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(54) Titre : COMPOSITIONS POUR RELIER DES MODULES EN DOIGT DE ZINC
(54) Title: COMPOSITIONS FOR LINKING ZINC FINGER MODULES

Portability Studies With Linkers Selected to Skip One Basepair

A

Linker		ELISA Score											
Sequence	Designation	ZFP1	ZFP2	ZFP3	ZFP4	ZFP5	ZFP6	ZFP7	ZFP8	ZFP9	ZFP10	ZFP11	ZFP12
TGGGGSQKP	flexible	0.75	1.08	0.21	0.26	0.08	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TVPRPTPPKP	1e	1.19	1.30	1.05	1.10	0.75	0.29	0.48	0.38	0.50	0.18	0.05	0.05
TYPRPIAAKP	1f	1.25	1.35	1.03	0.94	0.96	0.30	0.33	0.12	0.30	0.11	0.05	0.05
TPNRRPAPKP	1d	1.20	1.08	1.04	0.83	0.85	0.25	0.39	0.28	0.24	0.12	0.05	0.05
THPRAPIPKP	1c	0.92	1.18	0.66	0.56	0.91	0.23	0.21	0.26	0.18	0.05	0.05	0.05

B

Linker		ELISA Score Normalized to Flexible Linker												
Sequence	Designation	ZFP1	ZFP2	ZFP3	ZFP4	ZFP5	ZFP6	ZFP7	ZFP8	ZFP9	ZFP10	ZFP11	ZFP12	Average
TGGGGSQKP	flexible	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
TVPRPTPPKP	1e	1.6	1.2	4.9	4.1	9.2	5.9	9.6	7.6	10.1	3.6	1.0	1.0	5.0
TYPRPIAAKP	1f	1.7	1.2	4.9	3.5	11.7	5.9	6.5	2.3	6.0	2.2	1.0	1.0	4.0
TPNRRPAPKP	1d	1.6	1.0	4.9	3.1	10.4	4.9	7.8	5.7	4.8	2.4	1.0	1.0	4.0
THPRAPIPKP	1c	1.2	1.1	3.1	2.1	11.2	4.7	4.3	5.3	3.6	1.0	1.0	1.0	3.3

underlined values show >4-fold improvement

(57) Abrégé/Abstract:
Disclosed herein are compositions for linking DNA binding modules to allow for specific and selective binding to module subsites separated by 1 or more base pairs. Also described are methods of making and using compositions comprising these linkers.

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(54) **Title**: COMPOSITIONS FOR LINKING ZINC FINGER MODULES

FIG. 7

Portability Studies With Linkers Selected to Skip One Basepair

A

Linker Sequence	Designation	ELISA Score ZFP1	ZFP2	ZFP3	ZFP4	ZFP5	ZFP6	ZFP7	ZFP8	ZFP9	ZFP10	ZFP11	ZFP12
TGGGGSQKP	flexible	0.75	1.08	0.21	0.26	0.08	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TVPRPTPPKP	1e	1.19	1.30	1.05	1.10	0.75	0.29	0.48	0.38	0.50	0.18	0.05	0.05
TYPRPIAAKP	1f	1.25	1.35	1.03	0.94	0.96	0.30	0.33	0.12	0.30	0.11	0.05	0.05
TPNRRPAPKP	1d	1.20	1.08	1.04	0.83	0.85	0.25	0.39	0.28	0.24	0.12	0.05	0.05
THPRAPIPKP	1c	0.92	1.18	0.66	0.56	0.91	0.23	0.21	0.26	0.18	0.05	0.05	0.05

B

Linker Sequence	Designation	ELISA Score Normalized to Flexible Linker ZFP1	ZFP2	ZFP3	ZFP4	ZFP5	ZFP6	ZFP7	ZFP8	ZFP9	ZFP10	ZFP11	ZFP12	Average
TGGGGSQKP	flexible	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
TVPRPTPPKP	1e	1.6	1.2	4.9	4.1	9.2	5.9	9.6	7.6	10.1	3.6	1.0	1.0	5.0
TYPRPIAAKP	1f	1.7	1.2	4.9	3.5	11.7	5.9	6.5	2.3	6.0	2.2	1.0	1.0	4.0
TPNRRPAPKP	1d	1.6	1.0	4.9	3.1	10.4	4.9	7.8	5.7	4.8	2.4	1.0	1.0	4.0
THPRAPIPKP	1c	1.2	1.1	3.1	2.1	11.2	4.7	4.3	5.3	3.6	1.0	1.0	1.0	3.3

underlined values show >4-fold improvement

(57) **Abstract**: Disclosed herein are compositions for linking DNA binding modules to allow for specific and selective binding to module subsites separated by 1 or more base pairs. Also described are methods of making and using compositions comprising these linkers.

COMPOSITIONS FOR LINKING ZINC FINGER MODULES

[0001]

[0002]

TECHNICAL FIELD

[0003] The present disclosure is in the fields of genome and protein engineering.

BACKGROUND

[0004] Zinc-finger proteins with recognition regions that are engineered to bind to selected target sites are regularly linked to other zinc-finger proteins as well as to regulatory domains and used to modify gene expression and genomic target sites. For example, artificial nucleases comprising DNA binding domains operably linked to cleavage domains have been used for targeted alteration of genomic sequences, including, insertion of exogenous sequences, inactivation of one or more endogenous genes, creation of organisms (*e.g.*, animal or crops) and cell lines with altered gene expression patterns, and the like. See, *e.g.*, U.S. Patent Publication Nos. 20050064474; 20060063231; 20070134796; 20080015164 and International Publication No. 2007/139982.

[0005] Zinc-finger protein modules (*e.g.*, engineered zinc fingers of one or more fingers) are typically linked to each other using "canonical" linker sequences of 5 amino acids such as TGEKP (SEQ ID NO:1) or longer flexible linkers. See, U.S. Patent Nos. 6,479,626; 6,903,185; 7,153,949 and U.S. Patent Publication No. 20030119023. However, zinc-finger protein modules linked via these canonical linkers bind most effectively only when there is no gap between the linked module target subsites in the target nucleic acid molecule. Furthermore, previously-described long, flexible linkers designed to allow the linked modules to bind to target sites with 1, 2 or 3 base pair gaps do not distinguish between these different base pair gaps in terms of binding. See, U.S. Patent Nos. 6,479,626; 6,903,185; 7,153,949 and U.S. Patent Publication No. 20030119023. Thus, there remains a need for methods and compositions for linking zinc-finger modules to each other that improves both the affinity of proteins that span a 1, 2, or 3 bp intermodule gap, as well improve the

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selectivity of these proteins for binding targets that span a gap of a desired length and do not bind non-selectively to other targets without the gap of that desired length. Linkers for zinc-finger modules that distinguish between 0, 1, 2, 3 or even more base pair gaps between adjacent module subsites would allow for greater design capability of any zinc-finger fusion proteins, including zinc-finger transcription factors (ZFP-TFs) and zinc finger nucleases (ZFNs).

SUMMARY

[0006] Disclosed herein are linkers for use in linking DNA-binding modules (*e.g.*, zinc-finger modules) to each other. Also described are fusion proteins, for example zinc-finger proteins comprising these linkers which are in turn fused to regulatory domains such as transcriptional regulatory domains or to nucleases. The disclosure also provides methods of using these fusion proteins and compositions thereof for modulation of gene expression, targeted cleavage of cellular DNA (*e.g.*, endogenous cellular chromatin) in a region of interest and/or homologous recombination at a predetermined region of interest in cells.

[0006a] Certain exemplary embodiments provide a multi-finger zinc finger protein that specifically binds to a target site, the multi-finger zinc finger protein comprising non-naturally occurring zinc finger modules, wherein each zinc finger module binds to a target subsite and at least two of the non-naturally occurring zinc finger DNA-binding modules that bind to target subsites separated by 1 or 2 base pairs are joined by an amino acid linker of 5 to 20 amino acid residues between the last residue of the N-terminal zinc finger module and the first residue of C-terminal zinc finger module, the amino acid linker comprising an N-terminal amino acid linker residue adjacent to the N-terminal zinc finger module, a C-terminal amino acid linker residue adjacent to the C-terminal zinc finger module, and amino acid residues internal to the N- and C-terminal amino acid linker residues, wherein said amino acid linker is selected from the group consisting of: TPDAPKPKP, TPGLHRPKP, TEPRAKPPKP, TPSHTPRPKP, TGYSIPRPKP, TYPRPIAAKP, THPRAPIPKP, TPNRRPAPKP, TSPRLPAPKP, TCPRPPTRKP, TSSPRSNAKP, TVSPAPCRSKP, TPDRPISTCKP, TPRPPIPKP, TQRPQIPPKP, TPNRCPPTKP, TYPRPLLAKP, TPLCQRPMKQKP, TGLPKPKP, TSRPRPKP, TLPLPRPKP, TVPRPTPPKP, and TLPPCFRPKP when the target subsites are separated by 1 base pair, or are selected from the group consisting of TLAPRPYRPPKP, TPNPHRRTDPSHKP,

TPGGKSSRTDRNKP, TNTTRPYRPPKP, TGSLRPYRRPKP, TGEARPYRPPKP, TETTRPFRPPKP, TSINRPFRRPKP, and TASCPRPFRPPKP when the target subsites are separated by 2 base pairs.

[0007] In one aspect, described herein are linkers comprising 5 or more amino acids between the last residue of the amino (N)-terminal finger (typically the carboxy (C)-terminal zinc-coordinating residue) and the first residue of the C-terminal finger (typically the first (N-terminal)- conserved aromatic residue), for example 7-17 amino acids. In certain embodiments, the linker comprises an N-terminal residue, a C-terminal residue, and residues internal to the terminal residues, and further wherein the N-terminal residue or internal residues comprises at least one proline residue, for example a linker comprising the amino acid sequence $X^{N\text{-term}}$-X_n-$X^{C\text{-term}}$, wherein X is any amino acid residue, X_n comprises at least 3 amino acid residues and at least one of $X^{N\text{-term}}$ and X_n comprises a proline residue. In certain embodiments, the linker comprises at least two proline residues (*e.g.*, 2, 3, 4 or more). In other embodiments, where the linker comprises at least one proline residue and at least one basic residue

(*e.g.*, Arg, His or Lys). In other embodiments, where the linker comprises at least two basic residue (*e.g.*, Arg, His or Lys). In certain embodiments, the linker is one shown in any of Tables 4, 5, 6, 9, 10, 11 or 13.

[0008] In another aspect, fusion polypeptides comprising a linker as described herein are provided.

[0009] In another aspect, polynucleotides encoding any of the linkers or fusion proteins as described herein are provided.

[0010] In yet another aspect, cells comprising any of the polypeptides (*e.g.*, fusion polypeptides) and/or polynucleotides as described herein are also provided.

[0011] In a further aspect, organisms (*e.g.* mammals, fungi and plants) comprising the polypeptides (*e.g.* fusion polypeptides) and/or polynucleotides as described herein are also provided.

[0012] A fusion protein can be expressed in a cell, *e.g.*, by delivering the fusion protein to the cell or by delivering a polynucleotide encoding the fusion protein to a cell. If the polynucleotide is DNA, it is then transcribed and translated to generate the fusion protein. If delivered as an RNA molecule, it is then immediately translated, thus generating the fusion protein. Methods for polynucleotide and polypeptide delivery to cells are presented elsewhere in this disclosure.

[0013] These and other aspects will be readily apparent to the skilled artisan in light of disclosure as a whole.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] **Figure 1, panels A and B,** show amino sequences of exemplary zinc finger proteins and linkers. **Figure 1A** shows the amino acid sequence of each host ZFP (F1-F4 of ZFP 8196 shown in SEQ ID NO:130; SEQ ID NO:131; SEQ ID NO:132 and SEQ ID NO:133; F1 to F4 of ZFP 7263 shown in SEQ ID NO:134; SEQ ID NO:135; SEQ ID NO:136 and SEQ ID NO:137; F1 to F4 of ZFP 7264 shown in SEQ ID NO:138; SEQ ID NO:139; SEQ ID NO:140 and SEQ ID NO:141) used for these studies. Amino acids are designated by single letter code. Each sequence is listed in the amino terminal → carboxy terminal direction, so that the amino terminus of each protein is the first methionine of finger 1, and the carboxy terminus is the final serine of finger 4. "F1", "F2", "F3" and "F4" designate the first, second, third and fourth fingers, respectively, of each protein. Underlining denotes amino acid residues at finger junctions which are conventionally considered to be linker sequence.

Recognition helices are boxed. **Figure 1B** shows linker library designs in which each library was generated by replacing codons for two or three residues in the central linker with a mixture of two to twelve fully randomized codons. Library codons are denoted by $(NNS)_{2-12}$.

[0015] **Figure 2, panels A through D**, are graphs depicting gap selectivity of phage pools with the indicated zinc finger proteins and linkers. Figure 2A shows the phage pool selected from the ZFP8196 library using a target with a 1bp inserted base (ATAAACTGdCAAAAGGC (SEQ ID NO:33) (Table 2A)) that was tested for binding to each ZFP8196 target in Table 2C. Figure 2B shows the phage pool selected from the ZFP7263 library using a target with a 1bp inserted base (CCACTCTGhTGGAAGTG (SEQ ID NO:43) (Table 2A)) that was tested for binding to each ZFP7263 target in Table 2C. Figure 2C shows the phage pool selected from the ZFP7264 library using a target with a 1bp inserted base (TTAAAGCGhGCTCCGAA (SEQ ID NO:38) (Table 2A)) tested for binding to each ZFP7264 target in Table 2C. Figure 2D shows the phage pool selected from the ZFP8196 library using a target with a 2bp inserted base (ATAAACTGdbCAAAAGGC (SEQ ID NO:34) (Table 2A)) tested for binding to each ZFP8196 target in Table 2C. Each test also included two control targets for the other two host ZFPs to rule out nonspecific binding to DNA as well as a negative control sample which did not include a target site. The % of phage which successfully bound each target is indicated. Each phage pool was from the fifth round of selection. Retention efficiency was determined essentially as previously described (Rebar, *et al. Methods in Enzymology*, 1996 (267):129-149).

[0016] **Figure 3, panels A and B**, show linkers selected for target sites containing the indicated gap. Figure 3A shows linker sequences selected for skipping a 1 bp gap in the context of ZFP8196, ZFP7263, and ZFP7264 (SEQ ID NOs:142 to 166). Figure 3B shows linker sequences (SEQ ID NOs:167 to 174) for skipping a 2 bp gap in the context of ZFP8196. Selected linkers are enriched for proline and arginine (shaded). Length preferences are also apparent and depend on the number of skipped bases.

[0017] **Figure 4, panels A through E**, are graphs depicting gap selectivity for linkers selected to skip 1 basepair in the zinc finger protein designated ZFP8196. In each panel, ELISA scores were normalized to the parent, non-skipping linker on its non-gapped target site. "Gap sequence" refers to the identity of the base(s) between

the module subsites where (-) indicates the nongapped target. Figures 4A-4C depict results from three of the 1 bp gap skipping linkers, (linkers referred to as 1f (SEQ ID NO:54), 1d (SEQ ID NO:56) and 1c (SEQ ID NO:55)). Figure 4D shows results with a standard flexible linker that has previously been shown to enable modification of an endogenous locus in human cells (TGGGGSQKP, SEQ ID NO:2) (See Hockemeyer *et al.* (2009) *Nature Biotechnology* 27:851-857) and Figure 4E depicts the results for a previously published flexible linker (LRQKDERP, SEQ ID NO:3) (See Kim JS & Pabo CO (1998) *Proc Natl Acad Sci U S A* 95(6):2812-2817). The selected linkers 1c, 1d and 1f (Figures 4A-4C) all show clear preferences for the four target sites with a single base pair gap whereas the control linkers in Figures 4D and 4E show less effective overall binding and little gap selectivity.

[0018] **Figure 5, panels A through D,** are graphs depicting gap selectivity for linkers selected to skip 1 base pair in ZFP7264. Figures 5A to 5C depict the results from an ELISA testing of the 1e linker (SEQ ID NO:12) in the ZFP7264 background. Figure 5A shows the results for the 1e linker, selected to skip a 1 bp gap between the module subsites. Figure 5B shows the results for a standard flexible linker (TGGGGSQKP, SEQ ID NO:2), and Figure 5C shows the results for a the flexible linker LRQKDERP (SEQ ID NO:3). ELISA scores are normalized to the parent, nonskipping ZFP7264 on its non-gapped target. "Gap sequence" is the identity of the skipped base(s) between the module subsites where (-) indicates the nongapped target. Figure 5D shows an expanded version of the data from Figure 5B where the ELISA score range is 0-0.6 as compared to 0-5 in the other panels.

[0019] **Figure 6, panels A through F,** are graphs depicting gap selectivity for the linkers selected to skip 2bp in ZFP8196. Figures 6A to 6E depict the results from an ELISA testing the linkers selected to skip a 2 bp gap between the module subsites in the ZFP8196 background. Figures 6A through 6C show the results for the selected linkers 2f (SEQ ID NO:69), 2d (SEQ ID NO:70) and 2e (SEQ ID NO:71), whereas Figures 6D shows the results for a previously published flexible linker (LRQKDGGGSERP (SEQ ID NO: 68)) and Figure 6E shows the results for a standard flexible linker (TGGGGSGGSQKP (SEQ ID NO: 14)). Figure 6F shows an expanded version of the data shown in Figure 6E where the ELISA score range is 0-0.1 as compared to 0-1 in the other panels. "Gap sequence" is the identity of the base(s) between the module subsites where (-) indicates the nongapped target. The selected linkers (Figures 6A-6C) demonstrate a clear preference for a 2 bp gap as

compared to a 1 bp gap or no gap whereas the control linkers in Figures 6D and 6E show less effective overall binding and little gap selectivity.

[0020] **Figure 7, panels A and B,** depict a summary of ELISA data from a study designed to analyze the portability of the 1 bp skipping linkers to different ZFP backgrounds. Twelve different ZFPs were tested (indicated as ZFP1, ZFP2 *etc.*). Figure 7A shows ELISA scores normalized to standard positive control ZFPs that have been shown to efficiently modify an endogenous IL2Rγ locus when used as ZFNs (Urnov *et al.* (2005) *Nature* 435(7042):646-651). Figure 7B shows all scores further normalized to each parent ZFP bearing the standard flexible linker TGGGGSQKP (SEQ ID NO:2). Underlined values in Figure 7B indicate a >4-fold improvement in ELISA score for ZFPs with the selected linkers (1e (SEQ ID NO:12), 1f (SEQ ID NO:54), 1d (SEQ ID NO:56), and 1c (SEQ ID NO:55)) compared to the same host ZFP with the flexible linker TGGGGSQKP (SEQ ID NO:2). Overall, linkers 1e, 1f, 1d and 1c lead to a general increase in ELISA score of 3-5 fold over the flexible linker.

[0021] **Figure 8, panels A and B,** depict a summary of ELISA data from a study designed to analyze the portability of the 2 bp skipping linkers to different ZFP backgrounds. Six different ZFPs were tested (indicated as ZFP13, ZFP14 *etc.*). Figure 8A shows ELISA scores normalized to standard positive control ZFPs that have been shown to efficiently modify an endogenous IL2Rγ locus when used as ZFNs (Urnov *et al.* (2005) *Nature* 435(7042):646-651). Figure 8B shows all scores further normalized to each parent ZFP bearing the standard flexible linker TGGGGSGGSQKP (SEQ ID NO:14). Underlined values in Figure 8B indicate a >2-fold improvement in ELISA score for ZFPs with the selected linkers (2f (SEQ ID NO:69), 2d (SEQ ID NO:70) and 2e (SEQ ID NO:71)) compared to the same host ZFP with the flexible linker TGGGGSGGSQKP (SEQ ID NO:14). Overall, linkers 2f (SEQ ID NO:69), 2d (SEQ ID NO:70) and 2e (SEQ ID NO:71) led to a general increase in ELISA score of 1.9-2.4 fold over the flexible linker.

[0022] **Figure 9, panels A and B,** depict results of endogenous gene modification studies, as determined by CEL-I assays, with ZFNs containing selected linkers. Figures 9A and 9B depict example gels used to determine ZFN nuclease activity at endogenous loci by the CEL-I assay (measuring non-homologous end joining (NHEJ) activity, Surveyor™, Transkaryotic) to determine if linkers as

described herein can be used in the context of different ZFNs. The gel shown in Figure 9A depicts the results from the 1e (SEQ ID NO:12), 1f (SEQ ID NO:54), 1d (SEQ ID NO:56), and 1c (SEQ ID NO:55) linkers in the ZFN3 and ZFN4 backgrounds. The gel shown in Figure 9B depicts the results from the 2f, 2d and 2e linkers in the ZFN14 background. Percent gene modification by NHEJ, "Gene mod. (%)", is indicated at the bottom of the lanes. The negative control, "neg", is a sample transfected with a GFP bearing plasmid. The results from the ZFNs using a standard flexible linker (TGGGGSQKP (SEQ ID NO:2) for Figure 9A and TGGGGSGGSQKP (SEQ ID NO:14) for Figure 9B) are shown in the lanes labeled "C". Unlabeled lanes contain samples of ZFNs bearing other linkers that were not further developed in these studies. The data in the gels demonstrates that the linkers as described herein significantly increase levels of gene modification as compared to the flexible linkers.

[0023] **Figure 10, panels A and B,** depict a summary of gene modification studies for ZFNs as described above for Figure 9 containing the indicated linkers selected to skip 1bp. Figure 10A is the quantitation of the percent gene modification for each ZFN with the set of five linkers tested (flexible, 1e (SEQ ID NO:12), 1f (SEQ ID NO:54), 1d (SEQ ID NO:56), and 1c (SEQ ID NO:55)). Figure 10B shows this same data normalized to the flexible linker (TGGGGSQKP, SEQ ID NO:2) and also shows the average increase in gene modification across all the active ZFN pairs. Samples produced using high expression conditions (see Example 3) are highlighted in grey. ZFNs bearing exemplary linkers that improved the level of gene modification by >2-fold are underlined in Figure 10B. Overall, ZFNs bearing linkers 1e (SEQ ID NO:12), 1f (SEQ ID NO:54), 1d (SEQ ID NO:56), and 1c (SEQ ID NO:55) lead to an average increase in gene modification of 1.8 to 2.8 fold over their respective host ZFNs bearing the flexible linker.

[0024] **Figure 11, panels A and B,** depict a summary of gene modification studies as described for Figure 9, for ZFNs containing the indicated linkers selected to skip a 2 bp gap between the module subsites of the 6 host ZFNs. Figure 11A is the quantitation of the percent of gene modification for each ZFN with the set of four linkers tested (flexible, 2f (SEQ ID NO:69), 2d (SEQ ID NO:70) and 2e (SEQ ID NO:71)). Figure 11B shows this same data normalized to the flexible linker (TGGGGSGGSQKP, SEQ ID NO:14) and also shows the average increase across all the active ZFN pairs. Samples produced using high expression conditions (see Example 3) are highlighted in grey. ZFNs bearing exemplary linkers that improved

the level of gene modification by >2-fold are underlined in Figure 11B. ZFNs bearing linkers 2f (SEQ ID NO:69), 2d (SEQ ID NO:70) and 2e (SEQ ID NO:71) led to an average increase in gene modification of 1.5- 2.0 fold over their respective host ZFNs bearing the flexible linker.

[0025] **Figure 12,** shows the amino acid sequence of the host ZFP8196 used for the secondary selection for linkers spanning a 2-bp gap. Amino acids are designated by single letter code. The sequence is listed in the amino terminal → carboxy terminal direction, so that the amino terminus of the protein is the first methionine of finger 1, and the carboxy terminus is the final serine of finger 4. "F1" (SEQ ID NO:130), "F2" (SEQ ID NO:131), "F3" (SEQ ID NO:132) and "F4" (SEQ ID NO:133) designate the first, second, third and fourth fingers, respectively, of the protein. Recognition helices are boxed. The linker library was generated by replacing codons for two residues in the central linker with a mixture of five to seven fully randomized codons, followed by one codon randomized to obtain either phenylalanine (F), lysine (L), or tyrosine (Y) residues, and the final three codons were fixed to be arginine (R), proline (P), and proline (P). Library codons are denoted by $(NNS)_{5-7}$ and (F/L/Y).

[0026] **Figure 13, panels A and B,** depict the gap selectivity of the phage pool from the secondary selection for linkers spanning a 2-bp gap and the resulting amino acid sequences of the clones obtained in the selection. Figure 13A shows the phage pool selected from the ZFP8196 library using a target with a 2bp inserted gap (ATAAACTGdbCAAAAGGC (SEQ ID NO:34) (Table 2A)) tested for binding to each ZFP8196 target in Table 2C. Each test also included a control target for one other host ZFP to rule out nonspecific binding to DNA as well as a negative control sample which did not include a target site. The % of phage which successfully bound each target is indicated. The phage pool was from the sixth round of selection. Retention efficiency was determined essentially as previously described (Rebar, *et al. Methods in Enzymology*, 1996 (267):129-149). Figure 13B shows amino acid sequences (SEQ ID NO:175 to 210) of linkers selected for skipping a 2 bp gap from the secondary selection in the context of ZFP8196. Selected linkers are enriched for proline and arginine (shaded).

DETAILED DESCRIPTION

[0027] Described herein are compositions for linking DNA-binding domains, particularly zinc-finger modules, to other zinc-finger modules. Unlike previously described linkers, the linkers described herein allow preferential and/or selective binding of targets bearing gaps between module subsites of 1 or 2 bp. The linkers are also capable of binding targets bearing 1, or 2 bp gaps at higher affinities than current linker designs., Exemplary linkers are shown in Tables 11 and 13. Thus, certain linkers described herein significantly increase the ability to design zinc-finger proteins which bind to specific target sites, thereby increasing the activity of fusion proteins (*e.g.*, ZFP-TFs or ZFNs) comprising these linkers.

General

[0028] Practice of the methods, as well as preparation and use of the compositions disclosed herein employ, unless otherwise indicated, conventional techniques in molecular biology, biochemistry, chromatin structure and analysis, computational chemistry, cell culture, recombinant DNA and related fields as are within the skill of the art. These techniques are fully explained in the literature. *See*, for example, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, Second edition, Cold Spring Harbor Laboratory Press, 1989 and Third edition, 2001; Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, 1987 and periodic updates; the series METHODS IN ENZYMOLOGY, Academic Press, San Diego; Wolffe, CHROMATIN STRUCTURE AND FUNCTION, Third edition, Academic Press, San Diego, 1998; METHODS IN ENZYMOLOGY, Vol. 304, "Chromatin" (P.M. Wassarman and A. P. Wolffe, eds.), Academic Press, San Diego, 1999; and METHODS IN MOLECULAR BIOLOGY, Vol. 119, "Chromatin Protocols" (P.B. Becker, ed.) Humana Press, Totowa, 1999.

Definitions

[0029] The terms "nucleic acid," "polynucleotide," and "oligonucleotide" are used interchangeably and refer to a deoxyribonucleotide or ribonucleotide polymer, in linear or circular conformation, and in either single- or double-stranded form. For the purposes of the present disclosure, these terms are not to be construed as limiting with respect to the length of a polymer. The terms can encompass known analogues of natural nucleotides, as well as nucleotides that are modified in the base, sugar and/or phosphate moieties (*e.g.*,

phosphorothioate backbones). In general, an analogue of a particular nucleotide has the same base-pairing specificity; *i.e.*, an analogue of A will base-pair with T.

[0030] The terms "polypeptide," "peptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. The term also applies to amino acid polymers in which one or more amino acids are chemical analogues or modified derivatives of a corresponding naturally-occurring amino acids.

[0031] A polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. A "conservative substitution," when describing a protein, refers to a change in the amino acid composition of the protein that does not substantially alter the protein's activity. Thus, "conservatively modified variations" of a particular amino acid sequence refers to amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (*e.g.*, acidic, basic, positively or negatively charged, polar or non-polar, etc.) such that the substitutions of even critical amino acids do not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. See, *e.g.*, Creighton (1984) Proteins, W. H. Freeman and Company. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations."

[0032] "Binding" refers to a sequence-specific, non-covalent interaction between macromolecules (*e.g.*, between a protein and a nucleic acid). Not all components of a binding interaction need be sequence-specific (*e.g.*, contacts with phosphate groups in a DNA backbone), as long as the interaction as a whole is sequence-specific. Such interactions are generally characterized by a dissociation constant (K_d) of 10^{-6} M^{-1} or lower. "Affinity" refers to the strength of binding: increased binding affinity being defined by a lower K_d.

[0033] A "binding protein" is a protein that is able to bind non-covalently to another molecule. A binding protein can bind to, for example, a DNA molecule (a DNA-binding protein), an RNA molecule (an RNA-binding protein) and/or a protein molecule (a protein-binding protein). In the case of a protein-binding protein, it can bind to itself (to form homodimers, homotrimers, *etc.*) and/or it can bind to one or more molecules of a different protein or proteins. A binding protein can have more than one type of binding

activity. For example, zinc finger proteins have DNA-binding, RNA-binding and protein-binding activity.

[0034] A "zinc finger DNA binding protein" (or binding domain) is a protein, or a domain within a larger protein, that binds DNA in a sequence-specific manner through one or more zinc fingers, which are regions of amino acid sequence within the binding domain whose structure is stabilized through coordination of a zinc ion. The term zinc finger DNA binding protein is often abbreviated as zinc finger protein or ZFP.

[0035] Zinc finger binding domains (*e.g.*, recognition regions of zinc fingers) can be "engineered" to bind to a predetermined nucleotide sequence. Non-limiting examples of methods for engineering zinc finger proteins are design and selection. A designed zinc finger protein is a protein not occurring in nature whose design/composition results principally from rational criteria. Rational criteria for design include application of substitution rules and computerized algorithms for processing information in a database storing information of existing ZFP designs and binding data. See, for example, US Patents 6,140,081; 6,453,242; and 6,534,261; see also WO 98/53058; WO 98/53059; WO 98/53060; WO 02/016536 and WO 03/016496.

[0036] A "selected" zinc finger protein is a protein not found in nature whose production results primarily from an empirical process such as phage display, interaction trap or hybrid selection. See *e.g.*, US 5,789,538; US 5,925,523; US 6,007,988; US 6,013,453; US 6,200,759; WO 95/19431; WO 96/06166; WO 98/53057; WO 98/54311; WO 00/27878; WO 01/60970 WO 01/88197 and WO 02/099084.

[0037] A "regulatory domain" or "functional domain" refers to a protein or a protein domain that has transcriptional modulation activity when tethered to a DNA binding domain, *i.e.*, a ZFP. Typically, a regulatory domain is covalently or non-covalently linked to a ZFP (*e.g.*, to form a fusion molecule) to effect transcription modulation. Regulatory domains can be activation domains or repression domains. Activation domains include, but are not limited to, VP16, VP64 and the p65 subunit of nuclear factor Kappa-B. Repression domains include, but are not limited to, KOX, KRAB MBD2B and v-ErbA. Additional regulatory domains include, *e.g.*, transcription factors and co-factors (*e.g.*, MAD, ERD, SID, early growth response factor 1, and nuclear hormone receptors), endonucleases, integrases, recombinases, methyltransferases, histone acetyltransferases, histone deacetylases etc. Activators and repressors include co-activators and co-repressors (see, *e.g.*, Utley *et al.*, *Nature* 394:498-502 (1998)). Alternatively, a ZFP

can act alone, without a regulatory domain, to effect transcription modulation. Regulatory domains also can be nucleases, such as cleavage domains or cleavage half-domains.

[0038] "Cleavage" refers to the breakage of the covalent backbone of a DNA molecule. Cleavage can be initiated by a variety of methods including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible, and double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events. DNA cleavage can result in the production of either blunt ends or staggered ends. In certain embodiments, fusion polypeptides are used for targeted double-stranded DNA cleavage.

[0039] A "cleavage half-domain" is a polypeptide sequence which, in conjunction with a second polypeptide (either identical or different) forms a complex having cleavage activity (preferably double-strand cleavage activity). The terms "first and second cleavage half-domains;" "+ and – cleavage half-domains" and "right and left cleavage half-domains" are used interchangeably to refer to pairs of cleavage half-domains that dimerize.

[0040] An "engineered cleavage half-domain" is a cleavage half-domain that has been modified so as to form obligate heterodimers with another cleavage half-domain (*e.g.,* another engineered cleavage half-domain). *See, also,* U.S. Patent Publication No. 20050064474; and WO 2007/13989.

[0041] "Chromatin" is the nucleoprotein structure comprising the cellular genome. Cellular chromatin comprises nucleic acid, primarily DNA, and protein, including histones and non-histone chromosomal proteins. The majority of eukaryotic cellular chromatin exists in the form of nucleosomes, wherein a nucleosome core comprises approximately 150 base pairs of DNA associated with an octamer comprising two each of histones H2A, H2B, H3 and H4; and linker DNA (of variable length depending on the organism) extends between nucleosome cores. A molecule of histone H1 is generally associated with the linker DNA. For the purposes of the present disclosure, the term "chromatin" is meant to encompass all types of cellular nucleoprotein, both prokaryotic and eukaryotic. Cellular chromatin includes both chromosomal and episomal chromatin.

[0042] A "chromosome," is a chromatin complex comprising all or a portion of the genome of a cell. The genome of a cell is often characterized by its karyotype,

which is the collection of all the chromosomes that comprise the genome of the cell. The genome of a cell can comprise one or more chromosomes.

[0043] An "episome" is a replicating nucleic acid, nucleoprotein complex or other structure comprising a nucleic acid that is not part of the chromosomal karyotype of a cell. Examples of episomes include plasmids and certain viral genomes.

[0044] An "accessible region" is a site in cellular chromatin in which a target site present in the nucleic acid can be bound by an exogenous molecule which recognizes the target site. Without wishing to be bound by any particular theory, it is believed that an accessible region is one that is not packaged into a nucleosomal structure. The distinct structure of an accessible region can often be detected by its sensitivity to chemical and enzymatic probes, for example, nucleases.

[0045] A "target site" or "target sequence" is a nucleic acid sequence that defines a portion of a nucleic acid to which a binding molecule will bind, provided sufficient conditions for binding exist. For example, the sequence 5'-GAATTC-3' is a target site for the Eco RI restriction endonuclease.

[0046] A "module subsite" is a nucleic acid sequence that defines a portion of a nucleic acid to which a zinc-finger module (*e.g.* 1, 2, 3 or more zinc fingers) within a larger zinc-finger DNA binding protein will bind, provided sufficient conditions for binding exist.

[0047] An "exogenous" molecule is a molecule that is not normally present in a cell, but can be introduced into a cell by one or more genetic, biochemical or other methods. "Normal presence in the cell" is determined with respect to the particular developmental stage and environmental conditions of the cell. Thus, for example, a molecule that is present only during embryonic development of muscle is an exogenous molecule with respect to an adult muscle cell. Similarly, a molecule induced by heat shock is an exogenous molecule with respect to a non-heat-shocked cell. An exogenous molecule can comprise, for example, a functioning version of a malfunctioning endogenous molecule, a malfunctioning version of a normally-functioning endogenous molecule or an ortholog (functioning version of endogenous molecule from a different species).

[0048] An exogenous molecule can be, among other things, a small molecule, such as is generated by a combinatorial chemistry process, or a macromolecule such as a protein, nucleic acid, carbohydrate, lipid, glycoprotein, lipoprotein,

polysaccharide, any modified derivative of the above molecules, or any complex comprising one or more of the above molecules. Nucleic acids include DNA and RNA, can be single- or double-stranded; can be linear, branched or circular; and can be of any length. Nucleic acids include those capable of forming duplexes, as well as triplex-forming nucleic acids. See, for example, U.S. Patent Nos. 5,176,996 and 5,422,251. Proteins include, but are not limited to, DNA-binding proteins, transcription factors, chromatin remodeling factors, methylated DNA binding proteins, polymerases, methylases, demethylases, acetylases, deacetylases, kinases, phosphatases, integrases, recombinases, ligases, topoisomerases, gyrases and helicases.

[0049] An exogenous molecule can be the same type of molecule as an endogenous molecule, *e.g.*, an exogenous protein or nucleic acid. For example, an exogenous nucleic acid can comprise an infecting viral genome, a plasmid or episome introduced into a cell, or a chromosome that is not normally present in the cell. Methods for the introduction of exogenous molecules into cells are known to those of skill in the art and include, but are not limited to, lipid-mediated transfer (*i.e.*, liposomes, including neutral and cationic lipids), electroporation, direct injection, cell fusion, particle bombardment, calcium phosphate co-precipitation, DEAE-dextran-mediated transfer and viral vector-mediated transfer.

[0050] By contrast, an "endogenous" molecule is one that is normally present in a particular cell at a particular developmental stage under particular environmental conditions. For example, an endogenous nucleic acid can comprise a chromosome, the genome of a mitochondrion, chloroplast or other organelle, or a naturally-occurring episomal nucleic acid. Additional endogenous molecules can include proteins, for example, transcription factors and enzymes.

[0051] A "fusion" molecule is a molecule in which two or more subunit molecules are linked, preferably covalently. The subunit molecules can be the same chemical type of molecule, or can be different chemical types of molecules. Examples of the first type of fusion molecule include, but are not limited to, fusion proteins (for example, a fusion between a ZFP DNA-binding domain and a cleavage domain) and fusion nucleic acids (for example, a nucleic acid encoding the fusion protein described *supra*). Examples of the second type of fusion molecule include, but are not limited to, a fusion between a triplex-forming nucleic acid and a polypeptide, and a fusion between a minor groove binder and a nucleic acid.

[0052] Expression of a fusion protein in a cell can result from delivery of the fusion protein to the cell or by delivery of a polynucleotide encoding the fusion protein to a cell, wherein the polynucleotide is transcribed, and the transcript is translated, to generate the fusion protein. Trans-splicing, polypeptide cleavage and polypeptide ligation can also be involved in expression of a protein in a cell. Methods for polynucleotide and polypeptide delivery to cells are presented elsewhere in this disclosure.

[0053] A "gene," for the purposes of the present disclosure, includes a DNA region encoding a gene product (see *infra*), as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

[0054] "Gene expression" refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (*e.g.*, mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of an mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

[0055] "Modulation" of gene expression refers to a change in the activity of a gene. Modulation of expression can include, but is not limited to, gene activation and gene repression. Gene inactivation refers to any reduction in gene expression as compared to a cell that does not include a ZFP as described herein. Thus, gene inactivation may be partial or complete.

[0056] "Eukaryotic" cells include, but are not limited to, fungal cells (such as yeast), plant cells, animal cells, mammalian cells and human cells (*e.g.*, T-cells).

[0057] A "region of interest" is any region of cellular chromatin, such as, for example, a gene or a non-coding sequence within or adjacent to a gene, in which it is desirable to bind an exogenous molecule. Binding can be for the purposes of targeted DNA cleavage and/or targeted recombination. A region of interest can be present in a

chromosome, an episome, an organellar genome (*e.g.*, mitochondrial, chloroplast), or an infecting viral genome, for example. A region of interest can be within the coding region of a gene, within transcribed non-coding regions such as, for example, leader sequences, trailer sequences or introns, or within non-transcribed regions, either upstream or downstream of the coding region. A region of interest can be as small as a single nucleotide pair or up to 20,000 nucleotide pairs in length, or any integral value of nucleotide pairs, or up to the length of a chromosome. A region is interest does not need to comprise only contiguous nucleic acid sequences.

[0058] The terms "operative linkage" and "operatively linked" (or "operably linked") are used interchangeably with reference to a juxtaposition of two or more components (such as sequence elements), in which the components are arranged such that both components function normally and allow the possibility that at least one of the components can mediate a function that is exerted upon at least one of the other components. By way of illustration, a transcriptional regulatory sequence, such as a promoter, is operatively linked to a coding sequence if the transcriptional regulatory sequence controls the level of transcription of the coding sequence in response to the presence or absence of one or more transcriptional regulatory factors. A transcriptional regulatory sequence is generally operatively linked in *cis* with a coding sequence, but need not be directly adjacent to it. For example, an enhancer is a transcriptional regulatory sequence that is operatively linked to a coding sequence, even though they are not contiguous.

[0059] With respect to fusion polypeptides, the term "operatively linked" can refer to the fact that each of the components performs the same function in linkage to the other component as it would if it were not so linked. For example, with respect to a fusion polypeptide in which a ZFP DNA-binding domain is fused to a cleavage domain, the ZFP DNA-binding domain and the cleavage domain are in operative linkage if, in the fusion polypeptide, the ZFP DNA-binding domain portion is able to bind its target site and/or its binding site, while the cleavage domain is able to cleave DNA in the vicinity of the target site.

[0060] A "functional fragment" of a protein, polypeptide or nucleic acid is a protein, polypeptide or nucleic acid whose sequence is not identical to the full-length protein, polypeptide or nucleic acid, yet retains one of more of the functions of the full-length protein, polypeptide or nucleic acid. A functional fragment can possess more, fewer, or the same number of residues as the corresponding native molecule,

and/or can contain one ore more amino acid or nucleotide substitutions. Methods for determining the function of a nucleic acid (*e.g.*, coding function, ability to hybridize to another nucleic acid) are well-known in the art. Similarly, methods for determining protein function are well-known. For example, the DNA-binding function of a polypeptide can be determined, for example, by filter-binding, electrophoretic mobility-shift, or immunoprecipitation assays. DNA cleavage can be assayed by gel electrophoresis. See Ausubel *et al., supra*. The ability of a protein to interact with another protein can be determined, for example, by co-immunoprecipitation, two-hybrid assays or complementation, both genetic and biochemical. See, for example, Fields *et al.* (1989) *Nature* **340**:245-246; U.S. Patent No. 5,585,245 and PCT WO 98/44350.

Linkers

[0061] Described herein are amino acid sequences that fuse (link) DNA-binding modules (*e.g.*, zinc-finger modules) to each other. The zinc-finger modules fused using the linkers described herein may contain 1, 2, 3, 4 or even more zinc fingers. In certain embodiments, the zinc-finger modules contain 1, 2, or 3 zinc fingers, which when linked together form a 3 or more finger zinc-finger protein.

[0062] The linker sequences described herein extend between the last residue of the α-helix in a zinc finger and the first residue of the β-sheet in the next zinc finger . The linker sequence therefore joins together two zinc fingers. Typically, the last (C-terminal) amino acid in a zinc finger is the C-terminal zinc-coordinating residue, whereas an aromatic residue (*e.g.*, Phe) is typically the first amino acid of the following zinc finger. Accordingly, in a "wild type" zinc finger, threonine is the first residue in the linker, and proline is the last residue of the linker. Thus, for example, the canonical linker sequence for Zif268 is TG(E/Q)(K/R)P (SEQ ID NO:129). *See, e.g.*, U.S. Patent Nos. 6,479,626; 6,903,185 and 7,153,949.

[0063] Additional linkers are described for example in U.S. Patent Publication 20030119023, which describes linkers including multiple glycine residues (*e.g.*, TGGGGSQKP (SEQ ID NO:2), TGGGGSGGSQKP (SEQ ID NO:14) and TGGGGSGGSGGSQKP (SEQ ID NO:15), TGGEKP (SEQ ID NO:16), TGGQKP (SEQ ID NO:17), TGGSGEKP (SEQ ID NO:18), TGGSGQKP (SEQ ID NO:19), TGGSGGSGEKP (SEQ ID NO:20), and TGGSGGSGQKP (SEQ ID NO:21).

[0064] Typically, the linkers are made using recombinant nucleic acids encoding the linker and the nucleic acid binding modules, which are fused via the linker amino acid sequence. The linkers may also be made using peptide synthesis and then linked to the nucleic acid binding modules. Methods of manipulating nucleic acids and peptide synthesis methods are known in the art (see, for example, Maniatis, *et al.*, 1991. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press).

[0065] The linkers described herein are more rigid than the linkers previously used, and allow efficient binding of each zinc finger module to its target site only when subsites are separated by a specific number of base pairs.

[0066] Thus, unlike previous linkers, the linkers described herein include at least one internal or N-terminal proline residue, namely a proline residue not at the C-terminal of the linker. The linkers described herein have the following general amino acid structure:

$$X^{N\text{-term}}\text{-}X_n\text{-}X^{C\text{-term}}$$

where X is any amino acid residue, X_n comprises at least 3 amino acid residues and at least one of $X^{N\text{-term}}$ and X_n comprises a proline residue. Non-limiting examples of such linkers are shown in Tables 4, 5, 6, 9, 10, 11 or 13. Furthermore, the linkers described herein also typically include at least two basic residues, for example one or more arginine residues, one or more histidine residues, one or more lysine residues or combinations thereof.

[0067] The linkers of the invention can be any length, typically 5 or more amino acids in length. In certain embodiments, the linkers are 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or even more amino acids length.

DNA-binding modules

[0068] The linker sequences described herein are advantageously used to link DNA-binding modules.

[0069] Any DNA-binding domain can be used in the methods disclosed herein. In certain embodiments, the DNA binding domain comprises a zinc-finger protein. Preferably, the zinc-finger protein is non-naturally occurring in that it is engineered to bind to a target site of choice. *See*, for example, Beerli *et al.* (2002) *Nature Biotechnol.* **20**:135-141; Pabo *et al.* (2001) *Ann. Rev. Biochem.* **70**:313-340; Isalan *et al.* (2001) *Nature Biotechnol.* **19**:656-660; Segal *et al.* (2001) *Curr. Opin.*

Biotechnol. **12**:632-637; Choo *et al.* (2000) *Curr. Opin. Struct. Biol.* **10**:411-416. An engineered zinc-finger binding domain can have a novel binding specificity, compared to a naturally-occurring zinc-finger protein. Engineering methods include, but are not limited to, rational design and various types of selection. Rational design includes, for example, using databases comprising triplet (or quadruplet) nucleotide sequences and individual zinc finger amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers which bind the particular triplet or quadruplet sequence. See, for example, co-owned U.S. Patents 6,453,242 and 6,534,261.

[0070] Exemplary selection methods, including phage display and two-hybrid systems, are disclosed in US Patents 5,789,538; 5,925,523; 6,007,988; 6,013,453; 6,410,248; 6,140,466; 6,200,759; and 6,242,568; as well as WO 98/37186; WO 98/53057; WO 00/27878; WO 01/88197 and GB 2,338,237. In addition, enhancement of binding specificity for zinc finger binding domains has been described, for example, in co-owned WO 02/077227.

[0071] Selection of target sites; ZFPs and methods for design and construction of fusion proteins (and polynucleotides encoding same) are known to those of skill in the art and described in detail in U.S. Patent Application Publication Nos. 20050064474 and 20060188987.

[0072] In addition, as disclosed in these and other references, zinc-finger domains and/or multi-finger zinc-finger proteins may be linked together using any suitable linker sequences, including for example, linkers of 5 or more amino acids in length. See, also, U.S. Patent Nos. 6,479,626; 6,903,185; and 7,153,949 for exemplary linker sequences 6 or more amino acids in length. The proteins described herein may include any combination of suitable linkers between the individual zinc fingers of the protein.

[0073] Alternatively, the DNA-binding domain may be derived from a nuclease. For example, the recognition sequences of homing endonucleases and meganucleases such as I-*Sce*I, I-*Ceu*I, PI-*Psp*I, PI-*Sce*, I-*Sce*IV, I-*Csm*I, I-*Pan*I, I-*Sce*II, I-*Ppo*I, I-*Sce*III, I-*Cre*I, I-*Tev*I, I-*Tev*II and I-*Tev*III are known. *See* also U.S. Patent No. 5,420,032; U.S. Patent No. 6,833,252; Belfort *et al.* (1997) *Nucleic Acids Res.* **25**:3379–3388; Dujon *et al.* (1989) *Gene* **82**:115–118; Perler *et al.* (1994) Nucleic Acids Res. 22, 1125–1127; Jasin (1996) *Trends Genet.* **12**:224–228; Gimble

et al. (1996) *J. Mol. Biol.* **263:**163–180; Argast *et al.* (1998) *J. Mol. Biol.* **280:**345–353 and the New England Biolabs catalogue. In addition, the DNA-binding specificity of homing endonucleases and meganucleases can be engineered to bind non-natural target sites. *See*, for example, Chevalier *et al.* (2002) *Molec. Cell* **10:**895-905; Epinat *et al.* (2003) *Nucleic Acids Res.* **31:**2952-2962; Ashworth *et al.* (2006) *Nature* **441:**656-659; Paques *et al.* (2007) *Current Gene Therapy* **7:**49-66; U.S. Patent Publication No. 20070117128.

[0074] In some embodiments, the DNA binding domain is an engineered domain from a TAL effector (TALE) derived from the plant pathogen *Xanthomonas* (see, Miller *et al.* (2010) *Nature Biotechnology,* Dec 22 [Epub ahead of print]; Boch *et al*, (2009) *Science* 29 Oct 2009 (10.1126/science.117881) and Moscou and Bogdanove, (2009) *Science* 29 Oct 2009 (10.1126/science.1178817); *see, also,* U.S. Patent No. 8,586,526, US 2012/0109749, US 2013/0198878, U.S. Patent No. 9,322,005, U.S. Patent No. 9,493,750 and US 2017/0016030.

Regulatory Domains

[0075] Zinc-finger modules linked as described herein are often expressed with an exogenous domain (or functional fragment thereof) as fusion proteins. Common regulatory domains for addition to the ZFP include, *e.g.*, transcription factor domains (activators, repressors, co-activators, co-repressors), silencers, oncogenes (*e.g.*, myc, jun, fos, myb, max, mad, rel, ets, bcl, myb, mos family members etc.); DNA repair enzymes and their associated factors and modifiers; DNA rearrangement enzymes and their associated factors and modifiers; chromatin associated proteins and their modifiers (*e.g.* kinases, acetylases and deacetylases); and DNA modifying enzymes (*e.g.*, methyltransferases, topoisomerases, helicases, ligases, kinases, phosphatases, polymerases, endonucleases) and their associated factors and modifiers.

[0076] An exemplary functional domain for fusing with a DNA-binding domain such as, for example, a ZFP, to be used for repressing expression of a gene is a KRAB repression domain from the human KOX-1 protein (see, *e.g.*, Thiesen *et al.*, *New Biologist* 2, 363-374 (1990); Margolin *et al.*, *Proc. Natl. Acad. Sci.* USA 91, 4509-4513 (1994); Pengue *et al.*, *Nucl. Acids Res.* 22:2908-2914 (1994); Witzgall *et*

al., *Proc. Natl. Acad. Sci.* USA 91, 4514-4518 (1994). Another suitable repression domain is methyl binding domain protein 2B (MBD-2B) (see, also Hendrich *et al.* (1999) *Mamm. Genome* 10:906-912 for description of MBD proteins). Another useful repression domain is that associated with the v-ErbA protein. See, for example, Damm, *et al.* (1989) *Nature* 339:593-597; Evans (1989) *Int. J. Cancer Suppl.* 4:26-28; Pain *et al.* (1990) *New Biol.* 2:284-294; Sap *et al.* (1989) *Nature* 340:242-244; Zenke *et al.* (1988) *Cell* 52:107-119; and Zenke *et al.* (1990) *Cell* 61:1035-1049.

[0077] Additional exemplary repression domains include, but are not limited to, KRAB (also referred to as "KOX"), SID, MBD2, MBD3, members of the DNMT family (e.g., DNMT1, DNMT3A, DNMT3B), Rb, and MeCP2. See, for example, Bird *et al.* (1999) *Cell* 99:451-454; Tyler *et al.* (1999) *Cell* 99:443-446; Knoepfler *et al.* (1999) *Cell* 99:447-450; and Robertson *et al.* (2000) *Nature Genet.* 25:338-342. Additional exemplary repression domains include, but are not limited to, ROM2 and AtHD2A. See, for example, Chem *et al.* (1996) *Plant Cell* 8:305-321; and Wu *et al.* (2000) *Plant J.* 22:19-27.

[0078] Suitable domains for achieving activation include the HSV VP16 activation domain (see, *e.g.*, Hagmann *et al.*, *J. Virol.* 71, 5952-5962 (1997)) nuclear hormone receptors (see, *e.g.*, Torchia *et al.*, *Curr. Opin. Cell. Biol.* 10:373-383 (1998)); the p65 subunit of nuclear factor kappa B (Bitko and Barik, *J. Virol.* 72:5610-5618 (1998) and Doyle and Hunt, *Neuroreport* 8:2937-2942 (1997)); Liu *et al.*, *Cancer Gene Ther.* 5:3-28 (1998)), or artificial chimeric functional domains such as VP64 (Seifpal *et al.*, *EMBO J.* 11, 4961-4968 (1992)). Additional exemplary activation domains include, but are not limited to, VP16, VP64, p300, CBP, PCAF, SRC1 PvALF, AtHD2A and ERF-2. See, for example, Robyr *et al.* (2000) *Mol. Endocrinol.* 14:329-347; Collingwood *et al.* (1999) *J. Mol. Endocrinol.* 23:255-275; Leo *et al.* (2000) *Gene* 245:1-11; Manteuffel-Cymborowska (1999) *Acta Biochim. Pol.* 46:77-89; McKenna *et al.* (1999) *J. Steroid Biochem. Mol. Biol.* 69:3-12; Malik *et al.* (2000) *Trends Biochem. Sci.* 25:277-283; and Lemon *et al.* (1999) *Curr. Opin. Genet. Dev.* 9:499-504. Additional exemplary activation domains include, but are not limited to, OsGAI, HALF-1, C1, AP1, ARF-5,-6,-7, and -8, CPRF1, CPRF4, MYC-RP/GP, and TRAB1. See, for example, Ogawa *et al.* (2000) *Gene* 245:21-29; Okanami *et al.* (1996) *Genes Cells* 1:87-99; Goff *et al.* (1991) *Genes Dev.* 5:298-309; Cho *et al.* (1999) *Plant Mol. Biol.* 40:419-429; Ulmason *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:5844-5849; Sprenger-Haussels *et al.* (2000) *Plant J.* 22:1-8; Gong

et al. (1999) *Plant Mol. Biol.* 41:33-44; and Hobo *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:15,348-15,353.

[0079] In certain embodiments, the regulatory domain comprises a nuclease (*e.g.*, cleavage domain). Such engineered nucleases can be used to create a double-strand break (DSB) in a target nucleotide sequence, which increases the frequency of donor nucleic acid introduction via homologous recombination at the targeted locus (targeted integration) more than 1000-fold. In addition, the inaccurate repair of a site-specific DSB by non-homologous end joining (NHEJ) can also result in gene disruption. Nucleases can be used for a wide variety of purposes such as for cell line engineering as well as for therapeutic applications.

[0080] Cleavage domains of the fusion proteins disclosed herein can be obtained from any endonuclease or exonuclease. Exemplary endonucleases from which a cleavage domain can be derived include, but are not limited to, restriction endonucleases and homing endonucleases. *See*, for example, 2002-2003 Catalogue, New England Biolabs, Beverly, MA; and Belfort *et al.* (1997) *Nucleic Acids Res.* **25:**3379-3388. Additional enzymes which cleave DNA are known (*e.g.*, S1 Nuclease; mung bean nuclease; pancreatic DNase I; micrococcal nuclease; yeast HO endonuclease; *see* also Linn *et al.* (eds.) *Nucleases*, Cold Spring Harbor Laboratory Press,1993). One or more of these enzymes (or functional fragments thereof) can be used as a source of cleavage domains and cleavage half-domains.

[0081] Similarly, a cleavage half-domain can be derived from any nuclease or portion thereof, as set forth above, that requires dimerization for cleavage activity. In general, two fusion proteins are required for cleavage if the fusion proteins comprise cleavage half-domains. Alternatively, a single protein comprising two cleavage half-domains can be used. The two cleavage half-domains can be derived from the same endonuclease (or functional fragments thereof), or each cleavage half-domain can be derived from a different endonuclease (or functional fragments thereof).

[0082] In addition, the target sites for the two fusion proteins are preferably disposed, with respect to each other, such that binding of the two fusion proteins to their respective target sites places the cleavage half-domains in a spatial orientation to each other that allows the cleavage half-domains to form a functional cleavage domain, *e.g.*, by dimerizing. Thus, in certain embodiments, the near edges of the target sites are separated by 5-8 nucleotides or by 15-18 nucleotides. However any integral number of nucleotides or nucleotide pairs can intervene between two target

sites (*e.g.*, from 2 to 50 nucleotide pairs or more). In general, the site of cleavage lies between the target sites.

[0083] Restriction endonucleases (restriction enzymes) are present in many species and are capable of sequence-specific binding to DNA (at a recognition site), and cleaving DNA at or near the site of binding. Certain restriction enzymes (*e.g.*, Type IIS) cleave DNA at sites removed from the recognition site and have separable binding and cleavage domains. For example, the Type IIS enzyme *Fok* I catalyzes double-stranded cleavage of DNA, at 9 nucleotides from its recognition site on one strand and 13 nucleotides from its recognition site on the other. See, for example, US Patents 5,356,802; 5,436,150 and 5,487,994; as well as Li *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89:**4275-4279; Li *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90:**2764-2768; Kim *et al.* (1994a) *Proc. Natl. Acad. Sci. USA* **91:**883-887; Kim *et al.* (1994b) *J. Biol. Chem.* **269:**31,978-31,982. Thus, in one embodiment, fusion proteins comprise the cleavage domain (or cleavage half-domain) from at least one Type IIS restriction enzyme and one or more zinc finger binding domains, which may or may not be engineered.

[0084] An exemplary Type IIS restriction enzyme, whose cleavage domain is separable from the binding domain, is *Fok* I. This particular enzyme is active as a dimer. Bitinaite *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95:** 10,570-10,575. Accordingly, for the purposes of the present disclosure, the portion of the Fok I enzyme used in the disclosed fusion proteins is considered a cleavage half-domain. Thus, for targeted double-stranded cleavage and/or targeted replacement of cellular sequences using zinc finger-*Fok* I fusions, two fusion proteins, each comprising a *Fok*I cleavage half-domain, can be used to reconstitute a catalytically active cleavage domain. Alternatively, a single polypeptide molecule containing a zinc finger binding domain and two *Fok* I cleavage half-domains can also be used. Parameters for targeted cleavage and targeted sequence alteration using zinc finger-*Fok* I fusions are provided elsewhere in this disclosure.

[0085] A cleavage domain or cleavage half-domain can be any portion of a protein that retains cleavage activity, or that retains the ability to multimerize (*e.g.*, dimerize) to form a functional cleavage domain.

[0086] Exemplary Type IIS restriction enzymes are described in International Publication WO 07/014275. Additional restriction enzymes also contain separable

binding and cleavage domains, and these are contemplated by the present disclosure. See, for example, Roberts *et al.* (2003) *Nucleic Acids Res.* **31**:418-420.

[0087] In certain embodiments, the cleavage domain comprises one or more engineered cleavage half-domain (also referred to as dimerization domain mutants) that minimize or prevent homodimerization, as described, for example, in U.S. Patent Publication Nos. 20050064474 and 20060188987 and in U.S. Application No. 11/805,850 (filed May 23, 2007). Amino acid residues at positions 446, 447, 479, 483, 484, 486, 487, 490, 491, 496, 498, 499, 500, 531, 534, 537, and 538 of *Fok* I are all targets for influencing dimerization of the *Fok* I cleavage half-domains.

[0088] Exemplary engineered cleavage half-domains of *Fok* I that form obligate heterodimers include a pair in which a first cleavage half-domain includes mutations at amino acid residues at positions 490 and 538 of *Fok* I and a second cleavage half-domain includes mutations at amino acid residues 486 and 499.

[0089] Thus, in one embodiment, a mutation at 490 replaces Glu (E) with Lys (K); the mutation at 538 replaces Iso (I) with Lys (K); the mutation at 486 replaced Gln (Q) with Glu (E); and the mutation at position 499 replaces Iso (I) with Lys (K). Specifically, the engineered cleavage half-domains described herein were prepared by mutating positions 490 (E→K) and 538 (I→K) in one cleavage half-domain to produce an engineered cleavage half-domain designated "E490K:I538K" and by mutating positions 486 (Q→E) and 499 (I→L) in another cleavage half-domain to produce an engineered cleavage half-domain designated "Q486E:I499L". The engineered cleavage half-domains described herein are obligate heterodimer mutants in which aberrant cleavage is minimized or abolished. *See, e.g.,* Example 1 of WO 07/139898. In certain embodiments, the engineered cleavage half-domain comprises mutations at positions 486, 499 and 496 (numbered relative to wild-type *Fok*I), for instance mutations that replace the wild type Gln (Q) residue at position 486 with a Glu (E) residue, the wild type Iso (I) residue at position 499 with a Leu (L) residue and the wild-type Asn (N) residue at position 496 with an Asp (D) or Glu (E) residue (also referred to as a "ELD" and "ELE" domains, respectively). In other embodiments, the engineered cleavage half-domain comprises mutations at positions 490, 538 and 537 (numbered relative to wild-type *Fok*I), for instance mutations that replace the wild type Glu (E) residue at position 490 with a Lys (K) residue, the wild type Iso (I) residue at position 538 with a Lys (K) residue, and the wild-type His (H)

residue at position 537 with a Lys (K) residue or a Arg (R) residue (also referred to as "KKK" and "KKR" domains, respectively). In other embodiments, the engineered cleavage half-domain comprises mutations at positions 490 and 537 (numbered relative to wild-type *Fok*I), for instance mutations that replace the wild type Glu (E) residue at position 490 with a Lys (K) residue and the wild-type His (H) residue at position 537 with a Lys (K) residue or a Arg (R) residue (also referred to as "KIK" and "KIR" domains, respectively). (See U.S. Patent Application No: 12/931,660).

[0090] Engineered cleavage half-domains described herein can be prepared using any suitable method, for example, by site-directed mutagenesis of wild-type cleavage half-domains (*Fok* I) as described in U.S. Patent Publication No. 20050064474 (see, *e.g.*, Example 5); and WO 07/139898.

[0091] Alternatively, nucleases may be assembled *in vivo* at the nucleic acid target site using so-called "split-enzyme" technology (see *e.g.* U.S. Patent Publication No. 20090068164). Components of such split enzymes may be expressed either on separate expression constructs, or can be linked in one open reading frame where the individual components are separated, for example, by a self-cleaving 2A peptide or IRES sequence. Components may be individual zinc finger binding domains or domains of a meganuclease nucleic acid binding domain.

[0092] Fusion molecules are constructed by methods of cloning and biochemical conjugation that are well known to those of skill in the art. Fusion molecules comprise a DNA-binding domain and a functional domain (*e.g.*, a transcriptional activation or repression domain). Fusion molecules also optionally comprise nuclear localization signals (such as, for example, that from the SV40 medium T-antigen) and epitope tags (such as, for example, FLAG and hemagglutinin). Fusion proteins (and nucleic acids encoding them) are designed such that the translational reading frame is preserved among the components of the fusion.

[0093] For such applications, the fusion molecule is typically formulated with a pharmaceutically acceptable carrier, as is known to those of skill in the art. See, for example, Remington's Pharmaceutical Sciences, 17th ed., 1985; and co-owned WO 00/42219.

Kits

[0094] Also provided are kits comprising any of the linkers described herein and/or for performing any of the above methods. The kits typically contain a linker sequence as described herein (or a polynucleotide encoding a linker as described herein). The kit may supply the linker alone or may provide vectors into which a DNA-binding domain and/or nuclease of choice can be readily inserted into. The kits can also contain cells, buffers for transformation of cells, culture media for cells, and/or buffers for performing assays. Typically, the kits also contain a label which includes any material such as instructions, packaging or advertising leaflet that is attached to or otherwise accompanies the other components of the kit.

Applications

[0095] The disclosed linkers are advantageously used to enhance the repertoire of target sites for engineered zinc-finger proteins. For example, the linkers described herein facilitate binding to desired target sites when the module subsites are not adjacent. Thus, there would effectively be an increase the number of ZFPs that could be constructed to target a given nucleic acid sequence for a given repertoire size. Furthermore, because the linkers described distinguish between various module subsite separations (*e.g.*, 0, 1 and 2 base pair gaps), they reduce binding of ZFPs to improper target sites. For example, a ZFP with a flexible linker designed to skip 2 basepairs (*e.g.* TGGGGSGGSQKP (SEQ ID NO:14)) is able to bind to target sites with either 0, 1, or 2 basepairs between the module subsites. This same ZFP with a 2bp-skipping linker as described herein should bind well to a target with 2 basepairs between the module subsites, but should not be able to bind efficiently to targets with 0 or 1 basepairs between module subsites (improper or unintended target sites).

[0096] Thus, linkers described herein can be used in any application for which zinc-finger proteins are currently used, including, but not limited to zinc-finger transcription factors (ZFP-TFs) for modulation of gene *expression* and/or in zinc-finger nucleases (ZFNs) for cleavage. *See, e.g.,* U.S. Patent Nos. 6,534,261; 6,599,692; 6,689,558; 7,067,317; 7,262,054 and 7,253,273; U.S. Patent Publication Nos. 20050064474; 2006/0063231; 2007/0134796; 2007/0218528; 2008/0015164; 2008/0188000; 2008/0299580 and 2008/0159996.

[0097] Accordingly, the disclosed linkers can be used in any ZFP or ZFN for any method in which specifically targeted modulation or cleavage is desirable. For example, ZFP-TFs and ZFNs can be used to treat genetic diseases, infections (viral or bacterial), to generate cell lines, animals and or plants in which desired genes are activated, repressed, targeted by homologous recombination and/or knocked-in or out. Accordingly, the linkers described herein can also be used to more efficiently clone DNA and in genome modifications facilitated by ZFNs, which is broadly applicable in many areas of biotechnology and basic science.

EXAMPLES

Example 1: Selection of linkers

[0098] Linker selections were performed in the context of three different host ZFPs: "ZFP7263", "ZFP7264" and "ZFP8196" (see U.S. Patent Publication Nos. 20050064474 for 7263 and 7264 and 20080159996 for 8196), which each contained four fingers. Recognition helices of each finger for each host ZFP are provided in Table 1, while the full sequence of each host ZFP is provided in Figure 1A. Selections were carried out as follows: (i) first, a library was generated within each host ZFP that replaced codons in the central linker with a mixture of two to twelve fully randomized codons (Figure 1B).; Sequencing of naïve libraries showed good diversity of sequences with no clone represented more than once; (ii) next, the libraries were expressed on the surface of filamentous bacteriophage; (iii) phage-expressed ZFP libraries were then selected for binding to biotinylated target variants that contained a 1- or 2-bp insertion at the center of the host protein binding site (*i.e.* in the region spanned by the randomized linker) (Table 2A). Each insertion comprised a gap between the binding sequences for the second and third fingers of the host protein that must be bridged by a longer linker to enable efficient binding (Table 2A). Insertions consisted of a mixture of bases in order to favor the selection of linkers with no intrinsic base specificity. Five selection cycles were performed. During the final four cycles, a counterselection was employed with a 1000-fold molar excess of binding sites that were nonbiotinylated and that contained non-targeted gap lengths (*i.e.* if phage were selected using a target sequence with a 1bp gap length, the counterselection comprised targets with 0, 2, 3 and 4 bp gaps; if phage were selected using a target sequence with a 2bp gap length, the counterselection comprised targets with 0, 1 and 3 and 4 bp gaps– see Table 2B).

[0099] Phage pools from the fifth round of selection were screened for the ability to selectively bind sequences bearing the targeted gap length, and these studies revealed gap selective binding (Figure 2). In particular, phage pools selected to skip a 1 bp gap in the context of ZFP8196 showed a 25-fold preference for targets bearing a 1bp gap as compared to no gap. Phage pools selected to skip a 1 bp gap in the context of ZFP7263 showed a 26-fold preference for targets bearing a 1bp gap as compared to no gap. Phage pools selected to skip a 1 bp gap in the context of ZFP7264 showed a 5.5-fold preference targets bearing a 1bp gap as compared to no gap. Each of these pools also exhibited little or no binding to targets bearing longer gap lengths (2, 3 or 4 bp).

[0100] Phage pools selected to skip a 2 bp gap in the context of ZFP8196 showed a 7-fold preference for targets bearing 2 bp gap as compared to a 1bp gap as well as a >30-fold preference over targets bearing 0, 3 and 4 bp gaps.

Table 1: Host ZFP recognition helices

ZFP	Finger 1	Finger 2	Finger 3	Finger 4
8196	RSDNLSV (SEQ ID NO:22)	QKINLQV (SEQ ID NO:23)	RSDVLSE (SEQ ID NO:24)	QRNHRTT (SEQ ID NO:25
7264	RSDTLSE (SEQ ID NO:26)	ARSTRTT (SEQ ID NO:27)	RSDSLSK (SEQ ID NO:28)	QRSNLKV (SEQ ID NO:29)
7263	RSDNLSV (SEQ ID NO:22)	RNAHRIN (SEQ ID NO:30)	RSDTLSE (SEQ ID NO:26)	ARSTRTN (SEQ ID NO:31)

Table 2A: Target sites used for selection

ZFP w/randomized linker	Target sites
8196	ATAAACTGdCAAAAGGC (SEQ ID NO:33) ATAAACTGdbCAAAAGGC (SEQ ID NO:34)
7264	TTAAAGCGhGCTCCGAA (SEQ ID NO:38) TTAAAGCGhdGCTCCGAA (SEQ ID NO:39)
7263	CCACTCTGhTGGAAGTG (SEQ ID NO:43) CCACTCTGhhTGGAAGTG (SEQ ID NO:44)

Table 2A. Target sites used for selections. Duplex DNA target sites used in phage studies had the general form of: TATAAT$(X)_{17-18}$T*TCACAGTCAGTCCACACGTC*, (SEQ ID NO:67) where $(X)_{17-18}$ was replaced with sequences listed in the table. DNA duplexes were made by extending a primer that annealed to the italicized sequence and which was biotinylated at its 5' end. Underlined bases indicate the binding sequences for the four fingers of each host ZFP, while lowercase bases indicate inserted nucleotides (or "gap" bases) that must be spanned by the selected linkers. Degeneracy codes for gap bases are as follows: "d" denotes a mix of A, G, and T; "b" denotes a mix of C, G, and T; "h" denotes a mix of A, C, and T; and "v" denotes a mix of A, C, and G.

Table 2B: Competitor sites used during selection

ZFP w/randomized linker	Competitor sites
8196	ATAAACTGCAAAAGGC (SEQ ID NO:32) ATAAACTGdCAAAAGGC(SEQ ID NO:33) ATAAACTGdbCAAAAGGC (SEQ ID NO:34) ATAAACTGdbbCAAAAGGC (SEQ ID NO:35) ATAAACTGdbbbCAAAAGGC (SEQ ID NO:36)
7264	TTAAAGCGGCTCCGAA (SEQ ID NO:37) TTAAAGCGhGCTCCGAA (SEQ ID NO:38) TTAAAGCGhdGCTCCGAA (SEQ ID NO:39) TTAAAGCGhdvGCTCCGAA (SEQ ID NO:40) TTAAAGCGhdvdGCTCCGAA (SEQ ID NO:41)
7263	CCACTCTGTGGAAGTG (SEQ ID NO:42) CCACTCTGhTGGAAGTG (SEQ ID NO:43) CCACTCTGhhTGGAAGTG (SEQ ID NO:44) CCACTCTGhhhTGGAAGTG (SEQ ID NO:45) CCACTCTGhhhbTGGAAGTG (SEQ ID NO:46)

Table 2B. Competitor sites used during selections. Duplex DNA competitor sites had the general form of:TATAAT$(X)_{16\text{-}20}$*TTCACAGTCAGTCCACACGTC*, (SEQ ID NO:67) where $(X)_{16\text{-}20}$ was replaced with sequences listed in the table. DNA duplexes were made by extending a (non-biotinylated) primer that annealed to the italicized sequence. Underlined bases indicate the binding sequences for the four fingers of each host ZFP, while lowercase bases indicate inserted nucleotides (or "gap" bases). Degeneracy codes for gap bases are as follows: "d" denotes a mix of A, G, and T; "b" denotes a mix of C, G, and T; "h" denotes a mix of A, C, and T; and "v" denotes a mix of A, C, and G.

Table 2C: Targets used for phage pool gap selectivity studies

ZFP w/randomized linker	Gap	Target sites
8196	0 gap 1 gap 2 gap 3 gap 4 gap	ATAAACTGCAAAAGGC (SEQ ID NO:32) ATAAACTGdCAAAAGGC(SEQ ID NO:33) ATAAACTGdbCAAAAGGC (SEQ ID NO:34) ATAAACTGdbbCAAAAGGC (SEQ ID NO:35) ATAAACTGdbbbCAAAAGGC (SEQ ID NO:36)
7264	0 gap 1 gap 2 gap 3 gap 4 gap	TTAAAGCGGCTCCGAA (SEQ ID NO:37) TTAAAGCGhGCTCCGAA (SEQ ID NO:38) TTAAAGCGhdGCTCCGAA (SEQ ID NO:39) TTAAAGCGhdvGCTCCGAA (SEQ ID NO:40) TTAAAGCGhdvdGCTCCGAA (SEQ ID NO:41)
7263	0 gap 1 gap 2 gap 3 gap 4 gap	CCACTCTGTGGAAGTG (SEQ ID NO:42) CCACTCTGhTGGAAGTG (SEQ ID NO:43) CCACTCTGhhTGGAAGTG (SEQ ID NO:44) CCACTCTGhhhTGGAAGTG (SEQ ID NO:45) CCACTCTGhhhbTGGAAGTG (SEQ ID NO:46)

Table 2C. Targets used for phage pool gap selectivity studies. Duplex DNA sites used in phage pool gap selectivity studies had the general form of: TATAAT$(X)_{16\text{-}20}$*TTCACAGTCAGTCCACACGTC*, (SEQ ID NO:67) where $(X)_{16\text{-}20}$ was replaced with sequences listed in the table. DNA duplexes were made by extending a biotinylated primer that annealed to the italicized sequence. Underlined bases

indicate the binding sequences for the four fingers of each host ZFP, while lowercase bases indicate inserted nucleotides (or "gap" bases). Degeneracy codes for gap bases are as follows: "d" denotes a mix of A, G, and T; "b" denotes a mix of C, G, and T; "h" denotes a mix of A, C, and T; and "v" denotes a mix of A, C, and G.

Sequencing

[0100] Genes encoding the selected ZFPs were subcloned and sequenced. Figure 3A presents linkers selected for skipping 1 bp gaps in the context of all three host proteins, while Figure 3B shows linkers selected for skipping 2 bp in the context of the "ZFP8196" host. The sequencing results revealed a strong compositional bias in the selected linkers towards proline- and arginine-rich sequences. Clear linker length trends were also apparent: although the starting libraries encoded approximately equal proportions of 11 different linker lengths (2-12 residues), selected linkers featured narrower distributions of from 5-8 residues (for the 1bp gap) or 9-11 residues (for the 2bp gap).

Example 2: Initial characterization of selected ZFPs

[0101] As an initial functional assessment of the linkers selected to skip 1 bp, ZFPs bearing the linkers listed in Figure 3A were subcloned, expressed as free protein using an *in vitro* transcription-translation kit, and evaluated by ELISA for binding to targets bearing insertions of 0, 1 or 2 bp opposite the selected linker. Targets for these studies are listed in Table 3. Nine additional control proteins were generated by replacing the central linker of each host ZFP with three alternative, previously characterized, linker sequences which collectively represented the state of the art for spanning 1bp. The sequences of these control linkers were LRQKDERP (SEQ ID NO:3) (*see,* U.S. Patent No. 6,479,626), TGEGGKP (SEQ ID NO:48), TGGGGSQKP (SEQ ID NO:2),. These control proteins, as well as the host ZFPs, were also included in the ELISA studies.

[0102] Table 3 shows the targets used for ELISA studies of ZFPs selected to skip a 1bp gap. Duplex DNA sites used these studies had the general form $TTAG(X)_{16-18}TATC$, (SEQ ID NO:94) where $(X)_{16-18}$ was replaced with sequences listed in the table. Each duplex DNA target was made by annealing a complementary oligonucleotide bearing a biotin at its 5' end. Underlines indicate the binding sequences for the four fingers of each host ZFP, while lowercase letters indicate inserted nucleotides (or "gap" bases).

[0103] The results of these studies are provided in Tables 4, 5 and 6, with each table listing data for proteins derived from a different host ZFP. Table 4 provides data for ZFP8196-derived proteins; Table 5 provides data for ZFP7263-derived proteins; and Table 6 provides data for ZFP7264-derived proteins. In each table, binding data for the host ZFP is listed in the top row, followed by binding data for three control proteins in rows 2-4, followed by data for the ZFPs selected from the phage display libraries. The values are normalized to the ELISA signal obtained from the binding of the parent ZFP to its unmodified target.

[0104] Each set of proteins exhibited a similar pattern of binding behavior, in three key respects: First, each parent ZFP bound well to its unmodified target (the "0-bp gap" target in Tables 4, 5 and 6) but not to any variant bearing inserts of 1 or 2 bp. This was expected since the parental linkers (either TGEKP (SEQ ID NO:1) (for ZFP8196) or TGSQKP (SEQ ID NO:72) (for ZFP7263 and ZFP7264)) are too short to span any additional inserted base.

[0105] Second, in almost all cases the control proteins bound very poorly to targets with a 1bp insert (normalized ELISA values were 0.10 or less for 31 of 36 such measurements). This indicates the poor performance of the linkers available prior to these studies. Moreover, the linkers used by these proteins showed no consistent preference for targets bearing a 1bp insert (vs a 0bp insert).

[0106] Third, in contrast to the behavior of the control proteins, the phage-selected ZFPs bound with much higher affinity to targets bearing a 1 bp insert as well as with a much higher level of discrimination against binding targets containing no inserted base. These proteins were also very selective for binding targets with a 1bp insert vs targets bearing a 2bp insert.

Table 3: Targets used for ELISA studies of ZFPs selected to skip a 1bp gap

ZFP w/randomized linker	Gap Sequence	Target sites
8196	-	ATAAACTGCAAAAGGC (SEQ ID NO:32)
	A	ATAAACTGaCAAAAGGC (SEQ ID NO:73)
	C	ATAAACTGcCAAAAGGC (SEQ ID NO:74)
	G	ATAAACTGgCAAAAGGC (SEQ ID NO:75)
	T	ATAAACTGtCAAAAGGC (SEQ ID NO:76)
	TC	ATAAACTGtcCAAAAGGC (SEQ ID NO:77)
	AC	ATAAACTGacCAAAAGGC (SEQ ID NO:78)
	TG	ATAAACTGtgCAAAAGGC (SEQ ID NO:79)
7264	-	TTAAAGCGGCTCCGAA (SEQ ID NO:37)
	A	TTAAAGCGaGCTCCGAA (SEQ ID NO:80)
	C	TTAAAGCGcGCTCCGAA (SEQ ID NO:81)
	G	TTAAAGCGgGCTCCGAA (SEQ ID NO:82)

	T TT TA CT	TTAAAGCGtGCTCCGAA (SEQ ID NO:83) TTAAAGCGttGCTCCGAA (SEQ ID NO:84) TTAAAGCGtaGCTCCGAA (SEQ ID NO:85) TTAAAGCGctGCTCCGAA (SEQ ID NO:86)
7263	- A C G T AC AT CT	CCACTCTGTGGAAGTG (SEQ ID NO:42) CCACTCTGaTGGAAGTG (SEQ ID NO:87) CCACTCTGcTGGAAGTG (SEQ ID NO:88) CCACTCTGgTGGAAGTG (SEQ ID NO:89) CCACTCTGtTGGAAGTG (SEQ ID NO:90) CCACTCTGacTGGAAGTG (SEQ ID NO:91) CCACTCTGatTGGAAGTG (SEQ ID NO:92) CCACTCTGctTGGAAGTG (SEQ ID NO:93)

Table 4: ELISA results for variants of the ZFP "8196" with different center linkers

Sequence of the center linker	ELISA score for binding to targets having the indicated gap [score is normalized to 8196 bound to its non-gapped target (underlined entry)]								
	0-bp gap	1-bp gap					2-bp gap		
	-	A	C	G	T	average ratio of 1bp:0bp score	TC	AC	TG
TGEKP (SEQ ID NO:1)	1.00	0.01	0.01	0.03	0.01	0.02	0.00	0.00	0.00
TGGGGSQKP (SEQ ID NO:2)	0.00	0.00	0.00	0.00	0.00	1.20	0.00	0.00	0.00
LRQKDERP (SEQ ID NO:3)	0.01	0.01	0.01	0.04	0.08	3.49	0.00	0.00	0.00
TGEGGKP (SEQ ID NO:48)	0.10	0.00	0.00	0.03	0.03	0.15	0.00	0.00	0.00
TPDAPKPKP (SEQ ID NO:49)	0.02	0.16	0.13	0.68	0.95	23.75	0.01	0.00	0.01
TPGLHRPKP (SEQ ID NO:50)	0.04	0.19	0.10	0.65	0.81	10.94	0.01	0.00	0.01
TEPRAKPPKP (SEQ ID NO:51)	0.01	0.39	0.17	0.78	0.93	70.72	0.02	0.01	0.01
TPSHTPRPKP (SEQ ID NO:52)	0.02	0.30	0.13	0.84	0.80	25.10	0.02	0.01	0.01
TGYSIPRPKP (SEQ ID NO:53)	0.01	0.13	0.06	0.43	0.55	44.57	0.01	0.00	0.01
TYPRPIAAKP (SEQ ID NO:54) (designated 1f)	0.01	0.41	0.14	0.65	0.64	82.25	0.01	0.00	0.01
THPRAPIPKP (SEQ ID NO:55)	0.00	0.20	0.09	0.57	0.60	78.86	0.01	0.00	0.00

(designated 1c)									
TPNRRPAPKP (SEQ ID NO:56) (designated 1d)	0.00	0.23	0.09	0.52	0.52	90.27	0.01	0.01	0.01
TSPRLPAPKP (SEQ ID NO:57)	0.01	0.26	0.14	0.62	0.81	67.95	0.01	0.00	0.01
TCPRPPTRKP (SEQ ID NO:58)	0.00	0.18	0.05	0.48	0.62	70.16	0.01	0.00	0.01
TSSPRSNAKP (SEQ ID NO:59)	0.01	0.05	0.02	0.20	0.25	20.85	0.01	0.00	0.01
TVSPAPCRSKP (SEQ ID NO:60)	0.01	0.03	0.01	0.14	0.19	11.52	0.02	0.00	0.01
TPDRPISTCKP (SEQ ID NO:61)	0.01	0.11	0.05	0.29	0.47	15.41	0.03	0.01	0.02

Table 5: ELISA results for variants of the ZFP "7263" with different center linkers

Sequence of the center linker	ELISA score for binding to targets having the indicated gap [score is normalized to 7263 bound to its non-gapped target (underlined entry)]								
	0-bp gap	1-bp gap					2-bp gap		
	-	A	C	G	T	average ratio of 1bp:0bp score	AC	AT	CT
TGSQKP (SEQ ID NO:72)	<u>1.00</u>	0.01	0.01	0.03	0.02	0.02	0.01	0.01	0.00
TGGGGSQKP (SEQ ID NO:2)	0.51	0.06	0.05	0.41	0.39	0.44	0.01	0.01	0.02
LRQKDERP (SEQ ID NO:3)	0.25	0.03	0.02	0.18	0.13	0.36	0.01	0.01	0.01
TGEGGKP (SEQ ID NO:48)	1.30	0.02	0.02	0.05	0.04	0.03	0.01	0.01	0.01
TPRPPIPKP (SEQ ID NO:4)	0.14	0.97	0.67	1.85	2.09	10.20	0.02	0.01	0.01
TQRPQIPPKP (SEQ ID NO:62)	0.15	1.66	1.00	2.86	3.05	14.68	0.03	0.02	0.01
TPNRCPPTKP (SEQ ID NO:63)	0.31	1.68	1.13	2.62	3.16	7.53	0.03	0.02	0.01
TYPRPLLAKP (SEQ ID NO:7)	0.29	1.95	1.27	3.88	3.97	10.08	0.03	0.01	0.01
TPLCQRPMKQKP (SEQ ID NO:8)	0.28	1.82	1.28	3.44	4.00	10.88	0.08	0.05	0.02

Table 6: ELISA results for variants of the ZFP "7264" with different center linkers

Sequence of the center linker	ELISA score for binding to targets having the indicated gap [score is normalized to 7264 bound to its non-gapped target (underlined entry)]								
	0-bp gap	1-bp gap					2-bp gap		
	-	A	C	G	T	average ratio of 1bp:0bp score	TT	TA	CT
TGSQKP (SEQ ID NO:72)	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TGGGGSQKP (SEQ ID NO:2)	0.46	0.07	0.04	0.08	0.17	0.19	0.03	0.03	0.07
LRQKDERP (SEQ ID NO:3)	0.26	0.05	0.03	0.06	0.10	0.22	0.02	0.02	0.02
TGEGGKP (SEQ ID NO:48)	1.39	0.02	0.03	0.05	0.08	0.03	0.03	0.02	0.03
TGLPKPKP(SEQ ID NO:64)	0.14	0.19	0.11	0.38	0.86	2.84	0.03	0.02	0.02
TSRPRPKP (SEQ ID NO:11)	0.18	0.52	0.22	0.77	2.07	4.93	0.03	0.03	0.03
TLPLPRPKP (SEQ ID NO:65)	0.25	0.58	0.25	0.85	1.36	3.01	0.04	0.03	0.03
TVPRPTPPKP (SEQ ID NO:12) (designated 1e)	0.16	2.35	1.02	1.58	2.55	11.71	0.05	0.05	0.06
TLPPCFRPKP (SEQ ID NO:66)	0.36	0.72	0.25	0.77	2.72	3.11	0.06	0.06	0.05
TKHGTPKHREDKP (SEQ ID NO:13)	0.01	0.01	0.01	0.01	0.01	0.79	0.00	0.00	0.00

[0107] To further support and expand upon the results obtained in the ELISA studies, ZFPs with selected linkers were evaluated for *in vivo* cleavage activity at various target sites using the yeast screening assay described in International Patent Publication WO 2009/042163. As these experiments are more labor intensive, they were performed on fewer ZFPs. For these studies, six ZFPs from Table 5 and five ZFPs from Table 6 were assembled into constructs that enabled expression as zinc finger nucleases (ZFNs) as described in WO 2007/139982. *In vivo* activity was then measured by evaluating MEL-1 secretion from yeast strains having various target sites. The target sequences used for these studies are provided in Tables 7 and 8, and included variations of the 7263 and 7264 binding sites with central insertions of 0, 1-, or 2-bp.

Table 7: Targets used for yeast screening assay of ZFPs selected to skip a 1bp gap in ZFP7263

ZFP w/randomized linker	Gap Sequence	Target sites
7263	-	ACTCTGTGGAAG (SEQ ID NO:95)
	A	ACTCTGaTGGAAG (SEQ ID NO:96)
	C	ACTCTGcTGGAAG (SEQ ID NO:97)
	G	ACTCTGgTGGAAG (SEQ ID NO:98)
	T	ACTCTGtTGGAAG (SEQ ID NO:99)
	AC	ACTCTGacTGGAAG (SEQ ID NO:100)
	AT	ACTCTGatTGGAAG (SEQ ID NO:101)
	CT	ACTCTGctTGGAAG (SEQ ID NO:102)

[0108] Reporter plasmids bearing nuclease target sites were constructed essentially as described in International Patent Publication WO 2009/042163, except that nuclease target cassettes had the general form of

GATCTGTT*CGGAGCCGCTTT*AACCC$(X)_{12-14}$TGCTCGCG (SEQ ID NO:103)

where (1) the four underlined bases at either end represent the overhangs used for cloning into the BamHI/BssHII digested reporter plasmid, (2) the italicized sequence represents the binding site for the 7264 ZFN which binds to the antisense strand and was invariant for these screens, and (3) $(X)_{12-14}$ was replaced with sequences listed in the table. Capitalized bases indicate the binding sequences for the four fingers of each host ZFP, while lowercase letters indicate inserted nucleotides (or "gap" bases).

Table 8: Targets used for yeast screening assay of ZFPs selected to skip a 1bp gap in ZFP7264

ZFP w/randomized linker	Gap Sequence	Target sites
7264	-	AAAGCGGCTCCG (SEQ ID NO:104)
	A	AAAGCGaGCTCCG (SEQ ID NO:105)
	C	AAAGCGcGCTCCG (SEQ ID NO:106)
	G	AAAGCGgGCTCCG (SEQ ID NO:107)
	T	AAAGCGtGCTCCG (SEQ ID NO:108)
	TT	AAAGCGttGCTCCG (SEQ ID NO:109)
	TA	AAAGCGtaGCTCCG (SEQ ID NO:110)
	CT	AAAGCGctGCTCCG (SEQ ID NO:111)

[0109] Reporter plasmids bearing nuclease target sites were constructed essentially as described in International Patent Publication WO 2009/042163, except that nuclease target cassettes had the general form of GATCTGTT$(X)_{12-14}$AACCC*ACTCTGTGGAAG*TGCTCGCG (SEQ ID NO:112) where (1) the four underlined bases at either end represent the overhangs used for cloning into the BamHI/BssHII digested reporter plasmid, (2) the italicized sequence represents the binding site for the 7263 ZFN which was invariant for these screens, and (3) $(X)_{12-14}$ was replaced with sequences listed in the table. Capitalized bases indicate the binding sequences for the four fingers of each host ZFP, while lowercase letters indicate inserted nucleotides (or "gap" bases). Note that the target sites listed in the table are the reverse complement of what is present in the target cassette as the 7264 ZFN binds to the antisense strand.

[0110] Data for these experiments are shown in Tables 9 and 10, with each table listing data for proteins derived from a different host ZFN. Table 9 provides data for 7263-derived ZFNs and Table 10 provides data for 7264-derived ZFNs. In each table, nuclease activity data for the host ZFN is listed in the top row, followed by nuclease activity data for one control protein in row 2, followed by data for the ZFPs selected from the phage display libraries. Since ZFP7263 and ZFP7264 are two halves of the same zinc-finger nuclease dimer, the data for the host ZFN is the same in each table. The results of these studies broadly matched the patterns observed in the ELISA studies, in that the ZFNs bearing phage-selected linkers showed both higher activity and better preference for targets bearing a 1bp insert than ZFPs bearing control linkers.

Table 9: Yeast screening results for variants of ZFP7263 with different center linkers

Sequence of the center linker	MEL-1 score for nuclease activity at targets having the indicated gap [score is normalized to ZFP7263 bound to its non-gapped target (underlined entry)]								
	0-bp gap	1-bp gap					2-bp gap		
	-	A	C	G	T	average ratio of 1bp:0bp score	AC	AT	CT
TGSQKP (SEQ ID NO:72)	1.00	0.05	0.05	0.09	0.06	0.06	0.09	0.02	0.08
TGGGGSQKP (SEQ ID NO:2)	0.11	0.14	0.04	0.60	0.31	2.57	0.04	0.02	0.04
TPRPPIPKP (SEQ ID NO:4)	0.04	1.30	1.41	2.18	1.40	35.90	0.05	0.03	0.04
TQRPQIPPKP (SEQ ID NO:62)	0.04	1.05	0.43	2.18	1.19	34.18	0.03	0.02	0.03
TPNRCPPTKP (SEQ ID NO:63)	0.05	1.30	0.34	2.85	1.59	33.22	0.04	0.03	0.06
TYPRPLLAKP (SEQ ID NO:7)	0.05	0.69	0.37	2.21	1.06	20.15	0.10	0.05	0.05
TPLCQRPMKQKP (SEQ ID NO:8)	0.04	0.97	0.30	1.52	1.19	27.52	1.05	0.01	0.03

Table 10: Yeast screening results for variants of ZFP7264 with different center linkers

Sequence of the center linker	MEL-1 score for nuclease activity at targets having the indicated gap [score is normalized to ZFP7264 bound to its non-gapped target (underlined entry)]								
	0-bp gap	1-bp gap					2-bp gap		
	-	A	C	G	T	average ratio of 1bp:0bp score	TT	TA	CT
TGSQKP (SEQ ID NO:72)	1.00	0.05	0.05	0.09	0.06	0.06	0.09	0.02	0.08
TGGGGSQKP (SEQ ID NO:2)	0.28	0.08	0.07	0.14	0.34	0.57	0.12	0.09	0.15
TGLPKPKP(SEQ ID NO:64)	0.04	0.08	0.07	0.42	0.79	8.03	0.11	0.08	0.09
TSRPRPKP (SEQ ID NO:11)	0.08	0.18	0.08	0.61	3.38	13.33	0.03	0.07	0.10
TVPRPTPPKP (SEQ ID NO:12) (designated 1e)	0.08	1.24	0.14	1.40	2.34	20.28	0.12	0.09	0.11

[0111] Since the ELISA was in close concordance with the yeast screening data, we chose a set of exemplary 1bp-skipping linkers that performed among the best in the ELISA assay. These are listed in Table 11 and are designated "1c", "1d", "1e", and "1f". These designations are also included in the data presented in Tables 4, 6, and 10. The ELISA data for these exemplary linkers is also shown in Figures 4 and 5.

Table 11: Exemplary linker designs

Linker Sequence	Linker Designation	Number of Bases Skipped
THPRAPIPKP (SEQ ID NO:55)	1c	1
TPNRRPAPKP (SEQ ID NO:56)	1d	1
TVPRPTPPKP (SEQ ID NO:12)	1e	1
TYPRPIAAKP (SEQ ID NO:54)	1f	1

[0112] As stated previously in Example 1, target sites used for selection (Table 2a) contained degenerate bases in the gap in order to favor the selection of linkers that exhibited no inherent preference for particular gap sequences. The data shown in figures 4 and 5 suggest that this selection strategy was successful: proteins bearing the exemplary linkers exhibit little variation in binding among targets with gap bases of A, G, C or T. Moreover, the minor amount of variation that is observed is mirrored in the results obtained with control flexible liners (see, *e.g.*, Figure 5D for flexible linker (TGGGGSQKP) (SEQ ID NO:2)) indicating that variation is a property of the flanking fingers.

[0113] An analogous ELISA experiment was performed for linkers selected to skip a 2 basepair gap in the context of ZFP8196. Two additional control proteins were generated by replacing the central linker of each host ZFP with two alternative, previously characterized, linker sequences which collectively represented the state of the art for spanning 2bp. The sequences of these control linkers were TGGGGSGGSQKP (SEQ ID NO:14) and LRQKDGGGSERP (SEQ ID NO:68). These control proteins, as well as the host ZFPs, were also included in the ELISA studies. ZFPs were tested for binding to target sites containing either no gap, each of the 4 possible 1 basepair gaps, and each of the 16 possible 2 basepair gaps. Target sites are listed in Table 12. ELISA scores were normalized to the score of the 8196

ZFP bound to its non-gapped target site. Exemplary linkers were chosen based on their ELISA behavior in a similar fashion to the 1bp-skipping linkers. These exemplary linkers were designated "2d", "2e", and "2f". The ELISA results for the exemplary linkers in the 8196 ZFP and control ZFPs are shown in Figure 6. As seen in Figure 6, neither of the ZFPs bearing the control linkers shows a preference for a target site with a 2 basepair gap. However, the ZFPs with the selected linkers showed clear preferences for a 2 basepair gap over both the 1 basepair and non-gapped target sites.

Table 12: Target sites used for ELISA characterization of 2bp-skipping linkers

ZFP w/randomized linker	Gap Sequence	Target sites
8196	-	ATAAACTGCAAAAGGC (SEQ ID NO:32)
	A	ATAAACTGaCAAAAGGC (SEQ ID NO:73)
	C	ATAAACTGcCAAAAGGC (SEQ ID NO:74)
	G	ATAAACTGgCAAAAGGC (SEQ ID NO:75)
	T	ATAAACTGtCAAAAGGC (SEQ ID NO:76)
	AA	ATAAACTGaaCAAAAGGC (SEQ ID NO:113)
	AC	ATAAACTGacCAAAAGGC (SEQ ID NO:114)
	AG	ATAAACTGagCAAAAGGC (SEQ ID NO:115)
	AT	ATAAACTGatCAAAAGGC (SEQ ID NO:116)
	CA	ATAAACTGcaCAAAAGGC (SEQ ID NO:117)
	CC	ATAAACTGccCAAAAGGC (SEQ ID NO:118)
	CG	ATAAACTGcgCAAAAGGC (SEQ ID NO:119)
	CT	ATAAACTGctCAAAAGGC (SEQ ID NO:120)
	GA	ATAAACTGgaCAAAAGGC (SEQ ID NO:121)
	GC	ATAAACTGgcCAAAAGGC (SEQ ID NO:122)
	GG	ATAAACTGggCAAAAGGC (SEQ ID NO:123)
	GT	ATAAACTGgtCAAAAGGC (SEQ ID NO:124)
	TA	ATAAACTGtaCAAAAGGC (SEQ ID NO:125)
	TC	ATAAACTGtcCAAAAGGC (SEQ ID NO:126)
	TG	ATAAACTGtgCAAAAGGC (SEQ ID NO:127)
	TT	ATAAACTGttCAAAAGGC (SEQ ID NO:128)

Table 12: Duplex DNA target sites used in ELISA characterization studies had the general form of: $TTAG(X)_{16\text{-}18}TATC$, (SEQ ID NO:94) where $(X)_{16\text{-}18}$ was replaced with sequences listed in the table. DNA duplexes were made by annealing complementary oligonucleotides. Oligonucleotides complementary to the sequences listed in the table contained a 5' biotin. Underlined bases indicate the binding sequences for the four fingers of each host ZFP, while lowercase bases indicate inserted nucleotides (or "gap" bases).

[0114] As stated previously in Example 1, target sites used for selection (Table 2a) contained degenerate bases in the gap in order to favor the selection of linkers that exhibited no inherent preference for particular gap sequences. Shown in Figure 6 is an expansion of the scale for one of the flexible linkers (TGGGGSGGSQKP (SEQ ID NO:14)). This flexible linker should not have any interaction with the target site, and thus the pattern seen is likely due to the binding of

the zinc finger proteins. The fact that the exemplary linkers show a similar pattern of binding to 2 basepair gap target sites suggests that the selected linkers also should not impose any gap compositional bias in ZFP binding.

[0115] A more concise summary of this data is presented in Table 13, where ELISA scores were averaged over all of the 1 or 2 basepair gap compositions. Also reported is the fold preference for a 2 basepair gap over the 1 basepair gap and the non-gapped target. The most selective linker (TPNPHRRTDPSHKP (SEQ ID NO:69), "2f") represents an improvement in 2 basepair gap selectivity of >100-fold over a zero basepair gap and >20-fold over a 1 basepair gap compared to the control linkers.

Table 13: Summary of ELISA data for 2-bp skipping linkers

		Average Normalized ELISA Score (gap)			2bp-Gap Selectivity vs:	
Linker Sequence	Designation	0bp	1bp	2bp	0bp	1bp
TGGGGSGGSQKP (SEQ ID NO: 14)	flexible	0.019	0.041	0.010	0.6	0.2
LRQKDGGGSERP (SEQ ID NO:68)	Kim,Pabo	0.010	0.047	0.003	0.4	0.1
TPNPHRRTDPSHKP (SEQ ID NO:69)	2f	0.003	0.046	0.219	64.6	4.7
TLAPRPYRPPKP (SEQ ID NO:70)	2d	0.005	0.035	0.127	24.4	3.6
TPGGKSSRTDRNKP (SEQ ID NO:71)	2e	0.005	0.099	0.100	22.0	1.0

Example 3: ELISA characterization of linkers in various host ZFPs

[0116] To demonstrate the generality of the exemplary linkers, the four 1bp-skipping linkers listed in Table 11 (1c-1f) were cloned into twelve different host ZFPs. The host ZFPs were designated ZFP1, ZFP2 etc. The resultant proteins were expressed via *in vitro* transcription and translation and tested via ELISA, as described above. For comparison, we also tested the host ZFPs with a flexible linker (TGGGGSQKP (SEQ ID NO:2)), and the results are presented in Figure 7. This data demonstrates that relative to a standard flexible linker, the new linkers significantly increased the ELISA score of most host ZFPs, with the only exceptions being ZFPs that either saturate the assay (ZFP1 and ZFP2) or for which binding is undetectably low (ZFP 11 and ZFP 12). Average fold increases in ELISA score across all host ZFPs were from 3-5.

[0117] In a similar study, the three exemplary linkers selected to skip a 2 bp gap listed in Table 13 (2d-2f) were tested in six different host ZFPs as described

above (ZFP13, ZFP14, etc.), and these results are presented in Figure 8. In these experiments, average fold improvements in ELISA score across all host ZFPs ranged from 1.9 to 2.4.

Example 4: Characterization of ZFNs with exemplary linkers at endogenous loci in mammalian cells

[0118] ZFNs were then tested for their ability to induce double-stranded breaks at endogenous loci. Briefly, a plasmid encoding the 18 ZFNs (ZFP-*Fok*I fusions) described above (Example 3) were paired with their appropriate partner ZFNs and introduced into K562 cells by transfection using the Amaxa™ Nucleofection kit as specified by the manufacturer. To determine the ZFN activity at the target locus as measured by the level of non-homologous end joining (NHEJ), CEL-I mismatch assays were performed essentially as per the manufacturer's instructions (Transgenomic SURVEYOR™). Cells were harvested and chromosomal DNA prepared using a Quickextract™ Kit according to manufacturer's directions (Epicentre®). The appropriate region of the target locus was PCR amplified using Accuprime™ Taq High-fidelity DNA polymerase (Invitrogen) followed by treatment with the CEL-I enzyme.

[0119] Example gels generated for the CEL-1 assay are shown in Figure 9. Figure 9A shows screening data for ZFN3 and ZFN4 (ZFNs skipping 1 basepair) as the host ZFN whereas Figure 9B shows the screening data for ZFN14 (ZFN skipping 2 basepairs) as the host ZFN. The data for all the 1 bp skipping exemplary linkers is summarized in Figure 10 (ZFN1-ZFN12). Some of the ZFNs were expressed using a high expression condition. The high expression is obtained post-transfection by incubating cells at 37°C for 24 hours and then incubating at 30°C for 48 hours before genomic DNA was isolated. The ZFNs utilizing this condition are highlighted in Figure 10. Notably, three ZFNs that were inactive with the TGGGGSQKP (SEQ ID NO:2) linker ("flexible linker") (ZFNs 4, 9 and 10) become active when using a linker as described herein. For these cases, a value of 1.0% modification was assigned to the flexible linker for normalization purposes (the detection limit of the assay). In 85% of the ZFNs tested with the new linkers, an increase in the level of gene modification was observed, with an average increase in approximately 1.8- 2.8 fold across the nine active ZFN pairs.

[0120] Similarly, the ZFNs described above (Example 3) containing the 2bp skipping exemplary linkers (ZFN13-ZFN18) were tested at endogenous loci and the results are summarized in Figure 11. In this study, substitution of the linkers described herein improved activity as compared to the flexible linker for 3 out of 4 active ZFNs, and the average improvement was 1.5 – 2 fold across all active ZFN pairs.

Example 5: Secondary selections for a 2-bp skipping linker

[0121] A secondary set of libraries were constructed based on information obtained from the initial selections for a 2-bp skipping linker (Example 3 and Figure 3). These libraries fixed the three carboxy-terminal residues of the linker as RPP (lysine, proline, proline) and randomized the remaining amino-terminal residues. The library design is shown in Figure 12.

[0122] Selections were performed in the same manner as in Example 1 using ZFP8196 as the host protein. Gap selectivity of the selected phage pool is shown in Figure 13A, and the sequences of the linkers from individual clones are shown in Figure 13B.

[0123] An ELISA experiment was performed on each of the individual clones from the secondary selection (Figure 13B), similar to that of Example 2. ZFPs were tested for binding to target sites containing either no gap, a pool of the 4 possible 1 basepair gaps, and a pool of the 16 possible 2 basepair gaps. Target sites are listed in Table 12. ELISA scores were normalized to the score of the host ZFP8196 bound to its non-gapped target site. The ELISA results for ZFPs bearing linkers that showed both a good normalized ELISA score on the pool of 2-bp gap target sites and good gap selectivity are shown in Table 14.

Table 14: Summary of ELISA data for 2-bp skipping linkers

	Average Normalized ELISA Score (gap)			2bp-Gap Selectivity vs:	
Linker Sequence	0bp	1bp	2bp	0bp	1bp
TETTRPFRPPKP (SEQ ID NO:183)	0.001	0.001	0.570	570.0	570.0
TGSLRPYRRPKP (SEQ ID NO:177)	0.001	0.010	0.310	310.0	31.0
TSINRPFRRPKP (SEQ ID NO:184)	0.010	0.020	0.570	57.0	28.5
TNTTRPYRPPKP (SEQ ID NO:175)	0.001	0.010	0.410	410.0	41.0
TASCPRPFRPPKP (SEQ ID NO:194)	0.010	0.020	0.370	37.0	18.5
TGEARPYRPPKP (SEQ ID NO:178)	0.001	0.010	0.610	610.0	61.0

[0124] As shown, ZFPs with the selected linkers showed clear preferences for a 2 basepair gap over both the 1 basepair and non-gapped target sites.

[0125] Although disclosure has been provided in some detail by way of illustration and example for the purposes of clarity of understanding, it will be apparent to those skilled in the art that various changes and modifications can be practiced without departing from the scope of the disclosure. Accordingly, the foregoing descriptions and examples should not be construed as limiting.

CLAIMS

1. A multi-finger zinc finger protein that specifically binds to a target site, the multi-finger zinc finger protein comprising non-naturally occurring zinc finger modules, wherein each zinc finger module binds to a target subsite and at least two of the non-naturally occurring zinc finger DNA-binding modules that bind to target subsites separated by 1 or 2 base pairs are joined by an amino acid linker of 5 to 20 amino acid residues between the last residue of the N-terminal zinc finger module and the first residue of C-terminal zinc finger module, the amino acid linker comprising an N-terminal amino acid linker residue adjacent to the N-terminal zinc finger module, a C-terminal amino acid linker residue adjacent to the C-terminal zinc finger module, and amino acid residues internal to the N- and C-terminal amino acid linker residues, wherein said amino acid linker is selected from the group consisting of: TPDAPKPKP, TPGLHRPKP, TEPRAKPPKP, TPSHTPRPKP, TGYSIPRPKP, TYPRPIAAKP, THPRAPIPKP, TPNRRPAPKP, TSPRLPAPKP, TCPRPPTRKP, TSSPRSNAKP, TVSPAPCRSKP, TPDRPISTCKP, TPRPPIPKP, TQRPQIPPKP, TPNRCPPTKP, TYPRPLLAKP, TPLCQRPMKQKP, TGLPKPKP, TSRPRPKP, TLPLPRPKP, TVPRPTPPKP, and TLPPCFRPKP when the target subsites are separated by 1 base pair, or are selected from the group consisting of TLAPRPYRPPKP, TPNPHRRTDPSHKP, TPGGKSSRTDRNKP, TNTTRPYRPPKP, TGSLRPYRRPKP, TGEARPYRPPKP, TETTRPFRPPKP, TSINRPFRRPKP, and TASCPRPFRPPKP when the target subsites are separated by 2 base pairs.

2. The zinc finger protein of claim 1, wherein the amino acid linker further comprises at least two basic amino acid residues.

3. The zinc finger protein of claim 2, wherein the basic amino acid residues are each independently selected from the group consisting of: an arginine residue, a histidine residue, and a lysine residue.

4. A fusion protein comprising the zinc finger protein of any one of claims 1 to 3 and a regulatory domain.

5. The fusion protein of claim 4, wherein the regulatory domain is a transcriptional modulating domain.

6. The fusion protein of claim 5, wherein the regulatory domain is an activation domain or a repression domain.

7. The fusion protein of claim 4, wherein the regulatory domain is a cleavage domain or a cleavage half-domain.

8. A polynucleotide encoding any one of the proteins of any one of claims 1 to 7.

9. A cell comprising any one of the proteins of any one of claims 1 to 7 and/or a polynucleotide according to claim 8.

10. An *in vitro* method of modulating expression of a gene in a cell, the method comprising contacting with the cell, or expressing in the cell, a protein according to any one of claims 1 to 7 or a polynucleotide according to claim 8.

11. The *in vitro* method of claim 10, wherein the modulation comprises up or down regulation of the gene.

12. The *in vitro* method of claim 10, wherein the modulation comprises cleavage of the gene.

13. The *in vitro* method of any one of claims 10 to 12, wherein the gene is an endogenous cellular gene.

14. Use, to modulate expression of a gene in a cell, of a protein according to any one of claims 1 to 7 or a polynucleotide according to claim 8.

15. The use of claim 14, wherein the modulation comprises up or down regulation of the gene.

16. The use of claim 14, wherein the modulation comprises cleavage of the gene.

17. The use of any one of claims 14 to 16, wherein the gene is an endogenous cellular gene.

18. A protein according to any one of claims 1 to 7 or a polynucleotide according to claim 8, for use to modulate expression of a gene in a cell.

19. The protein or polypeptide for use according to claim 18, wherein the modulation comprises up or down regulation of the gene.

20. The protein or polynucleotide for use according to claim 18, wherein the modulation comprises cleavage of the gene.

21. The protein or polynucleotide for use according to any one of claims 18 to 20, wherein the gene is an endogenous cellular gene.

22. Use, in the manufacture of a medicament for modulating expression of a gene in a cell, of a protein according to any one of claims 1 to 7 or a polynucleotide according to claim 8.

23. The use of claim 22, wherein the modulating comprises up or down regulation of the gene.

24. The use of claim 22, wherein the modulating comprises cleavage of the gene.

25. The use of any one of claims 22 to 24, wherein the gene is an endogenous cellular gene.

FIG. 1A

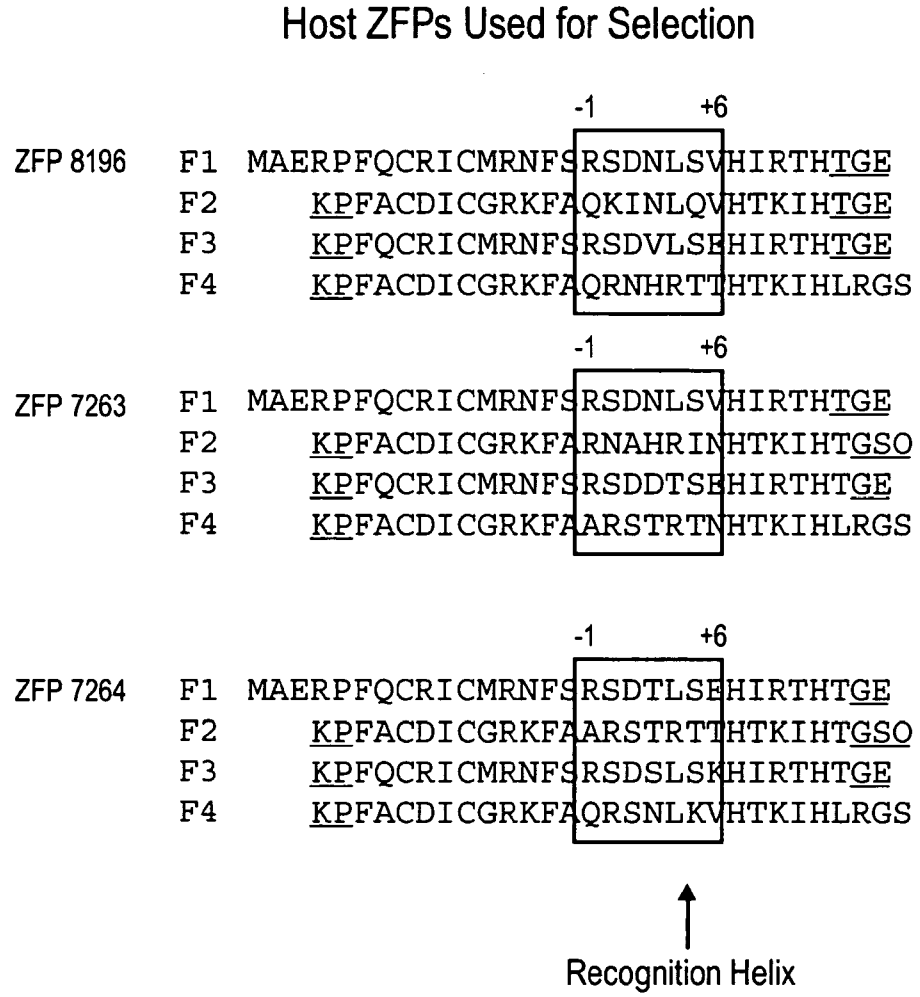
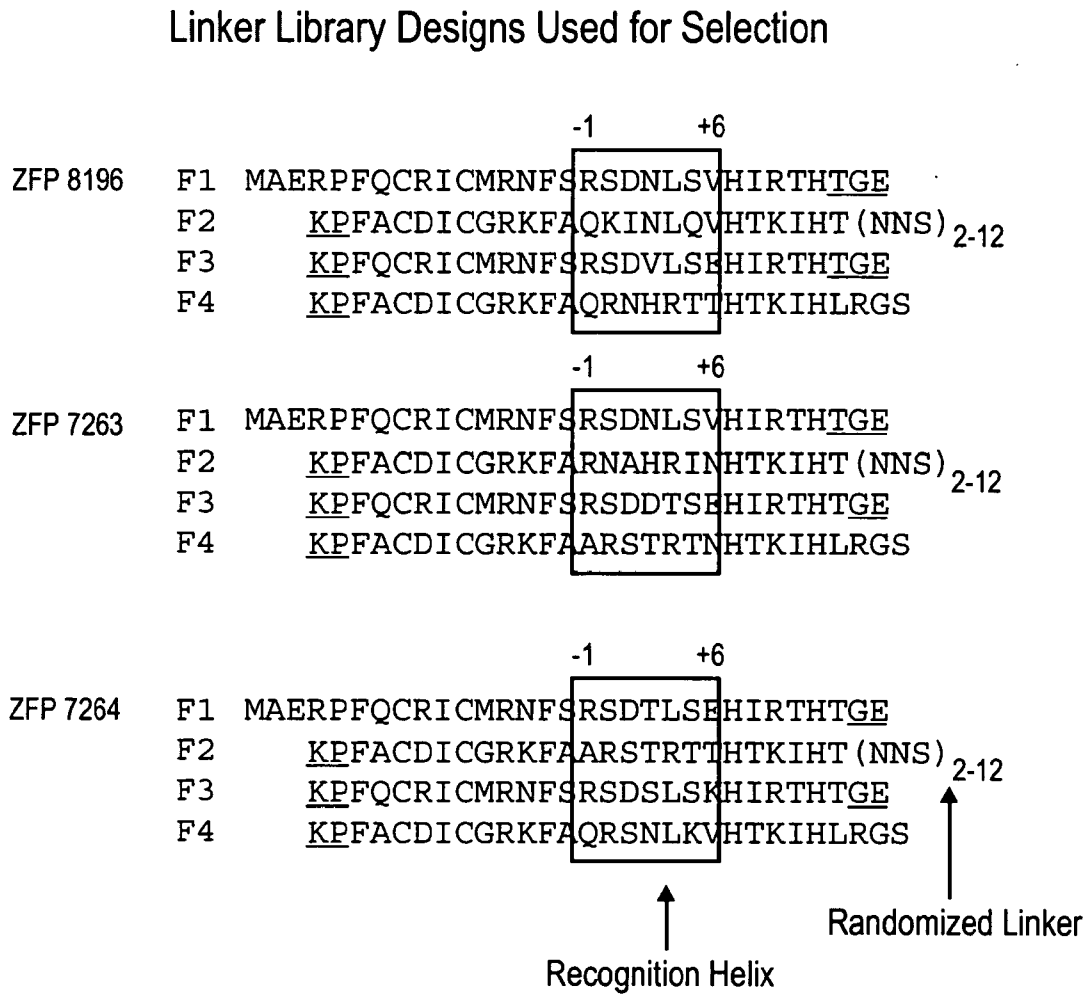
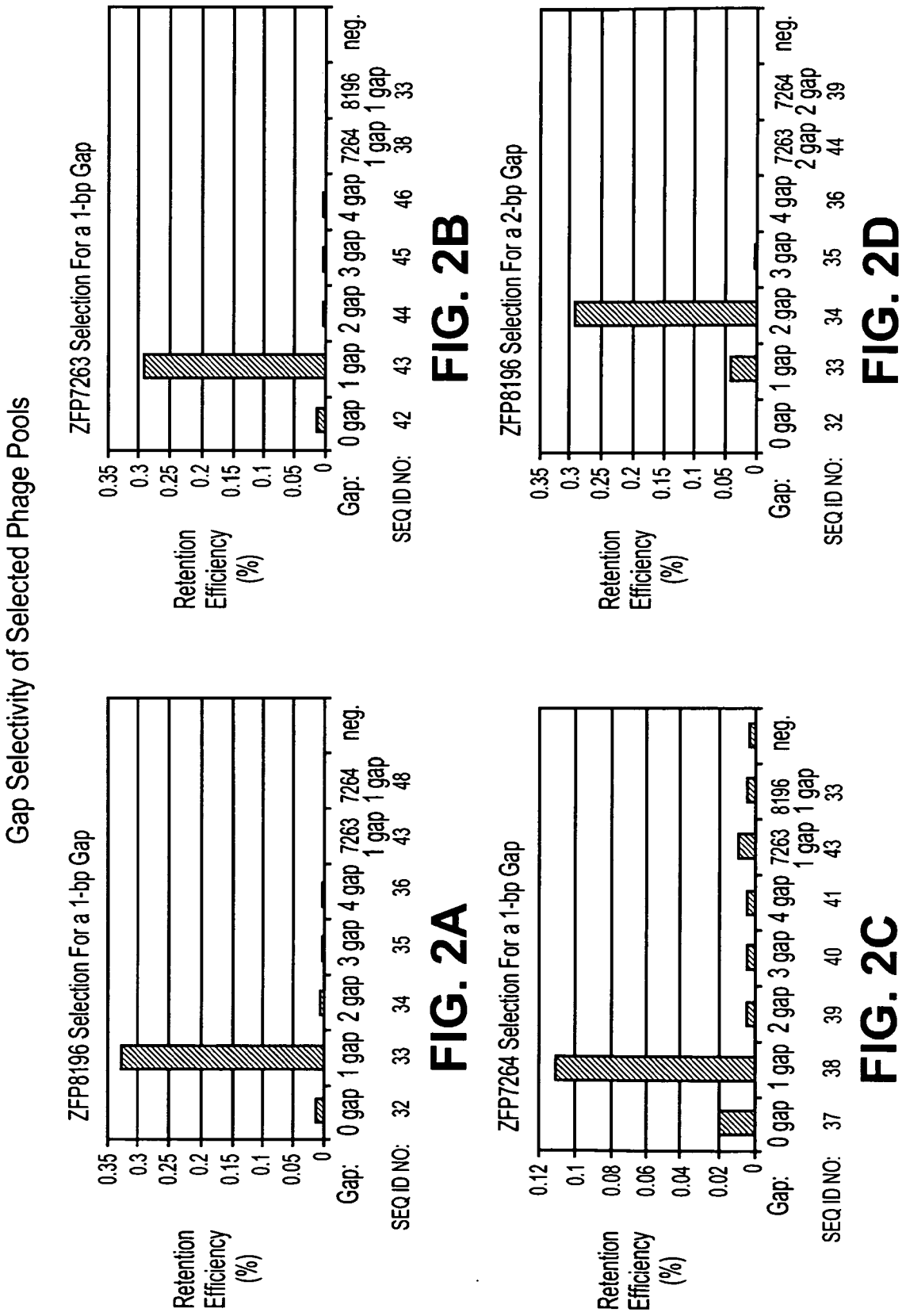

Host ZFPs Used for Selection
-1
+6
ZFP 8196
F1 MAERPFQCRICMRNFSRSDNLSVHIRTHTGE
F2 KPFACDICGRKFAQKINLQVHTKIHTGE
F3 KPFQCRICMRNFSRSDVLSEHIRTHTGE
F4 KPFACDICGRKFAQRNHRTTHTKIHLRGS
-1
+6
ZFP 7263
F1 MAERPFQCRICMRNFSRSDNLSVHIRTHTGE
F2 KPFACDICGRKFARNAHRINHTKIHTGSQ
F3 KPFQCRICMRNFSRSDDTSEHIRTHTGE
F4 KPFACDICGRKFAARSTRTNHTKIHLRGS
-1
+6
ZFP 7264
F1 MAERPFQCRICMRNFSRSDTLSEHIRTHTGE
F2 KPFACDICGRKFAARSTRTTHTKIHTGSQ
F3 KPFQCRICMRNFSRSDSLSKHIRTHTGE
F4 KPFACDICGRKFAQRSNLKVHTKIHLRGS
Recognition Helix

FIG. 1B


Linker Library Designs Used for Selection
-1
+6
ZFP 8196
F1 MAERPFQCRICMRNFSRSDNLSVHIRTHTGE
F2 KPFACDICGRKFAQKINLQVHTKIHT(NNS)2-12
F3 KPFQCRICMRNFSRSDVLSEHIRTHTGE
F4 KPFACDICGRKFAQRNHRTTHTKIHLRGS
-1
+6
ZFP 7263
F1 MAERPFQCRICMRNFSRSDNLSVHIRTHTGE
F2 KPFACDICGRKFARNAHRINHTKIHT(NNS)2-12
F3 KPFQCRICMRNFSRSDDTSEHIRTHTGE
F4 KPFACDICGRKFAARSTRTNHTKIHLRGS
-1
+6
ZFP 7264
F1 MAERPFQCRICMRNFSRSDTLSEHIRTHTGE
F2 KPFACDICGRKFAARSTRTTHTKIHT(NNS)2-12
F3 KPFQCRICMRNFSRSDSLSKHIRTHTGE
F4 KPFACDICGRKFAQRSNLKVHTKIHLRGS
Randomized Linker
Recognition Helix


Gap Selectivity of Selected Phage Pools
ZFP8196 Selection For a 1-bp Gap
Retention Efficiency (%)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Gap: 0 gap 1 gap 2 gap 3 gap 4 gap 7263 1 gap 7264 1 gap neg.
SEQ ID NO: 32 33 34 35 36 43 48
FIG. 2A
ZFP7263 Selection For a 1-bp Gap
Retention Efficiency (%)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Gap: 0 gap 1 gap 2 gap 3 gap 4 gap 7264 1 gap 8196 1 gap neg.
SEQ ID NO: 42 43 44 45 46 38 33
FIG. 2B
ZFP7264 Selection For a 1-bp Gap
Retention Efficiency (%)
0.12
0.1
0.08
0.06
0.04
0.02
0
Gap: 0 gap 1 gap 2 gap 3 gap 4 gap 7263 1 gap 8196 1 gap neg.
SEQ ID NO: 37 38 39 40 41 43 33
FIG. 2C
ZFP8196 Selection For a 2-bp Gap
Retention Efficiency (%)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Gap: 0 gap 1 gap 2 gap 3 gap 4 gap 7263 2 gap 7264 2 gap neg.
SEQ ID NO: 32 33 34 35 36 44 39
FIG. 2D

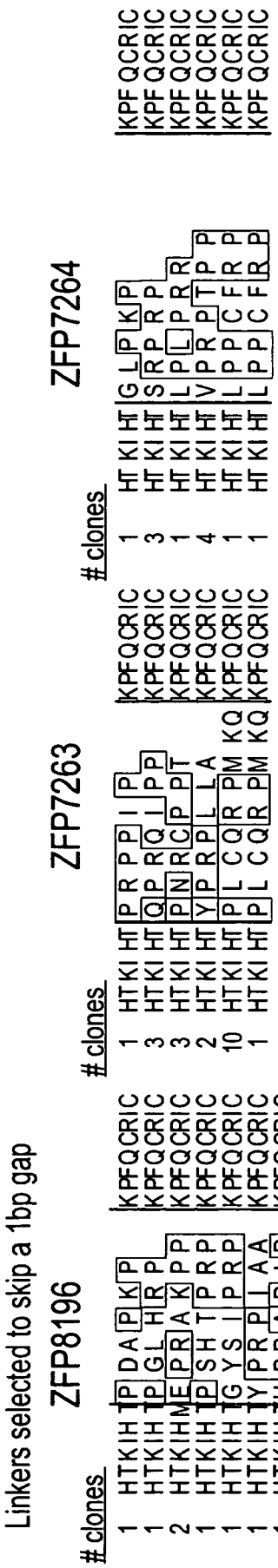

Linkers selected to skip a 1bp gap
ZFP8196
clones
1 HTKIHTPDAPKP KPFQCRIC
1 HTKIHTPGLHRP KPFQCRIC
2 HTKIHMEPRAKPP KPFQCRIC
1 HTKIHTPSHTPRP KPFQCRIC
1 HTKIHTGYSIPRP KPFQCRIC
1 HTKIHTYPRPIAA KPFQCRIC
1 HTKIHTHPRAPIP KPFQCRIC
1 HTKIHTPNRRPAP KPFQCRIC
1 HTKIHTSPRLPAP KPFQCRIC
1 HTKIHTCPRPPTR KPFQCRIC
1 HTKIHTSSPRSNA KPFQCRIC
1 HTKIHTVSPAPCR SKPFQCRIC
1 HTKIHMPDRPISTCKPFQCRIC
ZFP7263
clones
1 HTKIHTPRPPIP KPFQCRIC
3 HTKIHTQPRQIPP KPFQCRIC
3 HTKIHTPNRCPPT KPFQCRIC
2 HTKIHTYPRPLLA KPFQCRIC
10 HTKIHTPLCQRPM KQ KPFQCRIC
1 HTKIHTPLCQRPM KQ KPFQCRIC
ZFP7264
clones
1 HTKIHTGLPKP KPFQCRIC
3 HTKIHTSRPRP KPFQCRIC
1 HTKIHTLPLPRR KPFQCRIC
4 HTKIHTVPRPTPP KPFQCRIC
1 HTKIHTLPPCFRP KPFQCRIC
1 HTKIHTLPPCFRP KPFQCRIC

FIG. 3A

Linkers selected to skip a 2bp gap
ZFP8196
clones
13 HTKIHTNACKPYRTP KPFQCRIC
2 HTKIHTLAPRPYRPP KPFQCRIC
1 HTKIHTGSPHVRANS QPFQCRIC
1 HTKIHTDAAPRPRDT KPFQCRIC
1 HTKIHTEYCTRPFRPPI KPFQCRIC
1 HTKIHTPNPHRRYDPSH KPFQCRIC
1 HTKIHTNTPRPYRLRPP KPFQCRIC
1 HTKIHTPGGKSSRTDRN KPFQCRIC

FIG. 3B

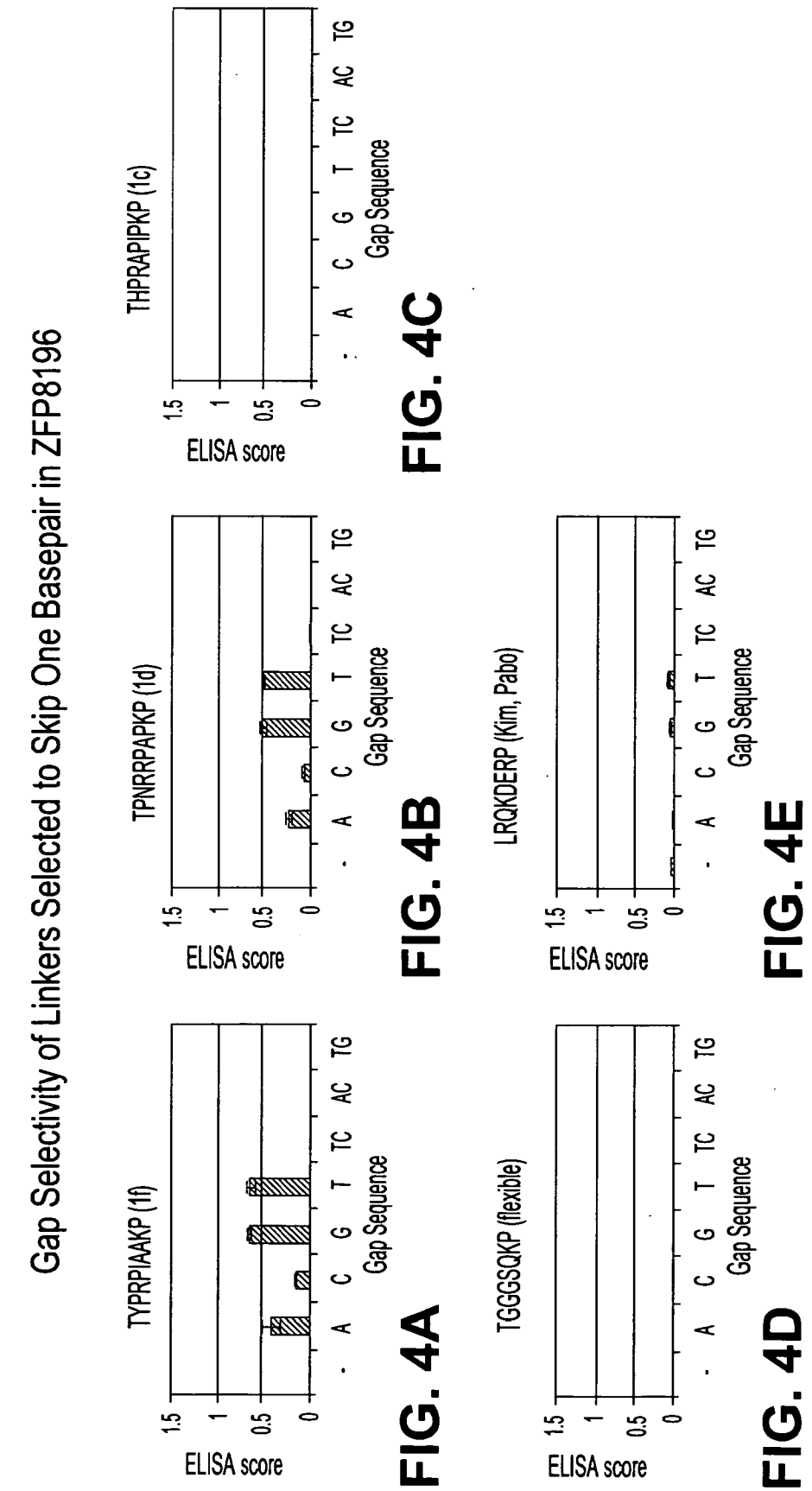


FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

FIG. 4E

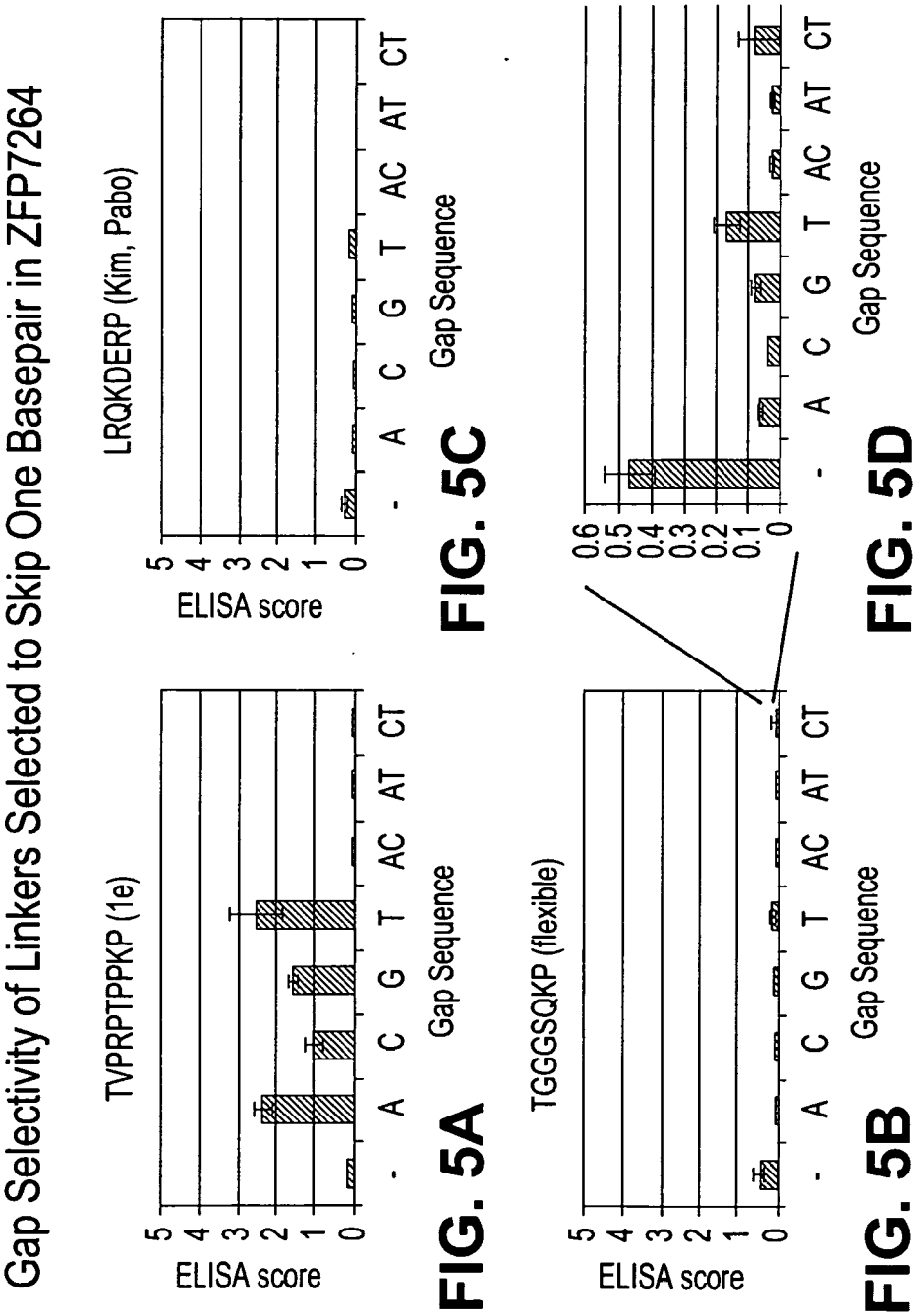


FIG. 5A

FIG. 5C

FIG. 5B

FIG. 5D

Gap Selectivity of Linkers Selected to Skip Two Basepairs in ZFP8196

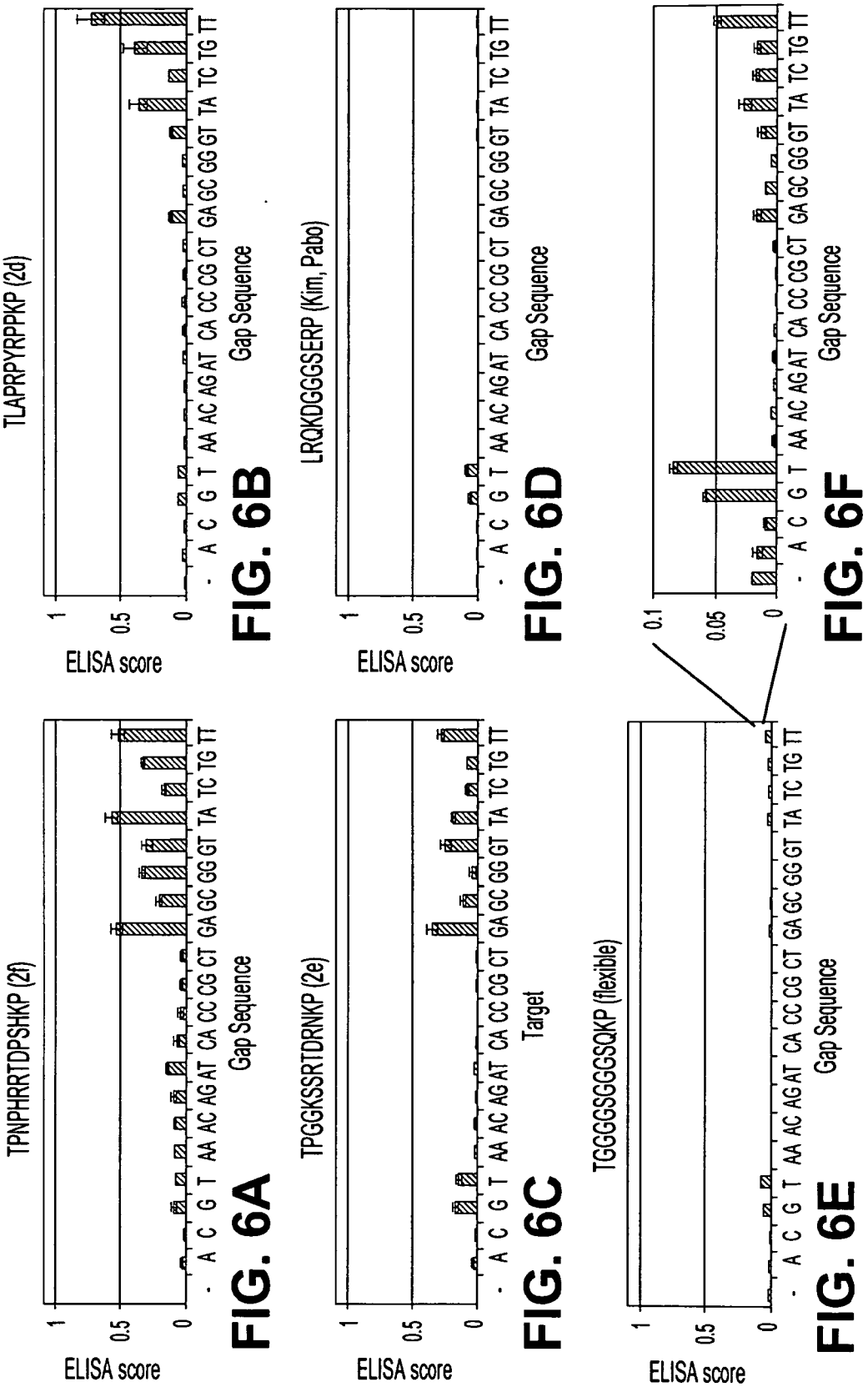


FIG. 6A FIG. 6B FIG. 6C FIG. 6D FIG. 6E FIG. 6F

FIG. 7

Portability Studies With Linkers Selected to Skip One Basepair

A

Linker Sequence	Designation	ELISA Score ZFP1	ZFP2	ZFP3	ZFP4	ZFP5	ZFP6	ZFP7	ZFP8	ZFP9	ZFP10	ZFP11	ZFP12
TGGGGSQKP	flexible	0.75	1.08	0.21	0.26	0.08	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TVPRPTPPKP	1e	1.19	1.30	1.05	1.10	0.75	0.29	0.48	0.38	0.50	0.18	0.05	0.05
TYPRPIAAKP	1f	1.25	1.35	1.03	0.94	0.96	0.30	0.33	0.12	0.30	0.11	0.05	0.05
TPNRRPAPKP	1d	1.20	1.08	1.04	0.83	0.85	0.25	0.39	0.28	0.24	0.12	0.05	0.05
THPRAPIPKP	1c	0.92	1.18	0.66	0.56	0.91	0.23	0.21	0.26	0.18	0.05	0.05	0.05

B

Linker Sequence	Designation	ELISA Score Normalized to Flexible Linker ZFP1	ZFP2	ZFP3	ZFP4	ZFP5	ZFP6	ZFP7	ZFP8	ZFP9	ZFP10	ZFP11	ZFP12	Average
TGGGGSQKP	flexible	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
TVPRPTPPKP	1e	1.6	1.2	4.9	4.1	9.2	5.9	9.6	7.6	10.1	3.6	1.0	1.0	5.0
TYPRPIAAKP	1f	1.7	1.2	4.9	3.5	11.7	5.9	6.5	2.3	6.0	2.2	1.0	1.0	4.0
TPNRRPAPKP	1d	1.6	1.0	4.9	3.1	10.4	4.9	7.8	5.7	4.8	2.4	1.0	1.0	4.0
THPRAPIPKP	1c	1.2	1.1	3.1	2.1	11.2	4.7	4.3	5.3	3.6	1.0	1.0	1.0	3.3

underlined values show >4-fold improvement

FIG. 8

Portability Studies With Linkers Selected to Skip Two Basepairs

A

Linker		ELISA Score					
Sequence	Designation	ZFP13	ZFP14	ZFP15	ZFP16	ZFP17	ZFP18
TGGGGSGGSQKP	flexible	0.25	0.61	0.05	0.13	0.35	0.51
TPNPHRRTDPSHKP	2f	0.48	1.13	0.27	0.31	0.79	0.47
TLAPRPYRPPKP	2d	0.44	1.13	0.11	0.34	0.82	0.43
TPGGKSSRTDRNKP	2e	0.42	0.75	0.17	0.27	0.68	0.52

B

Linker		ELISA Score Normalized to Flexible Linker						
Sequence	Designation	ZFP13	ZFP14	ZFP15	ZFP16	ZFP17	ZFP18	Average
TGGGGSGGSQKP	flexible	1.0	1.0	1.0	1.0	1.0	1.0	
TPNPHRRTDPSHKP	2f	1.9	1.9	<u>5.1</u>	<u>2.3</u>	<u>2.3</u>	0.9	<u>2.4</u>
TLAPRPYRPPKP	2d	1.7	1.9	2.1	2.5	2.4	0.8	1.9
TPGGKSSRTDRNKP	2e	1.7	1.2	<u>3.3</u>	<u>2.0</u>	<u>2.0</u>	1.0	1.9

underlined values show >2-fold improvement

FIG. 9

Example Assay Gels

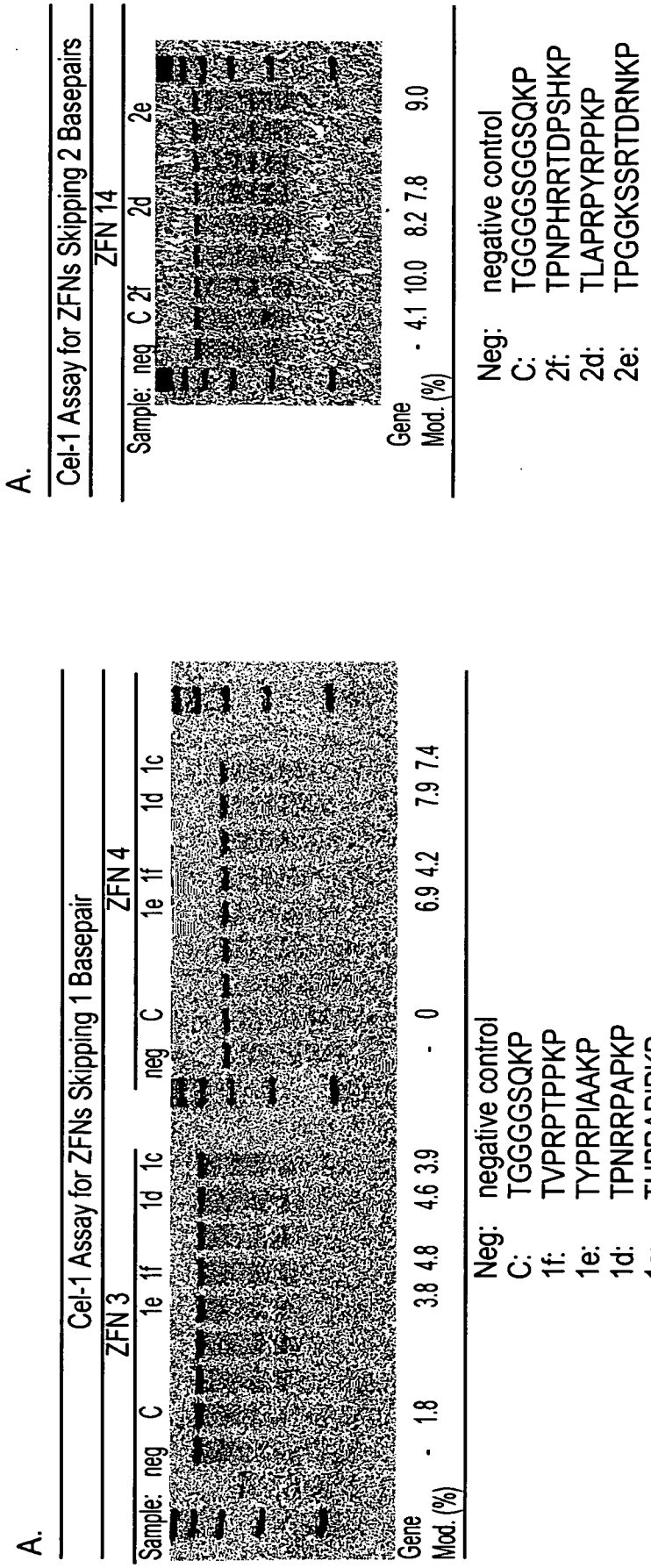


FIG. 10

Gene Modification for ZFNs Containing New Linkers Skipping One Basepairs

A

Linker		Gene Modification (%)											
Sequence	Designation	ZFN1	ZFN2	ZFN3	ZFN4	ZFN5	ZFN6	ZFN7	ZFN8	ZFN9	ZFN10	ZFN11	ZFN12
TGGGGSQKP	flexible	6.1	1.8	5.9	0.0	3.5	-	1.1	-	0.0	0.0	-	1.7
TVPRPTPPKP	1e	9.5	3.8	9.3	1.0	4.9	-	2.6	-	3.7	6.9	-	1.9
TYPRPIAAKP	1f	9.6	4.8	12.0	1.0	2.5	-	1.5	-	1.5	4.2	-	2.8
TPNRRPAPKP	1d	6.4	4.6	12.5	1.5	6.2	-	2.6	-	4.5	7.9	-	1.9
THPRAPIPKP	1c	9.9	3.9	12.8	1.4	5.1	-	2.7	-	NA	7.4	-	NA

B

Linker		Gene Modification Normalized to Flexible Linker												
Sequence	Designation	ZFN1	ZFN2	ZFN3	ZFN4	ZFN5	ZFN6	ZFN7	ZFN8	ZFN9	ZFN10	ZFN11	ZFN12	Average
TGGGGSQKP	flexible	1.0	1.0	1.0	-	1.0	-	1.0	-	-	-	-	1.0	
TVPRPTPPKP	1e	1.6	2.1	1.6	>1	1.4	-	2.4	-	>3.5	>7.0	-	1.1	2.4
TYPRPIAAKP	1f	1.6	2.7	2.0	>1	0.7	-	1.4	-	>1.5	>4.0	-	1.6	1.8
TPNRRPAPKP	1d	1.0	2.6	2.1	>1.5	1.8	-	2.4	-	>4.5	>8.0	-	1.1	2.8
THPRAPIPKP	1c	1.6	2.2	2.2	>1.5	1.5	-	2.5	-	NA	>7.0	-	NA	2.6

high expression condition

underlined values show >2-fold improvement

-- no quantifiable signal
NA not tested

FIG. 11

Gene Modification for ZFNs Containing New Linkers Skipping Two Basepairs

A

Linker		Gene Modification (%)					
Sequence	Designation	ZFN13	ZFN14	ZFN15	ZFN16	ZFN17	ZFN18
TGGGGSGGSQKP	flexible	-	7.5	2.5	2.9	5.3	-
TPNPHRRTDPSHKP	2f	-	17.0	5.6	7.3	4.7	-
TLAPRPYRPPKP	2d	-	11.3	5.0	4.8	4.9	-
TPGGKSSRTDRNKP	2e	-	11.7	5.0	4.3	6.3	-

B

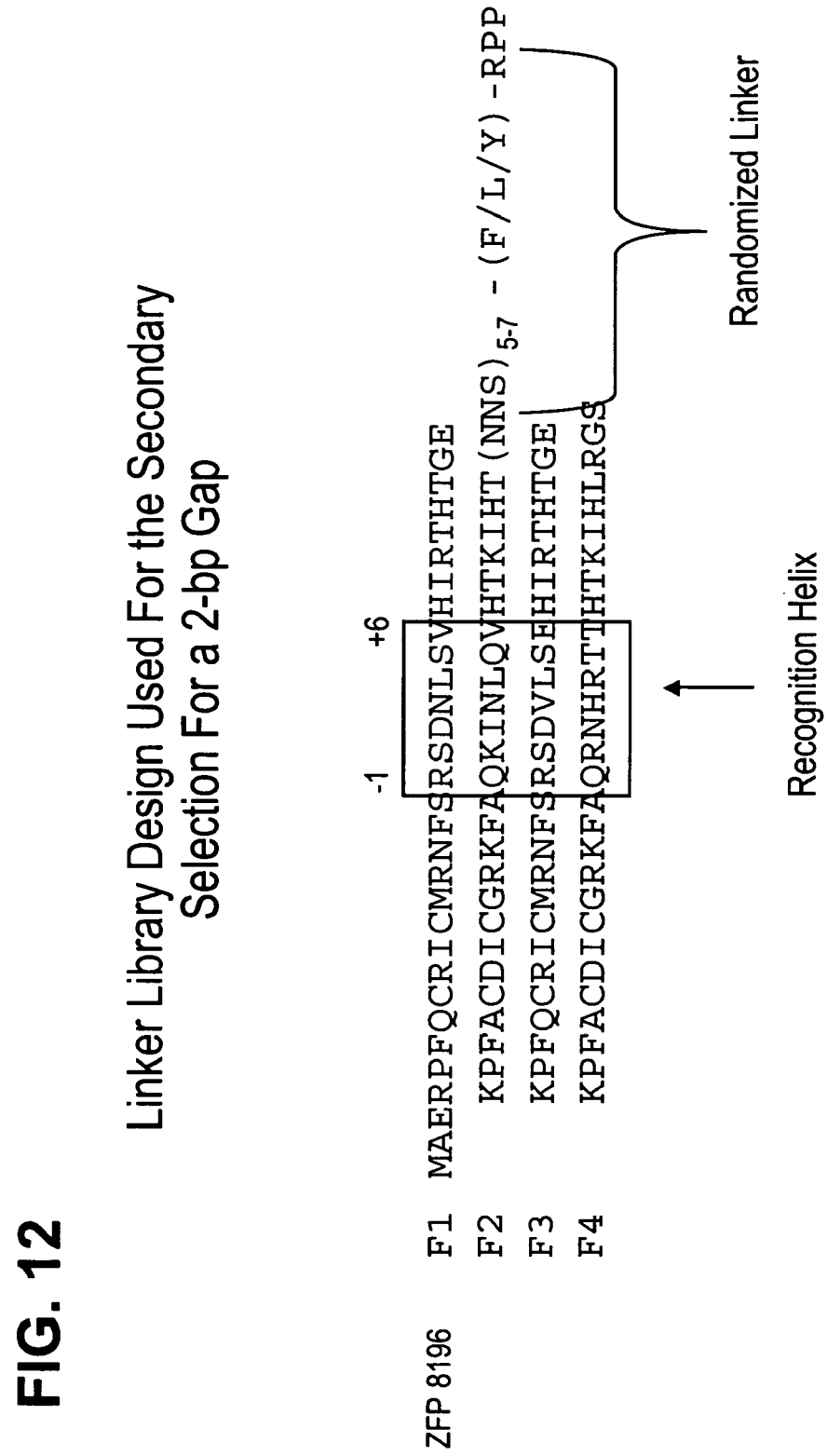
Linker		Gene Modification Normalized to Flexible Linker						
Sequence	Designation	ZFN13	ZFN14	ZFN15	ZFN16	ZFN17	ZFN18	Average
TGGGGSGGSQKP	flexible	-	1.0	1.0	1.0	1.0	-	
TPNPHRRTDPSHKP	2f	-	2.3	2.2	2.5	0.9	-	2.0
TLAPRPYRPPKP	2d	-	1.5	2.0	1.7	0.9	-	1.5
TPGGKSSRTDRNKP	2e	-	1.6	2.0	1.5	1.2	-	1.6

high expression condition

underlined values show >2-fold improvement

--|no quantifiable signal

FIG. 12


Linker Library Design Used For the Secondary Selection For a 2-bp Gap
ZFP 8196
F1 MAERPFQCRICMRNFSRSDNLSVHIRTHTGE
F2 KPFACDICGRKFAQKINLQVHTKIHT(NNS)5-7-(F/L/Y)-RPP
F3 KPFQCRICMRNFSRSDVLSEHIRTHTGE
F4 KPFACDICGRKFAQRNHRTTHTKIHLRGS
-1
+6
Randomized Linker
Recognition Helix

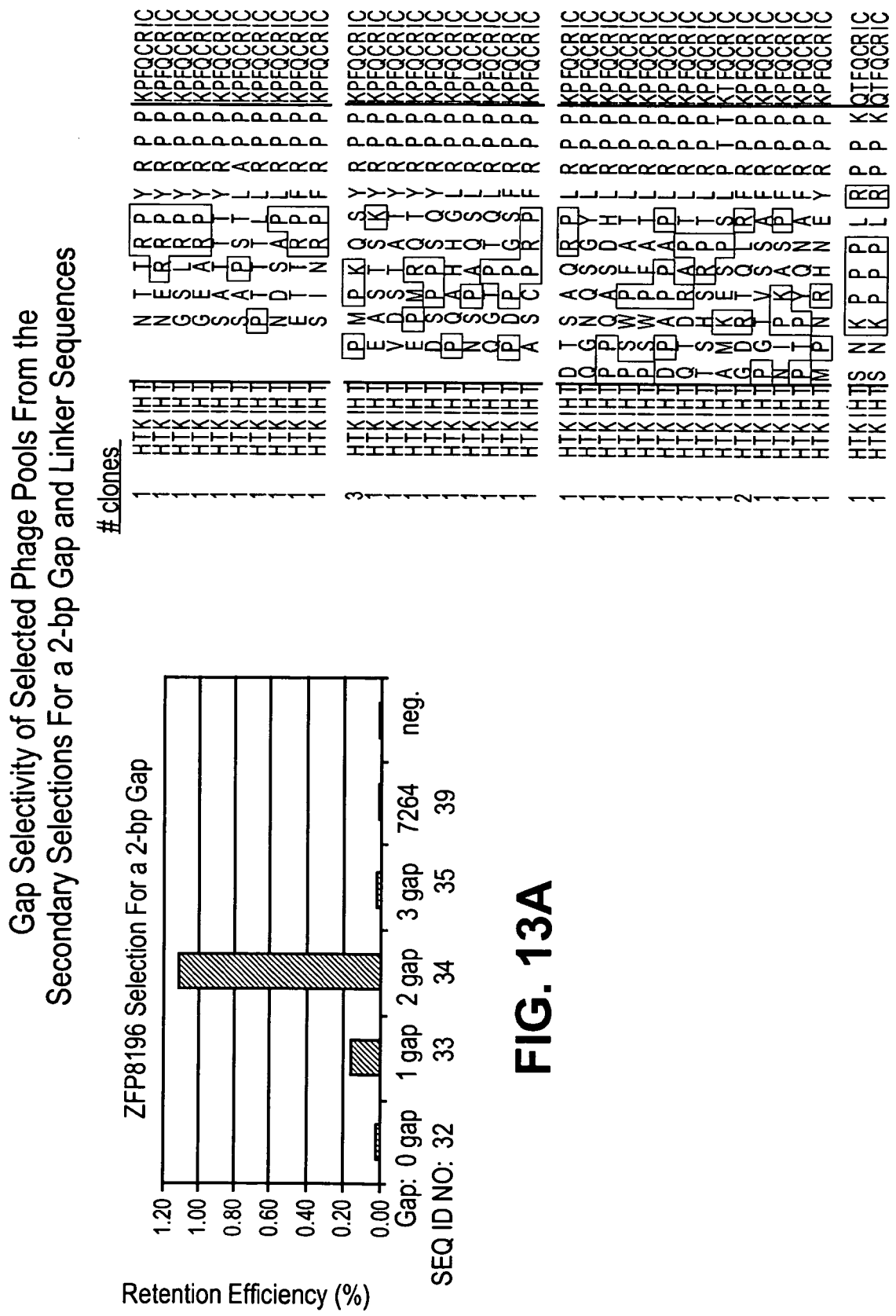


FIG. 13A

FIG. 13B

Portability Studies With Linkers Selected to Skip One Basepair

A

Linker		ELISA Score											
Sequence	Designation	ZFP1	ZFP2	ZFP3	ZFP4	ZFP5	ZFP6	ZFP7	ZFP8	ZFP9	ZFP10	ZFP11	ZFP12
TGGGGSQKP	flexible	0.75	1.08	0.21	0.26	0.08	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TVPRPTPPKP	1e	1.19	1.30	1.05	1.10	0.75	0.29	0.48	0.38	0.50	0.18	0.05	0.05
TYPRPIAAKP	1f	1.25	1.35	1.03	0.94	0.96	0.30	0.33	0.12	0.30	0.11	0.05	0.05
TPNRRPAPKP	1d	1.20	1.08	1.04	0.83	0.85	0.25	0.39	0.28	0.24	0.12	0.05	0.05
THPRAPIPKP	1c	0.92	1.18	0.66	0.56	0.91	0.23	0.21	0.26	0.18	0.05	0.05	0.05

B

Linker		ELISA Score Normalized to Flexible Linker												
Sequence	Designation	ZFP1	ZFP2	ZFP3	ZFP4	ZFP5	ZFP6	ZFP7	ZFP8	ZFP9	ZFP10	ZFP11	ZFP12	Average
TGGGGSQKP	flexible	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
TVPRPTPPKP	1e	1.6	1.2	4.9	4.1	9.2	5.9	9.6	7.6	10.1	3.6	1.0	1.0	5.0
TYPRPIAAKP	1f	1.7	1.2	4.9	3.5	11.7	5.9	6.5	2.3	6.0	2.2	1.0	1.0	4.0
TPNRRPAPKP	1d	1.6	1.0	4.9	3.1	10.4	4.9	7.8	5.7	4.8	2.4	1.0	1.0	4.0
THPRAPIPKP	1c	1.2	1.1	3.1	2.1	11.2	4.7	4.3	5.3	3.6	1.0	1.0	1.0	3.3

underlined values show >4-fold improvement