Global and Tunable Suppression of Zinc Finger Nuclease and ZFP-Transcription Factor Off-target Activity via Discrete Framework Substitutions

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Abstract

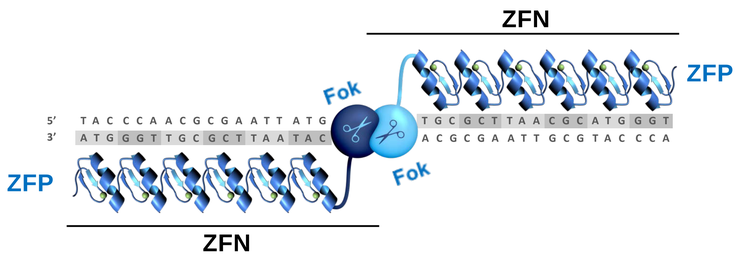
Designed sequence specific nucleases and transcription factors offer the prospect of treatments for currently intractable conditions by enabling the modification or regulated expression of targeted loci in disease-relevant cells. A considerable challenge in the development of these agents, however, involves the need to minimize off-target effects while retaining therapeutically sufficient on-target activity. Among the platforms available to develop nucleases and transcription factors, strategies for addressing this challenge typically involve either labor-intensive cycles of redesign of the base-sensing interface, or a tradeoff between activity and specificity that may compromise on-target performance. To realize the full potential of gene-targeted medicines, approaches for optimizing specificity will be needed that both avoid these limitations, and are ideally simple, global, tunable and selective. To our knowledge, approaches that combine these features have not been previously described.

In the work described here, we have addressed this issue in the context of designed zinc finger nucleases (ZFNs) and ZFP-transcription factors (ZFP-TFs) by developing a panel of single residue substitutions within otherwise invariant framework sequences that enable rapid optimization of specificity. These variants were developed by screening alternative residues at positions known or anticipated to nonspecifically contact DNA. Our studies proceeded in three stages. First, we examined substitutions within the zinc finger domain and identified a replacement – Arg(-8)Gln – that disrupts a highly-conserved phosphate contact and reduces nonspecific activity. Within the context of a well-characterized ZFN dimer, varying the number of fingers bearing this change provided an effective means for tuning total activity as well as on target preference. In the second stage of these studies, we examined substitutions within the Fok domain. In an analysis of 190 substitutions of 10 different DNA-proximal residues introduced into a previously characterized ZFN dimer (Nat Biotechnol. 2016 ;34(4):424-9), over twenty variants were identified that exhibited a broad spectrum of impacts on activity and specificity, including a single point mutant that reduced off-target cleavage 1000-fold while retaining full on-target activity. Finally, we combined approaches to generate nucleases targeted to the TCR alpha gene, and showed that the resultant ZFNs could introduce indels into the targeted locus in T cells at levels exceeding 99%, with little or no detectable off-target activity. In a parallel effort we have extended these studies to the optimization of ZFP-TFs. For our TAU program, we have shown that the introduction of three Arg(-8)Gln substitutions into a six-finger repressor enabled a 25-fold reduction in the level of off-target repression as gauged via microarray analysis. These results establish a new approach for optimizing ZFP specificity that should enable the development of highly specific ZFNs and ZFP-TFs for virtually any gene target.

Designed Zinc Finger Proteins

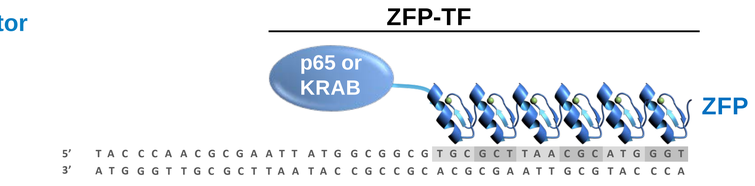
Zinc Finger Nuclease (ZFN):

- Programmable nuclease
- Contains two domains:
  - **nuclease domain of FokI**
  - **zinc finger protein (ZFP)**
- Cleaves only when dimerized
- Specifies an extended target (30 - 36bp)

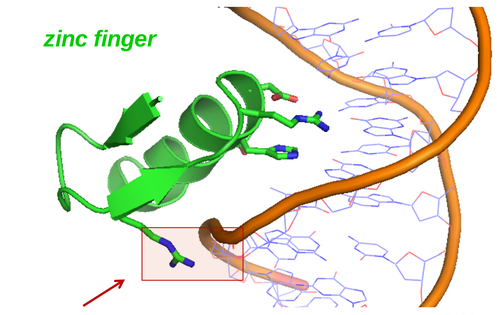


Zinc Finger Protein Transcription Factor (ZFP-TF):

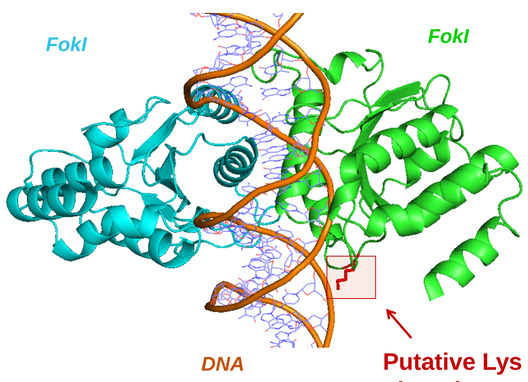
- Programmable transcription factor
- Contains two domains:
  - **Activation or repression domain**
  - **zinc finger protein (ZFP)**
- Binds 15-18 bp



Nonspecific Contacts Between ZFNs & DNA



Conserved Arg → phosphate contact

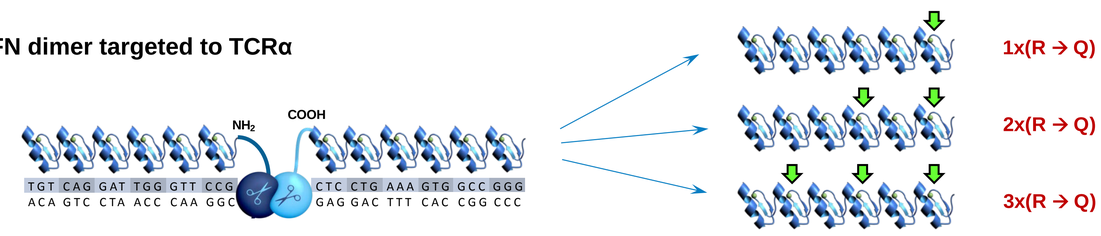


Putative Lys 142 → phosphate contact

Zinc finger – DNA structure is from Elrod-Erickson et al., *Structure*. 1996 Oct 15;4(10):1171-80. The structure of the DNA-bound FokI cleavage domain is based on homology modeling. For detail see Miller et al., *Nat Biotechnol*. 2007 Jul;25(7):778-85.

Finger Substitutions Improve ZFN Specificity

ZFN dimer targeted to TCRα



ZFN modification	
Left	**Right**
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-----	-----
-----	**1x(R → Q)**
-----	**2x(R → Q)**
-----	**3x(R → Q)**

%indels at 6 ug dose

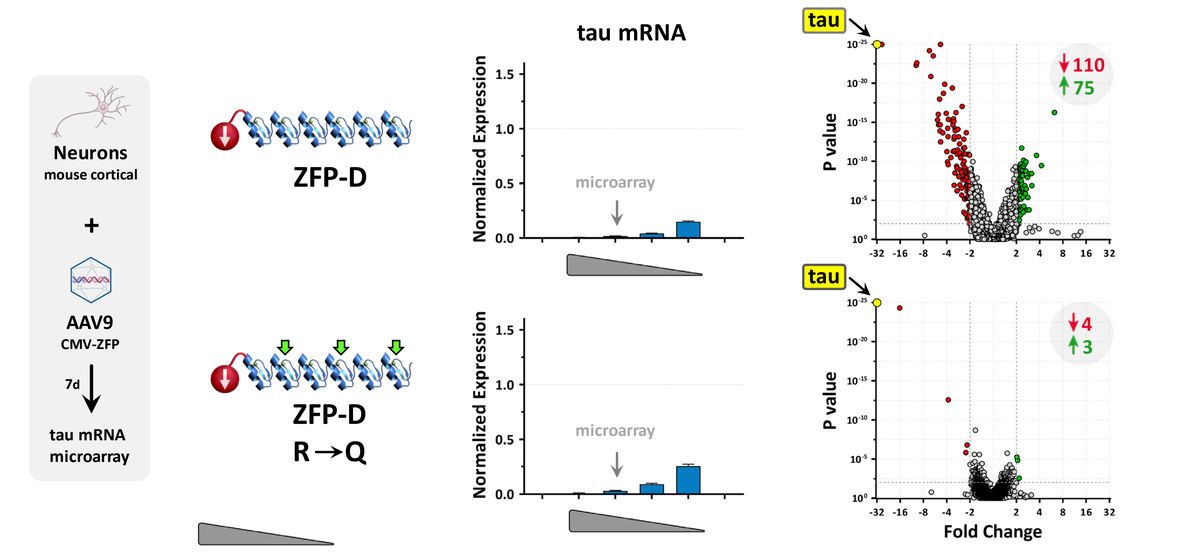
TCRα	OT1	OT2
62.6	19.2	4.3
62.8	15.3	4.3
68.6	14.5	4.2
65.7	5.1	1.1
70.0	1.4	0.5

%indels at 2 ug dose

TCRα	OT1	OT2
32.1	6.9	2.4
25.9	5.3	2.6
35.4	8.3	2.8
30.1	1.9	1.2
36.1	1.2	0.4

Study performed in CD34+ cells using BTX-mediated electroporation of RNA for ZFN delivery. Three days after delivery cells were harvested and analyzed for indels via deep sequencing of PCR-amplified loci.

Finger Substitutions Also Improve ZFP-TF Specificity



ZFP-TFs were delivered via AAV9 transduction and cells were harvested after 9 days. RNA was prepared and analyzed via qrt-PCR for TAU transcript or microarray analysis using Affymetrix Genechip Clariom S arrays as indicated. Fold-change analysis was performed using Transcriptome Analysis Console 4.0 (Affymetrix) with "Analysis Type – Expression (Gene)" and "Summarization – RMA" options selected.

Fok Substitutions Improve ZFN Specificity

Position # 142 in Fok domain

Residue Type	% indels On-target	OT1	OT2	OT3	OT4	On:off ratio
Lys	57.3	10.92	1.63	1.79	1.4	3.6
half dose	38.3	3.01	.31	.45	.24	9.5
Thr	84.7	.68	.07	.21	.21	72.3
Val	83.3	.48	.04	.06	.09	123.0
Cys	82.1	.55	.12	.12	.19	83.1
Ile	80.4	.51	.04	.13	.12	100.5
Ser	77.7	.11	.02	.06	.05	316.5
Ala	73.8	.07	.01	.04	.03	494.0
Gly	56.5	.07	.04	.04	.02	358.6
Arg	53.6	14.69	2.18	1.97	2.52	2.5
Gln	42.0	.1	.03	.07	.04	169.3
Asn	36.5	.23	.04	.05	.04	102.8
His	36.3	.27	.04	.06	.07	81.3
Met	27.6	.09	.02	.04	.03	157.0
Tyr	25.8	.83	.07	.11	.11	23.0
Glu	23.6	.02	.01	.03	.02	288.2
Leu	14.3	.02	.01	.04	.04	119.7
Phe	12.7	.28	.07	.08	.04	27.1
Trp	8.6	.1	.02	.03	.05	44.2
Asp	5.0	.02	.01	.02	.04	56.7
Pro	.0	.03	.03	.02	.02	.1
Mock (GFP)	.0	.03	.01	.03	.03	

parent amino acid

Position # 98 in Fok domain

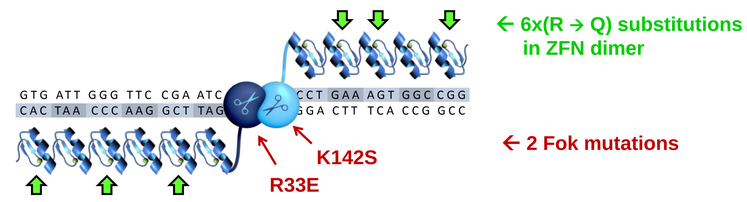
Residue Type	% indels On-target	OT1	OT2	OT3	OT4	On:off ratio
Gln	47.0	4.03	1.73	.98	.96	6.1
half dose	30.0	1.0	.32	.22	.17	17.5
Asp	90.5	.12	.05	.1	.16	214.4
Ala	86.3	.02	.04	.04	.09	462.9
His	84.3	22.17	.65	4.71	1.07	2.9
Cys	66.1	.05	.03	.05	.09	300.2
Glu	57.0	.04	.03	.04	.05	343.3
Ser	55.0	.02	.04	.07	.05	308.6
Thr	33.3	.02	.02	.0	.07	290.9
Pro	29.4	.2	.05	.13	.09	62.0
Gly	18.1	.34	.13	.18	.12	23.5
Arg	3.8	.03	.04	.03	.03	28.9
Leu	3.4	.03	.05	.02	.1	17.1
Asn	1.9	.03	.03	.09	.08	8.3
Gln	.4	.03	.03	.08	.1	1.8
Tyr	.4	.03	.01	.02	.05	3.5
Met	.1	.03	.01	.03	.06	1.0
Val	.1	.03	.01	.03	.09	.7
Ile	.1	.03	.01	.02	.06	.5
Phe	.0	.04	.02	.08	.05	.1
Trp	.0	.02	.04	.04	.07	.1
Mock (GFP)	.0	.03	.02	.03	.06	

Reanalysis via ultra-deep sequencing and background correction reveals 1000-fold reduction in off-target cleavage

Gln	52.0	5.71	1.13	.88	.77	6.1
Ala	91.5	.001	.001	.008	.001	8475.0

Studies performed in K562 cells using ZFNs targeted to the AAVS1 safe harbor (Ravin et al. *Nat Biotechnol*. 2016 ;34(4):424-9). Delivery via RNA transfection. Quantitation via deep sequencing.

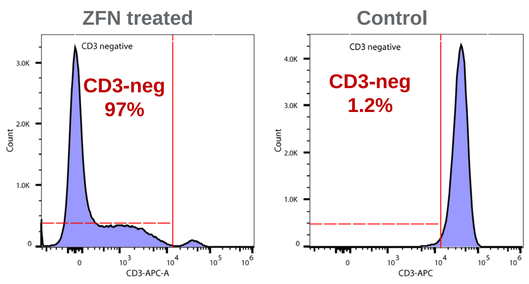
Combining Approaches Enables Highly Efficient Knockout w/No Off-target Cleavage



Oligo duplex capture assay followed by indel analysis yields no evidence of off-target cleavage

Locus rank	Genome coordinates	Total capture events	% indels ZFN	% indels control	pval if <0.05
1	chr14 22550604	4307	98.09	0.24	0.00
2	chr21 8990878	3	0.24	0.28	ns
3	chr2 15345444	3	ND	ND	ns
4	chr10 93837534	2	0.05	0.11	ns
5	chr11 93673350	2	0.03	0.13	ns
6	chr12 55375448	2	0.08	0.05	ns
7	chr14 50686876	2	0.07	0.02	ns
8	chr14 50686966	2	0.24	0.23	ns
9	chr14 63848848	2	0.05	0.09	ns
10	chr1 143199522	2	ND	ND	ns
11	chr1 143199578	2	0.18	0.21	ns

Flow cytometry confirms highly efficient target knockout



Candidate off-target sites identified and ranked using an oligonucleotide capture assay similar to that described in Tsai et al., (*Nat Biotechnol*. 2015 Feb;33(2):187-197.). Follow-up indel and cell sorting analyses performed in CD4 T-cells with ZFNs delivered via electroporation of RNA (BTX).

Summary

- **Mutating a conserved zinc finger-phosphate contact can substantially improve ZFN and ZFP-TF specificity**
- **Substituting FokI domain residues provides a powerful approach for eliminating ZFN off-target cleavage.**
- **Replacement of a single FokI domain residue can suppress off-target cleavage by 1000-fold.**
- **Combining approaches has yielded TRAC-targeted ZFNs that can achieve near-quantitative levels of functional knockout with no detectable off-target activity.**