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Restricted spacer tolerance of a zinc finger nuclease with a six amino acid linker

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ABSTRACT

Zinc finger nucleases can be engineered to create highly efficient and precise changes to the genetic information within living cells. We report the investigation of an important parameter that defines the type of target site the nuclease can cleave. The active nuclease is a dimer, requiring that the DNA target site contain two zinc finger binding sites separated by a short spacer. Using a plasmid-based recombination assay in HEK 293T cells, we show that a 6 amino acid linker between the zinc finger DNA-binding domain and the FokI cleavage domain restricts nuclease activity to sites containing a 6 bp spacer. These observations concur with other recent studies, suggesting this information will be useful in the design of new potent and accurate zinc finger nucleases.

The C2H2 class of zinc finger proteins has proven to be the most useful scaffold for engineering custom DNA-binding proteins.[1,2] Several elegant and successful zinc finger engineering methods have been and continue to be developed.[3–8] The Modular Assembly methodology developed by Barbas and co-workers[3,4] was one of the earliest and easiest to use, and has therefore played a seminal role in the development of number of targetable gene regulation and gene modification applications, including artificial transcription factors[9] and zinc finger nucleases (ZFNs).[10,11] Among these, ZFNs have generated particular excitement because of their potential to catalyze the targeted disruption or correction of genetic information within living cells, at efficiencies that can not currently be achieved using other methods.[12]

ZFNs create double-strand breaks (DSBs) on DNA in cells, stimulating endogenous DNA repair mechanisms to repair the breaks. In mammalian cells, such breaks are primarily repaired by a non-homologous end-joining pathway (NHEJ), which frequently leads to mutations at the cleavage site. Mutagenesis in as highs as 54% of targeted loci has been reported,[13] presenting ZFNs as a potent tool for targeted gene disruption. The presence of a repair template, typically an exogenous DNA with homology to the target locus, additional allows some of the breaks to be repaired by homologous recombination (HR). Targeted replacements and gene insertions in as high as 50% of target loci have been reported,[8,14] representing a >100,000-fold improvement over uncatalyzed events and presenting ZFNs also a potent tool for targeted gene correction and addition. However, the specificity of engineered ZFNs is not perfect in many cases, leading to DSBs at off-target sites.[10–12] Such off-target events could produce mutations in non-target genes, or could overwhelm the repair machinery leading to apoptotic cell death.

In this study, we investigated an important parameter in the protein design that defines what type of target site a ZFN can cleave. The current design of virtually all ZFNs to date has been to link engineered zinc finger DNA-binding domains to the C-terminal cleavage domain of the restriction endonuclease FokI. Pioneering work by the Carroll and Chandrasegaran groups[15] demonstrated that the active form of this chimeric nuclease was a dimer, requiring two zinc finger binding sites to be present on the target DNA in everted orientation (Fig. 1A). The earliest protein constructions employed a long (22 aa) flexible linker between the terminal zinc finger domain and the FokI domain. Herein, the ZF-FokI linker is defined as the amino acids immediately following the final H in the a-helix of the terminal zinc finger and the first amino acid of the FokI domain, conventionally Q in the sequence QLVKSEL. For example, the linker shown in Figure 1A is considered a 6 aa linker. (Note that this nomenclature adds 4 aa to the original definition of the ZF-FokI linker,[15] but is more consistent with the conventional definition of the linkers between neighboring zinc finger domains.[16]) Early studies in *Xenopus* oocytes, using a plasmid-based single-strand annealing (SSA) HR reporter assay, showed that a ZFN with a 22 aa linker was active on target sites in which the two zinc finger binding sites were separated by spacers in a broad range of 6–18 bp, with an optimum at 8 bp.[15] By

Abbreviations: DSB, double-strand break; HR, homologous recombination; NHEJ, nonhomologous end-joining; SSA, single-strand annealing; ZFN, zinc finger nuclease.

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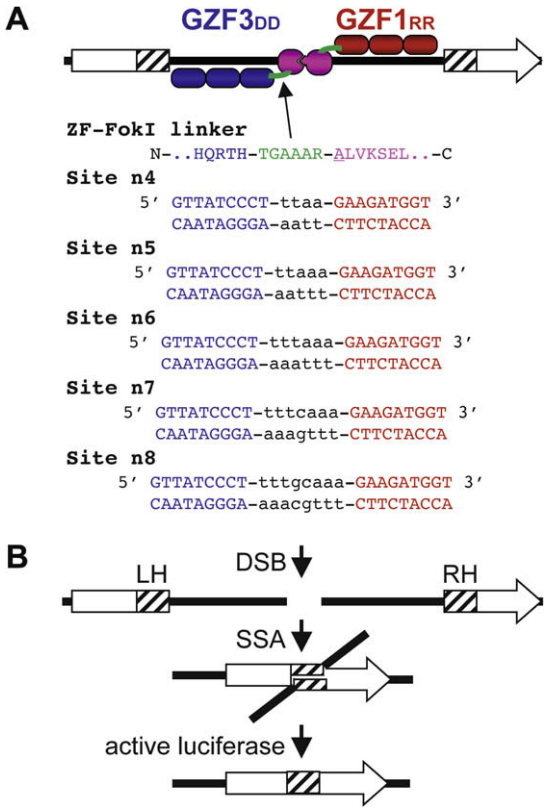

Figure 1. The plasmid-based SSA assay for testing ZFN activity in human cells. (A) The heterodimeric ZFN is shown as a cartoon. The 'left' zinc finger domain of the ZFN (blue) recognizes primarily the bottom strand of the DNA to deliver a C-terminal FokI cleavage domain (purple). The 'right' monomer (red) recognizes a site on the top strand to deliver the other cleavage domain. The two zinc finger binding sites are separated by a spacer region, within which the cleavage domains form an active dimer and cut the DNA. The sequences of the ZF-FokI protein linker and DNA spacers used in this study are shown. Note that the underlined A in the FokI sequence is more typically a Q in most other studies. (B) The SSA mechanism for reporter gene activation upon a ZFN-induced DSB is shown. Single-strand resection of the DNA ends allows the left (LH) and right (RH) homology regions of the split luciferase gene to anneal and recombine to form an active luciferase gene.

shortening the linker, the range of effective site separations narrowed significantly. Using purified proteins in vitro, ZFNs with 4 aa linkers (QNKK) were active on DNA targets with spacers of 5, 6 and 7 bp, as well as spacers of 14 and 16 bp (one full turn of the DNA double helix away). However, using the SSA assay in oocytes, the 4 aa linker greatly restricted activity to only sites with 6 bp spacers. This work thus established the canonical parameters for all subsequent ZFN experiments, that ZFNs with 4 aa linkers would be most active on everted zinc finger binding sties separated by 6 bp spacers.

These canonical parameters were soon challenged by a study by Sangamo Biosciences, which reported highly-efficient HR in human cells using a ZFN with a 4 aa linker on a target site with a 5 bp spacer.[17] The implications of this result are significant. Flexibility in the DNA spacer length has the practical advantage of expanding the repertoire of targetable sites, allowing ZFNs to be designed to sites that do not contain a canonical 6 bp spacer. However, this feature also has the disadvantage of expanding the number of off-target sites, potentially increasing geno- and cytotoxicity.

We have been studying ZFNs with a 6 aa linker (TGAAAR, used because it contains convenient restriction sties in the encoding DNA sequence).[18] To determine the range of DNA spacer lengths allowed by this linker in human cells, we used a plasmid-based SSA reporter assay in HEK 293T cells (Fig. 1B).[18] Briefly, a gene encoding luciferase was divided into two segments, separated by a stop codon and a ZFN target site. Both segment contained an 870 bp region of homology in direct repeat orientation. A ZFN-induced DSB between the segments allows efficient SSA HR, resulting in an active luciferase gene. Luciferase activity is therefore proportional to ZFN activity. The dimeric ZFN used in this study contained the zinc finger domains GZF1 and GZF3, which were designed to recognize the sites indicated in Figure 1A.[19] The ZFN also contained the FokI cleavage domain variants RR and DD, respectively.[18] These variants restrict activity to only GZF1/GZF3 heterodimers, reducing cytotoxicity presumably due to off-target DSBs at perfect or imperfect GZF1 or GZF3 homodimer sites. We have previously shown this ZFN to be active on SSA reporter targets containing a 6 bp spacer.[18] In this study, we examined spacers in the range of 4–8 bp. Assays were performed essentially as described previously.[18] HEK 293T cells were co-transfected with ZFN expression plasmids and a SSA reporter plasmid. ZFN expression (Fig. 2A) and luciferase activity (Fig. 2B) were measured at 48 h post-transfection.[20] We observed a strong peak of ZFN activity on targets with 6 bp DNA spacers, but sixfold less activity on targets with 4, 5, 7, and 8 bp spacers. The results suggest the 6 aa linker strongly restricts ZFN activity to 6 bp spacers.

Our results are also in good agreement with an early study in *Xenopus* oocytes, which found a 6 aa linker of somewhat different composition to that used here also demonstrated a strong restriction of activity to a 6 bp spacer in a similar plasmid-based SSA assay.[15] In addition, during the course of this study, a much more extensive and systematic study of ZFN linker and DNA spacer lengths in human cells was reported.[21] That study examined the same TGAAAR linker (among others) in the context of a different zinc finger domain and a wild type FokI nuclease domain. Our results using a plasmid-based SSA assay are largely in agreement with their results using a plasmid-based gene correction assay, although they observed a slightly higher activity with a 7 bp spacer. The authors also noted that the linkers were generally less restrictive in a chromosome-based assay compared to a plasmid-based assay. The activity with the 6 aa linker was generally similar; on a 6 bp spacer the activity was about fivefold greater than on the 7 bp spacer in the episomal assay, compared to only about fourfold greater in the chromosomal one. More dramatically, the activity on a 16 bp spacer was negligible in the episomal assay, but similar to the 7 bp spacer in the chromosomal one. Nonetheless, the authors concluded that, among the linkers they investigated, the 6 aa linker was the most specific for a 6 bp spacer. It is not immediately obvious why we failed to observe any increased activity on a 7 bp

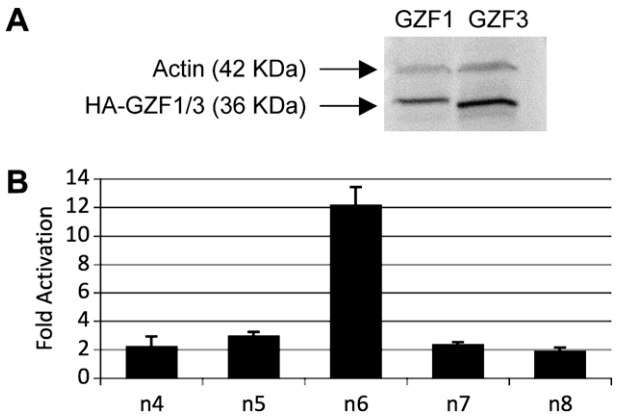

Figure 2. The activity of GZF1-DD and GZF2-RR on SSA reporters with different spacers in the ZFN target site. (A) A Western blot demonstrating expression of both ZFN component proteins in HEK 293T cells. Actin expression is also shown as a loading control. (B) The fold activation of recombination by the ZFN represents the luciferase expression level in cells co-transfected with ZFN and SSA reporter compared to those transfected with SSA reporter only. Error bars represent the standard deviation of at least two experiments.

spacer in our assay. One interpretation is that, despite similar trends, the exact spectrum of activities may depend on a variety of factors including the assay, the cell type, and the specific compositions and concentrations of the ZFNs and target sites.

There are several critical parameters to maximize the success of a ZFN experiment. Some parameters may be beyond the investigator's ability to manipulate. The accessibility of the target site on the chromosome is one example, although the use of chromatin remodeling drugs may be useful in some cases.[22] The most crucial design parameter is the ability to engineer zinc finger DNA-binding proteins that have both high affinity and high specificity for their target sites.[10–12] The zinc finger domains used here were created using the Modular Assembly methods of Barbas.[19] Methods that have come in the wake of Modular Assembly, such a Bipartite Selection[7] and OPEN,[8] have made improvements by including additional combinatorial selection steps at the level of the full target site. Such methods have shown tremendous potential for generating high quality ZFNs. However, the need for additional combinatorial steps after more than a decade of research on engineering zinc fingers illustrates that the structural determinants of affinity and specificity are still largely elusive. The generation of high quality zinc fingers remains a significant challenge, and is the most important consideration when selecting a target site. Off-target events can be partly ameliorated by the use of obligate heterodimer variants of the FokI cleavage domains, such as the DD/RR variants used in this study.[18,23] In some cases, such variants have turned cytotoxic ZFNs into catalysts capable of achieving >10% gene correction. Also useful is the ability to target sites that contain 5 bp, 7 bp or other length spacers beyond the canonical 6 bp spacer. However, this ability may come at the loss of some specificity, since linkers that can narrowly restrict activity to these spacings have not been identified.[21]

The current study concurs with others[21] that a 6 aa linker most narrowly restricts ZFN activity to sites with a 6 bp spacer. These results suggest that the ideal ZFN would target a site with a 6 bp spacer using this linker. However, we agree with the authors of the recent linker study[21] that the best strategy for optimization will likely require some empirical testing of ZFN parameters on the actual target site in the appropriate cell.

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