Gene Targeting is a Precise Recombination Event

Definition: Gene targeting is the replacement of genomic DNA with exogenous DNA by homologous recombination.

Commonly Used For Experimental Purposes in certain cell types (yeast, murine embryonic stem cells…….)

In addition to its usefulness for mammalian somatic cell genetics, it could also be an ideal way to treat genetic diseases.

Two Components for DSB-Induced Homologous Recombination

1. **Repair Substrate: Fragment of DNA that serves as template for repair of DSB by homologous recombination.**

2. **Nuclease: Enzyme to create DSB in target gene.**

Endogenous Genes Do Not have Recognition Sites for Homing Endonucleases

1. Modify Homing Endonucleases to Recognize new target sites.

Molecular mechanisms for new therapeutic approaches

Gene Editing (Correction/Insertion):

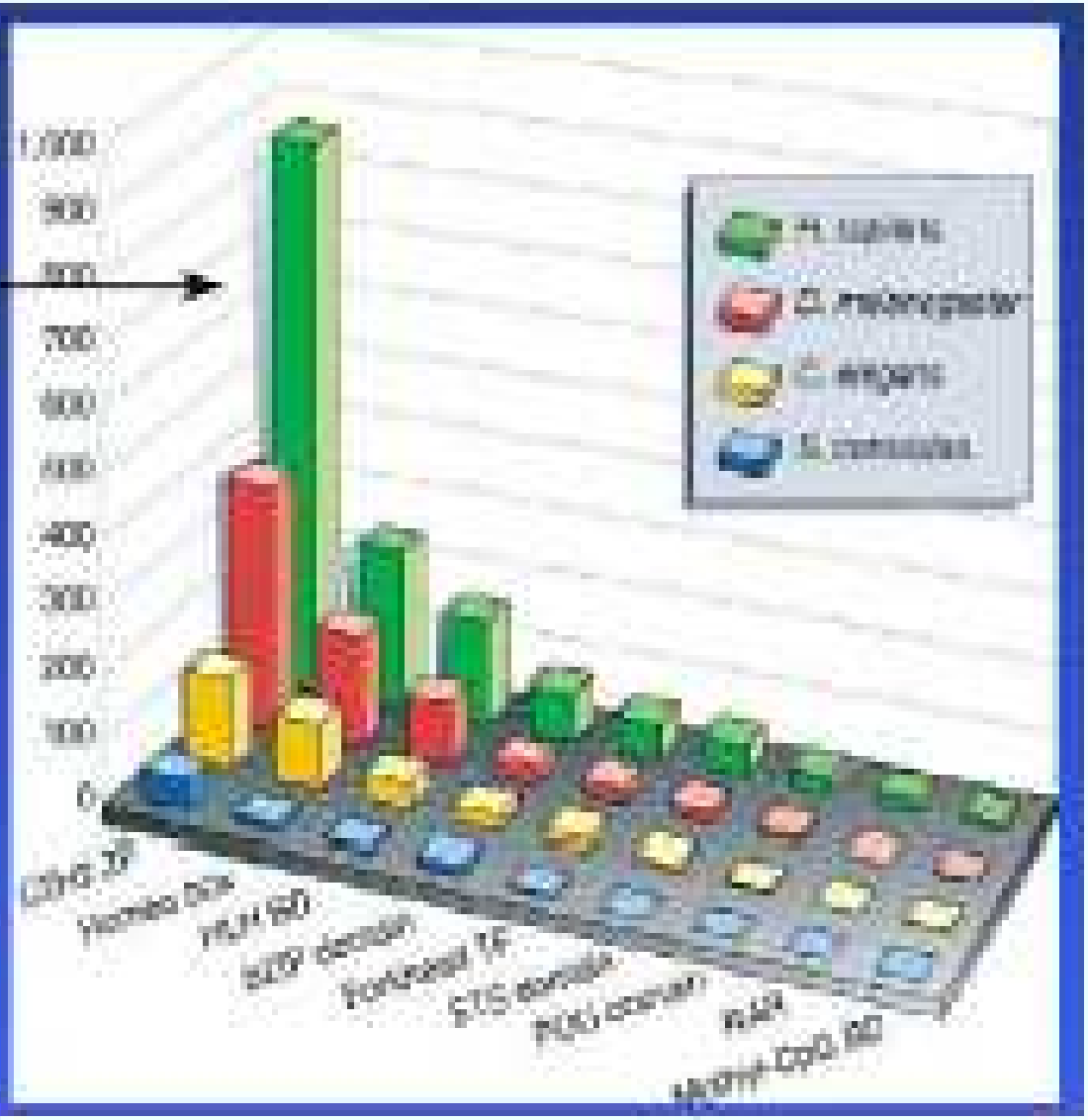
- Zinc Finger Nuclease
- TALE Nuclease
- CRISPR/Cas9
- Without nuclease

Endogenous Genes Do Not have Recognition Sites for Homing Endonucleases

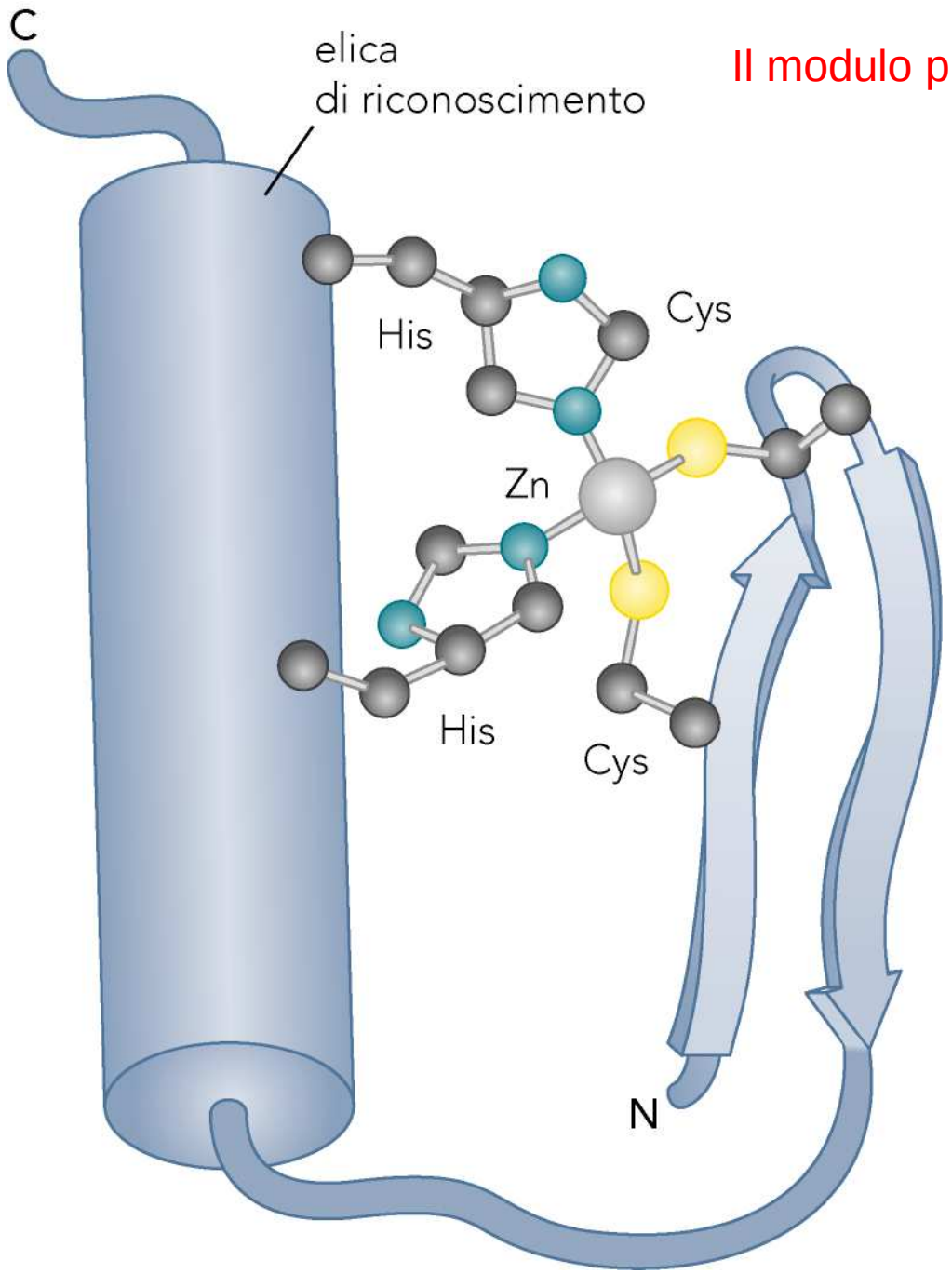
Use Zinc Finger Nucleases

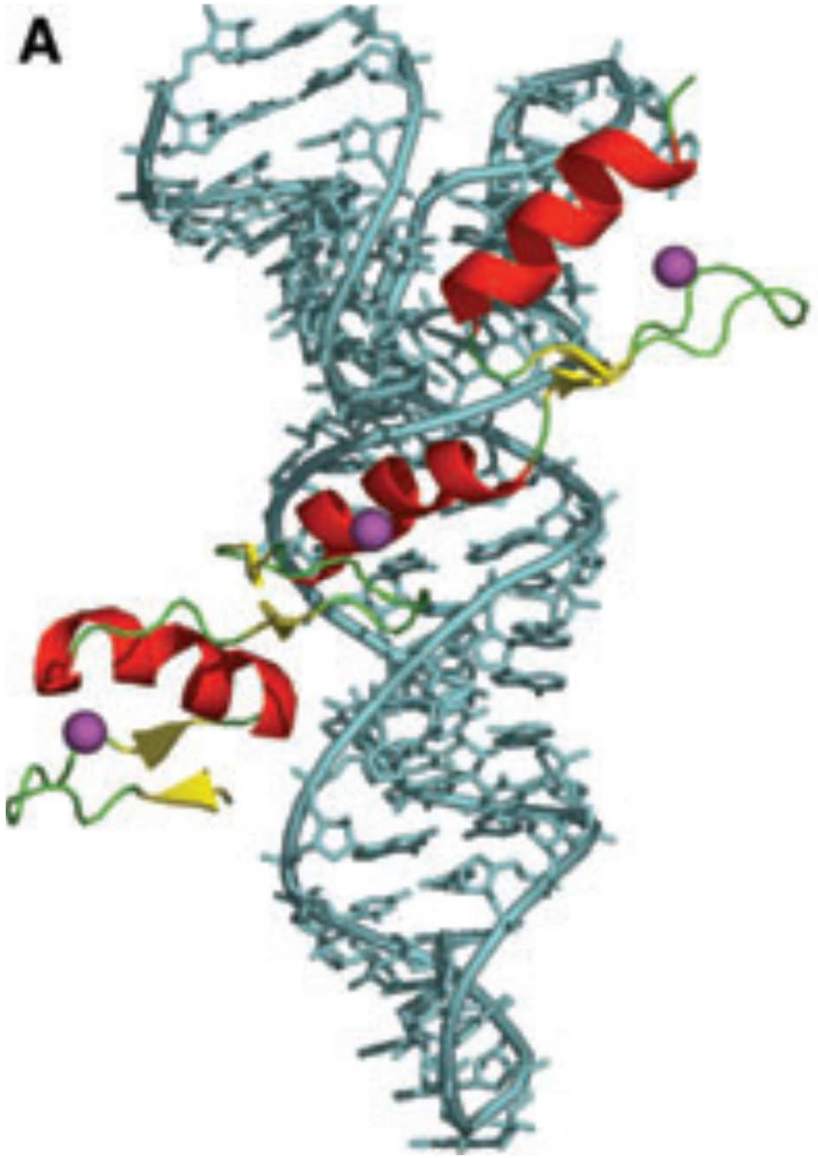
Zinc finger proteins

DNA binding protein families



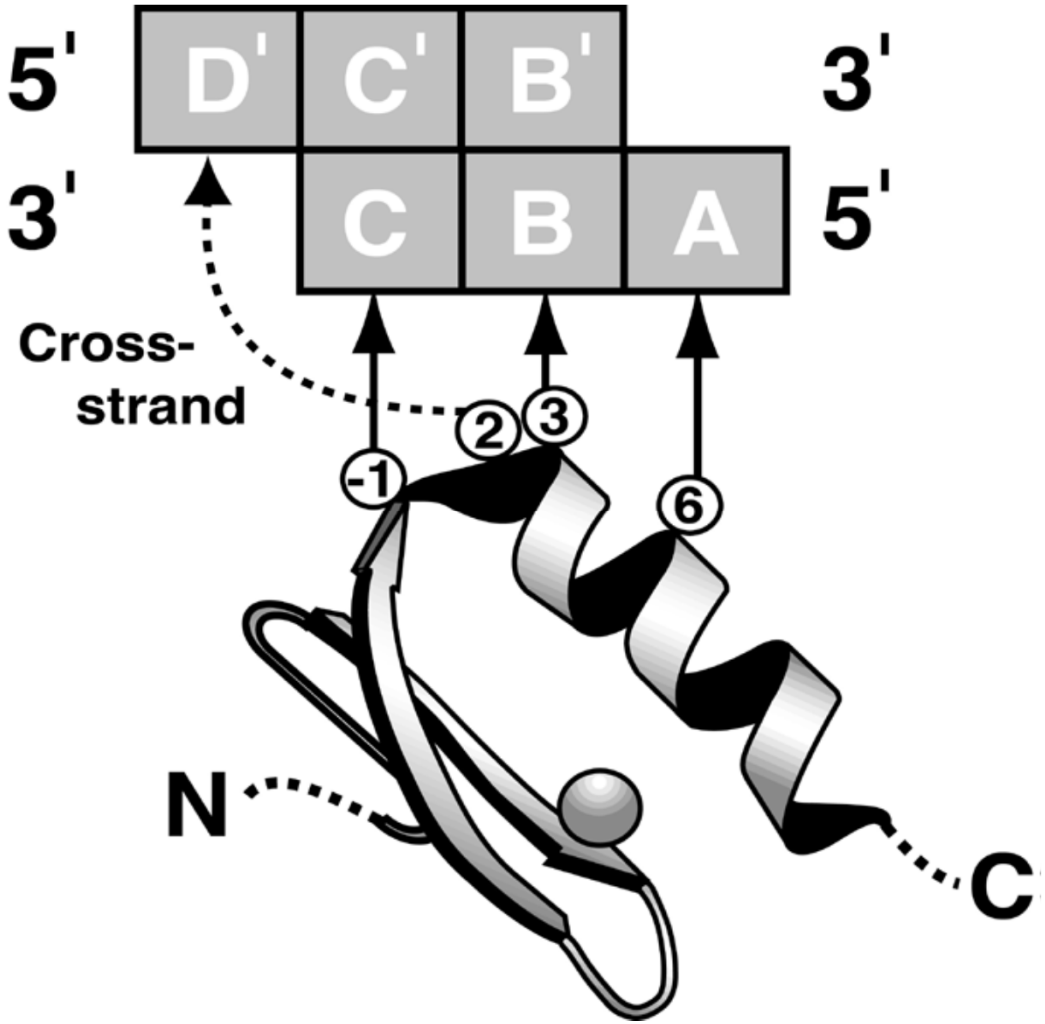
Il modulo proteico Zinc finger



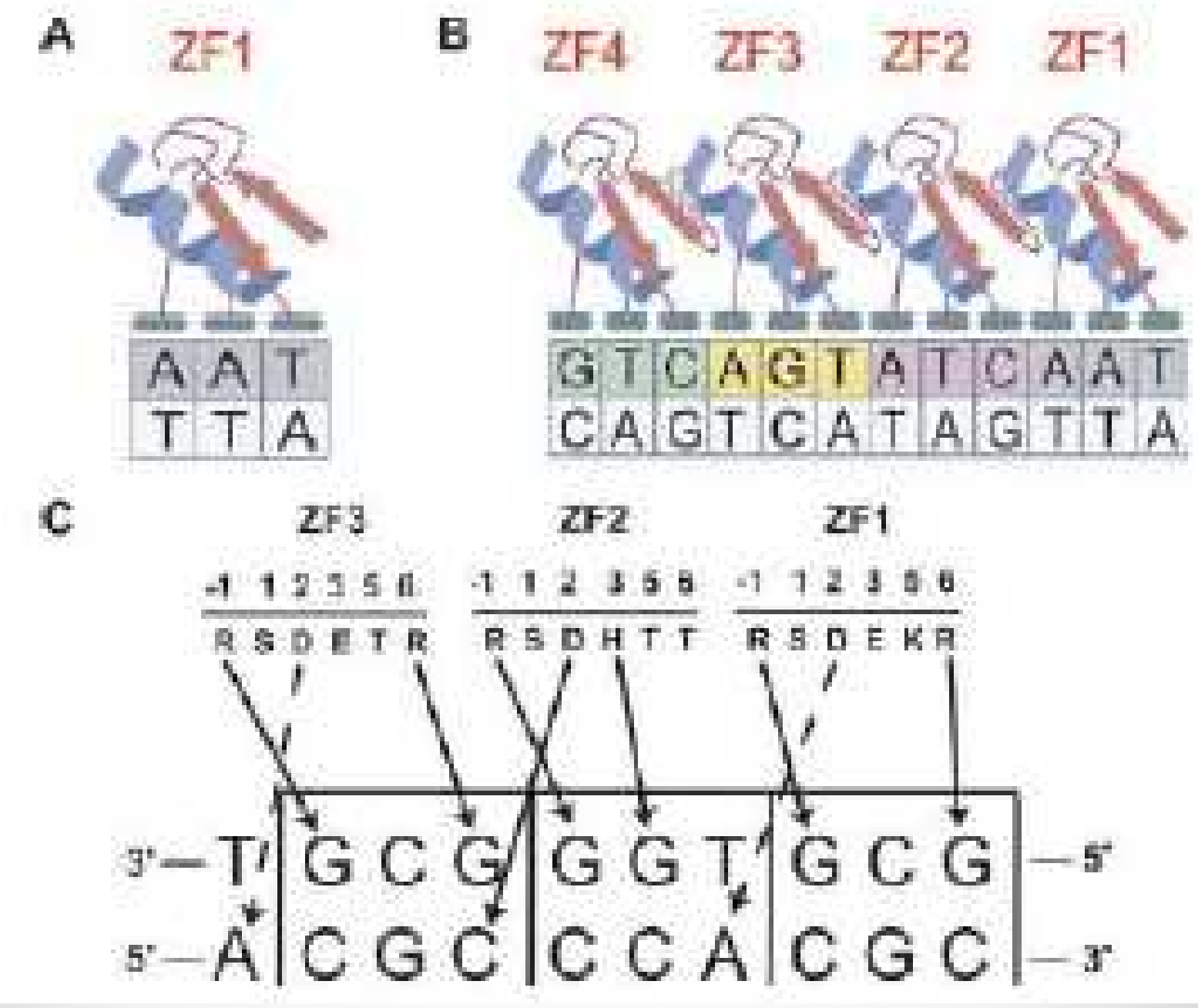


Interazione in tandem con il DNA

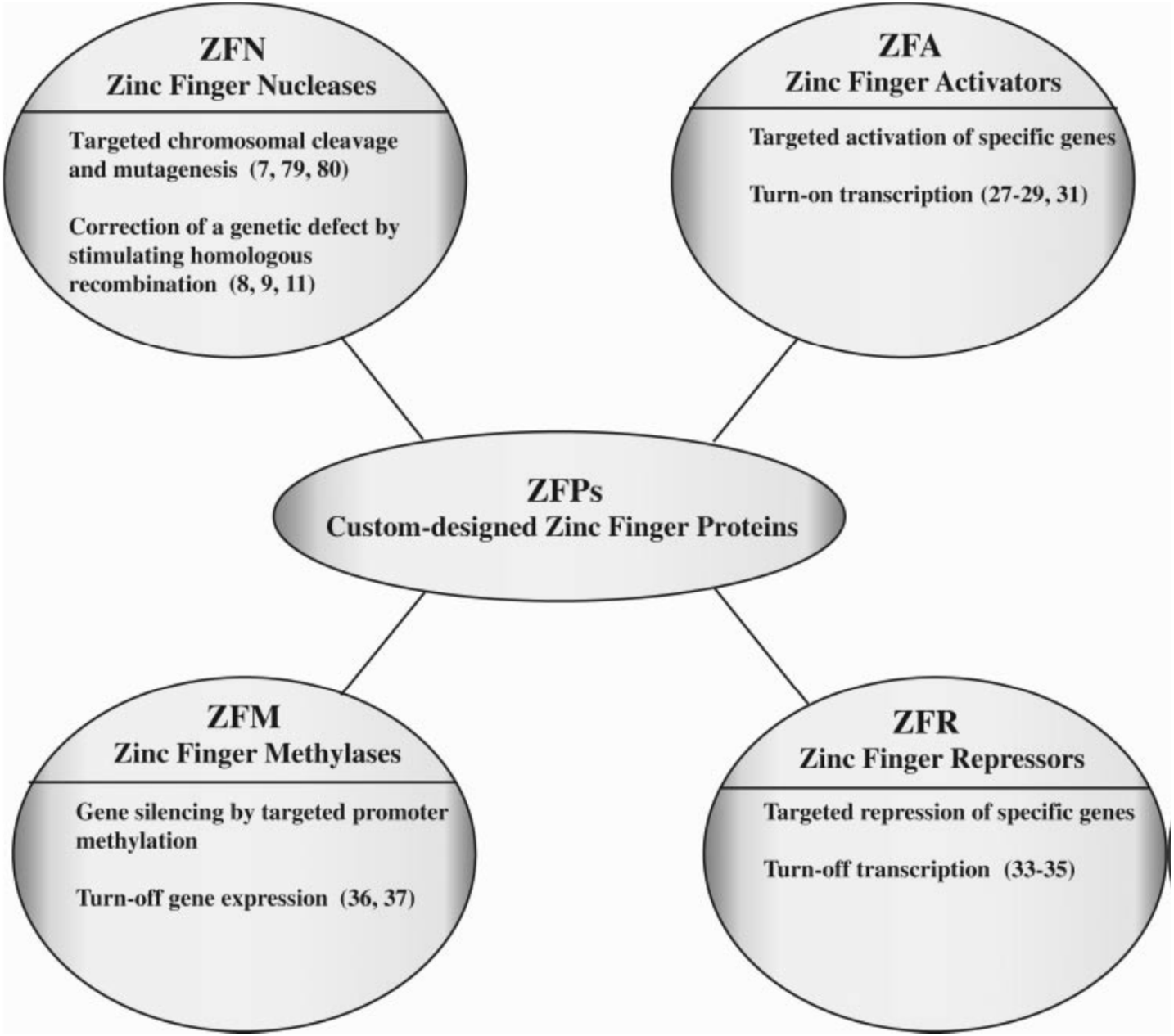
Interazione aminoacidi / basi del DNA



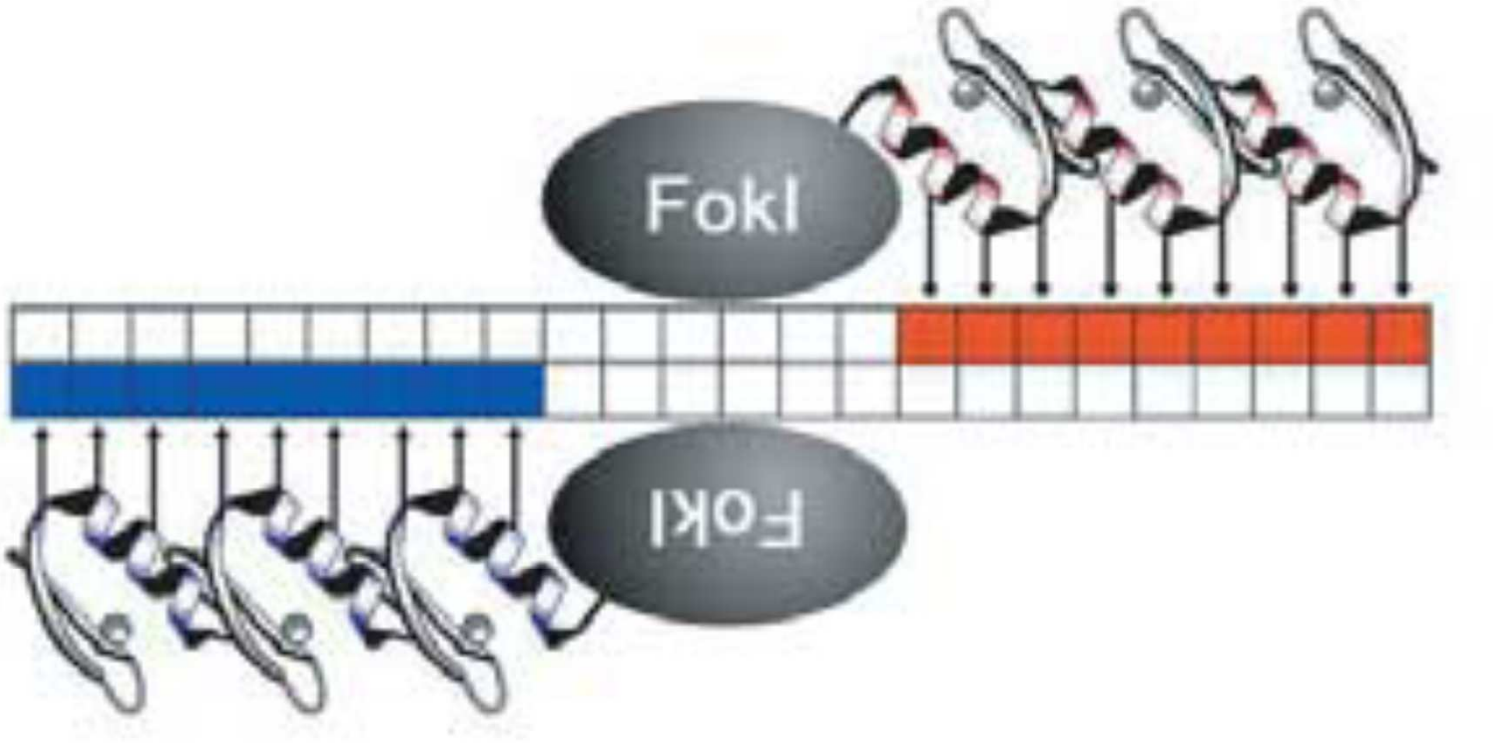
Interazione aminoacidi / basi del DNA



ZF Uno strumento versatile

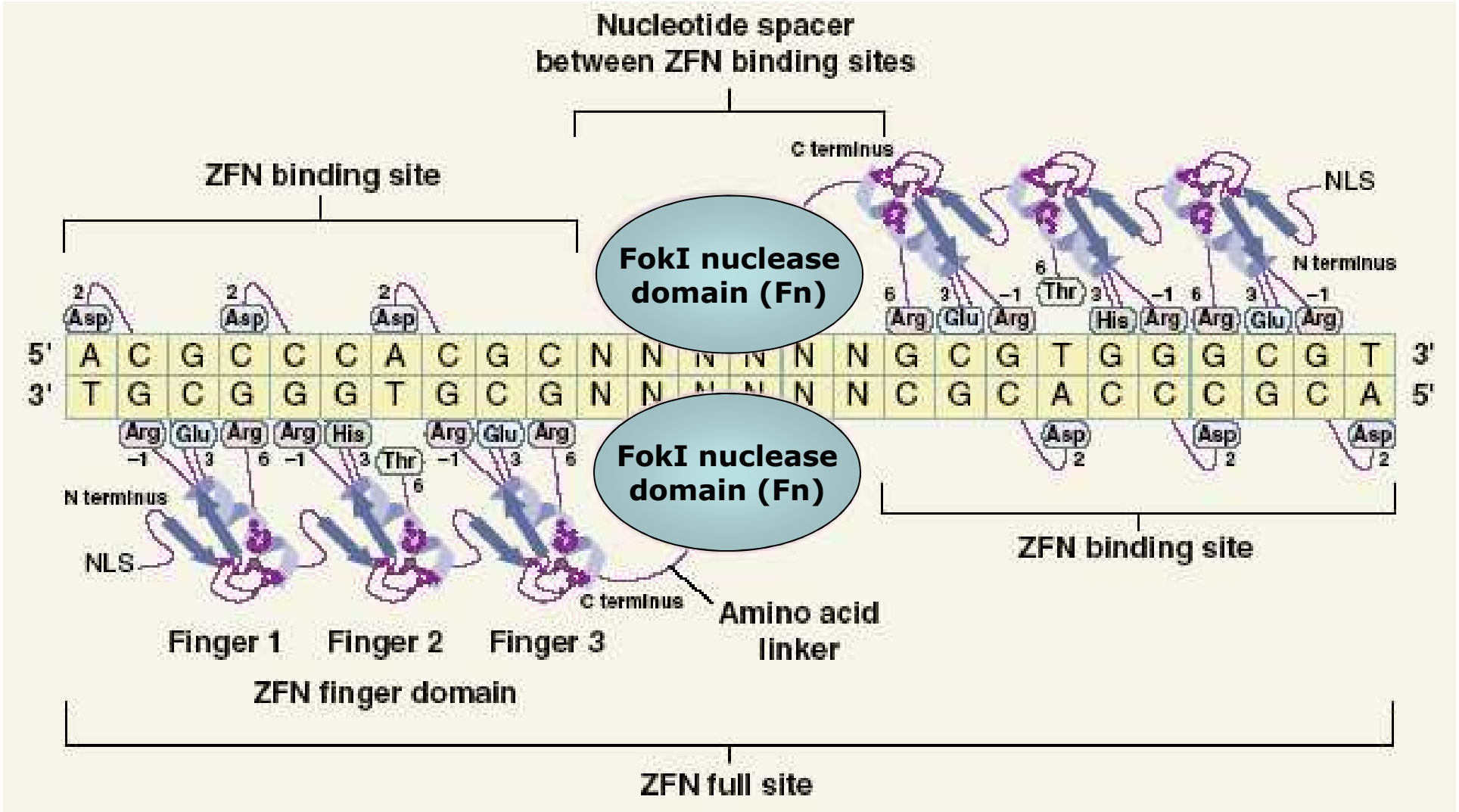


Zinc Finger Nuclease ZFN



Dimerization of FokI domains is required for its DNA binding-dependent endonuclease activity

Zinc Finger Nucleases as Potential Reagents to Create Double-Strand Breaks in Normal Genes



Initially developed by labs of Srinivasan Chandrasegaran (Johns Hopkins) and Dana Carroll (Univ. Utah)

Table 1 Potential applications of zinc finger nucleases

Experimental uses	Drug development
Create knockout genes (cell lines, primary cells, transgenic animals)	Create humanized cell lines
Create point mutations or small deletions in permanent or primary cell lines	Create cell lines for drug target validation
Improve efficiency of gene targeting in ES cells	Create cell lines for high-throughput screening for novel compounds
Create targeted transgenics with insertions into precise genomic locations	
Genome manipulation in model organisms currently without gene targeting mechanism (worms, zebrafish)	

Therapeutics
Correction of genes in monogenic diseases (e.g., Huntington disease)
Inserting genes into precise (safe and permissive) locations for correcting complex mutations (hemophilia A) and introducing RNAi, for example
Altering alleles; for example, the CCR5 gene to create resistance to HIV.
Designer immunotherapeutics
Modification of stem cells

ZF
strumenti versatili

zinc finger protein (ZFP) engineering

- Two approaches were originally used for zinc finger protein (ZFP) engineering

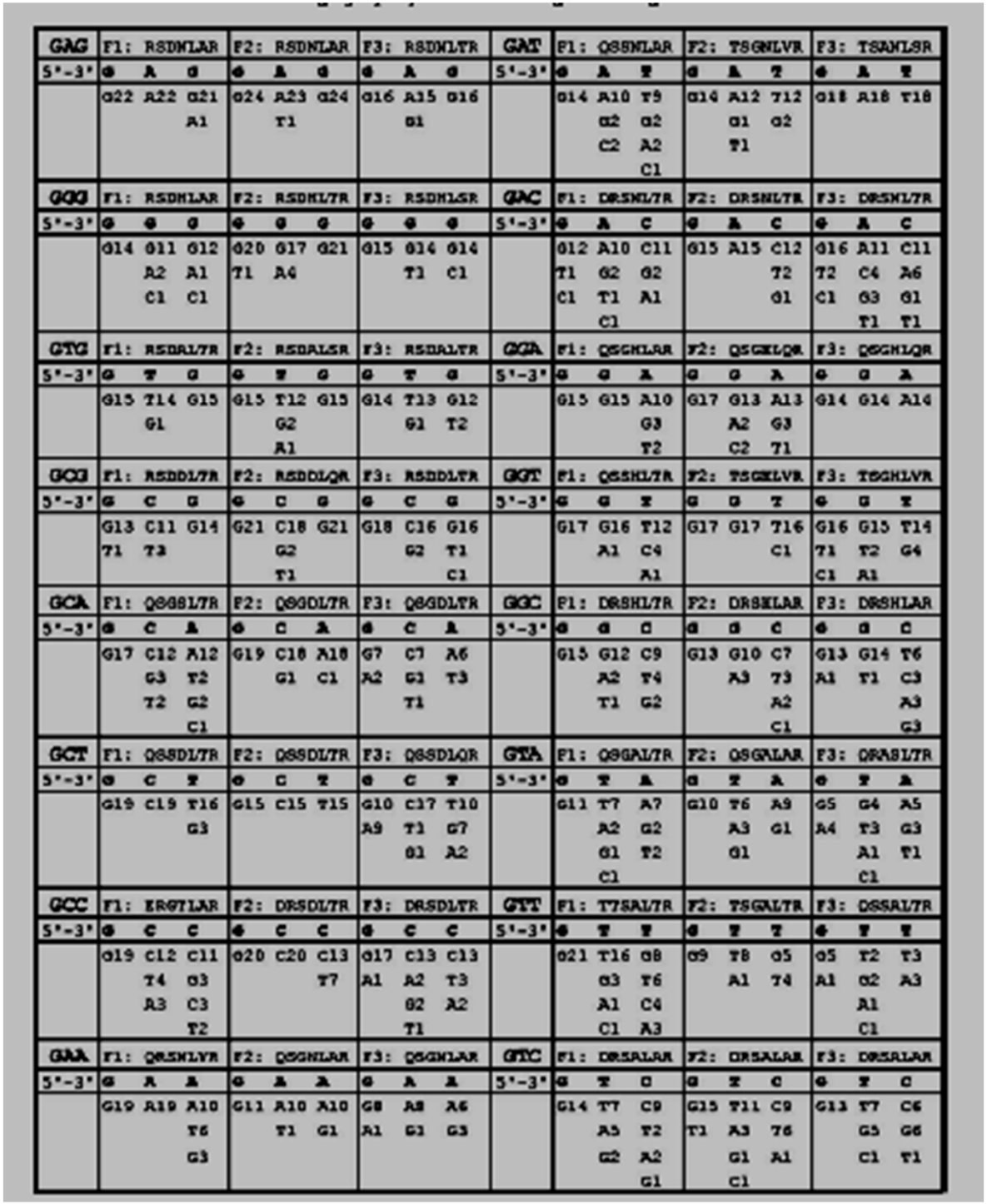
expand the DNA recognition code and create zinc fingers that bind desired base triplets

- 1 a combinatorial approach using libraries of zinc fingers displayed on the surface of filamentous phage that were selected against target DNA sequences
- 2 a rational design approach that used databases to predict rules for amino acid–base interactions.

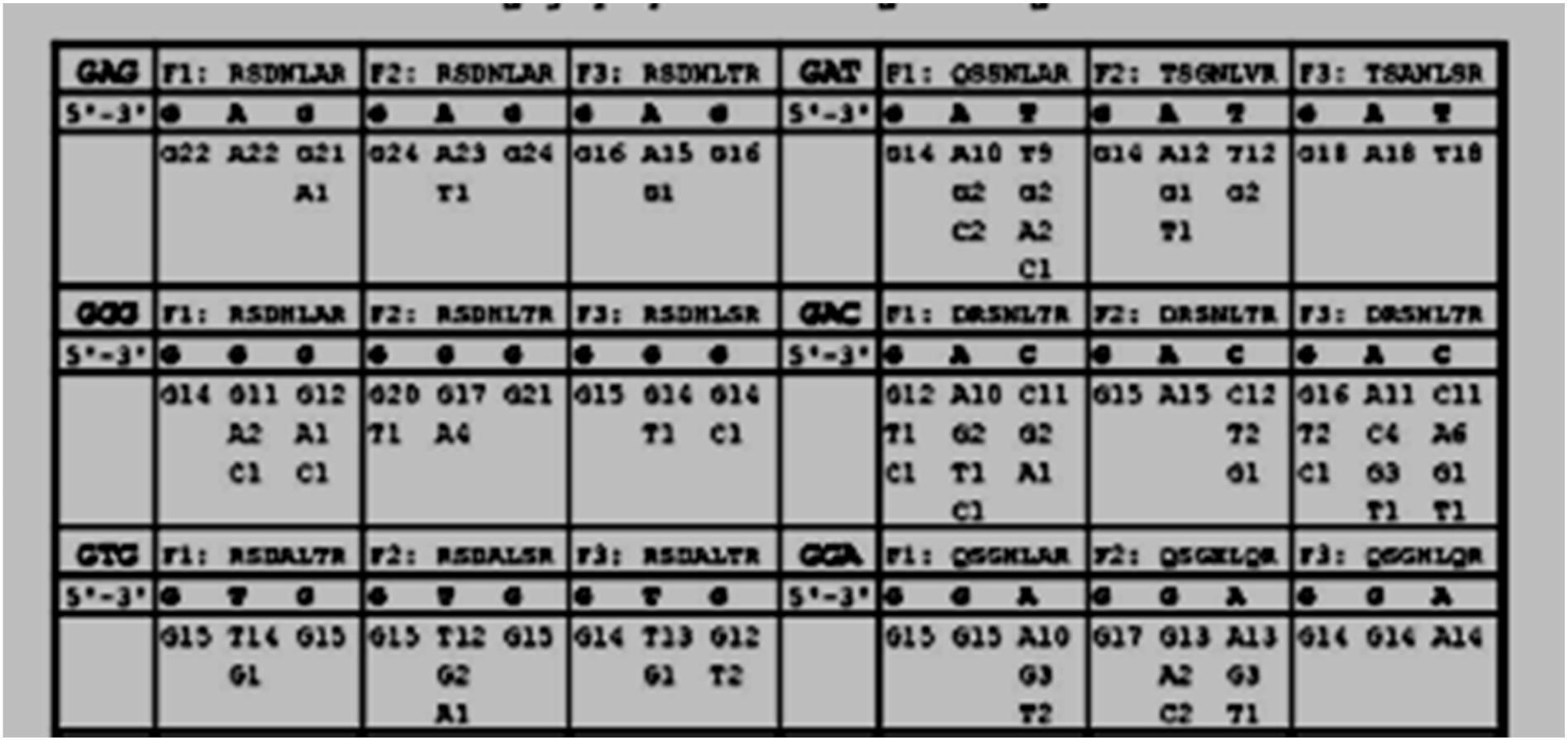
Empiric Design of Zinc Finger Nucleases

(assembly approach)

From Liu et al. (2002)



GAG	F1: RSDNLAR			F2: RSDNLAR			F3: RSDNLTR			GAT	F1: QSSNLAR			F2: TSGNLVR			F3: TSANLSR		
5'-3'	G	A	G	G	A	G	G	A	G	5'-3'	G	A	T	G	A	T	G	A	T
	G22	A22	G21	G24	A23	G24	G16	A15	G16		G14	A10	T9	G14	A12	T12	G18	A18	T18
			A1		T1			G1				G2	G2		G1	G2			
												C2	A2		T1				
													C1						
GGG	F1: RSDNLAR			F2: RSDNLTR			F3: RSDNLSR			GAC	F1: DRSNLTR			F2: DRSNLTR			F3: DRSNLTR		
5'-3'	G	G	G	G	G	G	G	G	G	5'-3'	G	A	C	G	A	C	G	A	C
	G14	G11	G12	G20	G17	G21	G15	G14	G14		G12	A10	C11	G15	A15	C12	G16	A11	C11
		A2	A1	T1	A4			T1	C1		T1	G2	G2			T2	T2	C4	A6
		C1	C1								C1	T1	A1			G1	C1	G3	G1
												C1						T1	T1
GTG	F1: RSDALTR			F2: RSDALSR			F3: RSDALTR			GCA	F1: QSGNLAR			F2: QSGNLQR			F3: QSGNLQR		
5'-3'	G	T	G	G	T	G	G	T	G	5'-3'	G	G	A	G	G	A	G	G	A
	G15	T14	G15	G15	T12	G15	G14	T13	G12		G15	G15	A10	G17	G13	A13	G14	G14	A14
		G1			G2			G1	T2				G3		A2	G3			
					A1								T2		C2	T1			
GCG	F1: RSDDLTR			F2: RSDDLQR			F3: RSDDLTR			GGT	F1: QSSNLTR			F2: TSGNLVR			F3: TSGNLVR		
5'-3'	G	C	G	G	C	G	G	C	G	5'-3'	G	G	T	G	G	T	G	G	T
	G13	C11	G14	G21	C18	G21	G18	C16	G16		G17	G16	T12	G17	G17	T16	G16	G15	T14
	T1	T3			G2			G2	T1			A1	C4			C1	T2	T2	G4
					T1				C1				A1				C1	A1	
GCA	F1: QSGSLTR			F2: QSGDLTR			F3: QSGDLTR			GGC	F1: DRSNLTR			F2: DRSNLAR			F3: DRSNLAR		
5'-3'	G	C	A	G	C	A	G	C	A	5'-3'	G	G	C	G	G	C	G	G	C
	G17	C12	A12	G19	C18	A18	G7	C7	A6		G15	G12	C9	G13	G10	C7	G13	G14	T6
		G3	T2		G1	C1	A2	G1	T3			A2	T4		A3	T3	A1	T1	C3
		T2	G2					T1				T1	G2			A2			A3
			C1													C1			G3
GCT	F1: QSSDLTR			F2: QSSDLTR			F3: QSSDLQR			GTA	F1: QSGALTR			F2: QSGALAR			F3: QRASLTR		
5'-3'	G	C	T	G	C	T	G	C	T	5'-3'	G	T	A	G	T	A	G	T	A
	G19	C19	T16	G15	C15	T15	G10	C17	T10		G11	T7	A7	G10	T6	A9	G5	G4	A5
			G3				A9	T1	G7			A2	G2		A3	G1	A4	T3	G3
								G1	A2			G1	T2		G1			A1	T1
												C1						C1	
GCC	F1: ERGTLAR			F2: DRSDLTR			F3: DRSDLTR			GTT	F1: TTSALTR			F2: TSGALTR			F3: QSSALTR		
5'-3'	G	C	C	G	C	C	G	C	C	5'-3'	G	T	T	G	T	T	G	T	T
	G19	C12	C11	G20	C20	C13	G17	C13	C13		G21	T16	G8	G9	T8	G5	G5	T2	T3
		T4	G3			T7	A1	A2	T3			G3	T6		A1	T4	A1	G2	A3
		A3	C3					G2	A2			A1	C4					A1	
			T2					T1				C1	A3					C1	
GAA	F1: QRSNLVR			F2: QSGNLAR			F3: QSGNLAR			GTC	F1: DRSALAR			F2: DRSALAR			F3: DRSALAR		
5'-3'	G	A	A	G	A	A	G	A	A	5'-3'	G	T	C	G	T	C	G	T	C
	G19	A19	A10	G11	A10	A10	G8	A8	A6		G14	T7	C9	G15	T11	C9	G13	T7	C6
			T6		T1	G1	A1	G1	G3			A5	T2	T1	A3	T6		G5	G6
			G3									G2	A2		G1	A1		C1	T1
													G1		C1				



GAG	F1: RSDNLAR			F2: RSDNLAR			F3: RSDNLTR			GAT	F1: QSSNLAR			F2: TSGNLVR			F3: TSANLSR		
5'-3'	G	A	G	G	A	G	G	A	G	5'-3'	G	A	T	G	A	T	G	A	T
	G22	A22	G21	G24	A23	G24	G16	A15	G16		G14	A10	T9	G14	A12	T12	G18	A18	T18
			A1		T1			G1				G2	G2		G1	G2			
												C2	A2		T1				
													C1						
GCG	F1: RSDNLAR			F2: RSDNLTR			F3: RSDNLSR			GAC	F1: DRSNLTR			F2: DRSNLTR			F3: DRSNLTR		
5'-3'	G	G	G	G	G	G	G	G	G	5'-3'	G	A	C	G	A	C	G	A	C
	G14	G11	G12	G20	G17	G21	G15	G14	G14		G12	A10	C11	G15	A15	C12	G16	A11	C11
		A2	A1	T1	A4			T1	C1		T1	G2	G2			T2	T2	C4	A6
		C1	C1								C1	T1	A1			G1	C1	G3	G1
												C1						T1	T1
GTC	F1: RSDALTR			F2: RSDALSR			F3: RSDALTR			GCA	F1: QSGNLAR			F2: QSGNLGR			F3: QSGNLGR		
5'-3'	G	T	G	G	T	G	G	T	G	5'-3'	G	G	A	G	G	A	G	G	A
	G15	T14	G15	G15	T12	G15	G14	T13	G12		G15	G15	A10	G17	G13	A13	G14	G14	A14
		G1			G2			G1	T2				G3		A2	G3			
					A1								T2		C2	T1			

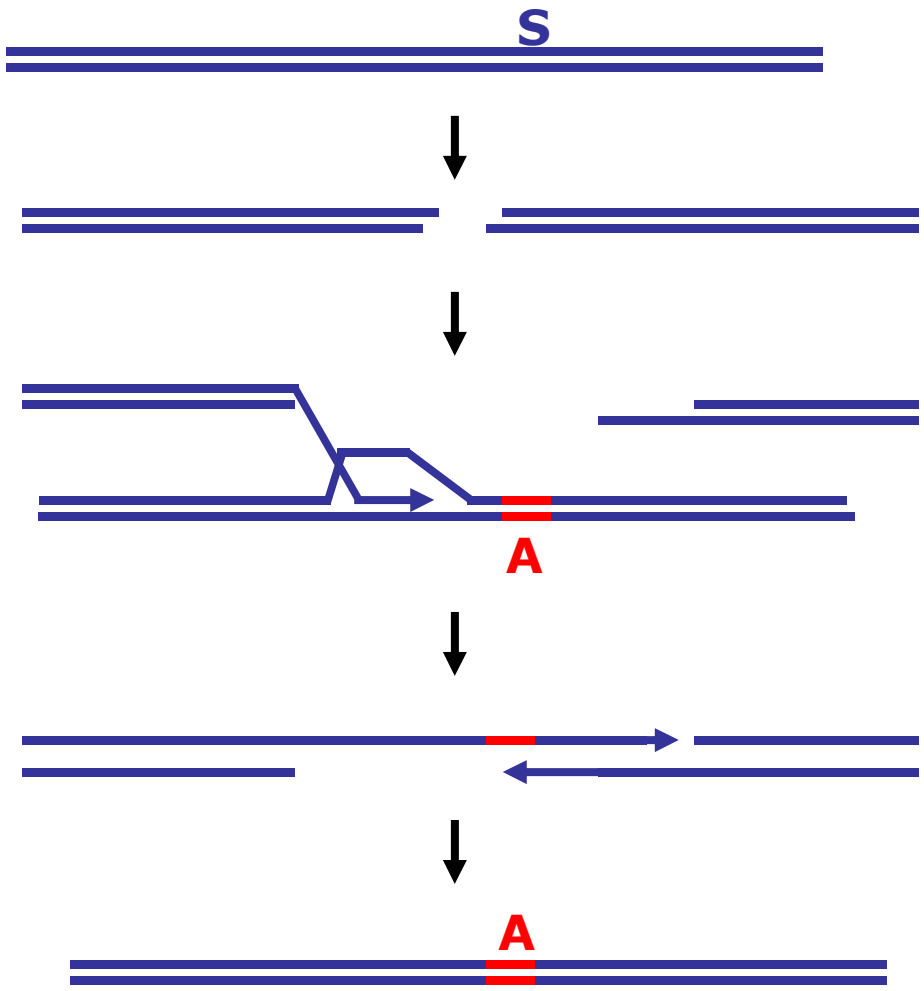
From Liu et al. (2002)

Highly efficient endogenous human gene correction using designed zinc-finger nucleases

Fyodor D. Urnov[1], Jeffrey C. Miller[1], Ya-Li Lee[1], Christian M. Beausejour[1], Jeremy M. Rock[1], Sheldon Augustus[1], Andrew C. Jamieson[1], Matthew H. Porteus[2], Philip D. Gregory[1] & Michael C. Holmes[1]

Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases

Schema of DSB-Induced Gene Conversion



Undamaged DNA (Allele S)

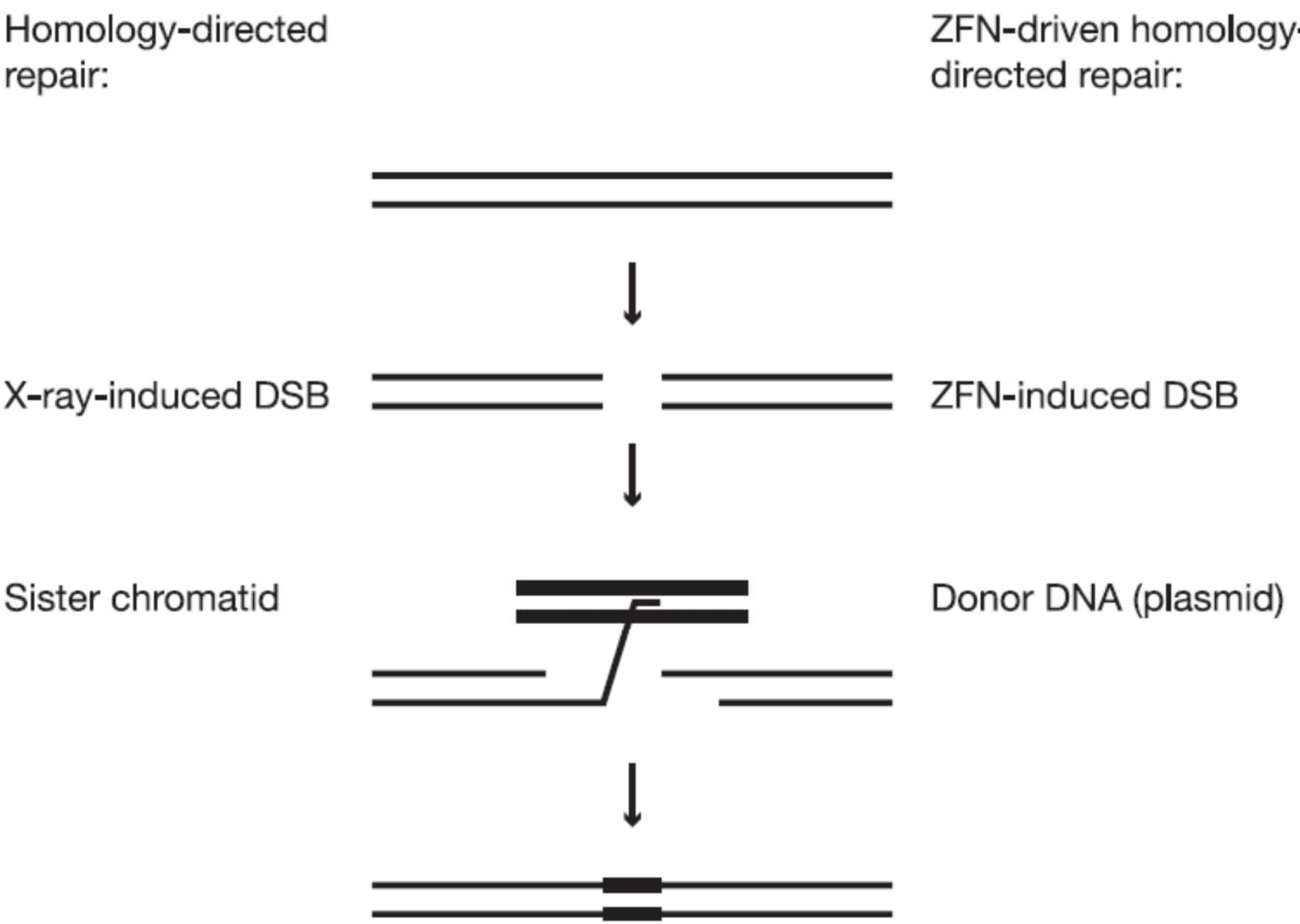
DSB Created (spontaneous or induced, e.g. by ZFN)

Strand Invasion into Undamaged Homologous DNA (Allele A)

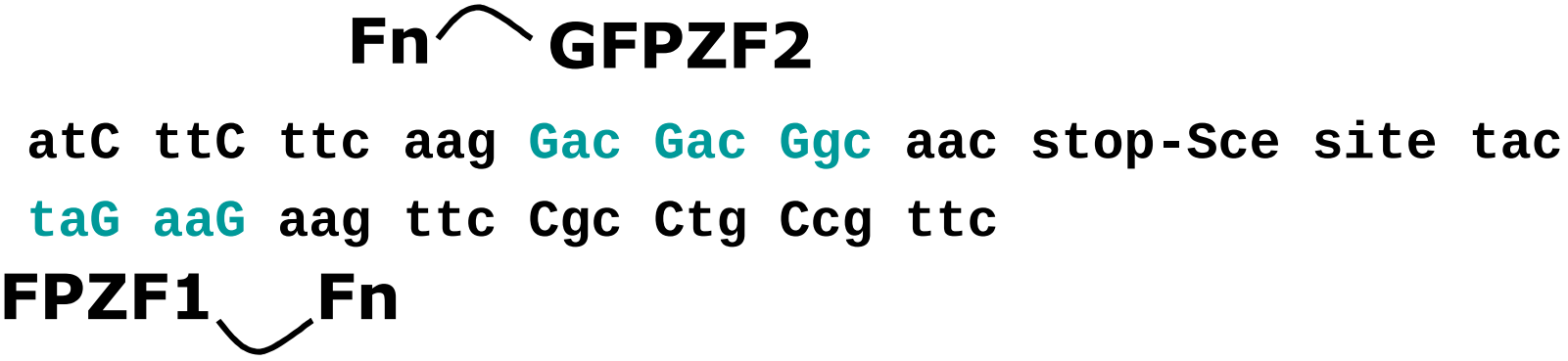
In gene targeting exogenous DNA serves as homologous DNA donor.

Repairing of original strands of DNA. Gaps filled by DNA polymerase and nicks sealed by DNA ligase.

Conversion of Blue Allele("S") into Red Allele ("A") in region of DSB

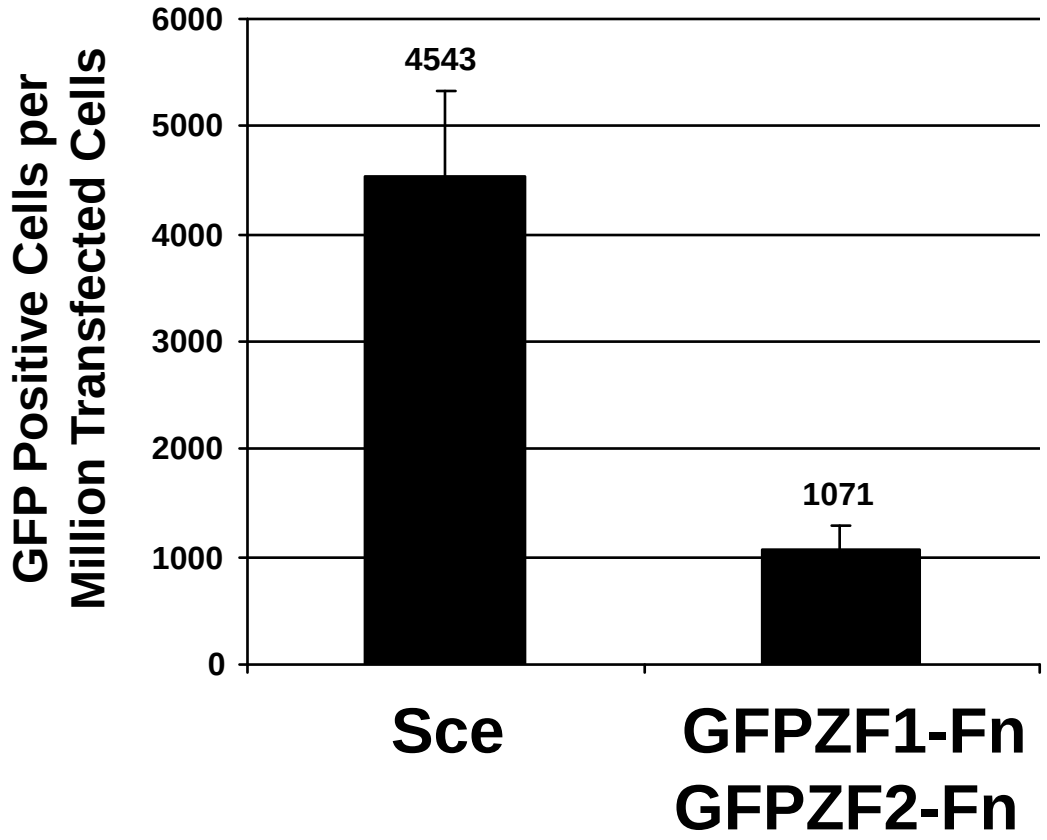

Homology-directed repair:
ZFN-driven homology-directed repair:
X-ray-induced DSB
ZFN-induced DSB
Sister chromatid
Donor DNA (plasmid)

Gene Targeting with Zinc Finger Nucleases to GFP

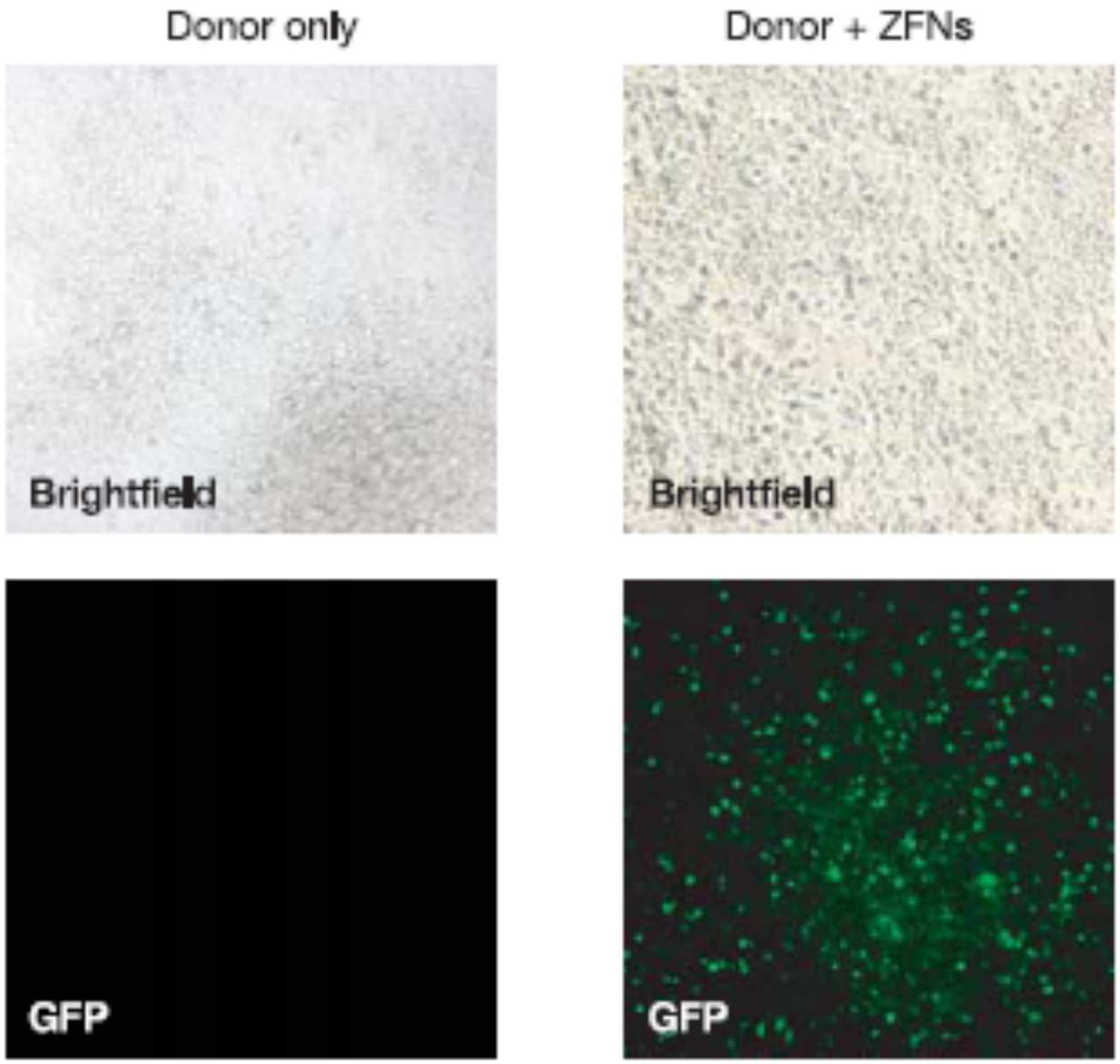


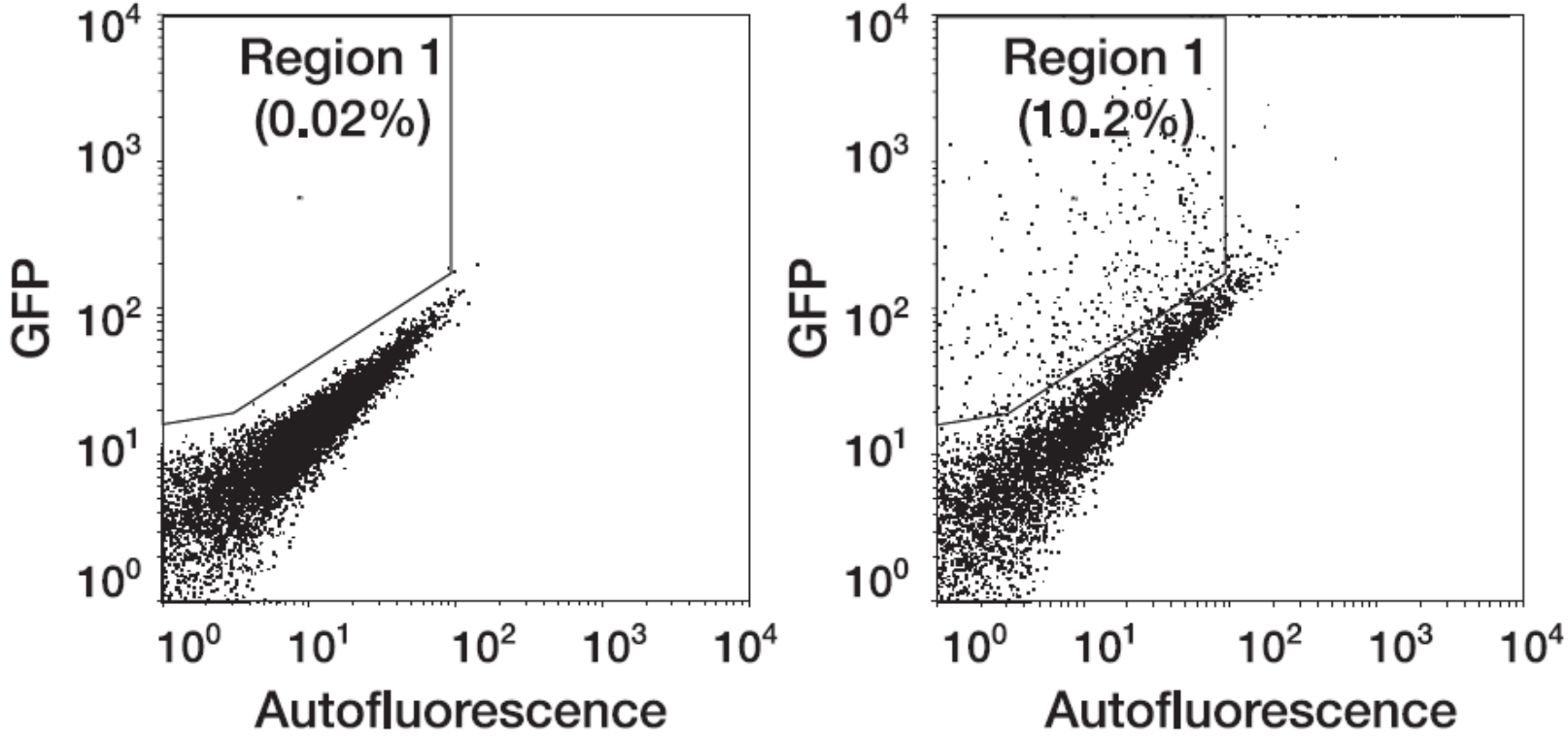
	Finger1	Finger2	Finger3
GFPZFN-1	QSSHLTR (ggt)	TRGNLVR (gat)	QSGNLAR (gaa)
GFPZFN-2	DRSHLTR (ggc)	DRSNLTR (gac)	DRSNLTR (gac)

Gene Targeting with Zinc Finger Nucleases to GFP



Cells carrying a mutated GFP reporter were transiently transfected with a donor plasmid carrying a fragment of wild-type GFP (left column), orthe donor plasmid and the ZFNs (right column).




Region 1
(0.02%)
Region 1
(10.2%)
GFP
Autofluorescence
10^0
10^1
10^2
10^3
10^4

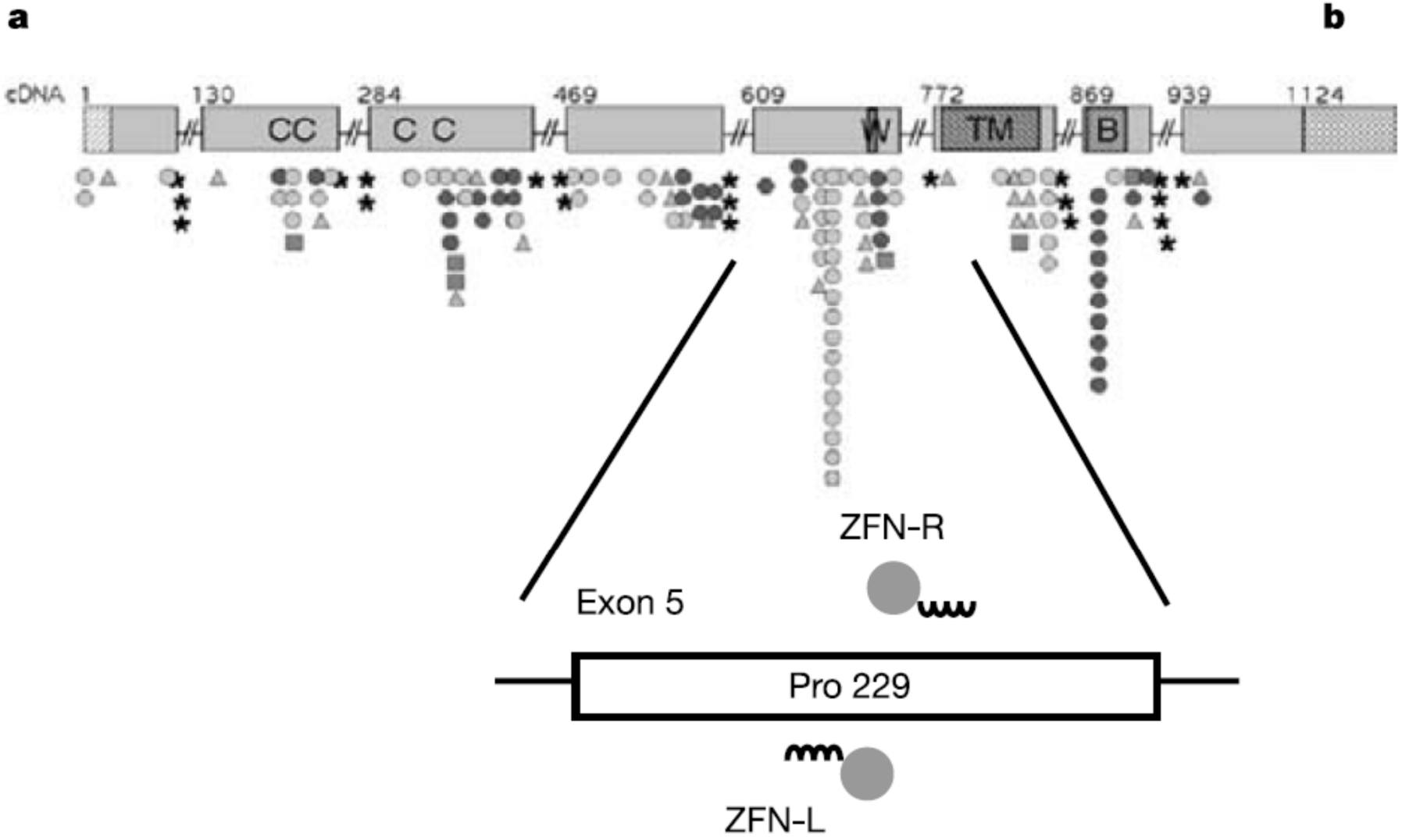
design zinc finger nucleases to stimulate gene targeting in a gene that causes human disease

Sangamo Biosciences (Richmond, CA)

Human Interleukin-2 Receptor Common Gamma Chain Deficiency (IL2RG)

1. Part of Receptor Complex for IL-2, IL-4, IL-7, IL-9, IL-15, IL-21. . .
2. On X-chromosome
3. Mutations in which are the most common cause of SCID (severe combined immunodeficiency)
   -25% of mutations lie in Exon 5.
4. Selective Advantage for corrected cells.
5. Treatment
   - -Bone Marrow Transplantation
     - : Allogeneic (sibling)
     - : Haploidentical (parent)
   - -Gene Therapy
     - : Alain Fischer trial in France
     - : Ooops, leukemia.

IL2Rg gene and its mutations



X-linked severe combined immune deficiency (SCID)

ZFN Gene Correction at the IL2RG gene

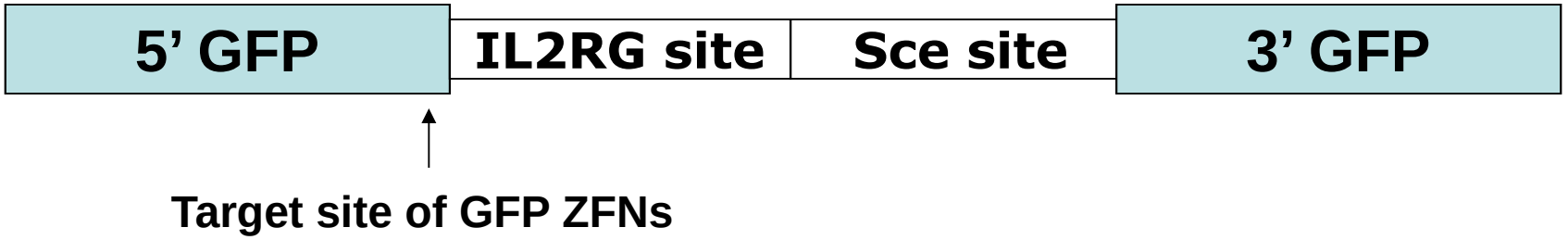
IL2RG ZFN-R

5′CTACACGTTTCGTGTTCGGAGCCGCTTTAACCCACTCTGTGGAAGTGCTC 3′

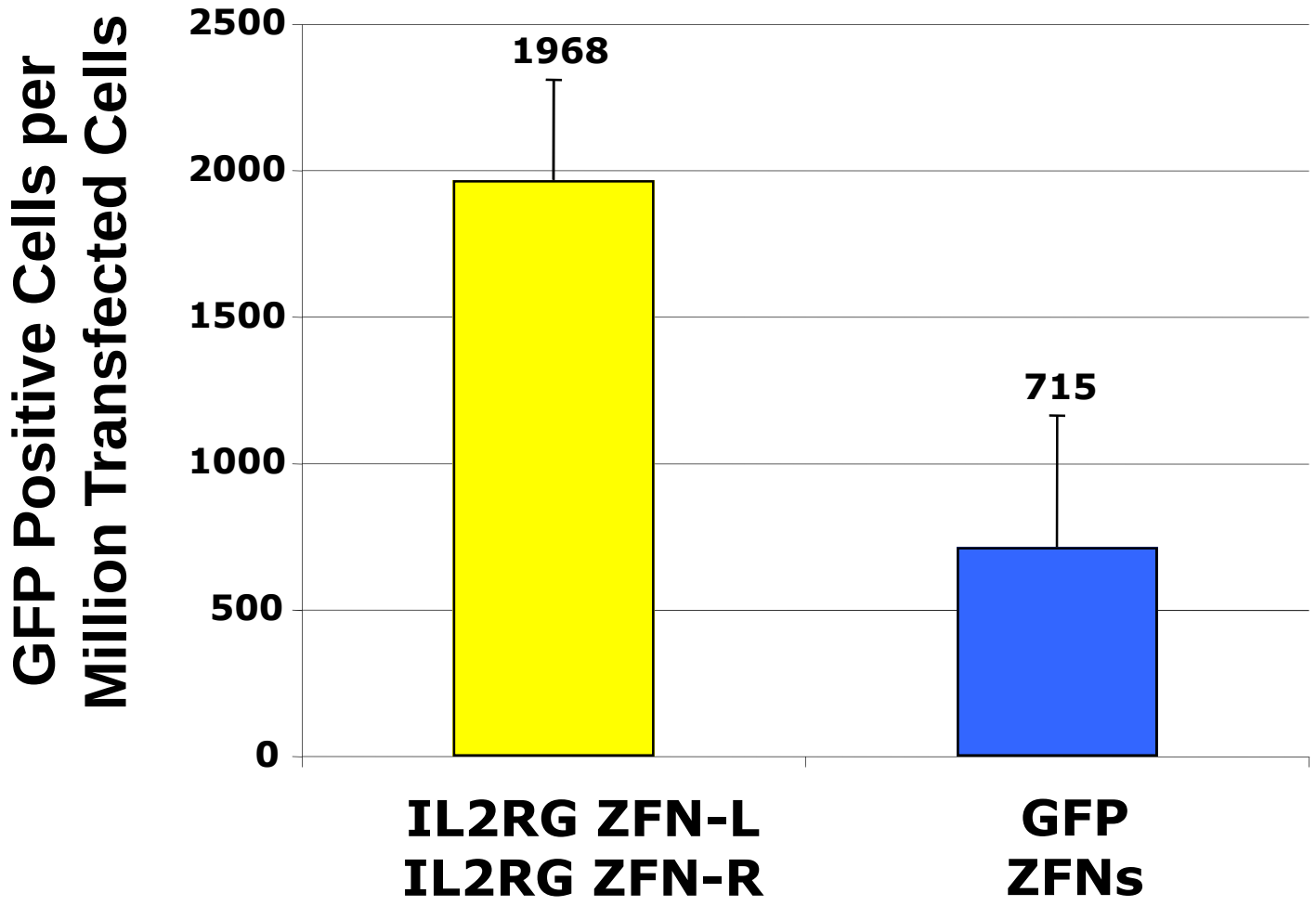
3′GATGTGCAAAGCACAAGCCTCGGCGAAATTGGGTGAGACACCTTCACGAG 5′

IL2RG ZFN-L

GFP Gene Targeting Reporter for IL2RG ZFNs

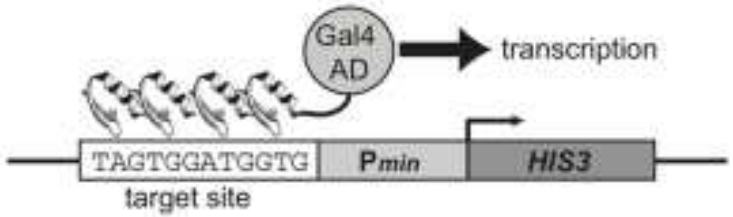


Stimulation of Gene Targeting Using ZFNs for the IL2RG Gene

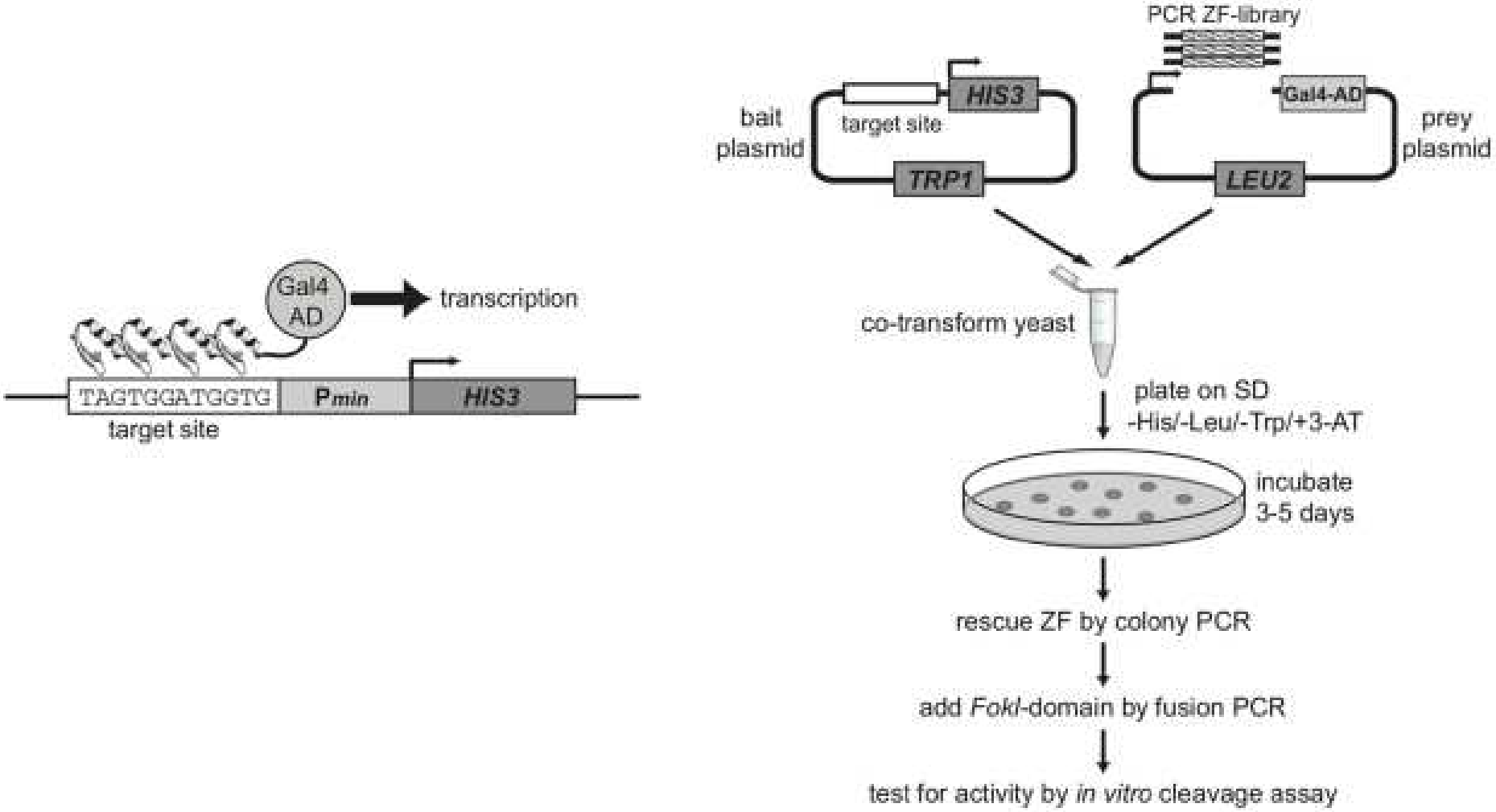


Selection/optimization of ZNF

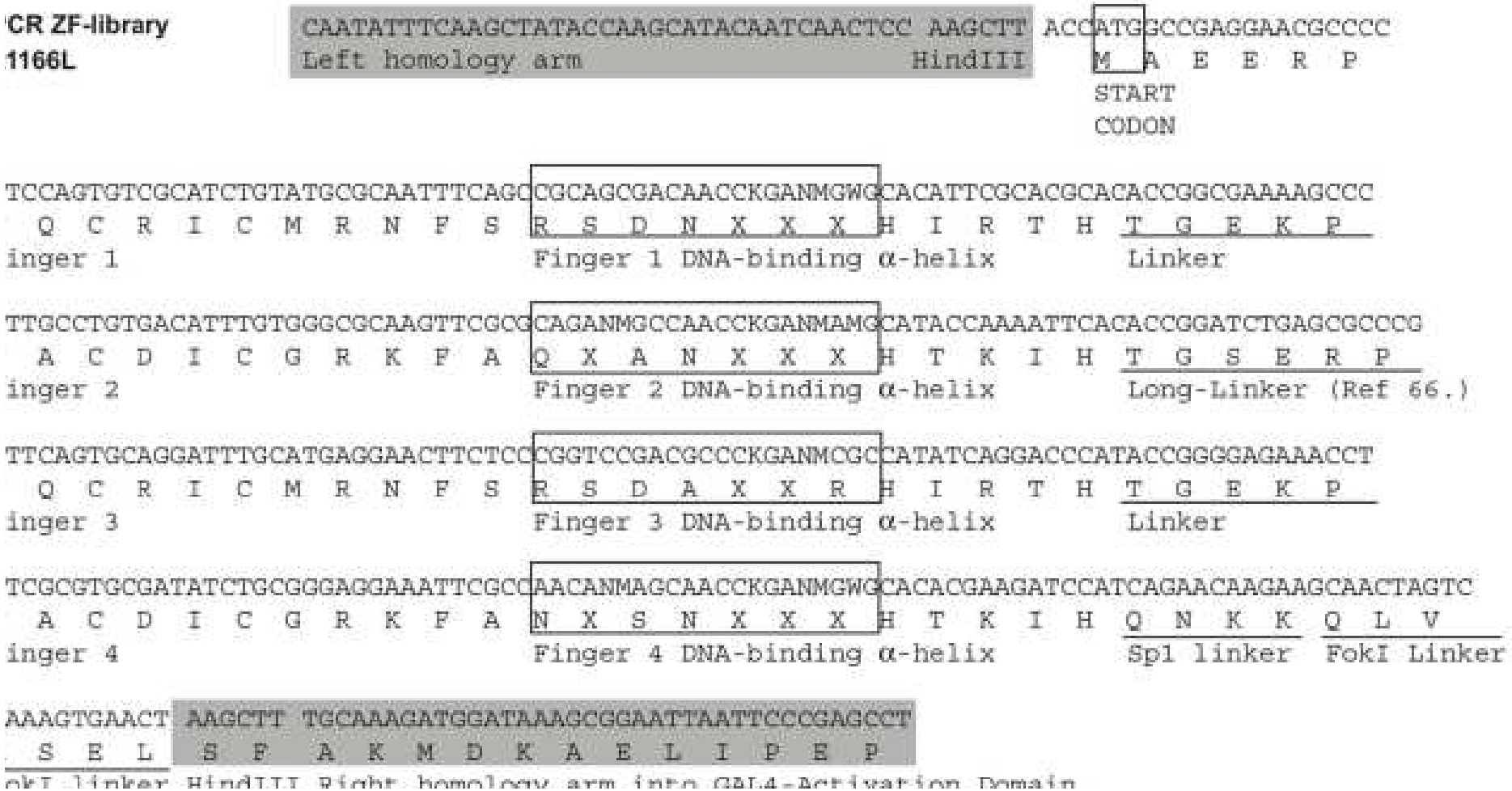
A



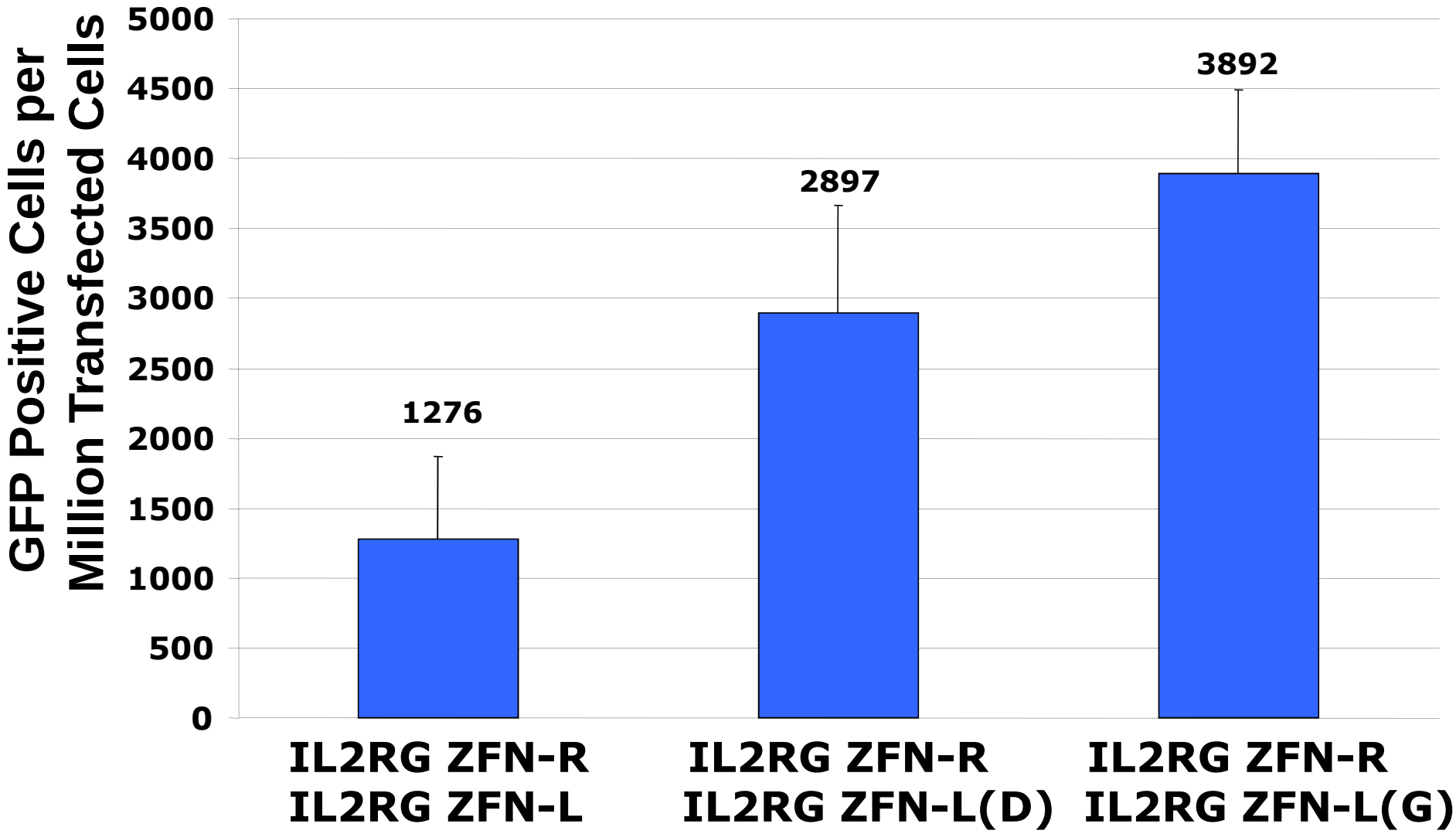
Selection/optimization of ZNF



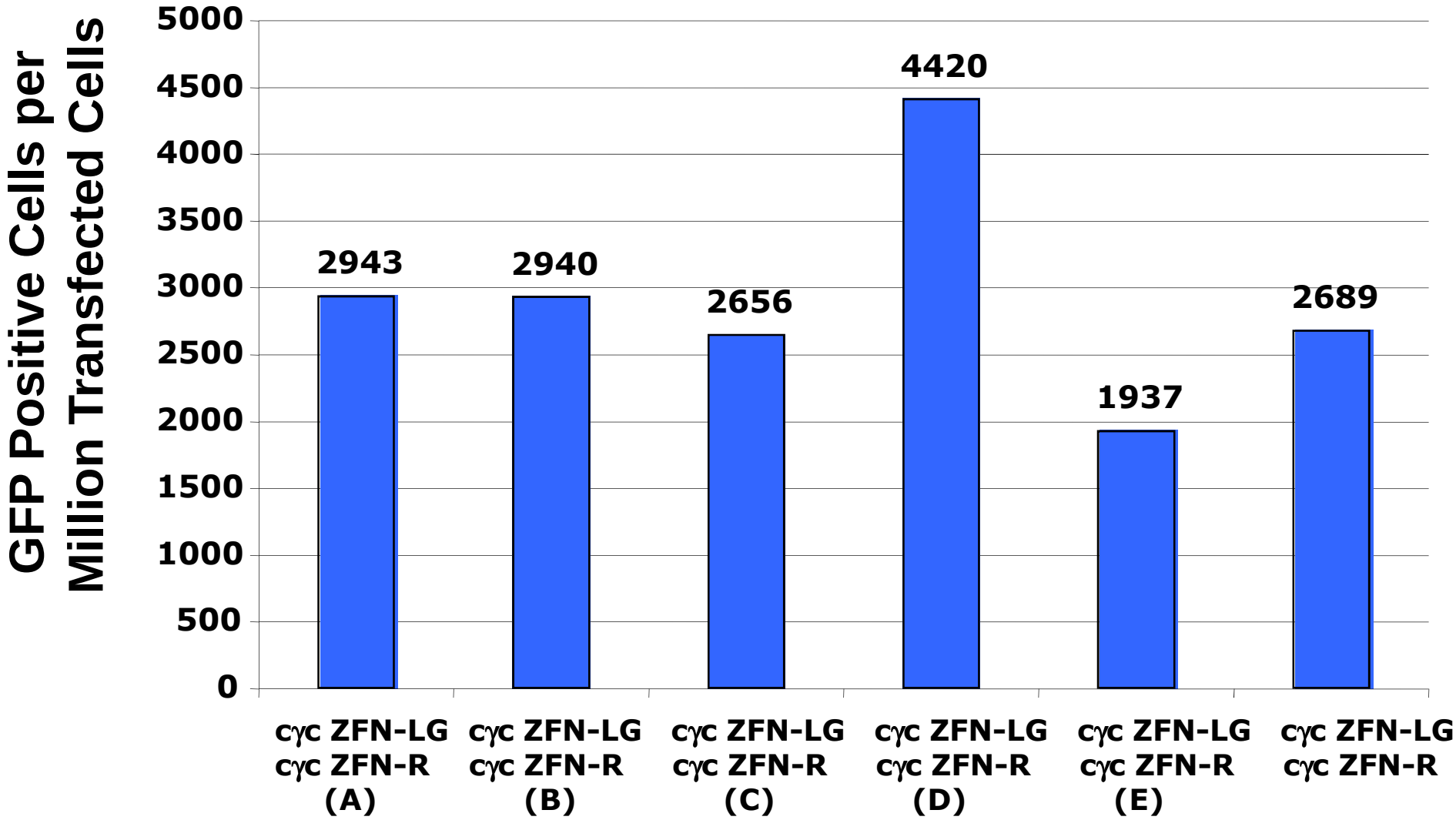
Selection/optimization of ZNF



Optimization of IL2RG ZFN-L



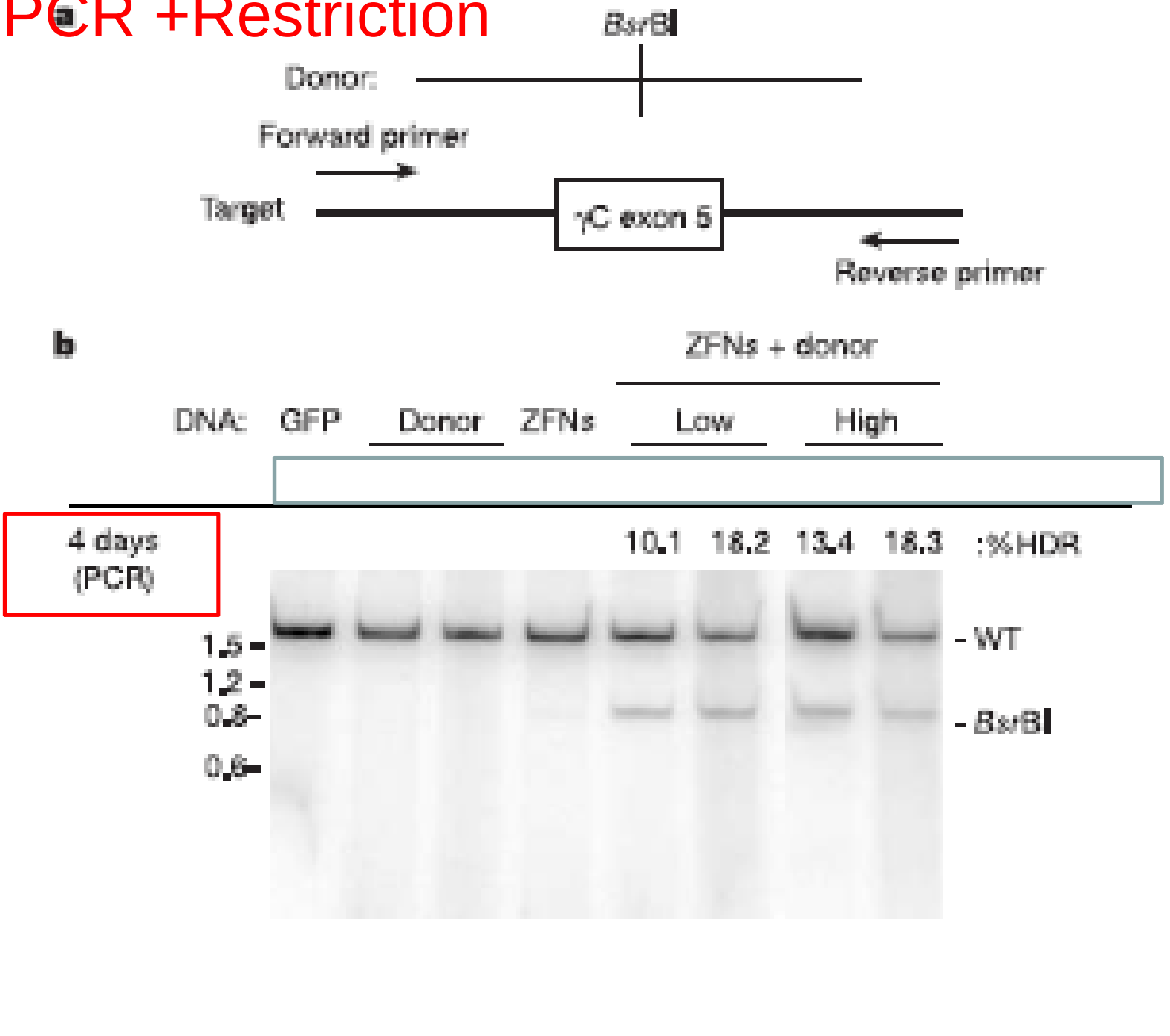
Optimization of cγc ZFN-R



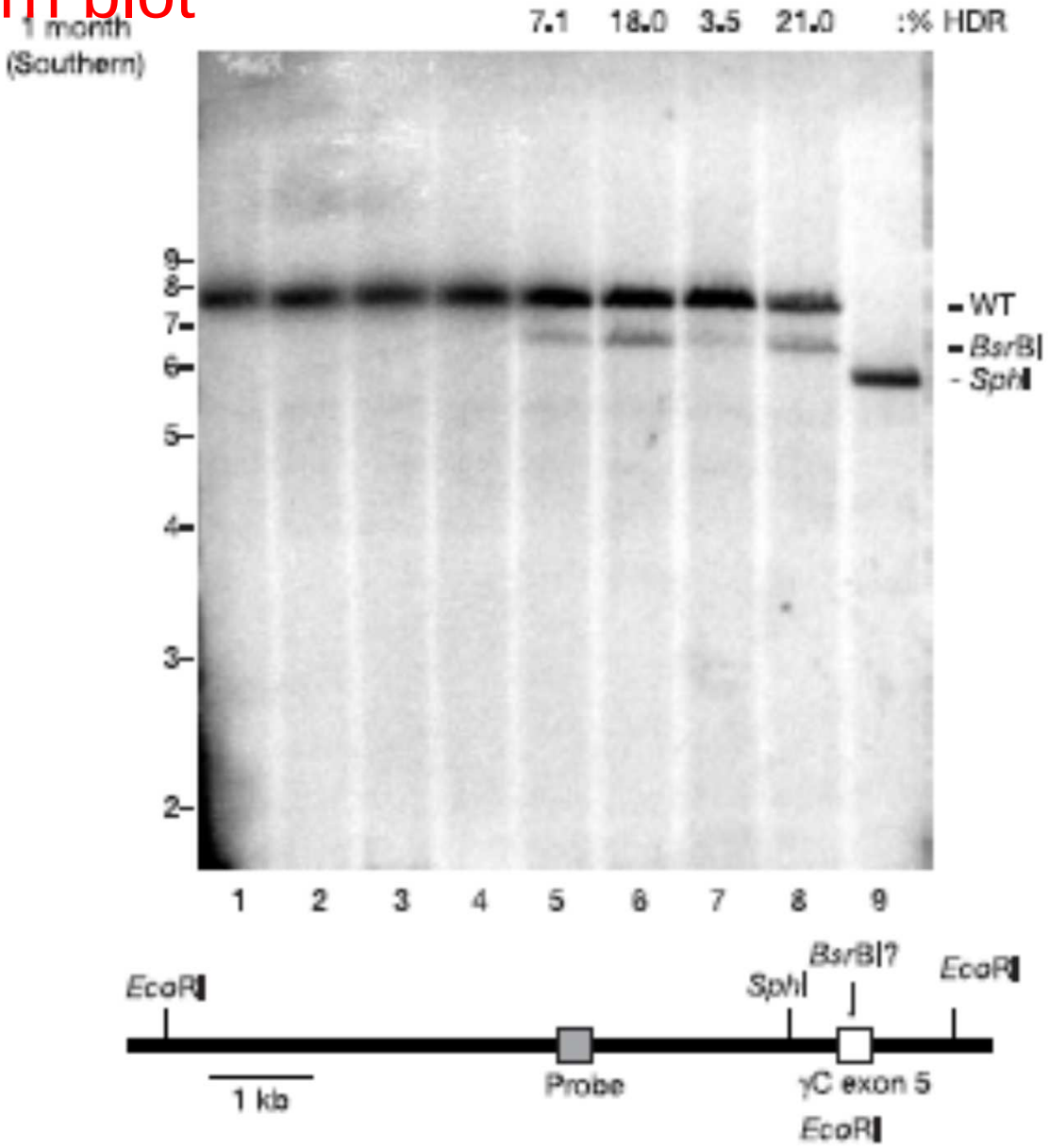
Experimental Design to Detect Targeting at Endogenous IL2RG Locus

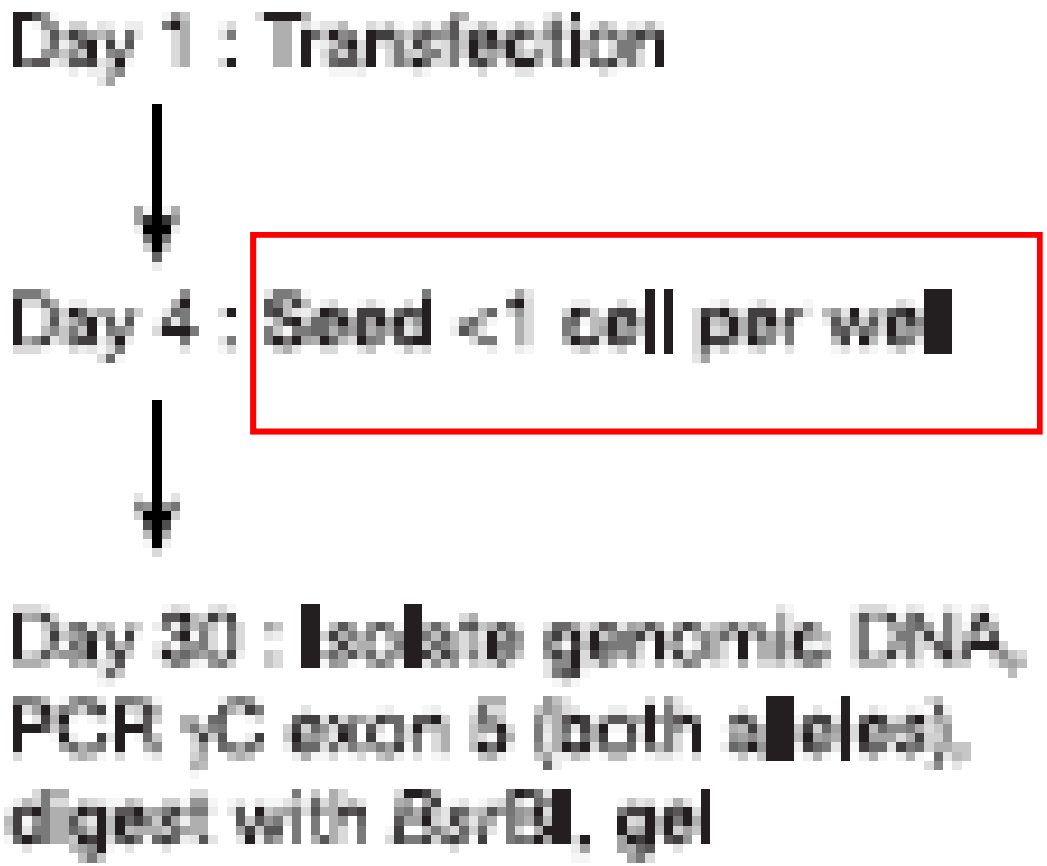
1. **Transfect K562 cells with IL2RG ZFNs with repair substrate that contains BsrBI polymorphism.**
2. **Isolate and expand individual clones individual clones**
3. **Harvest genomic DNA from individual clones.**
4. **Analyze genomic DNA for BsrBI polymorphism.**

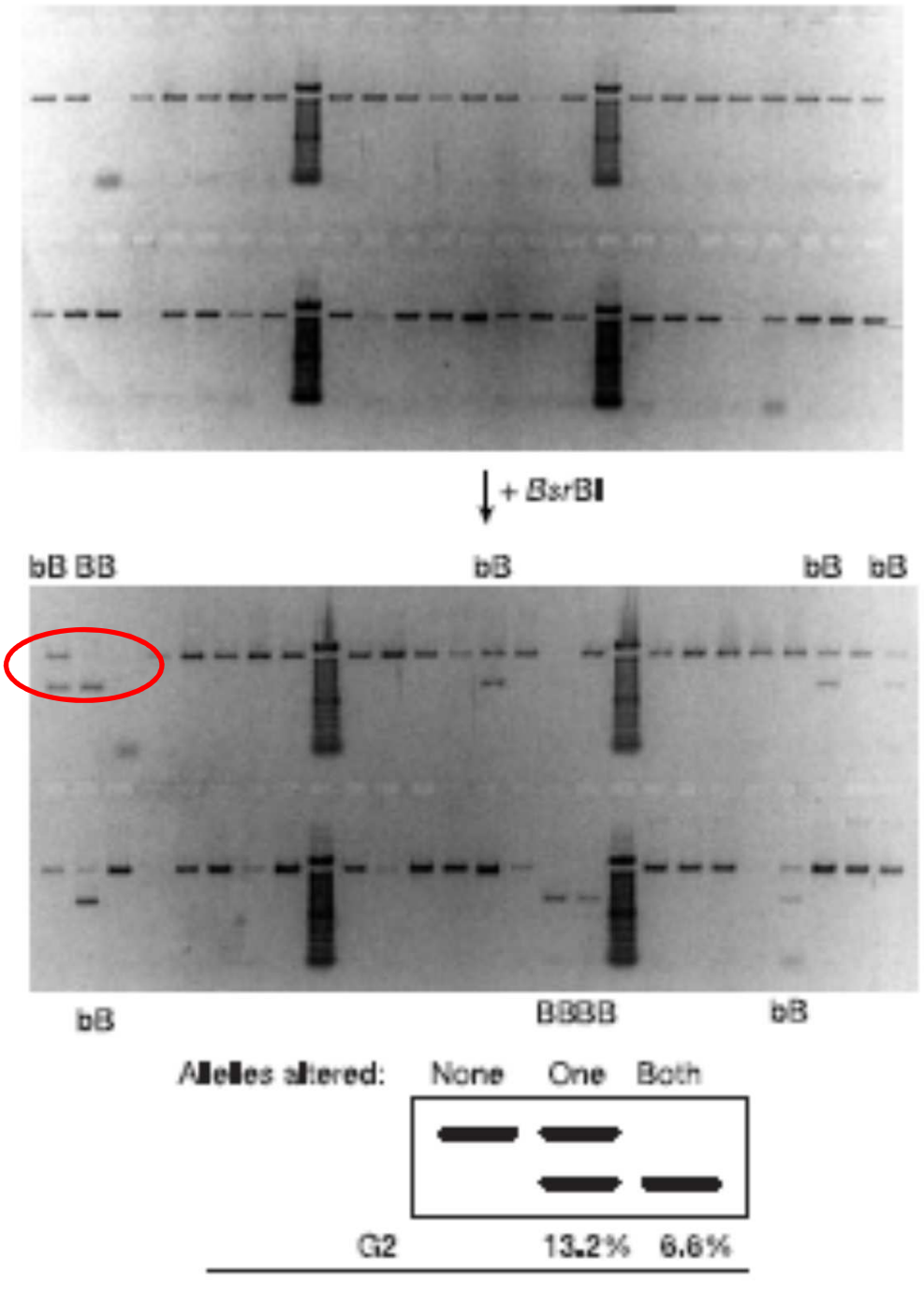
PCR +Restriction



Southern blot



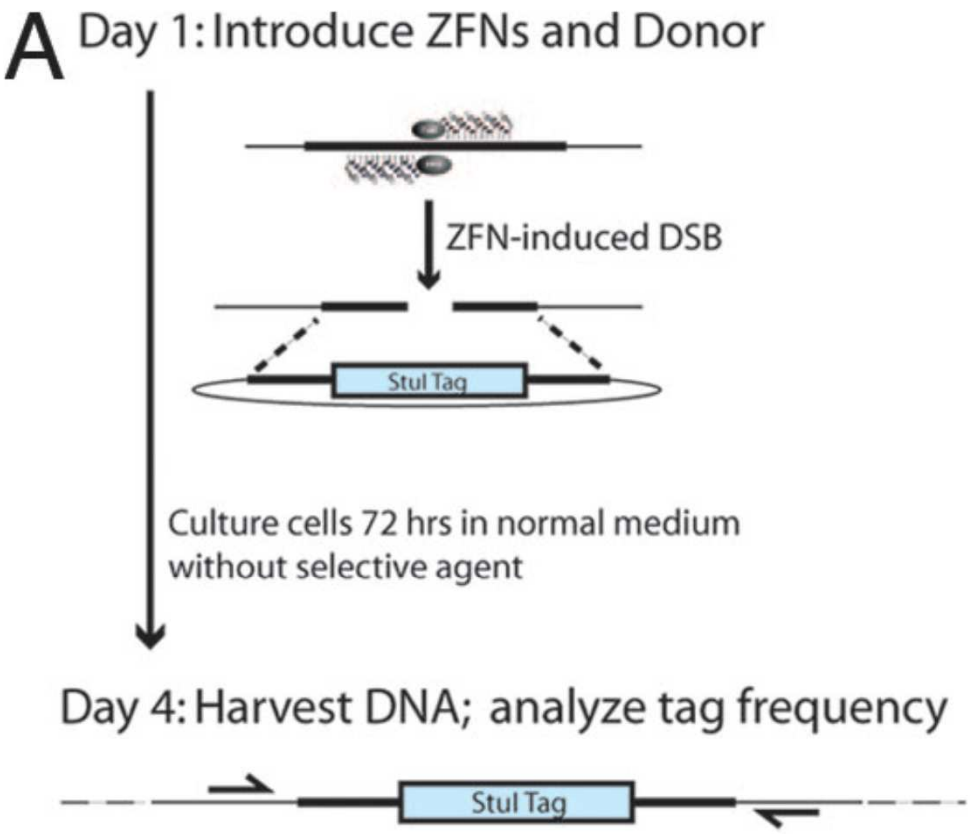

Day 1 : Transfection
Day 4 : Seed <1 cell per well
Day 30 : Isolate genomic DNA, PCR γC exon 5 (both alleles), digest with BsrBI, gel


+ BsrBI
bB BB
bB
bB bB
bB
BBBB
bB
Alleles altered:
None
One
Both
G2
13.2%
6.6%

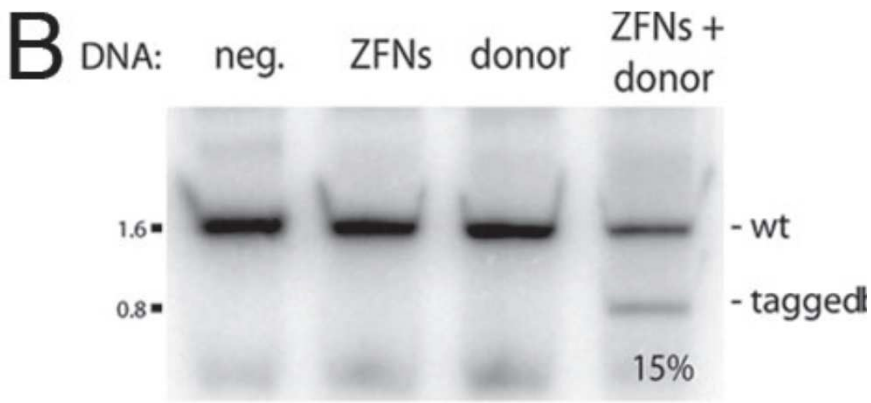
Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases Moehle PNAS 2007

- A precisely placed double-strand break induced by engineered zinc finger nucleases (ZFNs) can stimulate integration of long DNA stretches into a predetermined genomic location, resulting in high-efficiency site-specific gene addition.
- Using an extrachromosomal DNA donor carrying a 12-bp tag, a 900-bp ORF, or a 1.5-kb promoter-transcription unit flanked by locus-specific homology arms, we find targeted integration frequencies of 15%, 6%, and 5%, respectively, within 72 h of treatment, and with no selection for the desired event.
- The integration event occurs in a homology-directed manner and leads to the accurate reconstruction of the donor specified genotype at the endogenous chromosomal locus, and hence presumably results from synthesis-dependent strand annealing repair of the break using the donor DNA as a template.
- This site-specific gene addition occurs with no measurable increase in the rate of random integration. Remarkably, we also find that ZFNs can drive the addition of an 8-kb sequence into an endogenous locus at a frequency of 6%, also in the absence of any selection.
- Surprising versatility of the specialized polymerase machinery involved in double-strand break repair
- Powerful approach to mammalian cell engineering
- Possibility of ZFN-driven gene addition therapy for human genetic disease.

Experimental outline and a schematic of the process whereby a ZFN-induced DSB is repaired by using an extrachromosomal donor as a template



PCR-based measurements of ZFN-driven tag integration frequency into the IL2R locus

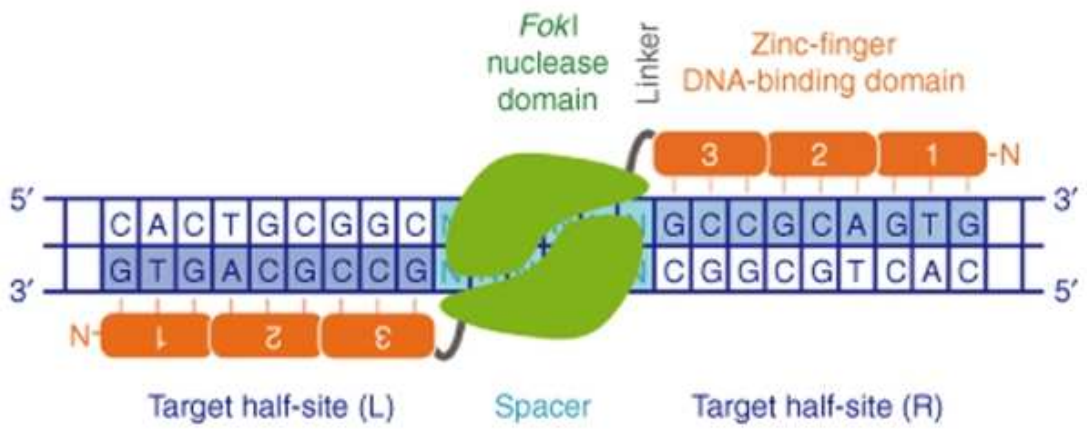


PCR products were digested with StuI

Selettività e tossicità

the inter-domain linker as a major determinant of target site selectivity.

a

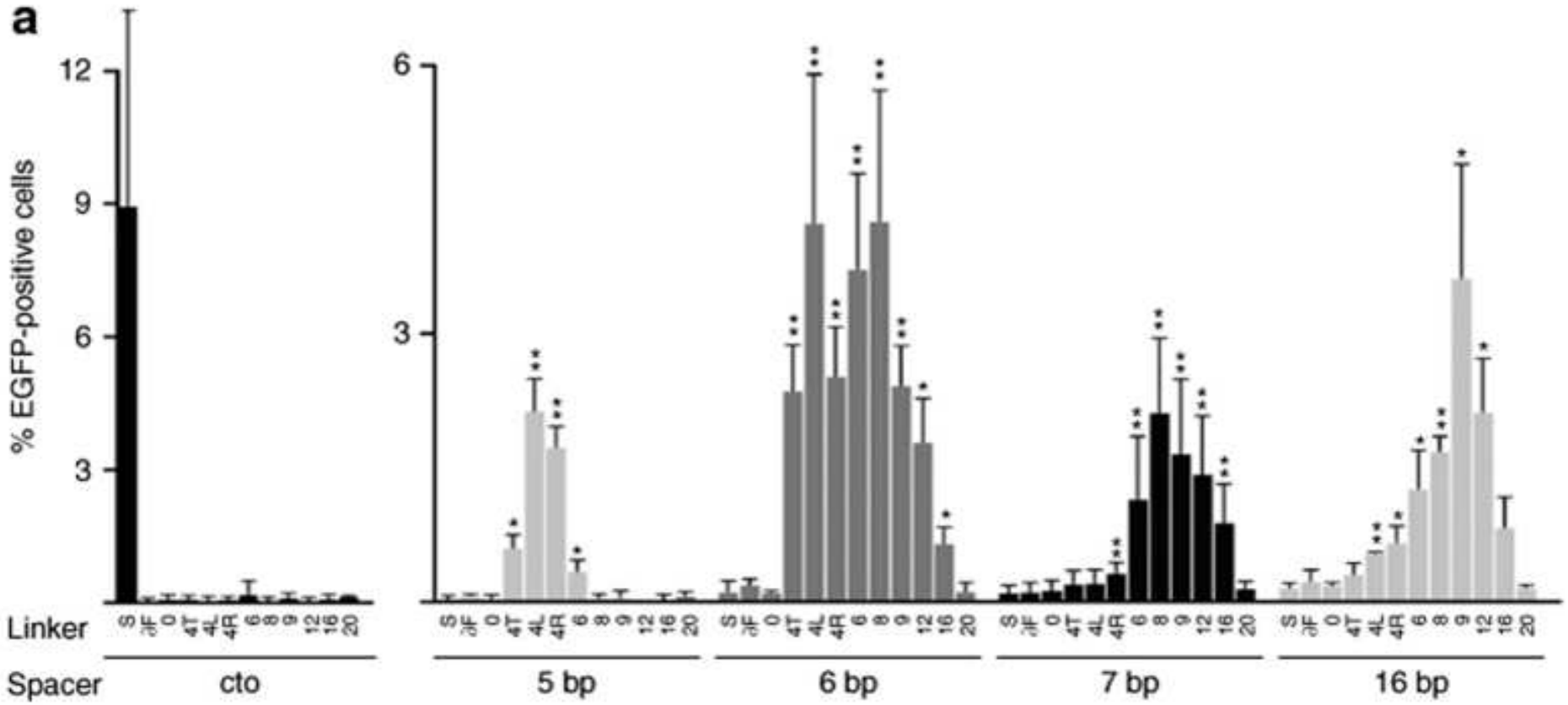


b

Name	Length	F3	Linker	*Fok*I
∂F	0aa	..H..HTG		V..
0	0aa	..H..H		QLV..
4T	4aa	..H..H**T**GGS		QLV..
4L	4aa	..H..H**L**GGS		QLV..
4R	4aa	..H..HL**R**GS		QLV..
6	6aa	..H..HTGAAARA		LV..
8	8aa	..H..HTGPGAAARA		LV..
9	9aa	..H..HTGPNRGVTK		QLV..
12	12aa	..H..HTGPGAAARAASG		QLV..
16	16aa	..H..HTGPGAAARAGSSGASG		QLV..
20	20aa	..H..HTGPGAAARAASPKKKRKVGRA		LV..

the last conserved histidine in the third zinc-finger (F3)

the inter-domain linker as a major determinant of target site selectivity.

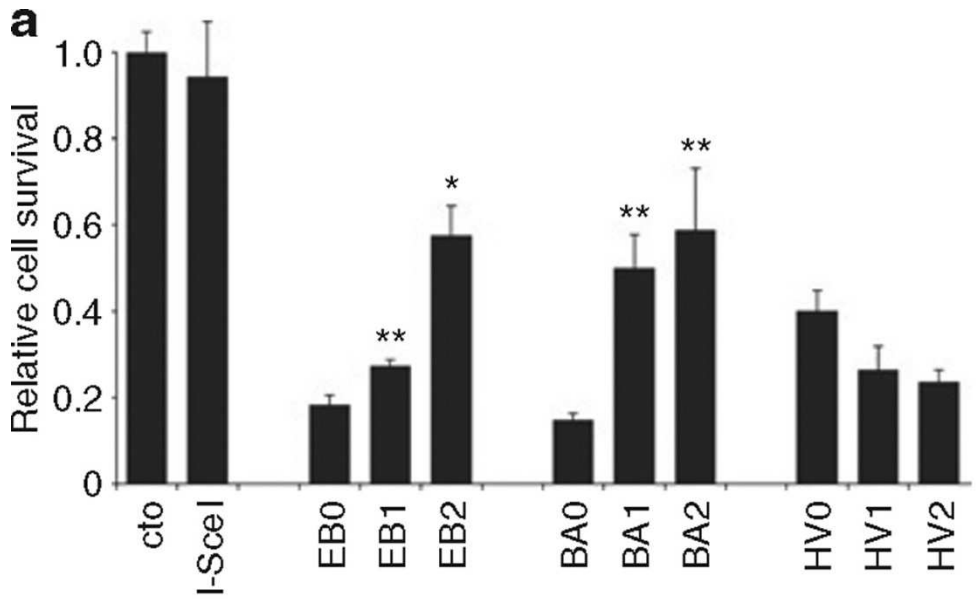


Mol Ther. 2009;17(1):104-11

Continuous Expression of ZFNs causes Cytotoxicity

293T cells co-transfected with pEGFP and ZFN expression vectors

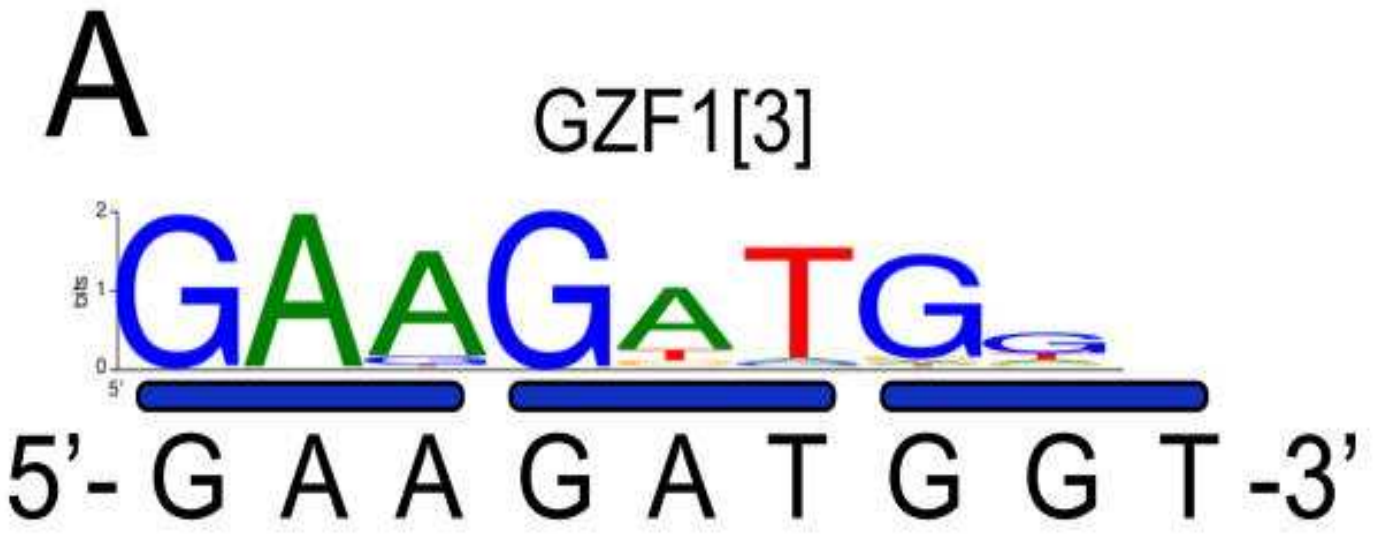
fraction of positive cells
at day 5 as compared to
30 hours post-transfection



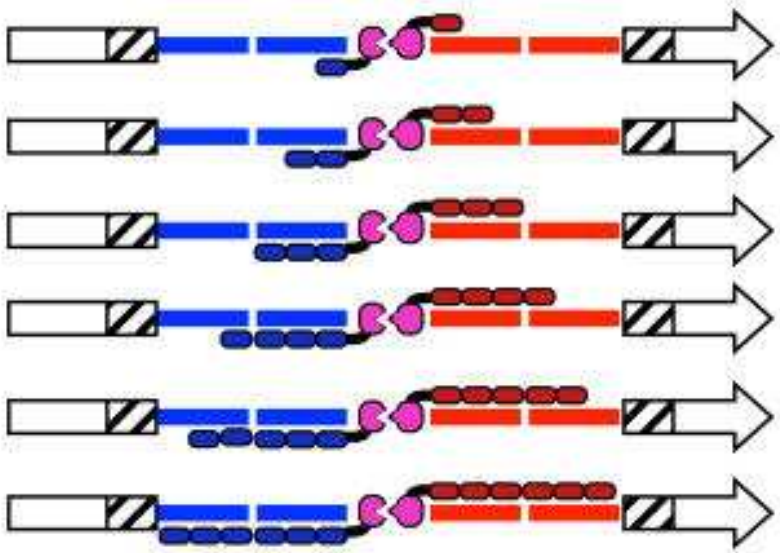
Zinc Fingers pairs

(cto) plasmid encoding a non-functional nuclease

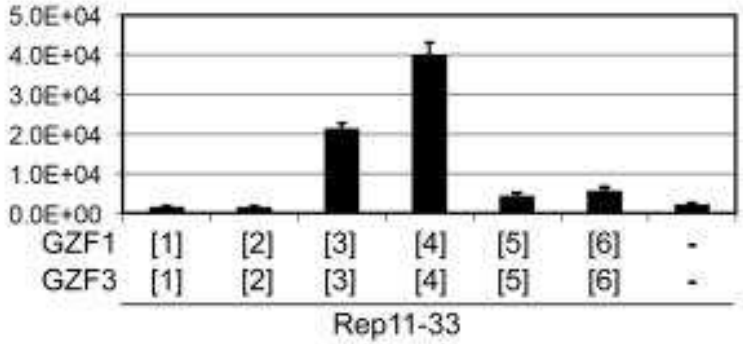
Binding specificity of ZF



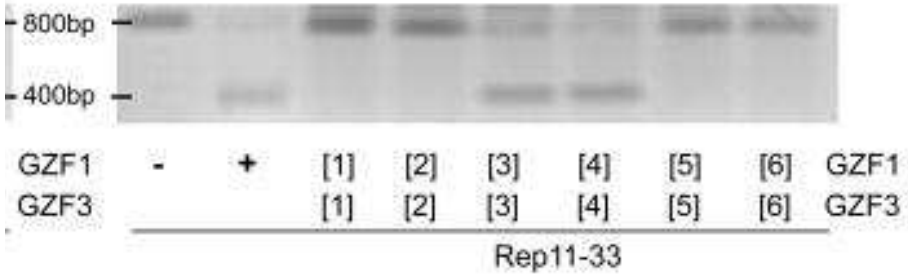
“target site composition” and ZFN activity



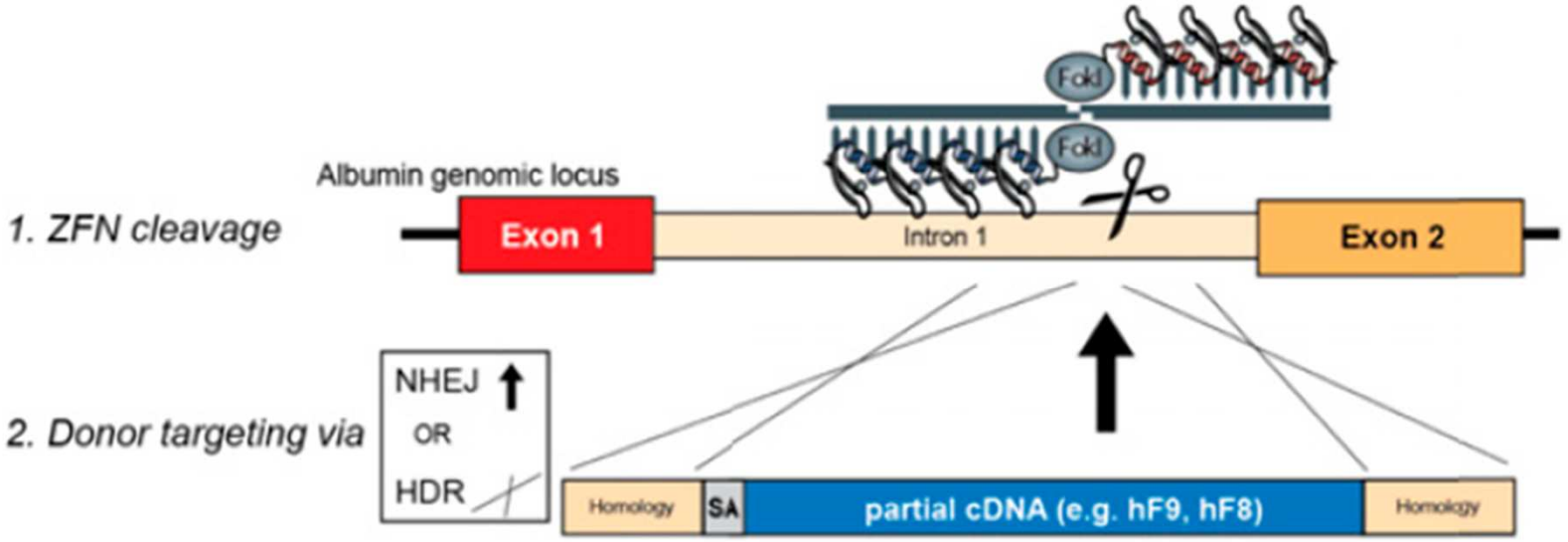
luciferase activity



In vitro cleavage activity

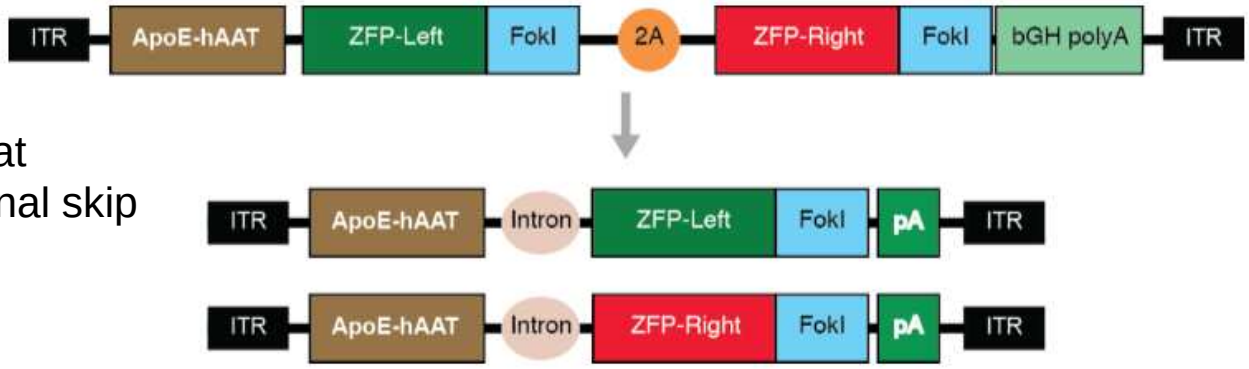


Targeting albumin supports production of therapeutic levels of FVIII and functional correction of hemophilia A phenotype.



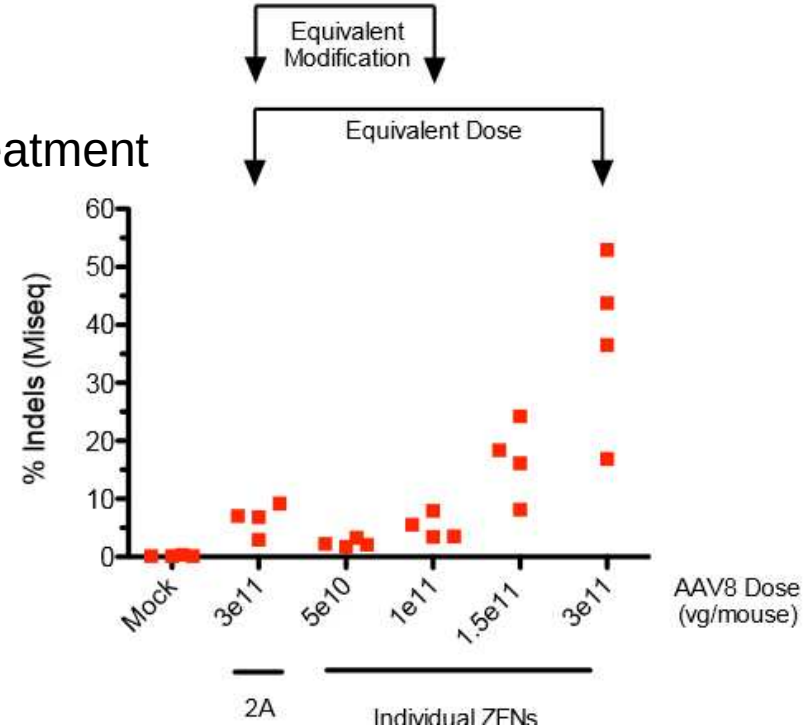
Sharma et al.
Blood 2015

ZFN vectors and Indels at ZFN target

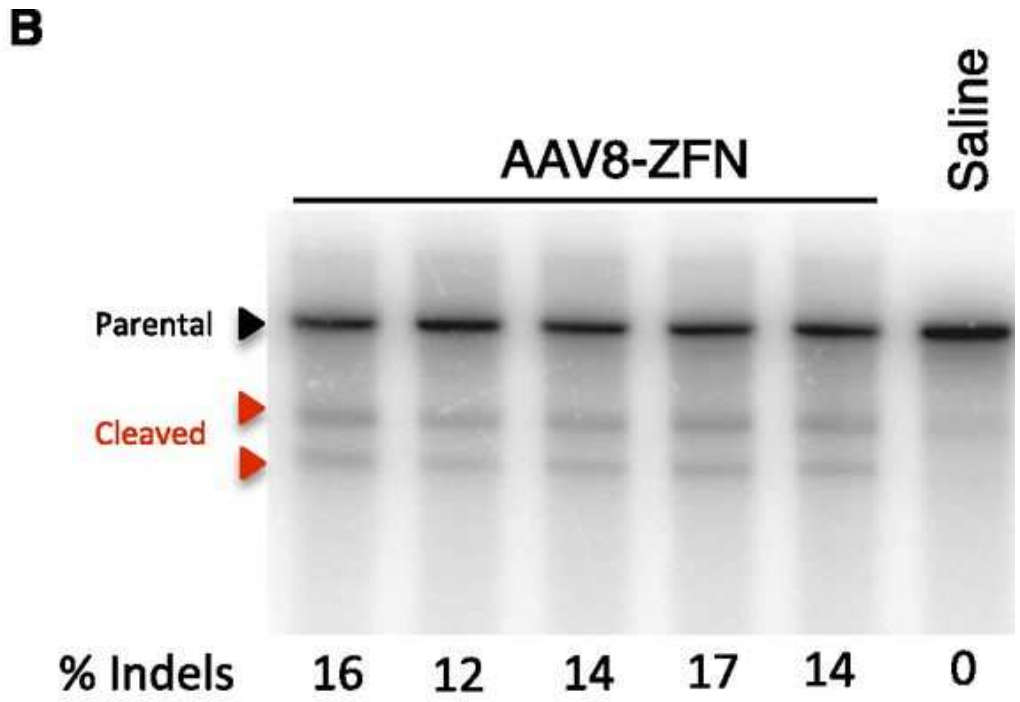


2A short peptide that mediates translational skip

week 2 post treatment



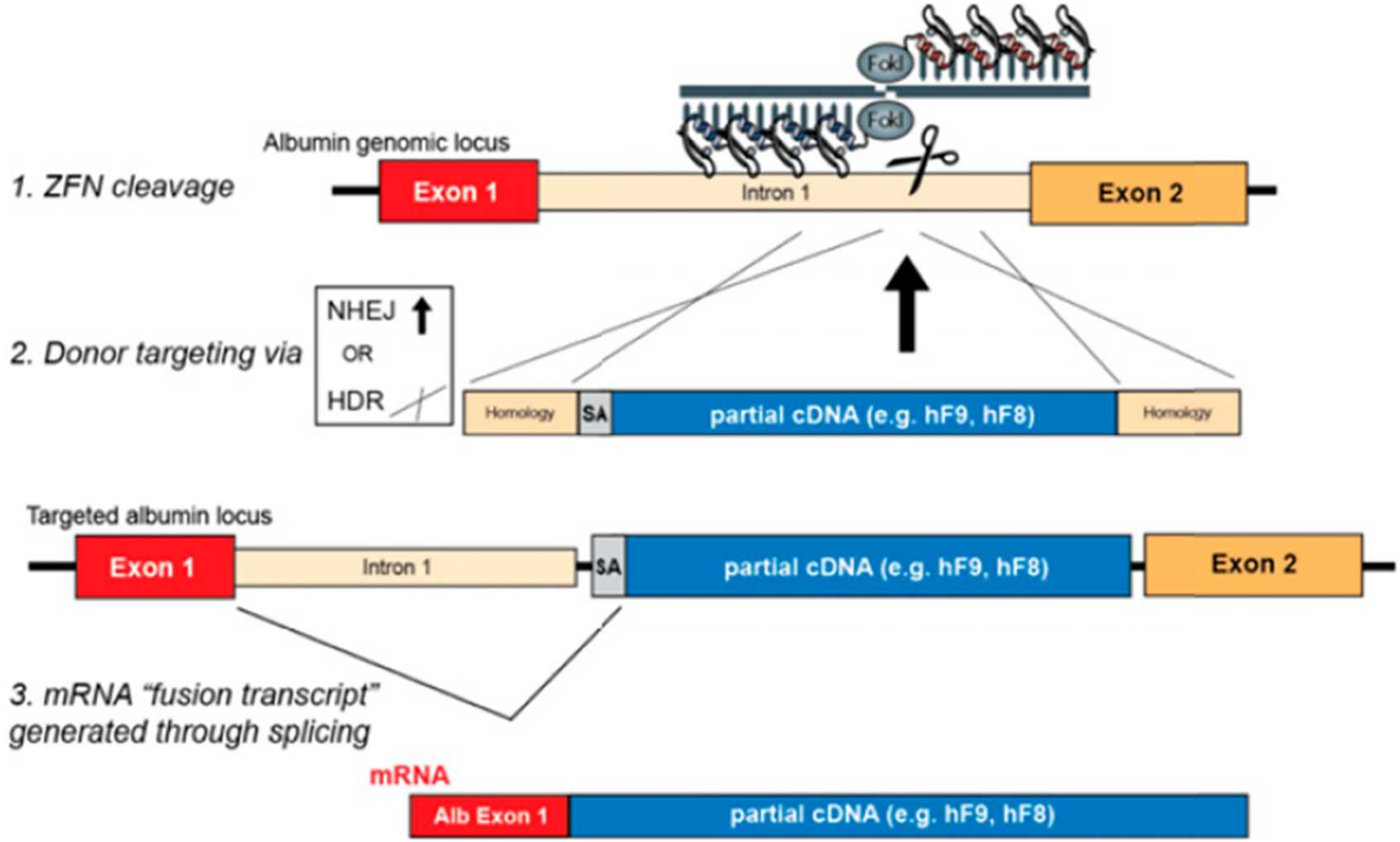
albumin gene (intron 1) targeting strategy



Cel I nuclease assay from liver DNA measuring ZFN-induced indels within albumin intron 1.
Lanes represent individual mice at day 7 after AAV8-ZFN treatment.

Rajiv Sharma et al. Blood 2015;126:1777-1784

Targeting albumin supports production of therapeutic levels of FVIII and functional correction of hemophilia A phenotype.

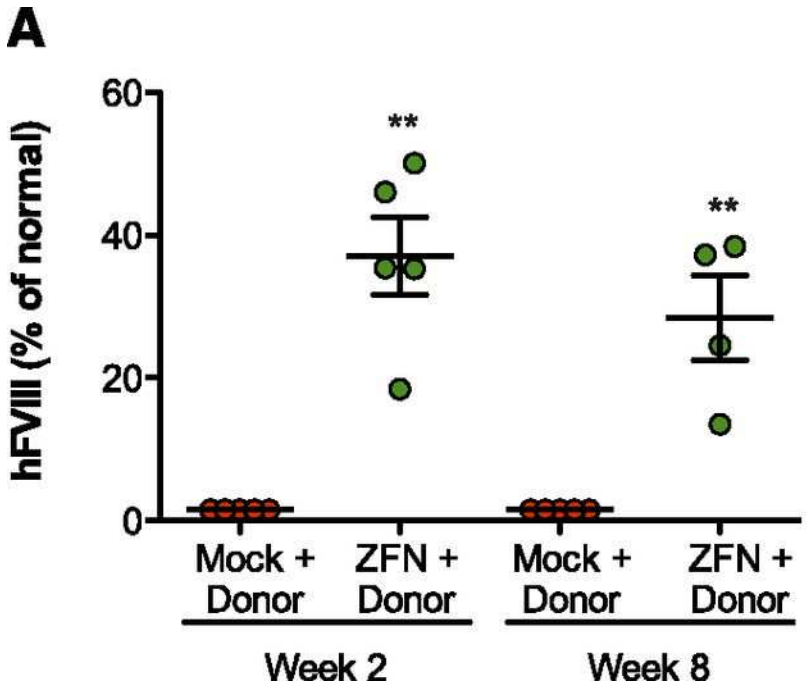


Sharma et al.
Blood 2015

Targeting albumin supports production of therapeutic levels of FVIII and functional correction of hemophilia A phenotype.

Rajiv Sharma et al. Blood 2015;126:1777-1784

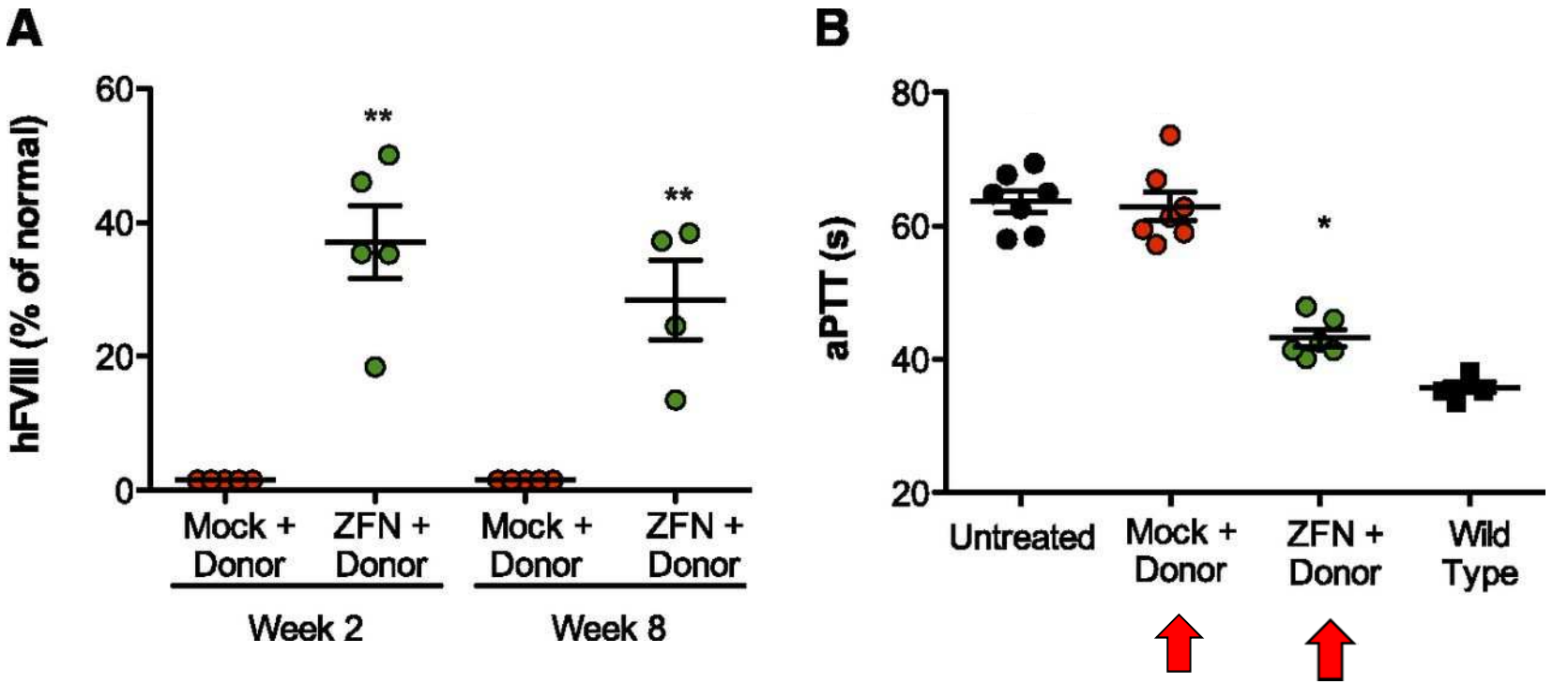
blood



Targeting albumin supports production of therapeutic levels of FVIII and functional correction of hemophilia A phenotype.

Rajiv Sharma et al. Blood 2015;126:1777-1784

blood



TRIAL CLINICI

Company	Transgene	Vector	
Sangamo Bioscience (SB-FIX)	Codon optimized FIX	AAV6/Zinc-finger–mediated targeted integration into the albumin locus in hepatocytes	Study has US Food and Drug Administration approval

Future Directions

1. Design ZFNs to other target genes.
2. Develop efficient method to make specific ZFNs that recognize a broad range of sequences.
3. Refine ZFNs for use in primary cells, including stem cells.
4. Assess possible induction of genomic rearrangements by ZFNs.
    I. Eliminate
5. Develop as a therapeutic tool.