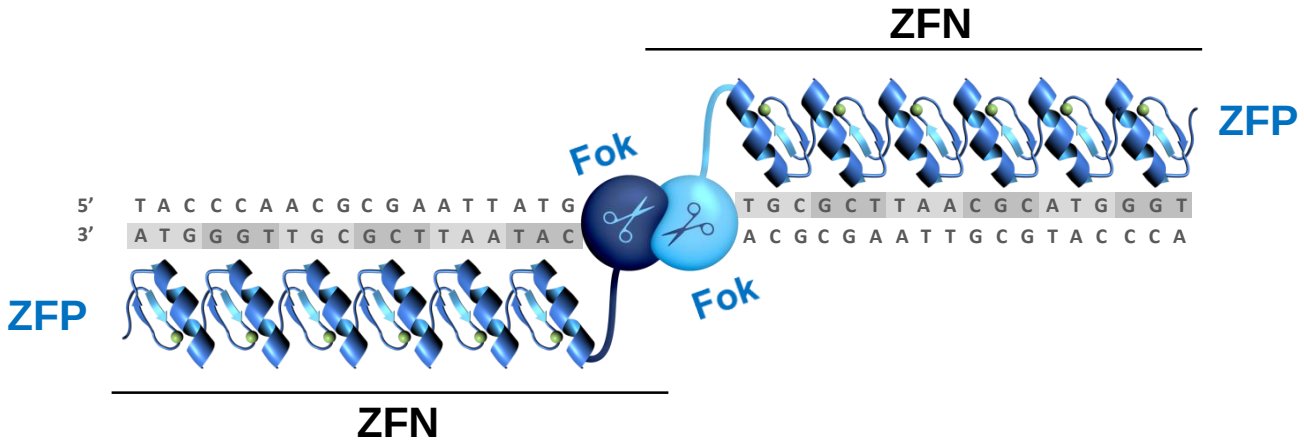
New Zinc Finger Nuclease Architectures for Highly Efficient Genome Engineering in Primary Cells at Large Scale with No Detectable Off-Target Effects

Ed Rebar

Sangamo Therapeutics

Zinc Finger Nuclease (ZFN)



- Programmable nuclease
- Must dimerize to cleave
- Contains two domains:
  - **nuclease domain of FokI**
  - **zinc finger protein (ZFP)**

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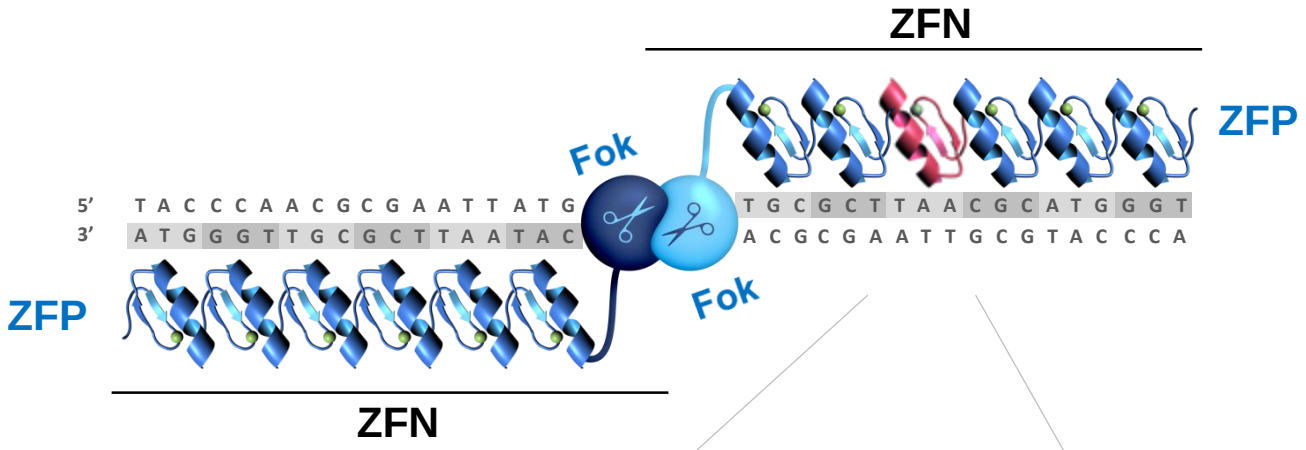
Zinc Finger – DNA Recognition



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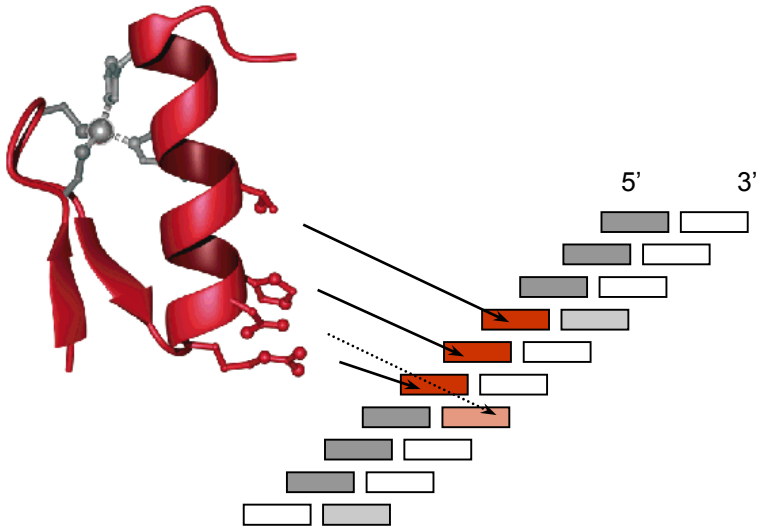

- One finger binds 3-4 bp
- Helix residues determine base preference

Helix sequence -1 +2+3 +6	Binding preference
LKQNLCM	CAT
AQCCLFH	AGC
DQSNLRA	AAC
RSDELTR	GCGG
… etc	…

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ZFN Specificity

Key consideration for therapeutic applications

Historically – addressed via redesign of helices

- Approach frequently yields very high on-target preference
- But it can require multiple design / characterization cycles
- Success not guaranteed

Can we identify orthogonal approaches for improving specificity?

Talk Overview

Three new approaches for improving ZFN specificity

- Skewing delivery ratios
- Removal of conserved zinc finger-phosphate contacts
- Mutation of presumptive Fok-DNA contacts

Combining approaches to achieve highly specific editing at clinical scale

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Rationale for Testing Skewed Delivery Ratios

ZFNs must dimerize to cleave DNA

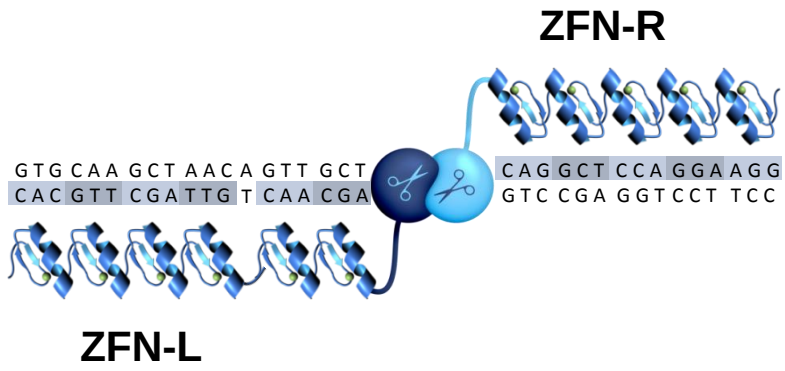
Binding events are highly independent

- Due to very weak dimerization interface

If monomer affinities differ, then delivery as a 1:1 ratio over-doses the tighter ZFN, which may reduce specificity

Test System for These Studies

ZFNs targeted to BCL11A erythroid enhancer
(*Nat Methods* 12, 927)
$CD34^+$ cells
Delivery via RNA transfection (MaxCyte OC100)



One-armed Titrations Identify ZFN-L as the More Active Monomer

ZFNs targeted to BCL11A erythroid enhancer
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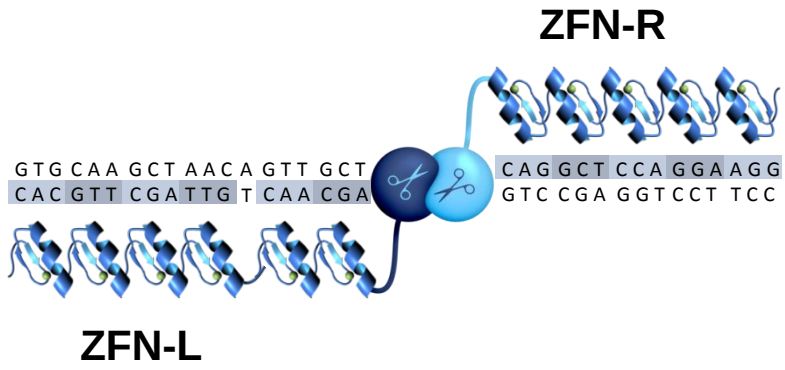

ug of RNA		% indels
ZFN-L	ZFN-R	
6.0	6.0	85.9
2.0		85.5
0.67		86.4
6.0	6.0	85.9
	2.0	83.0
	0.67	71.6

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Skewed Dosing Reduces Off-Target Cleavage

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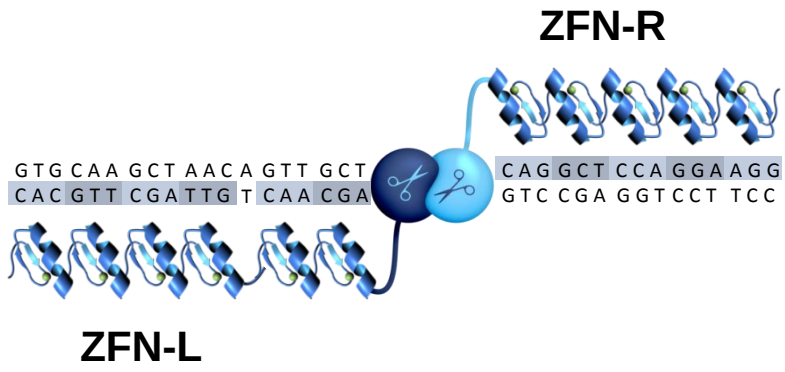

ug of RNA		% indels	
ZFN-L	ZFN-R	on-target	off-target "A"
6.0	6.0	85.9	27.3
2.0	↓	85.5	12.1
0.67		86.4	4.2
6.0	6.0	85.9	27.3
↓	2.0	83.0	17.0
	0.67	71.6	5.6

Titrations Confirm Improved Specificity vs 1:1 Mix

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(*Nat Methods* 12, 927)
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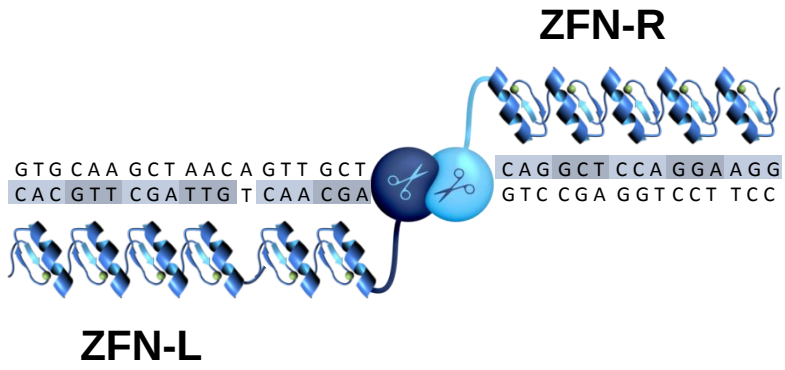
Delivery via RNA transfection (MaxCyte OC100)



	ug of RNA		% indels at each locus				
	ZFN-L	ZFN-R	on-target	OT-A	OT-B	OT-C	
Standard titration (ratio fixed at 1:1)	6.0	6.0	76.1	24.6	2.81	3.06	
	4.0	4.0	76.5	14.6	1.69	1.77	← **At on-target saturation thresholds-**
	2.0	2.0	71.5	4.9	0.62	0.36	
	1.0	1.0	62.0	1.3	0.10	0.14	
One-armed titration	6.0	6.0	76.1	24.6	2.81	3.06	
	2.0	6.0	78.3	11.8	1.38	1.32	**- off-target cleavage reduced about 5-fold**
	0.66	6.0	78.9	2.8	0.27	0.36	←
	0.22	6.0	73.7	0.9	0.15	0.29	

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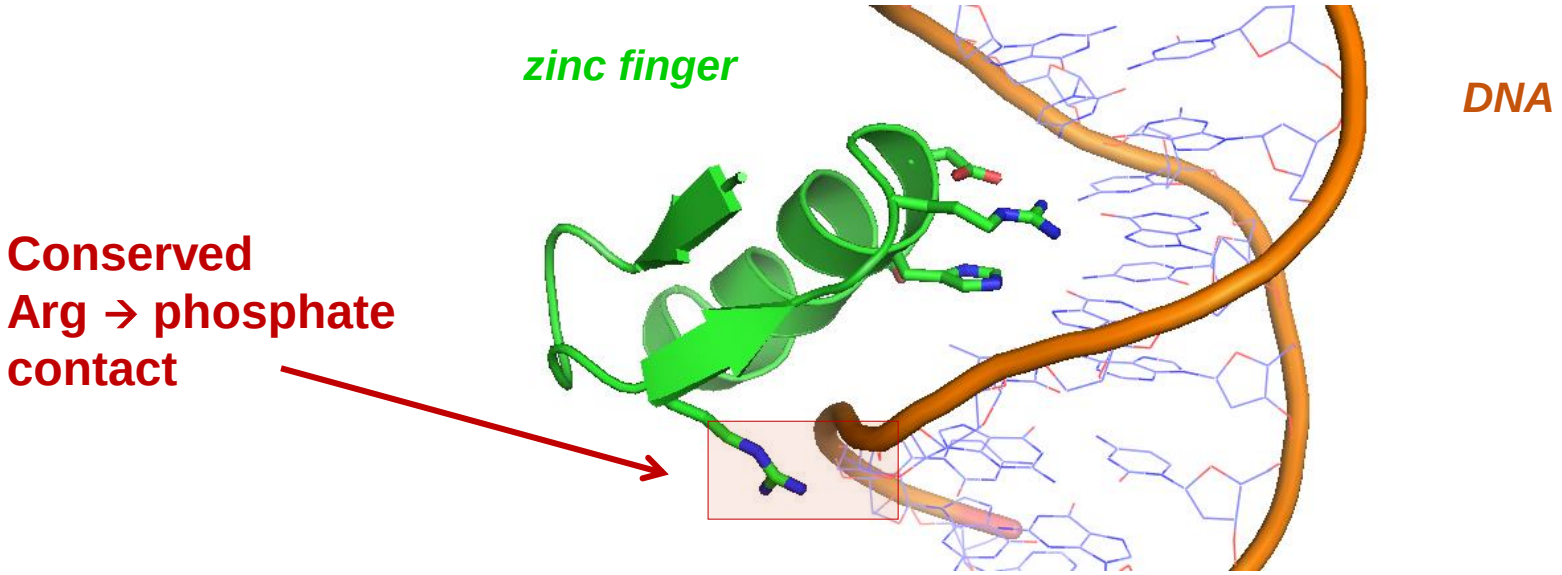
Second Approach: Remove Key Finger-phosphate Contacts

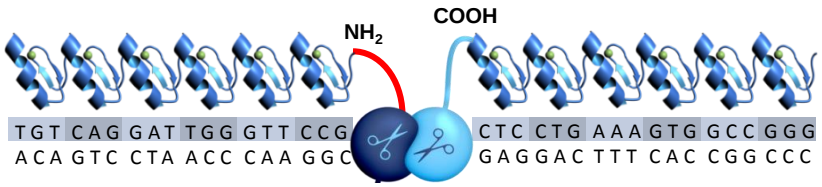
- **Most zinc fingers make a conserved phosphate contact from their β-sheet**
  - including all Sangamo designs
- **This may provide increased activity at the expense of specificity**
- **Test by removing contact and gauging specificity**



Elrod-Erickson *et al*., *Structure* 1996 – 1AAY.pdb

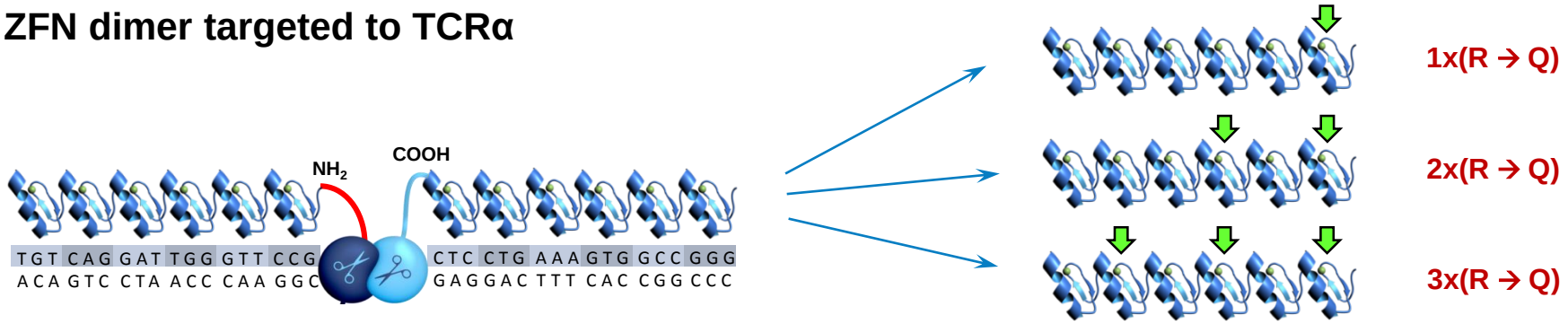
Test System for This Study

ZFN dimer targeted to TCRα



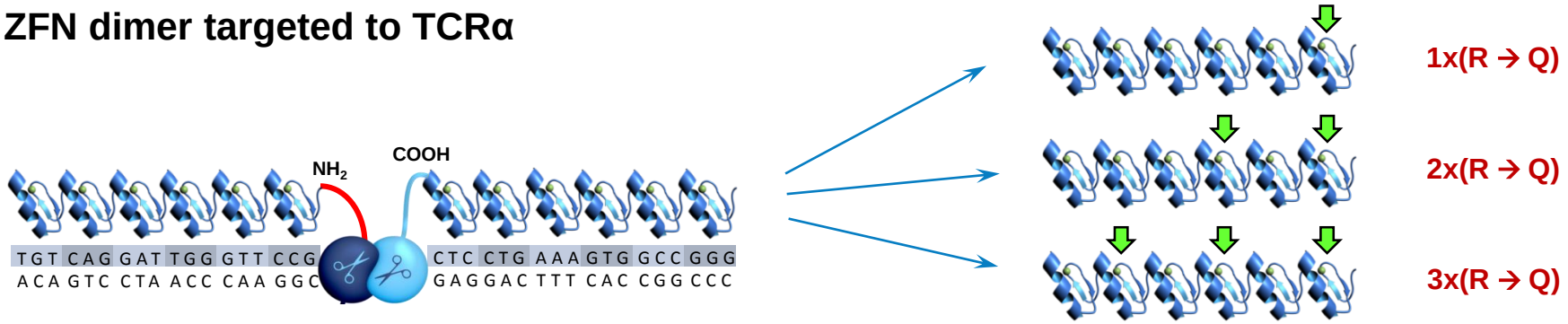
Arginine Replaced By Glutamine in 1 to 3 Fingers of the Right ZFN

ZFN dimer targeted to TCRα



Removing Phosphate Contacts Can Improve Specificity

ZFN dimer targeted to TCRα



ZFN modification	
Left	**Right**
-----	-----
-----	-----
-----	**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

CD34+ cells
BTX electroporation
2 or 6 ug RNA / ZFN

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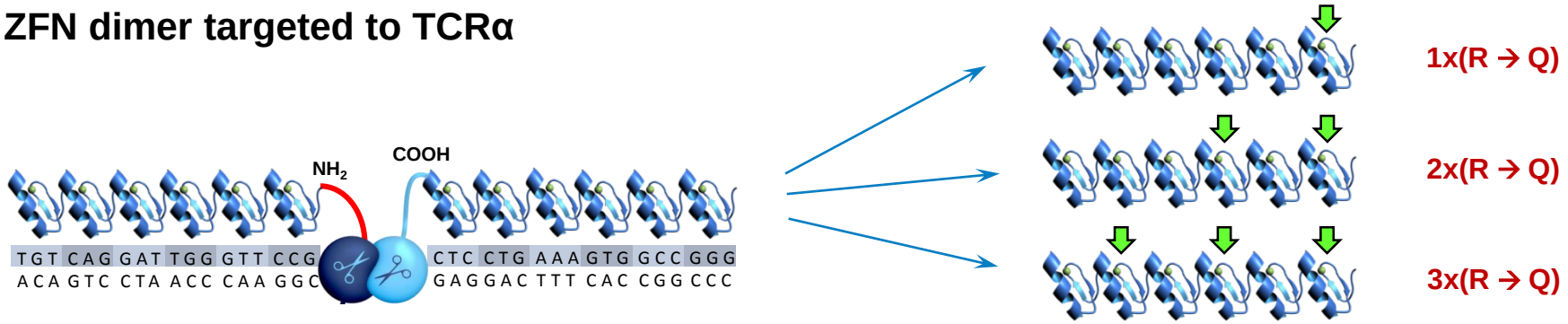
Benefit Retained at Nonsaturating Dose

ZFN dimer targeted to TCRα



ZFN modification	
Left	**Right**
-----	-----
-----	-----
-----	**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

CD34+ cells
BTX electroporation
2 or 6 ug RNA / ZFN

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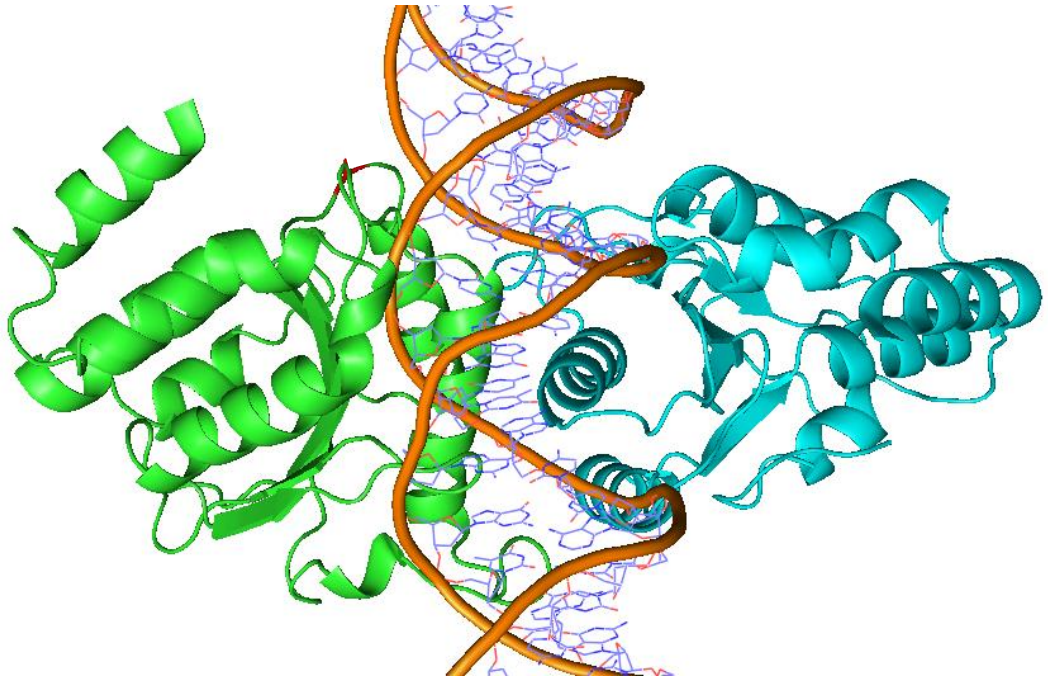
Third Approach: Mutate Presumptive Fok-DNA Contacts

- **Residues identified via examination of a structural model of Fok cleavage domain docked with DNA**
  - Based on a crystal structures of isolated FokI protein and also DNA-bound homologue (BamH1)



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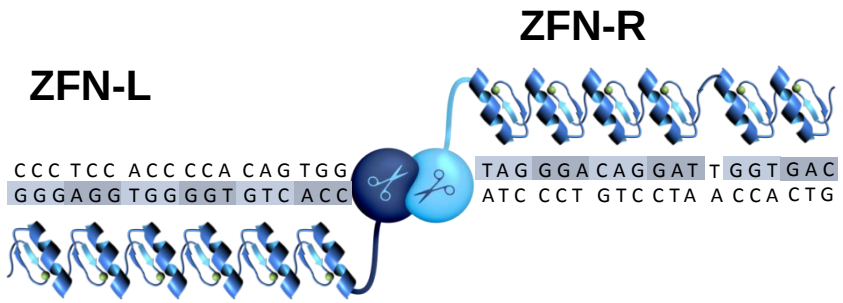
Test System

ZFNs targeted to the AAVS1 safe harbor
Substitutions generated via shotgun cloning in ZFN-R
K562 cells
Delivery via RNA transfection (Amaxa)



Many Alternative Residues Maintain Full On-Target Activity

ZFNs targeted to the AAVS1 safe harbor
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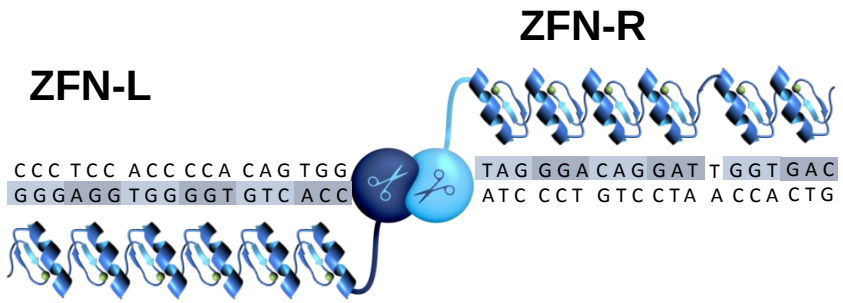
K562 cells
Delivery via RNA transfection (Amaxa)



Residue Type	% indels
	On-target
Lys	57.4
Thr	66.7
Ser	63.3
Cys	62.9
Ala	61.3
Val	58.8
Ile	57.4
Gly	53.1
Gln	52.7
Arg	52.4
Met	46.7
Asn	44.8
His	41.4
Asp	36.3
Tyr	35.2
Leu	30.4
Phe	25.5
Trp	18.0
Pro	2.7

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Off-target Cleavage Substantially Decreased

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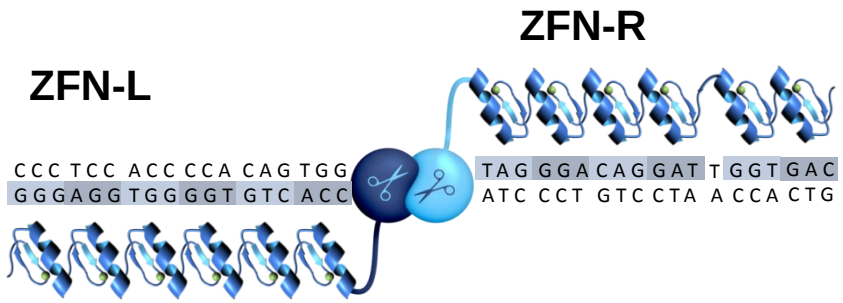

Residue Type	% indels				On:off ratio
	On-target	OT1	OT2	OT3	
Lys	57.4	1.42	1.80	1.42	12.4
Thr	66.7	0.24	0.35	0.48	62.6
Ser	63.3	0.11	0.13	0.36	105.4
Cys	62.9	0.15	0.31	0.40	73.4
Ala	61.3	0.11	0.12	0.24	130.2
Val	58.8	0.19	0.20	0.20	98.7
Ile	57.4	0.20	0.32	0.27	73.8
Gly	53.1	0.04	0.12	0.35	103.2
Gln	52.7	0.09	0.13	0.28	103.4
Arg	52.4	1.73	1.62	1.20	11.5
Met	46.7	0.16	0.21	0.24	76.1
Asn	44.8	0.16	0.27	0.31	59.8
His	41.4	0.28	0.36	0.28	45.1
Asp	36.3	0.03	0.05	0.06	257.6
Tyr	35.2	0.56	0.42	0.18	30.3
Leu	30.4	0.09	0.19	0.07	86.8
Phe	25.5	0.57	0.36	0.17	23.2
Trp	18.0	0.08	0.24	0.07	46.5
Pro	2.7	0.05	0.02	0.05	22.9

Parent ZFN

Off-target activity reduced by 5-10x

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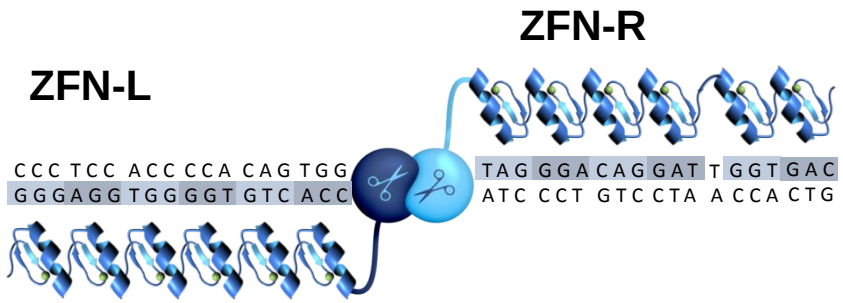
Half-dose Control Demonstrates Lack of Saturation Artifact

ZFNs targeted to the AAVS1 safe harbor
Substitutions generated via shotgun cloning in ZFN-R
K562 cells
Delivery via RNA transfection (Amaxa)



Residue Type		% indels				On:off ratio
		On-target	OT1	OT2	OT3	
Lys	full dose	57.4	1.42	1.80	1.42	12.4
	half dose	38.4	0.30	0.47	0.27	37.3
Thr		66.7	0.24	0.35	0.48	62.6
Ser		63.3	0.11	0.13	0.36	105.4
Cys		62.9	0.15	0.31	0.40	73.4
Ala		61.3	0.11	0.12	0.24	130.2
Val		58.8	0.19	0.20	0.20	98.7
Ile		57.4	0.20	0.32	0.27	73.8
Gly		53.1	0.04	0.12	0.35	103.2
Gln		52.7	0.09	0.13	0.28	103.4
Arg		52.4	1.73	1.62	1.20	11.5
Met		46.7	0.16	0.21	0.24	76.1
Asn		44.8	0.16	0.27	0.31	59.8
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Trp		18.0	0.08	0.24	0.07	46.5
Pro		2.7	0.05	0.02	0.05	22.9

Parent ZFN

Off-target activity reduced by 5-10x

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Combining Fok and ZFP Variants Further Reduces OT Cleavage

# modified fingers (Arg --> Gln)	Fok domain substitution	% Indels								
		BCL11A			Off-target A			Off-target C		
		6 ug	2 ug	0.5ug	6 ug	2 ug	0.5ug	6 ug	2 ug	0.5ug
---	---	82.7	87.0	76.5	28.00	5.02	.34	4.77	.32	.03
---	33S	86.4	86.8	77.6	5.07	.58	.10	.75	.07	.03
6	33S	88.3	86.4	78.0	.55	.08	.01	.04	.01	.02
7	33S	85.4	86.3	73.4	.17	.03	.03	.02	.00	.01
8	33S	83.6	86.5	71.7	.03	.01	.02	.02	.03	.00
7	142S	85.4	83.6	60.6	.01	.02	.02	.01	.00	.01
7	142S	85.7	84.3	40.1	.02	.00	.01	.00	.00	.00

← Parent ZFN

← Off-target activity reduced > 100x

Notes:

grey font = no evidence of ZFN cleavage

Test system: ZFNs targeted to BCL11A erythroid enhancer (*Nat Methods* 12, 927)
$CD34^+$ cells; delivery via RNA transfection (BTX)

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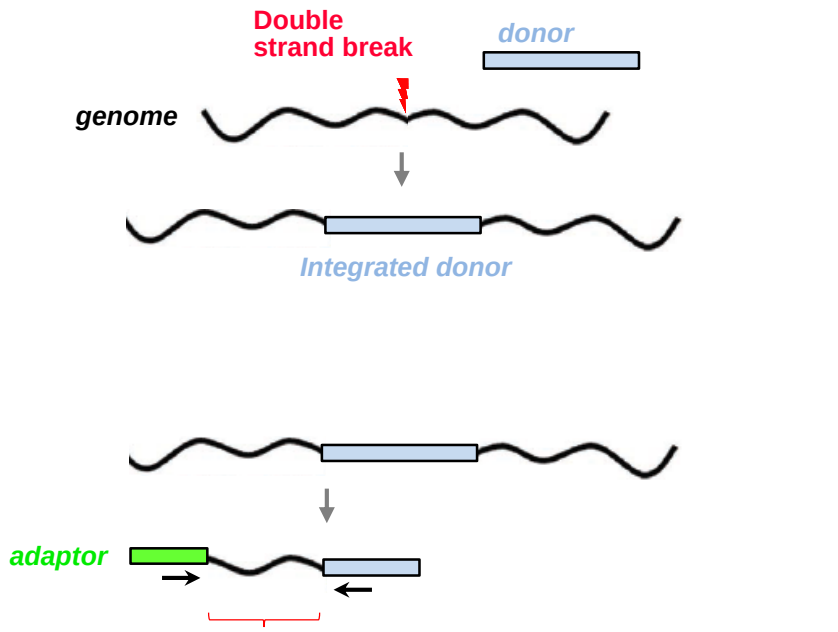
Candidate Off-Target Sites Identified Via End-Capture Study

Treat cells with ZFNs + donor oligonucleotide duplex

K562 cells, delivery via nucleofection
400 ng RNA / ZFN, 1 uM oligo duplex

Sequence genome segments adjacent to integrated donors

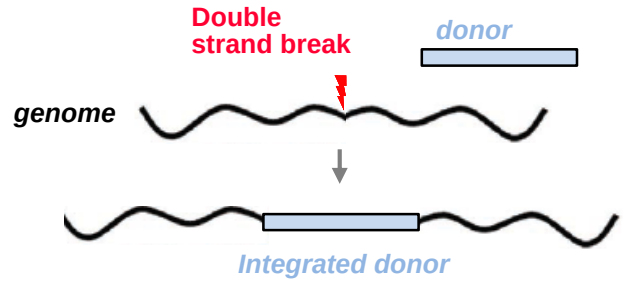


Sequence reveals candidate off-target site

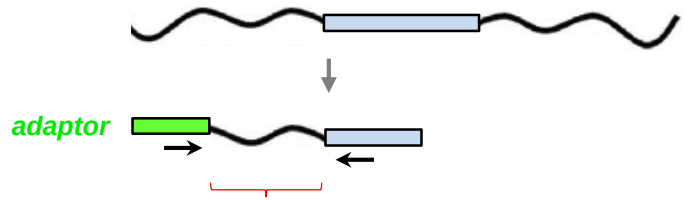
Indels Assessed Via Follow-up Study in CD34+ Cells At Clinical Scale

Treat cells with ZFNs + donor oligonucleotide duplex

K562 cells, delivery via nucleofection
400 ng RNA / ZFN, 1 uM oligo duplex



Sequence genome segments adjacent to integrated donors



Sequence reveals candidate off-target site

Treat CD34+ cells with ZFNs at clinical scale
PCR-amplify candidate off-target sites
Assess for indels

10^8 CD34+ cells, RNA delivery, MaxCyte CL1.1

High ZFN Levels Used for Both Stages of Study

ZFNs	On-target modification (% indels)	
	Capture study	Clinical scale follow-up
Parent pair	79.8%	72.5%
Variant pair	86.7%	82.0%

Capture Results Suggest Improved Specificity for Variant Pair

Parent ZFNs

Locus rank in capture assay		Integrant count
1	BCL11A	1064
2		939
3		156
4		105
5		102
6		93
7		88
8		85
9		82
10		75
11		68
12		66
13		66
14		64
15		58
16		57
17		57
18		54
19		53
20		53
21		52
22		52
23		48
24		40
25		39
26		36
27		35
28		35
29		34
30		33

< 32 loci not shown >

↓

Variant ZFNs

Locus rank in capture assay		Integrant count
1	BCL11A	1863
2		85
3		33
4		16
5		13
6		12
7		12
8		10
9		9
10		9
11		9
12		9
13		9
14		8
15		7
16		7
17		7
18		7
19		7
20		7
21		7
22		7
23		6
24		6
25		6
26		6
27		6
28		6
29		6
30		5

< 42 loci not shown >

↓

Variant ZFNs Yield No Evidence of Significant Off-target Cleavage in CD34+ Follow-up

Parent ZFNs

Locus rank in capture assay		Integrant count	% indels ZFN	% indels control	pval if < 0.05
1	BCL11A	1064	72.53	.09	<0.001
2		939	24.81	.06	<0.001
3		156	2.89	.03	<0.001
4		105	nd2	nd2	
5		102	.62	.04	<0.001
6		93	.07	.03	NS
7		88	1.5	.01	<0.001
8		85	.17	.03	<0.001
9		82	nd1	nd1	
10		75	1.68	.05	<0.001
11		68	.53	.03	<0.001
12		66	.29	.05	<0.001
13		66	.56	.03	<0.001
14		64	.13	.04	NS
15		58	nd2	nd2	
16		57	.57	.03	<0.001
17		57	nd2	nd2	
18		54	nd2	nd2	
19		53	1.15	.04	<0.001
20		53	.04	.05	NS
21		52	1.75	.04	<0.001
22		52	.39	.03	<0.001
23		48	.09	.01	.003
24		40	.5	.05	<0.001
25		39	.6	.06	<0.001
26		36	.33	.03	<0.001
27		35	.08	.03	.039
28		35	nd2	nd2	
29		34	.49	.04	<0.001
30		33	1.41	.02	<0.001

< 32 loci not shown >

36 loci modified significantly

Aggregate indel level = 44.8%

Variant ZFNs

Locus rank in capture assay		Integrant count	% indels ZFN	% indels control	pval if < 0.05
1	BCL11A	1863	81.99	.06	<0.001
2		85	.08	.05	NS
3		33	.05	.02	NS
4		16	nd2	nd2	
5		13	.03	.03	NS
6		12	.04	.01	NS
7		12	.03	.01	NS
8		10	nd1	nd1	
9		9	.03	.03	NS
10		9	.06	.04	NS
11		9	nd2	nd2	
12		9	.07	.03	NS
13		9	.03	.02	NS
14		8	.02	.05	NS
15		7	.04	.02	NS
16		7	.03	.03	NS
17		7	.02	.05	NS
18		7	.02	.04	NS
19		7	.02	.03	NS
20		7	nd2	nd2	
21		7	.1	.04	NS
22		7	.03	.03	NS
23		6	.03	.02	NS
24		6	.06	.03	NS
25		6	nd2	nd2	
26		6	nd2	nd2	
27		6	nd2	nd2	
28		6	nd2	nd2	
29		6	.05	.04	NS
30		5	.04	.04	NS

< 42 loci not shown >

No loci modified significantly

Take Home Points

- **Eliminating a key zinc finger-phosphate contact and mutating key Fok domain residues can substantially increase ZFN specificity**
- **Skewing delivery ratio also provides a simple strategy for improving specificity**
- **Combining approaches can increase on-target cleavage preference by >100 fold**
- **Using these approaches $CD34^+$ cells may be modified to high efficiency at clinical scale with no significant off-target cleavage**

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Thank you..

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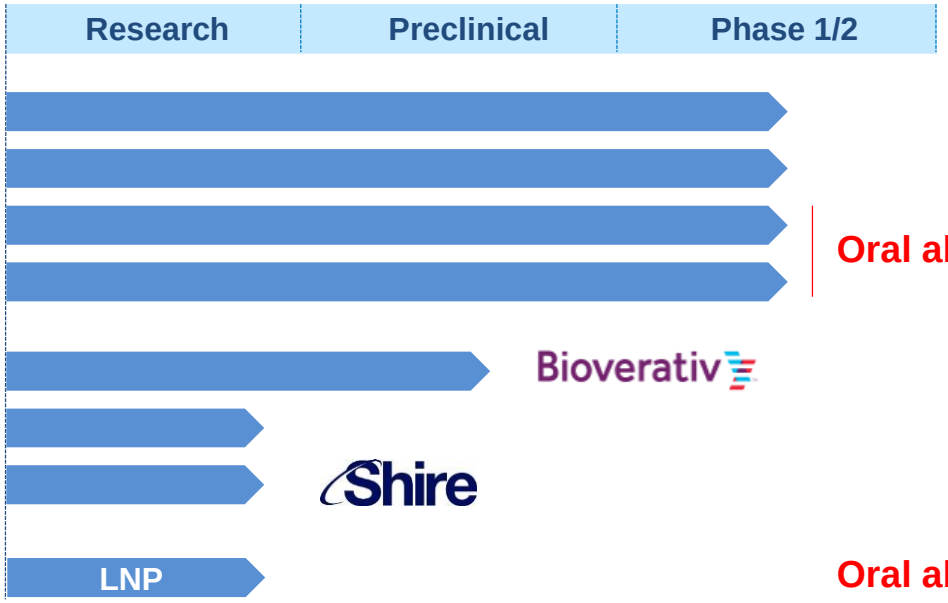
For More Information..



Oral abstract 30

Oral abstracts 509 & 510

Fabry
In vivo editing of SerpinA1
In vivo selection of engineered CD34 cells
SCID-X1 and RAG1
Assay development
Platform development

Oral abstract 27
Oral abstract 511
Oral abstract 512
Oral abstract 747; posters 163 & 654
Posters 396 & 644
Poster 170

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