vo,T.D., Mendel,M.C., Greenberg,S.G., Wang,J., Xia,D.F., Miller,J.C., Urnov,F.D., Gregory,P.D. and Holmes,M.C. (2011) Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. *Nat. Methods*, **8**, 74–79.
48. Guo,J., Gaj,T. and Barbas,C.F. III (2010) Directed evolution of an enhanced and highly efficient FokI cleavage domain for zinc finger nucleases. *J. Mol. Biol.*, **400**, 96–107.
49. Pruett-Miller,S.M., Reading,D.W., Porter,S.N. and Porteus,M.H. (2009) Attenuation of zinc finger nuclease toxicity by small-molecule regulation of protein levels. *PLoS Genet.*, **5**, e1000376.
50. Doyon,Y., Choi,V.M., Xia,D.F., Vo,T.D., Gregory,P.D. and Holmes,M.C. (2010) Transient cold shock enhances zinc-finger nuclease-mediated gene disruption. *Nat. Methods*, **7**, 459–460.
51. Cornu,T.I., Thibodeau-Beganny,S., Guhl,E., Alwin,S., Eichtinger,M., Joung,J.K. and Cathomen,T. (2008) DNA-binding specificity is a major determinant of the activity and toxicity of zinc-finger nucleases. *Mol. Ther.*, **16**, 352–358.
52. Pruett-Miller,S.M., Connelly,J.P., Maeder,M.L., Joung,J.K. and Porteus,M.H. (2008) Comparison of zinc finger nucleases for use in gene targeting in mammalian cells. *Mol. Ther.*, **16**, 707–717.
53. Gupta,A., Meng,X., Zhu,L.J., Lawson,N.D. and Wolfe,S.A. (2011) Zinc finger protein-dependent and -independent contributions to the in vivo off-target activity of zinc finger nucleases. *Nucleic Acids Res.*, **39**, 381–392.
54. Mussolino,C. and Cathomen,T. (2011) On target? Tracing zinc-finger-nuclease specificity. *Nat. Methods*, **8**, 725–726.
55. Gabriel,R., Lombardo,A., Arens,A., Miller,J.C., Genovese,P., Kaeppel,C., Nowrouzi,A., Bartholomae,C.C., Wang,J.,

Friedman,G. *et al.* (2011) An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat. Biotechnol.*, **29**, 816–823.
56. Pattanayak,V., Ramirez,C.L., Joung,J.K. and Liu,D.R. (2011) Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. *Nat. Methods*, **8**, 765–770.
57. Meister,G.E., Chandrasegaran,S. and Ostermeier,M. (2010) Heterodimeric DNA methyltransferases as a platform for creating designer zinc finger methyltransferases for targeted DNA methylation in cells. *Nucleic Acids Res.*, **38**, 1749–1759.
58. Gaj,T., Mercer,A.C., Gersbach,C.A., Gordley,R.M. and Barbas,C.F. III (2011) Structure-guided reprogramming of serine recombinase DNA sequence specificity. *Proc. Natl Acad. Sci. USA*, **108**, 498–503.
59. Gersbach,C.A., Gaj,T., Gordley,R.M. and Barbas,C.F. III (2010) Directed evolution of recombinase specificity by split gene reassembly. *Nucleic Acids Res.*, **38**, 4198–4206.
60. Proudfoot,C., McPherson,A.L., Kolb,A.F. and Stark,W.M. (2011) Zinc finger recombinases with adaptable DNA sequence specificity. *PLoS One*, **6**, e19537.
61. Feng,X., Bednarz,A.L. and Colloms,S.D. (2010) Precise targeted integration by a chimaeric transposase zinc-finger fusion protein. *Nucleic Acids Res.*, **38**, 1204–1216.
62. Mineta,Y., Okamoto,T., Takenaka,K., Doi,N., Aoyama,Y. and Sera,T. (2008) Enhanced cleavage of double-stranded DNA by artificial zinc-finger nuclease sandwiched between two zinc-finger proteins. *Biochemistry*, **47**, 12257–12259.
63. Stahl,F., Wende,W., Jeltsch,A. and Pingoud,A. (1996) Introduction of asymmetry in the naturally symmetric restriction endonuclease EcoRV to investigate intersubunit communication in the homodimeric protein. *Proc. Natl Acad. Sci. USA*, **93**, 6175–6180.
64. Zhu,Z., Blanchard,A., Xu,S.-Y., Wei,H., Zhang,P., Sun,D. and Chan,S.-H. (2009), High fidelity restriction endonucleases. US patent no. 20090029376.
65. Simoncsits,A., Tjornhammar,M.L., Rasko,T., Kiss,A. and Pongor,S. (2001) Covalent joining of the subunits of a homodimeric type II restriction endonuclease: single-chain PvuII endonuclease. *J. Mol. Biol.*, **309**, 89–97.
66. Kirsch,R.D. and Joly,E. (1998) An improved PCR-mutagenesis strategy for two-site mutagenesis or sequence swapping between related genes. *Nucleic Acids Res.*, **26**, 1848–1850.
67. Pace,C.N., Vajdos,F., Fee,L., Grimsley,G. and Gray,T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.*, **4**, 2411–2423.
68. Nastri,H.G., Evans,P.D., Walker,I.H. and Riggs,P.D. (1997) Catalytic and DNA binding properties of PvuII restriction endonuclease mutants. *J. Biol. Chem.*, **272**, 25761–25767.
69. Pingoud,A., Fuxreiter,M., Pingoud,V. and Wende,W. (2005) Type II restriction endonucleases: structure and mechanism. *Cell. Mol. Life Sci.*, **62**, 685–707.
70. Athanasiadis,A., Vlassi,M., Kotsifaki,D., Tucker,P.A., Wilson,K.S. and Kokkinidis,M. (1994) Crystal structure of PvuII endonuclease reveals extensive structural homologies to EcoRV. *Nat. Struct. Biol.*, **1**, 469–475.
71. Horton,J.R. and Cheng,X. (2000) PvuII endonuclease contains two calcium ions in active sites. *J. Mol. Biol.*, **300**, 1049–1056.
72. Xie,F., Qureshi,S.H., Papadakos,G.A. and Dupureur,C.M. (2008) One- and two-metal ion catalysis: global single-turnover kinetic analysis of the PvuII endonuclease mechanism. *Biochemistry*, **47**, 12540–12550.
73. Spyridaki,A., Matzen,C., Lanio,T., Jeltsch,A., Simoncsits,A., Athanasiadis,A., Scheuring-Vanamee,E., Kokkinidis,M. and Pingoud,A. (2003) Structural and biochemical characterization of a new Mg(2+) binding site near Tyr94 in the restriction endonuclease PvuII. *J. Mol. Biol.*, **331**, 395–406.
74. Catto,L.E., Ganguly,S., Milsom,S.E., Welsh,A.J. and Halford,S.E. (2006) Protein assembly and DNA looping by the FokI restriction endonuclease. *Nucleic Acids Res.*, **34**, 1711–1720.
75. Sanders,K.L., Catto,L.E., Bellamy,S.R. and Halford,S.E. (2009) Targeting individual subunits of the FokI restriction endonuclease to specific DNA strands. *Nucleic Acids Res.*, **37**, 2105–2115.
76. Halford,S.E., Catto,L.E., Pernstich,C., Rusling,D.A. and Sanders,K.L. (2011) The reaction mechanism of FokI excludes the possibility of targeting zinc finger nucleases to unique DNA sites. *Biochem. Soc. Trans.*, **39**, 584–588.
77. Metzger,M.J., McConnell-Smith,A., Stoddard,B.L. and Miller,A.D. (2011) Single-strand nicks induce homologous recombination with less toxicity than double-strand breaks using an AAV vector template. *Nucleic Acids Res.*, **39**, 926–935.
78. Fonfara,I., Curth,U., Pingoud,A. and Wende,W. (2011) Creating highly specific nucleases by fusion of active restriction endonucleases and catalytically inactive homing endonucleases. *Nucleic Acids Res.*, doi: 10.1093/nar/gkr1788.
79. Silanskas,A., Foss,M., Wende,W., Urbanke,C., Lagunavicius,A., Pingoud,A. and Siksnys,V. (2011) Photocaged variants of the MunI and PvuII restriction enzymes. *Biochemistry*, **50**, 2800–2807.
80. Schierling,B., Noel,A.J., Wende,W., Hien le,T., Volkov,E., Kubareva,E., Oretskaya,T., Kokkinidis,M., Rompp,A., Spengler,B. *et al.* (2010) Controlling the enzymatic activity of a restriction enzyme by light. *Proc. Natl Acad. Sci. USA*, **107**, 1361–1366.