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

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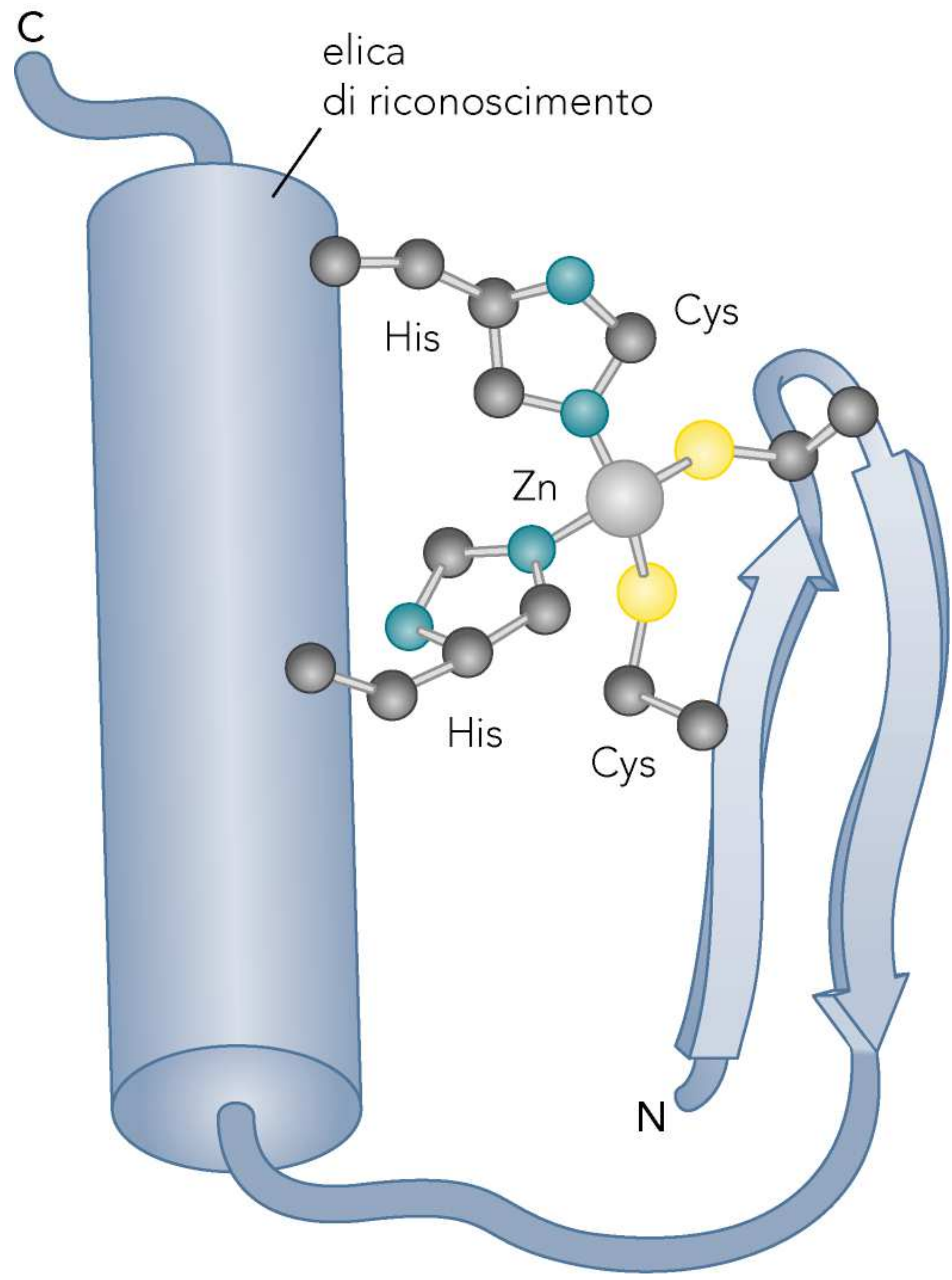
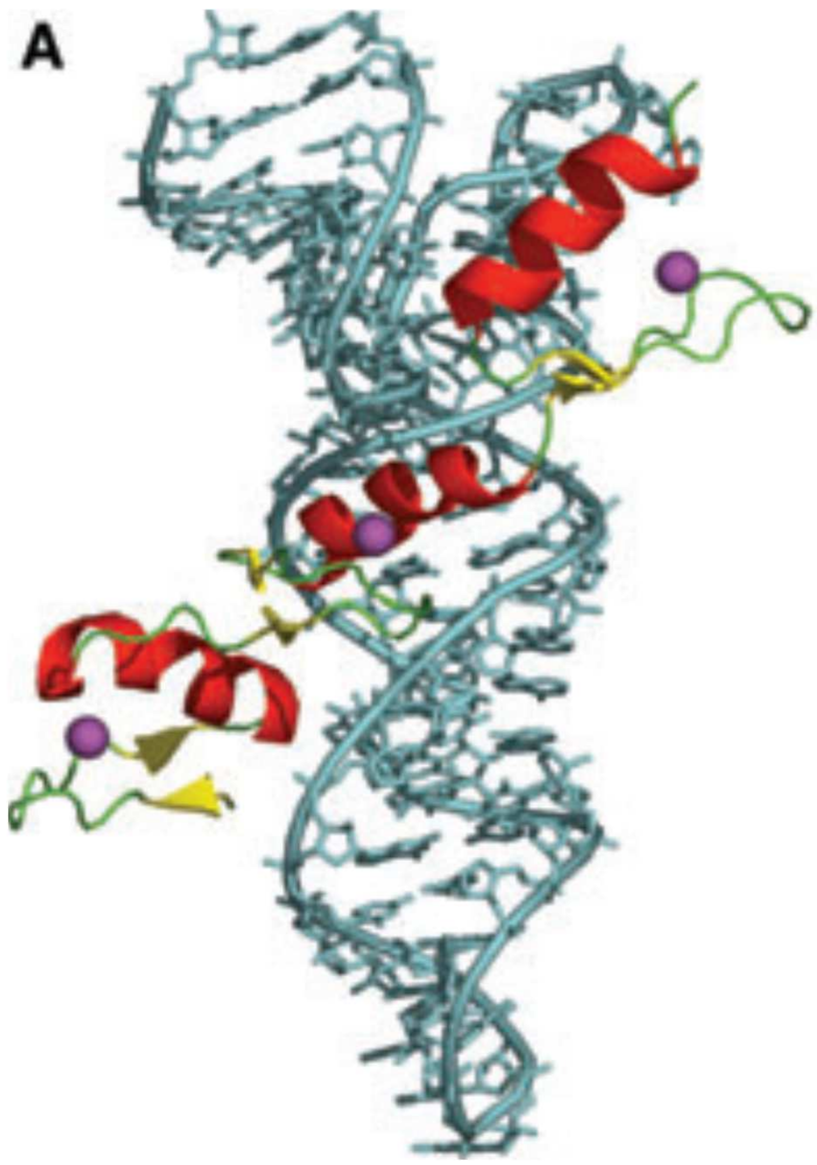
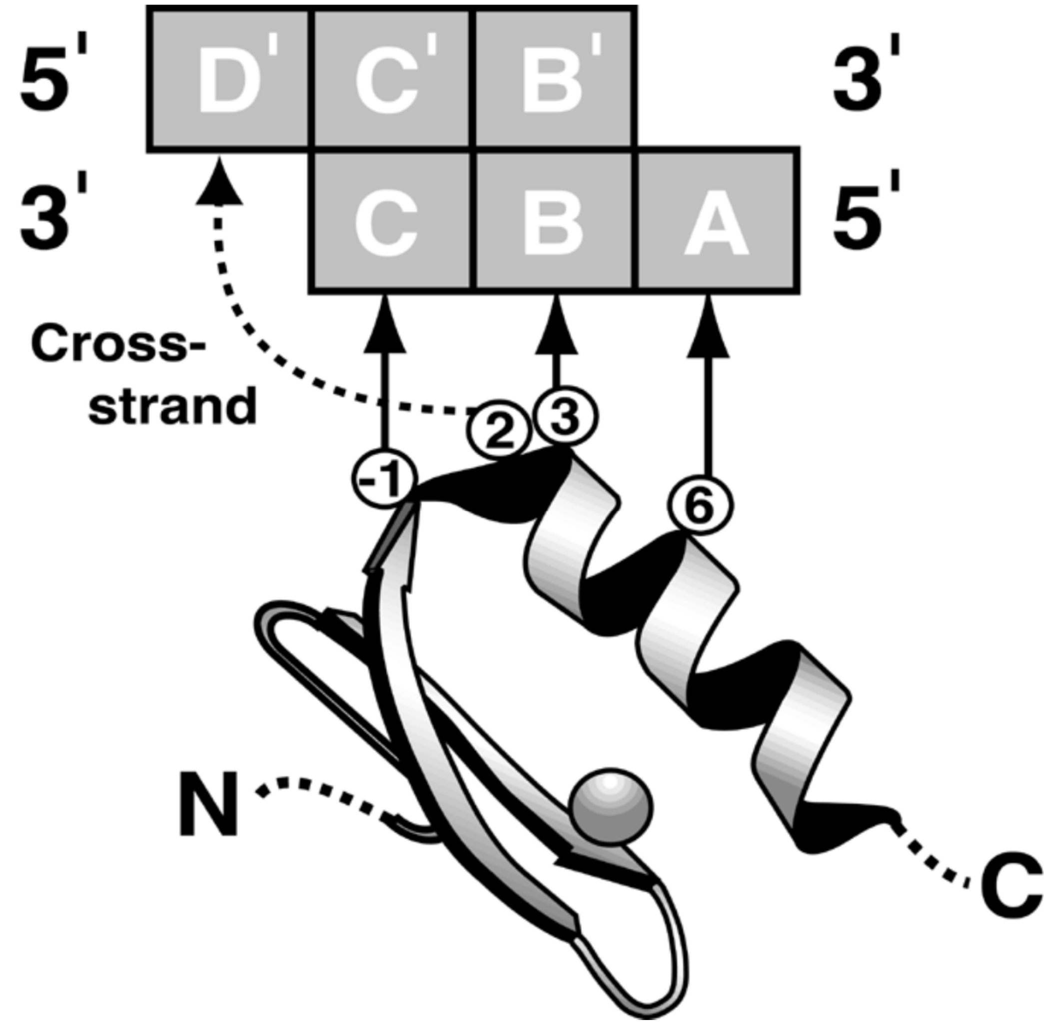
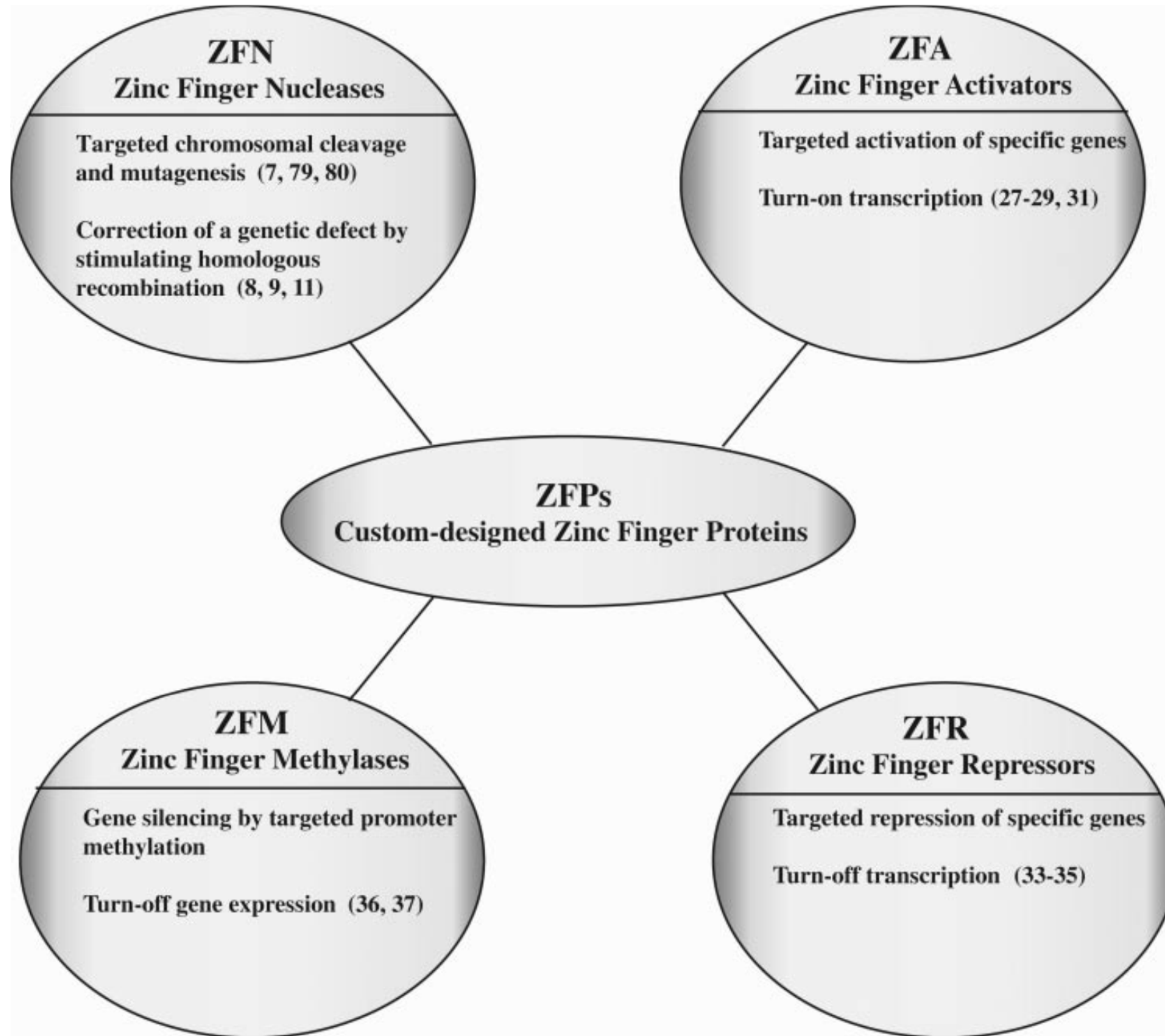
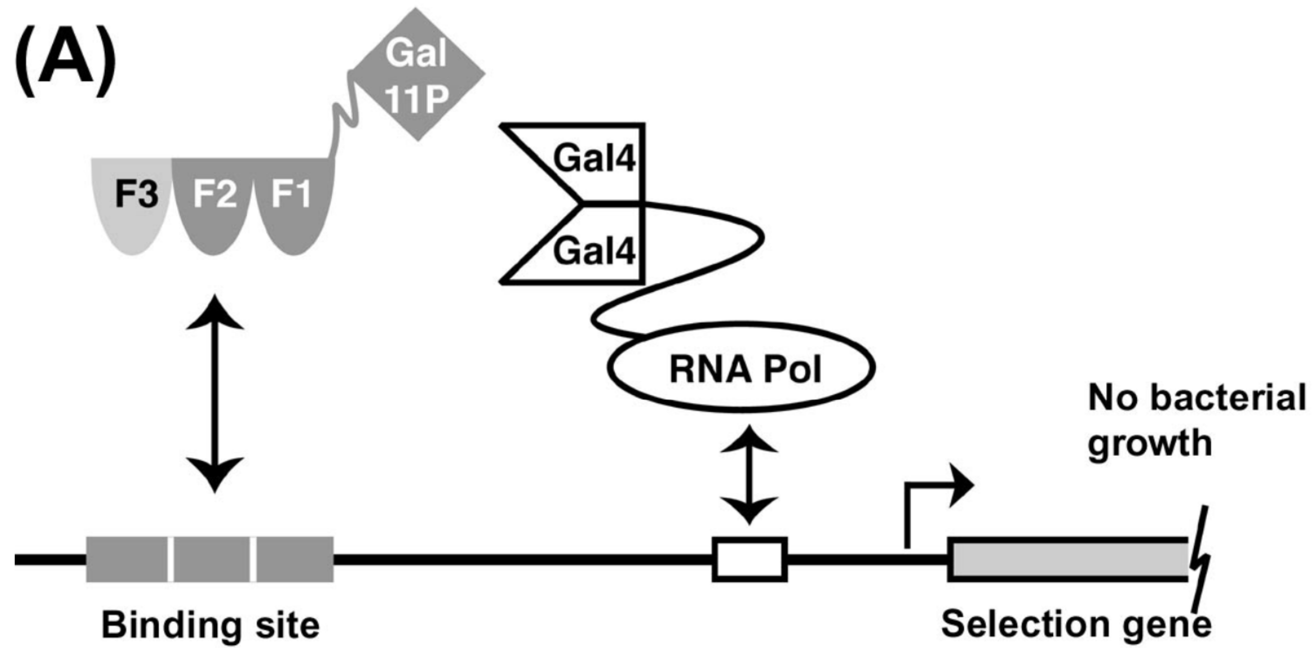

C
elica
di riconoscimento
His
Cys
Zn
His
Cys
N

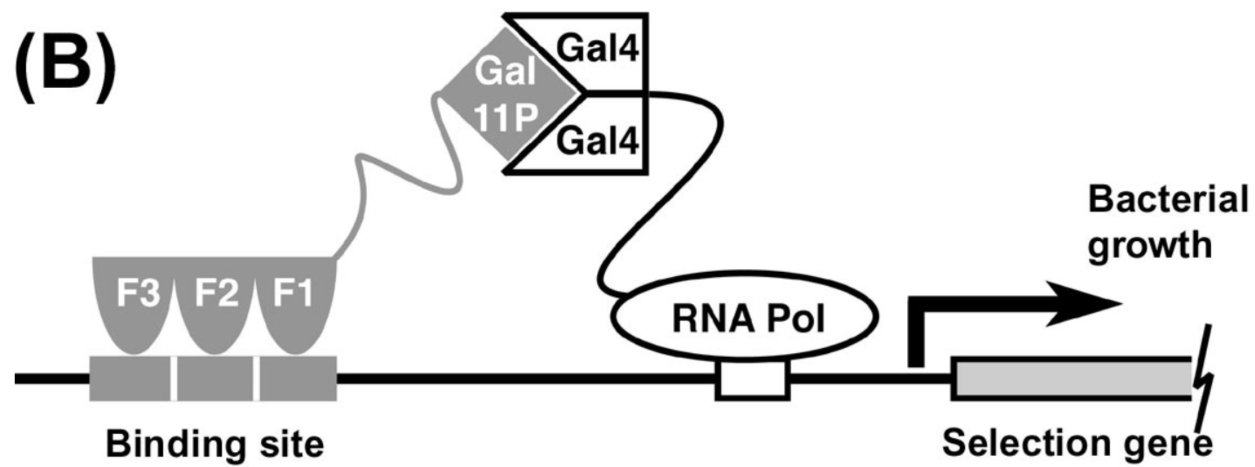

A


5'
D'
C'
B'
3'
3'
C
B
A
5'
Cross-
strand
-1
2
3
6
N
C


ZFN
Zinc Finger Nucleases
Targeted chromosomal cleavage and mutagenesis (7, 79, 80)
Correction of a genetic defect by stimulating homologous recombination (8, 9, 11)
ZFA
Zinc Finger Activators
Targeted activation of specific genes
Turn-on transcription (27-29, 31)
ZFPs
Custom-designed Zinc Finger Proteins
ZFM
Zinc Finger Methylases
Gene silencing by targeted promoter methylation
Turn-off gene expression (36, 37)
ZFR
Zinc Finger Repressors
Targeted repression of specific genes
Turn-off transcription (33-35)

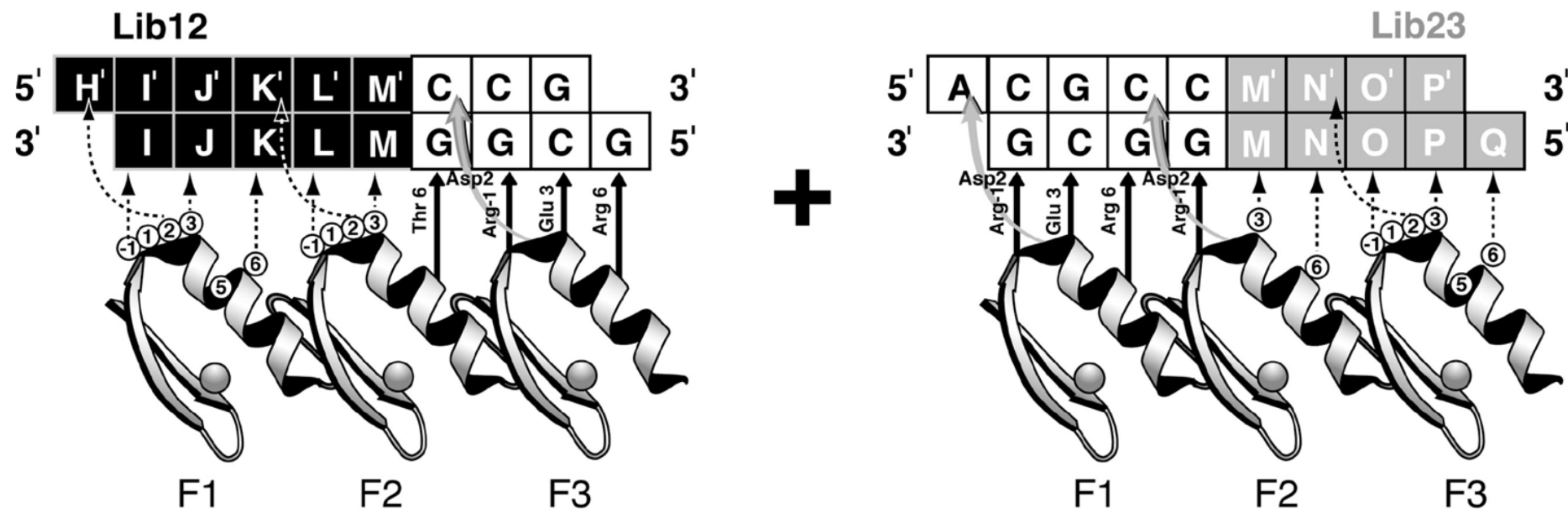
- Two approaches were originally used for zinc finger protein (ZFP) engineering in order to expand the DNA recognition code and to create zinc fingers that bind desired base triplets
- a combinatorial approach using libraries of zinc fingers displayed on the surface of filamentous phage that were selected against target DNA sequences
- a rational design approach that used databases to predict rules for amino acid–base interactions.


(A)
Gal
11P
Gal4
Gal4
F3
F2
F1
RNA Pol
No bacterial
growth
Binding site
Selection gene

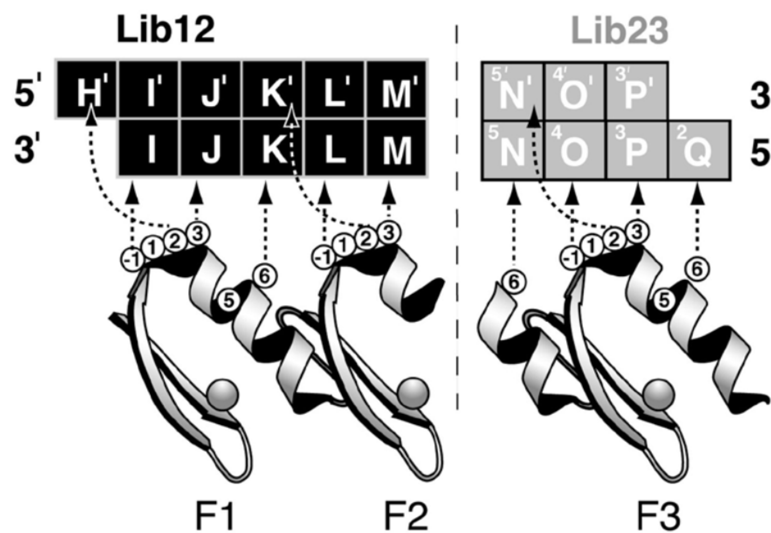

(B)
Gal
11P
Gal4
Gal4
Bacterial
growth
F3
F2
F1
RNA Pol
Binding site
Selection gene

A simple representation of the bacterial two-hybrid system for selecting zinc finger variants from large randomised libraries. In the example described, finger three (F3) of the three-finger protein is randomised at positions –1, 1, 2, 3, 5 and 6.

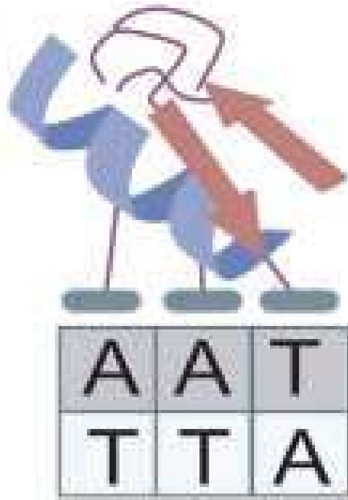
- In (A), F3 is unable to bind the DNA subsite, the binding of RNA polymerase to its promoter is not stabilised and insufficient selection marker is produced to allow bacterial growth on selective media.
- In (B), bacteria grow on the selective media when F3 binds tightly to the DNA subsite causing RNA polymerase to be recruited to theDNA complex and the selection marker is expressed



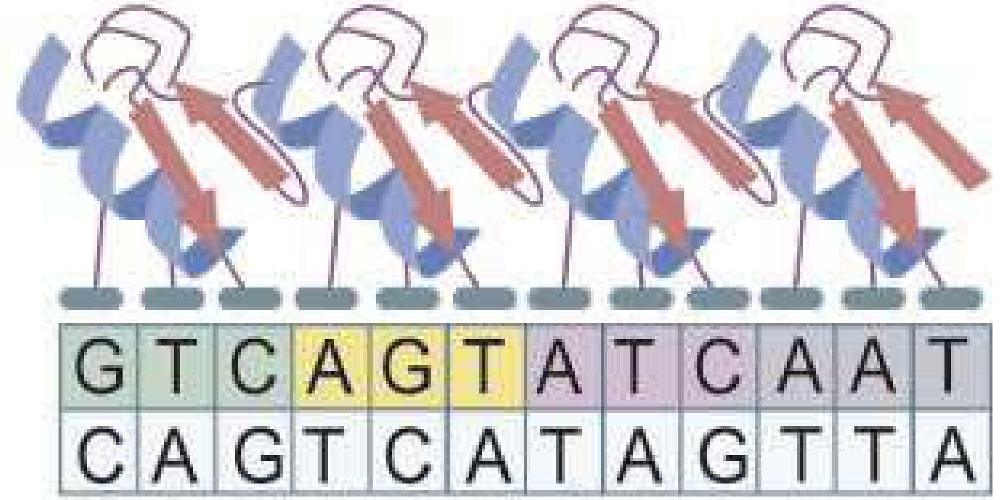
Recombine



A



B



C

ZF3 ZF2 ZF1

-1 1 2 3 5 6 | -1 1 2 3 5 6 | -1 1 2 3 5 6

R S D E T R | R S D H T T | R S D E K R

3' — T | G C G | G G T | G C G | — 5'

5' — A | C G C | C C A | C G C | — 3'

D

ZF3 ZF2 ZF1

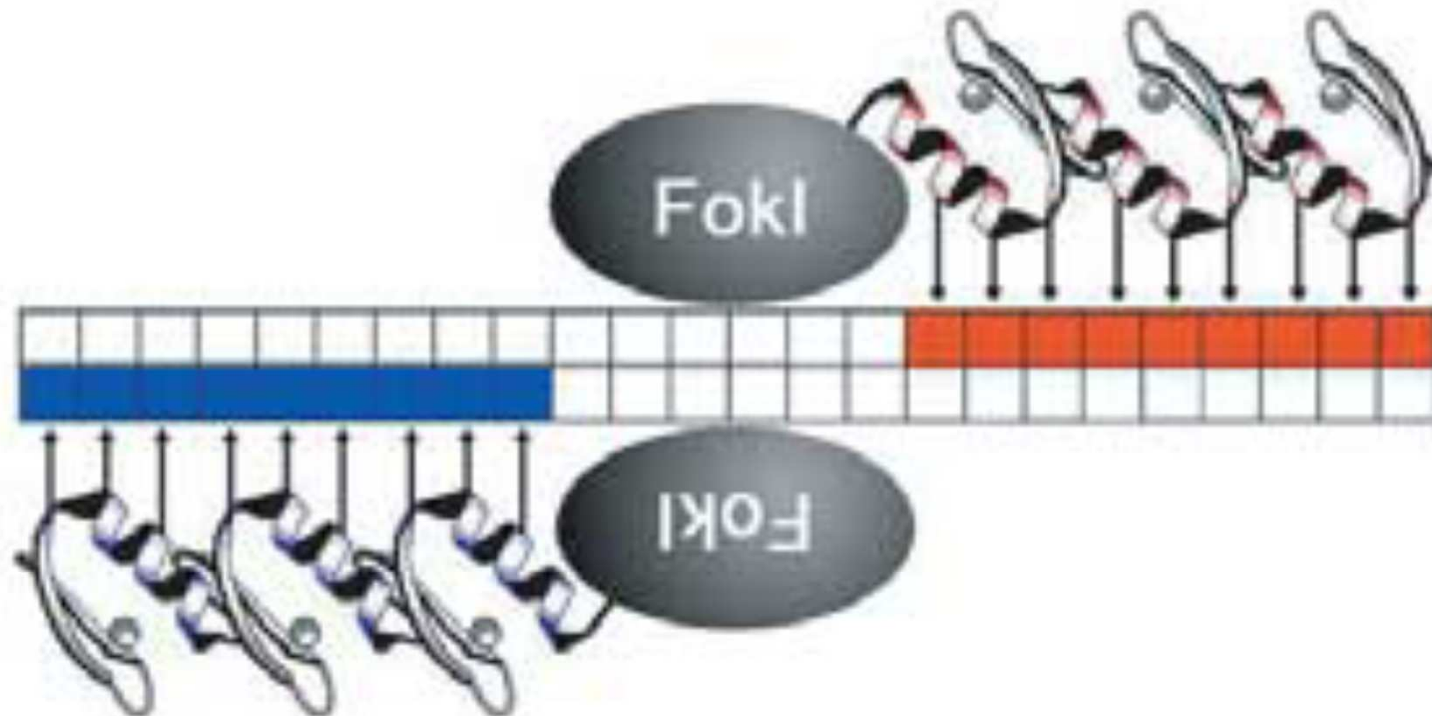
-1 1 2 3 5 6 | -1 1 2 3 5 6 | -1 1 2 3 5 6

R S D E T R | R S D H T T | R S D E K R

3' — T | G **N** G | G G T | G **N** G | — 5'

5' — A | C **N** C | C C A | C **N** C | — 3'

where N = G, A or T



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

A

7844

ACGGAGACCTTCCCTGGCCAATGCTCAGGTACTGG
TGCCTCTGGAAGGGACCGGTTACGAGTCCATGACC

7843

B

	GGA	AGG	TCT	CCG	
7843	QSGHLSR	RSDHLSA	NNRDRTK	RSDTLSE	
	AAT	GCT	CAG	GTA	CTG
7844	TSSNRKT	QSSDLSR	RSDNLRE	QSGALAR	

- Section of the DHFR gene targeted by ZFNs. TheDNAsequence of the primary binding site for each ZFN is boxed.
- ZFN7843 and ZFN9461 bind the same 12-bp site. ZFN7844 binds the 12-bp site AATGCTCAGGTA, whereas ZFN9684 binds the 15-bp site AATGCTCAGGTACTG.
- (*B*) Recognition helix sequences of ZFNs. The sequence of the recognition helix from position 1 to 6 is listed below its target triplet.

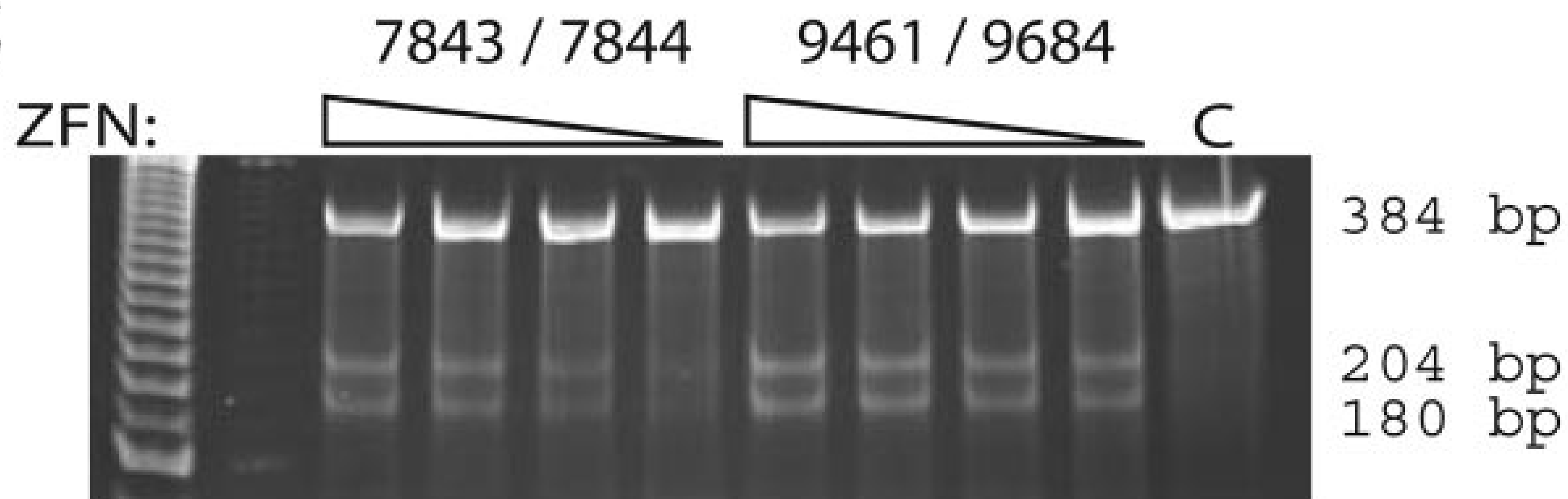
A

```
                         7844
ACGGAGACCTTCCCTGGCCAATGCTCAGGTACTGG
TGCCTCTGGAAGGGACCGGTTACGAGTCCATGACC
   7843
```

B

```
       GGA     AGG     TCT     CCG
7843   QSGHLSR RSDHLSA NNRDRTK RSDTLSE
9461*  QSGHLSR RSDHLSA NNRDRTK RSDTLSE

       AAT     GCT     CAG     GTA     CTG
7844   TSSNRKT QSSDLSR RSDNLRE QSGALAR
9684*  TSSNRKT QSSDLSR RSDNLRE TSSARTT RSDALSN
```


C
7843 / 7844
9461 / 9684
ZFN:
C
384 bp
204 bp
180 bp

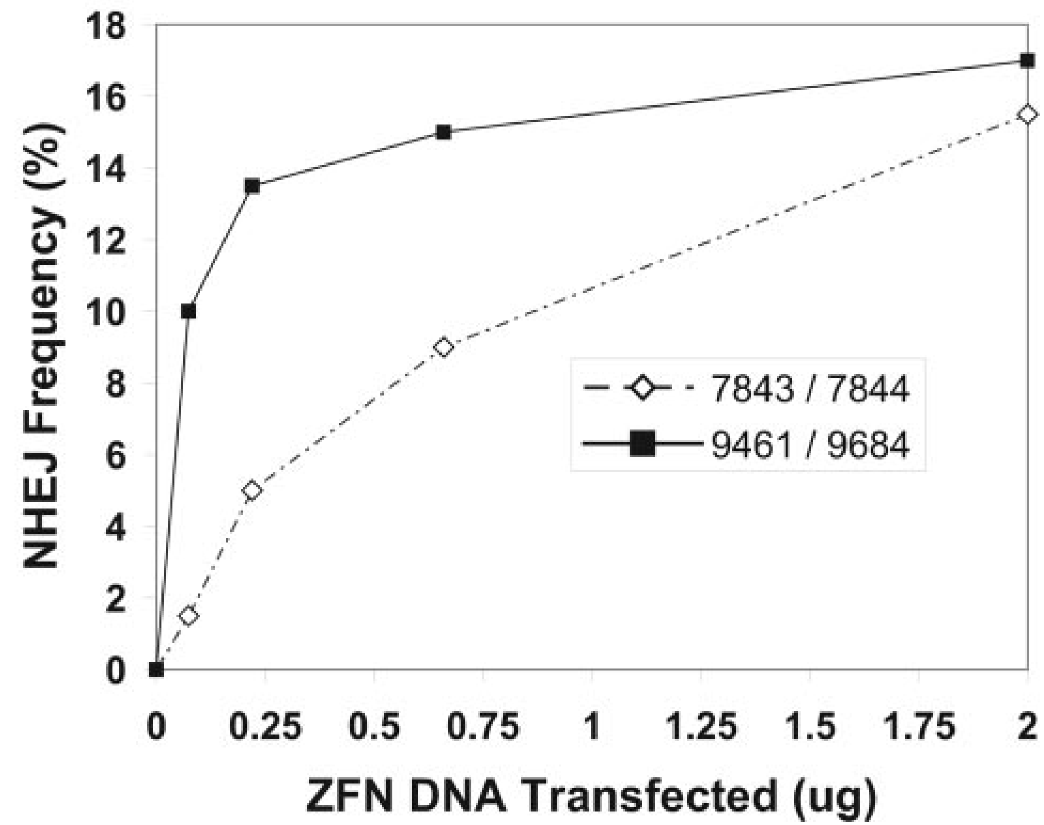
- Comparison of ZFN activity. Plasmids encoding each pair of ZFNs (ZFN7843/ZFN7844 and ZFN9461/ZFN9684) containing the ELKK FokI variants were delivered in the amounts shown to CHO-S cells in suspension culture. The frequency of allelic mutation in each pool of treated cells was determined by using the CEL-I assay (gel). Bands migrating at 384, 204, and 180 bp represent the parent amplicon and the two CEL-I digestion products, respectively.

CEL-I Nuclease Mismatch Assay

- .
- The frequency of targeted gene mutation in ZFN-treated pools of cells was determined by using the CEL-1 nuclease assay
- This assay detects alleles of the target locus that deviate from WT as a result of NHEJ-mediated imperfect repair of ZFN-induced DNA double strand breaks.
- PCR amplification of the targeted region from a pool of ZFN-treated cells generates a mixture of WT and mutant amplicons. Melting and annealing of this mixture results in mismatches forming between heteroduplexes of the WT and mutant alleles.
- A DNA “bubble” formed at the site of mismatch is cleaved by the surveyor nuclease CEL-I, and the cleavage products can be resolved by gel electrophoresis and quantitated by densitometry.

- The relative intensity of the cleavage products compared with the parental band is a measure of the level of CEL-I cleavage of the heteroduplex.
- This, in turn, reflects the frequency of ZFN-mediated cleavage of the endogenous target locus that has subsequently undergone imperfect repair by NHEJ.

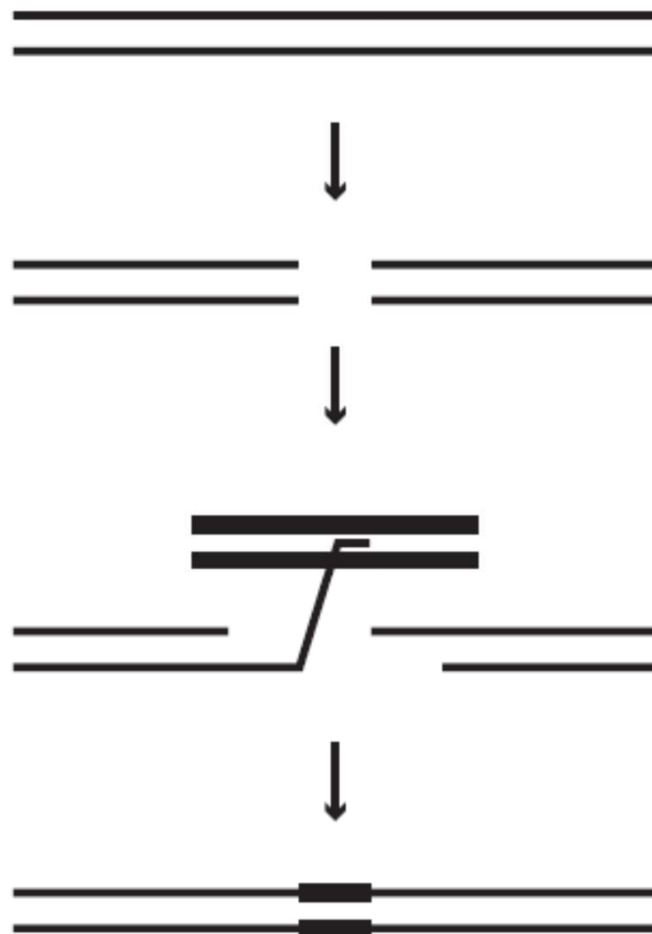
Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases



NHEJ Frequency (%)
18
16
14
12
10
8
6
4
2
0
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
ZFN DNA Transfected (ug)
7843 / 7844
9461 / 9684

Homology-directed repair:

ZFN-driven homology-directed repair:

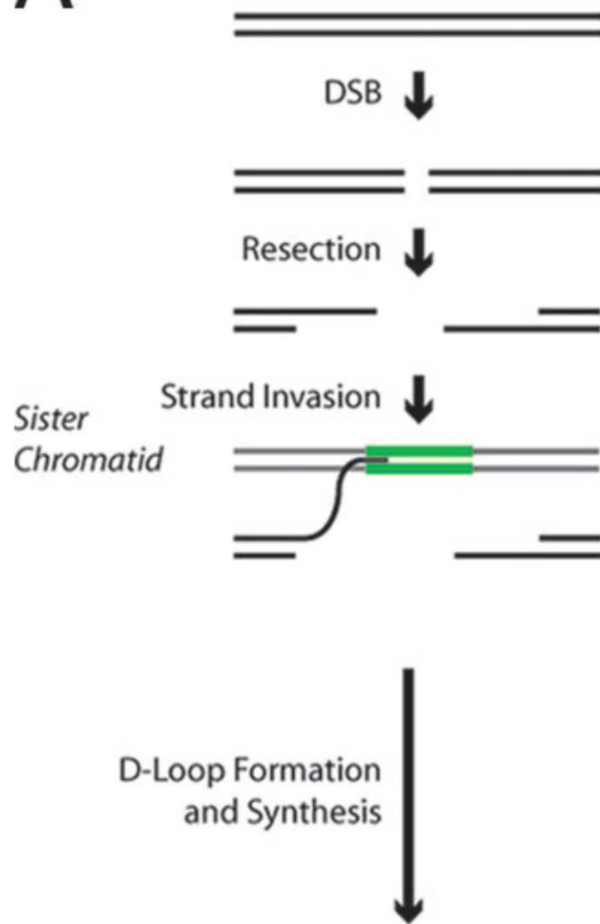


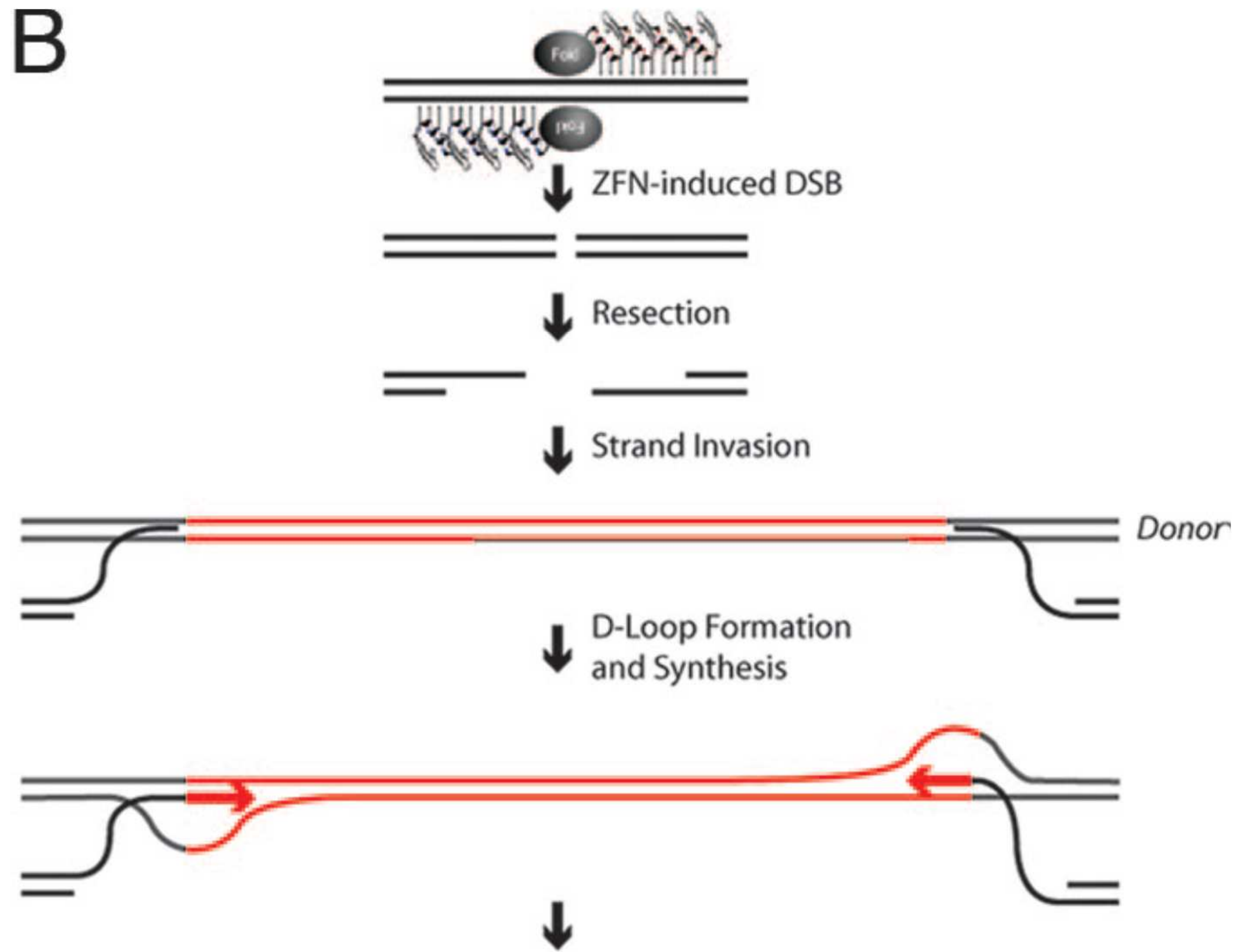
X-ray-induced DSB

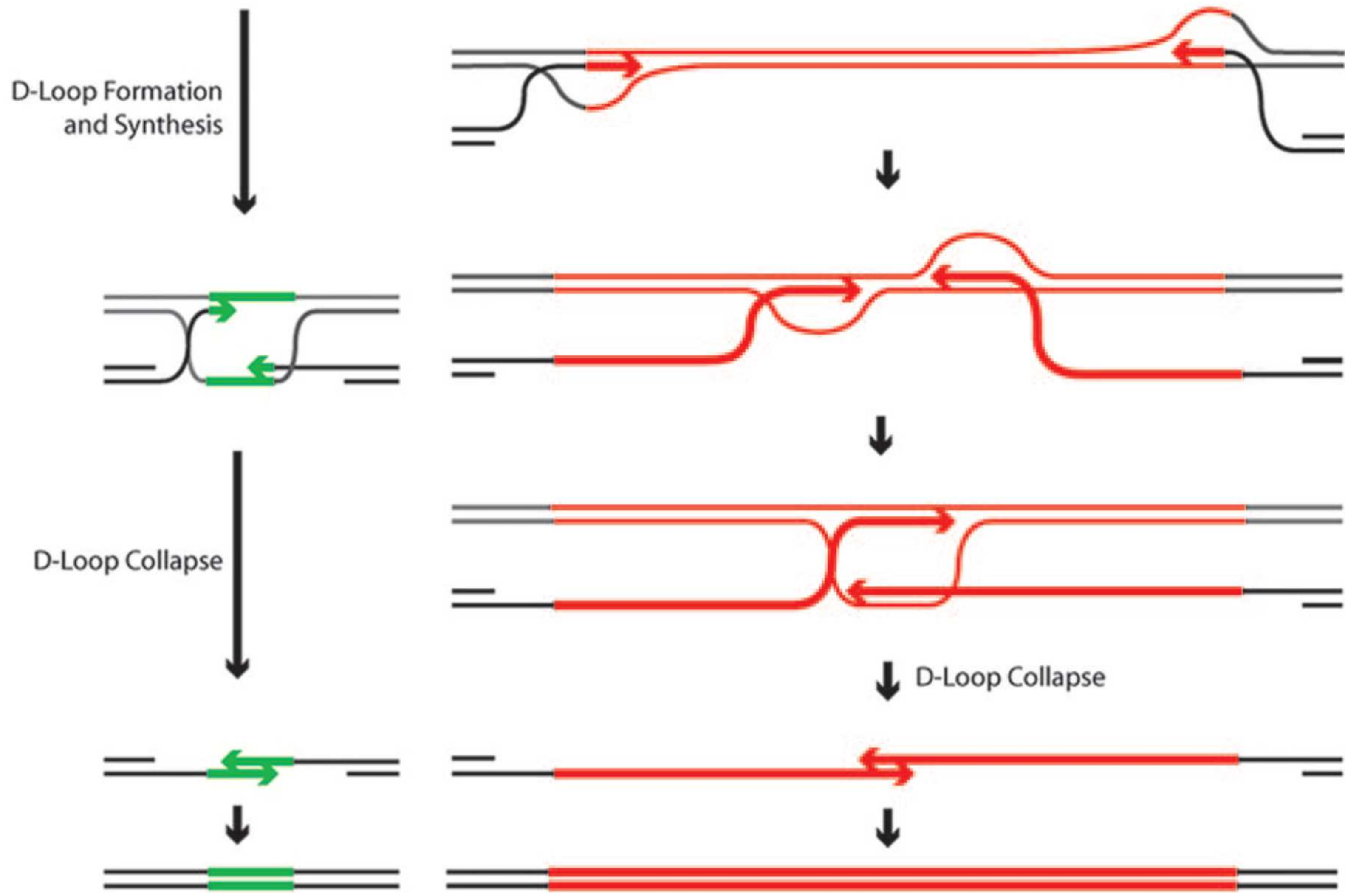
ZFN-induced DSB

Sister chromatid

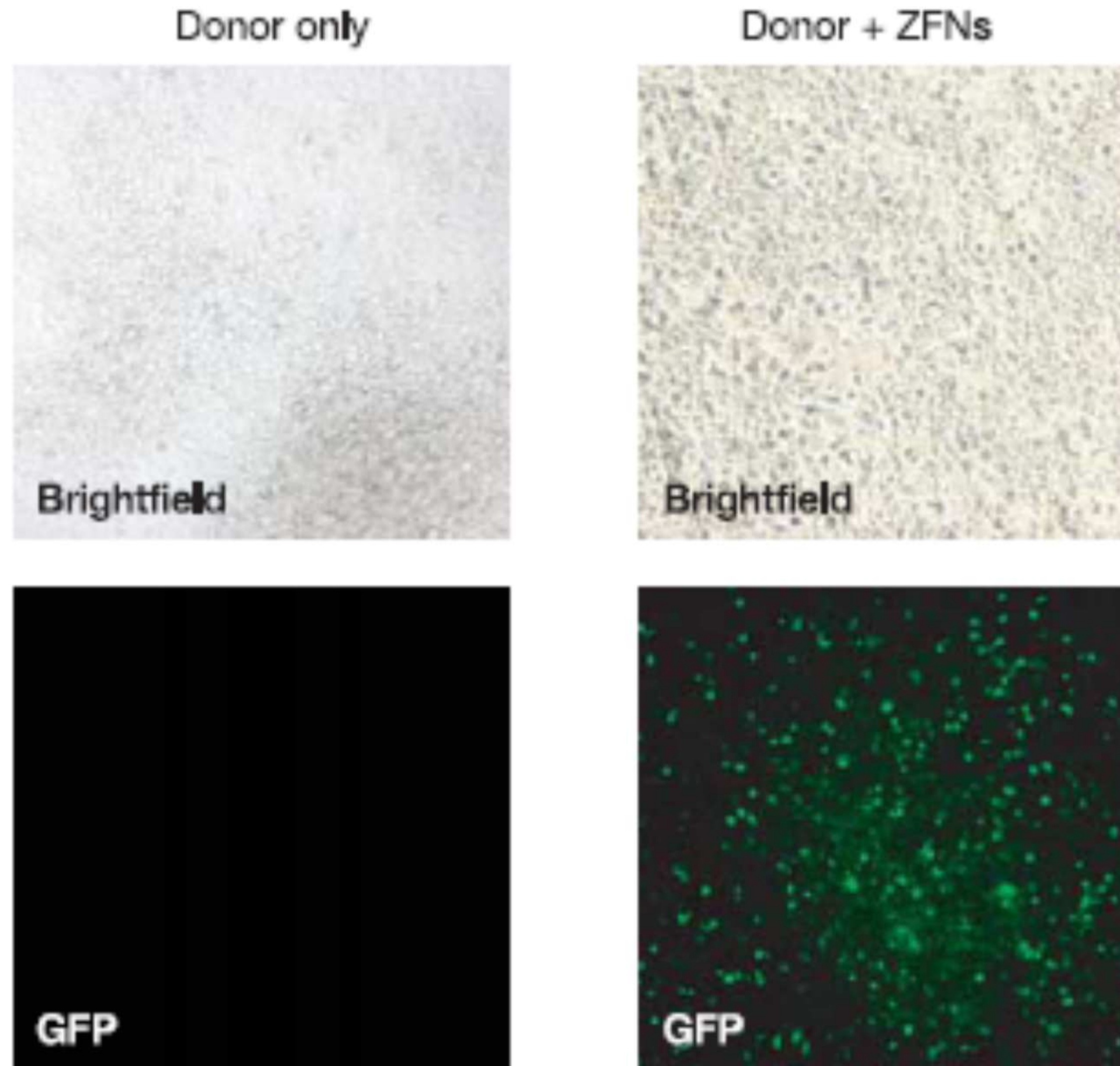
Donor DNA (plasmid)

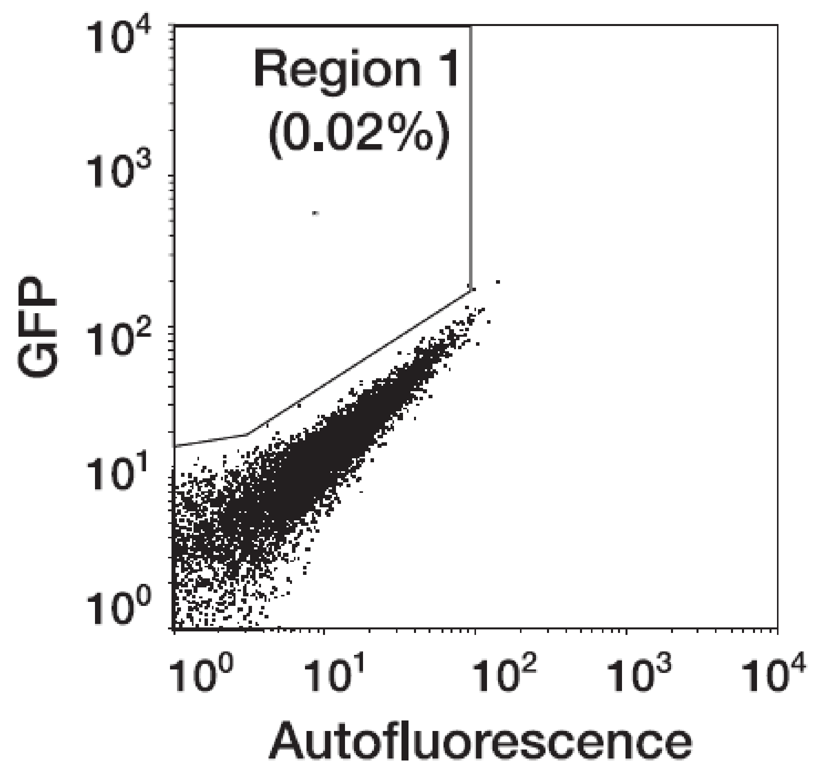

A
DSB
Resection
Strand Invasion
Sister
Chromatid
D-Loop Formation
and Synthesis

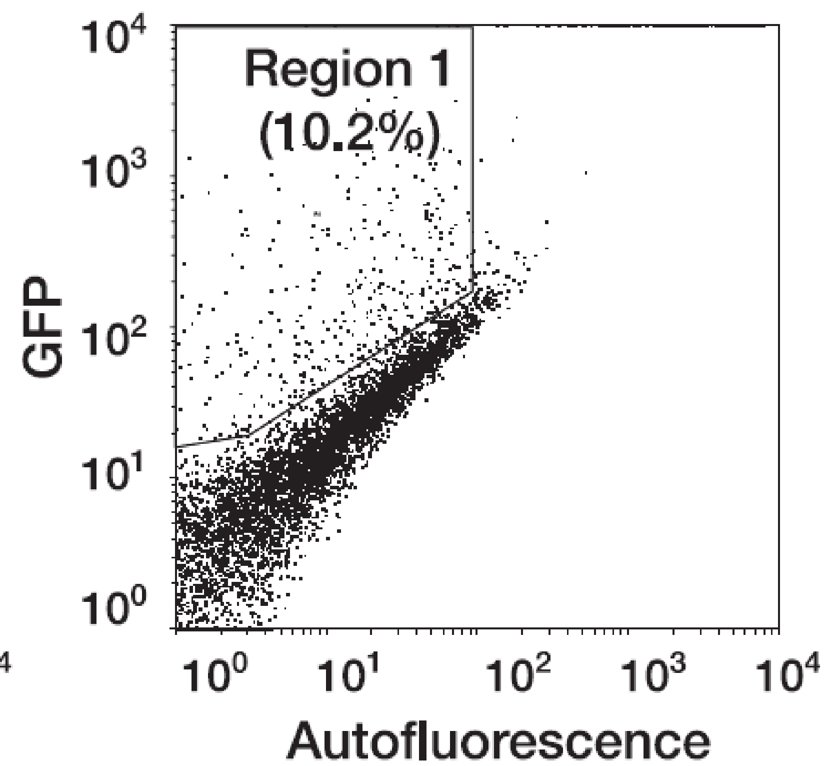

B
ZFN-induced DSB
Resection
Strand Invasion
Donor
D-Loop Formation
and Synthesis

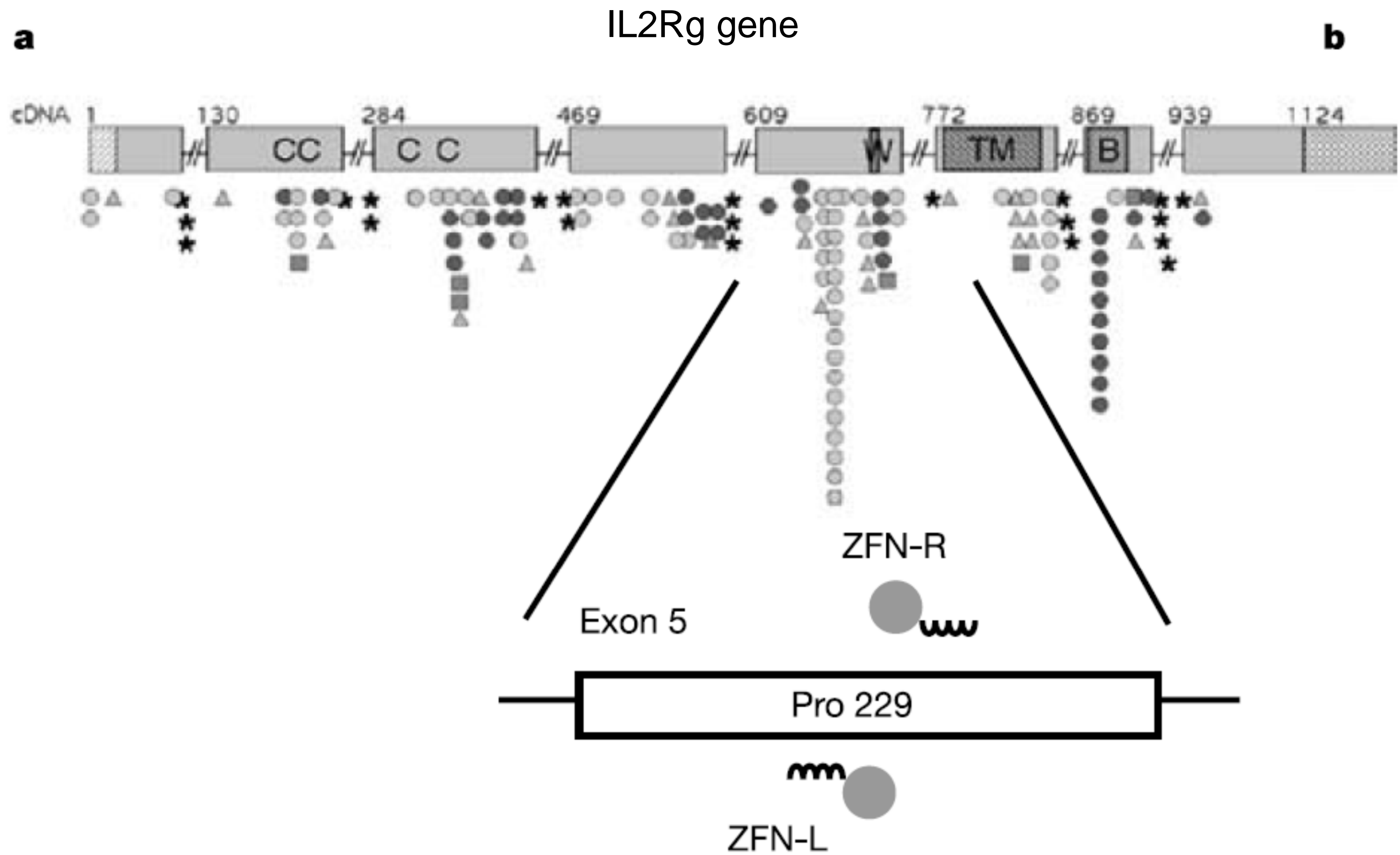

D-Loop Formation
and Synthesis
D-Loop Collapse
D-Loop Collapse

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%)
GFP
10^0
10^1
10^2
10^3
10^4
Autofluorescence


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



X-linked severe combined immune deficiency (SCID)

PCR +Restriction

a

*Bsr*BI

Donor:

Forward primer

Target

γC exon 5

Reverse primer

b

ZFNs + donor

DNA: GFP Donor ZFNs Low High

4 days (PCR)

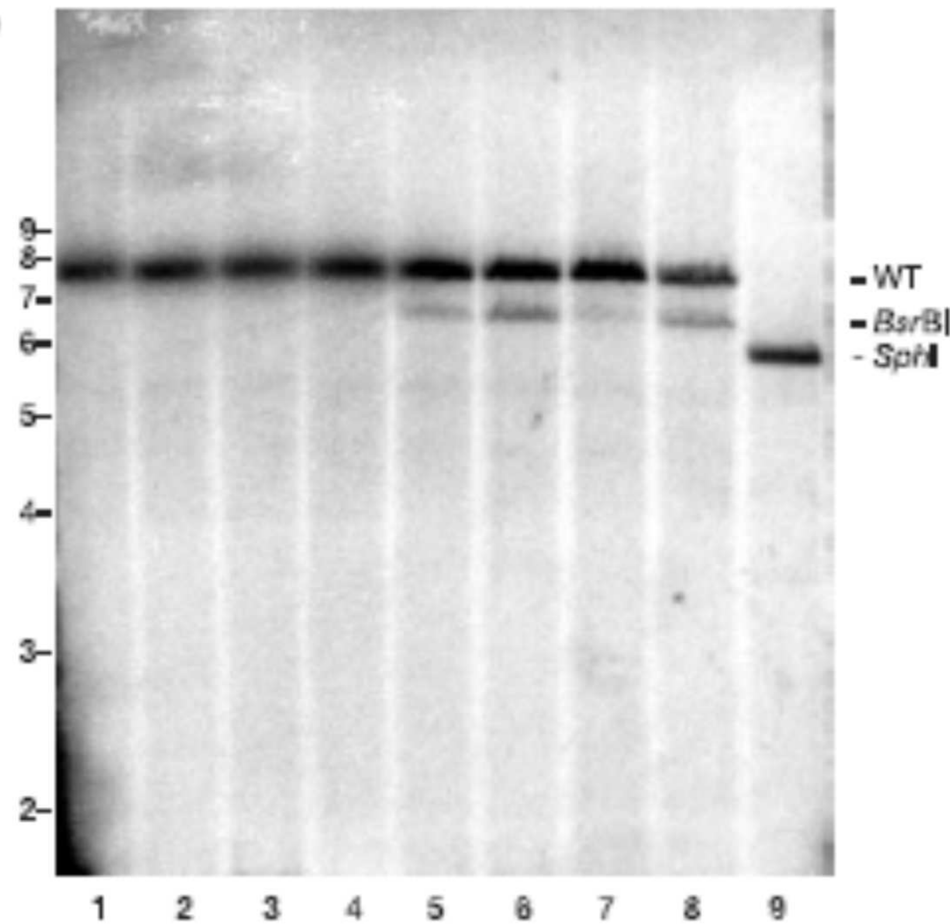
10.1 18.2 13.4 18.3 :%HDR

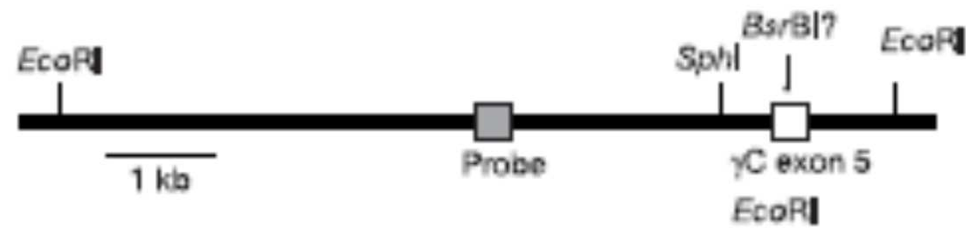
1.5
1.2
0.8
0.6

WT

*Bsr*BI

Southern blot

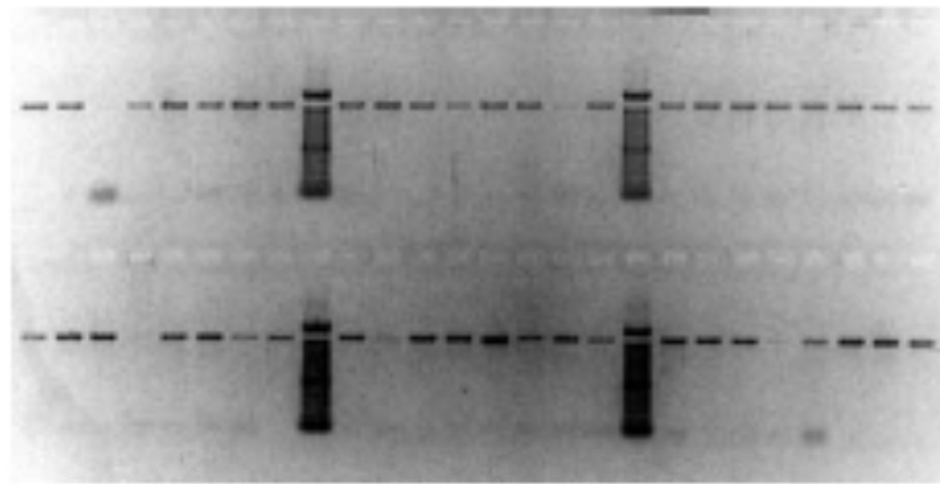




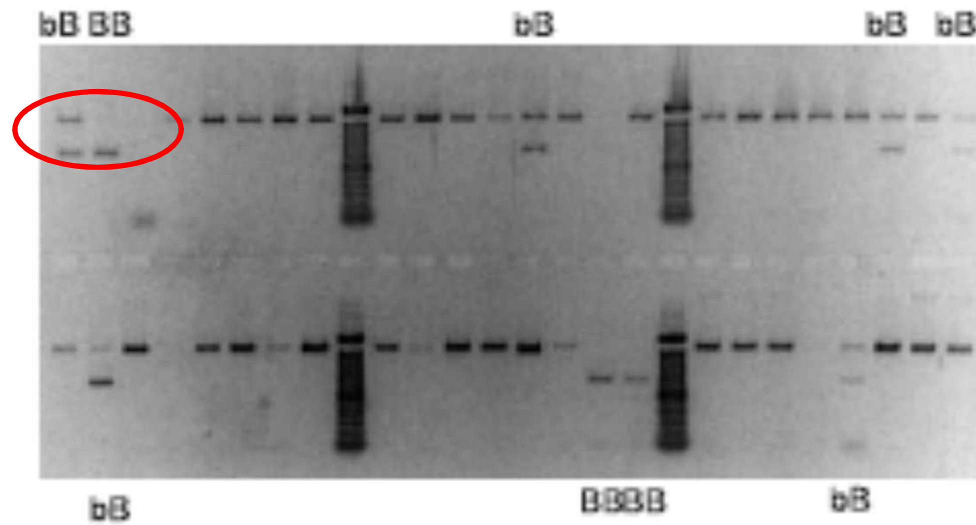
Day 1 : Transfection

Day 4 : Seed <1 cell per well

Day 30 : Isolate genomic DNA,
PCR γC exon 5 (both alleles),
digest with *BsrBI*, gel



+ *BsrBI*



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.

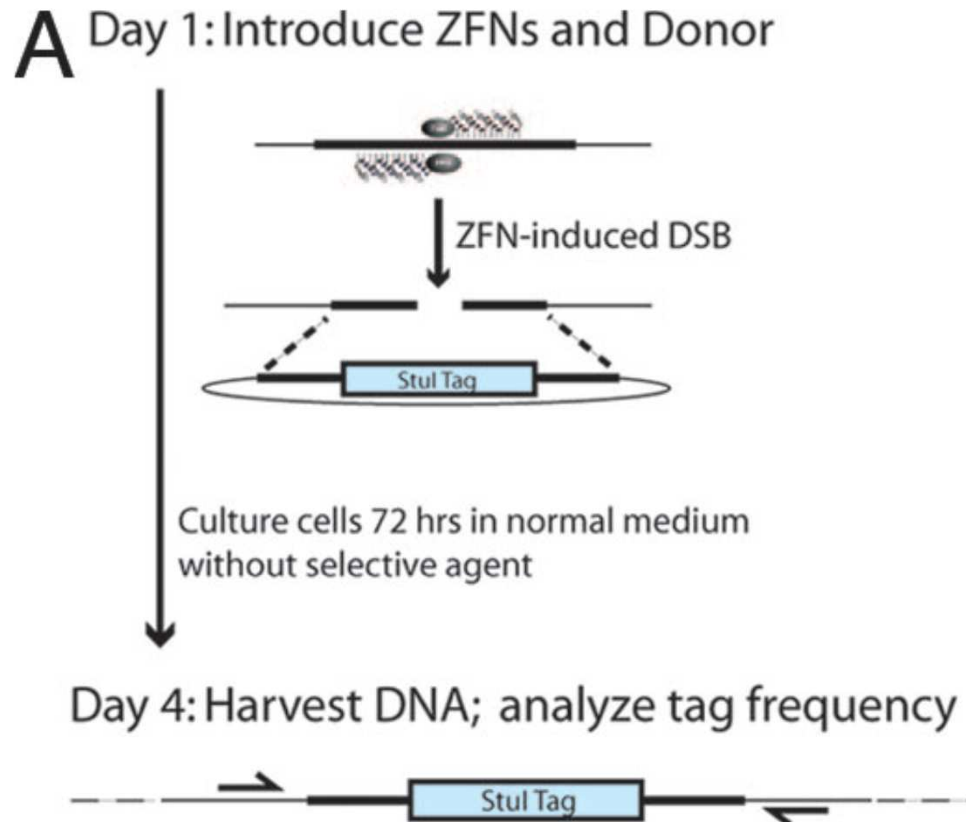
Surprising versatility of the specialized polymerase machinery involved in double-strand break repair

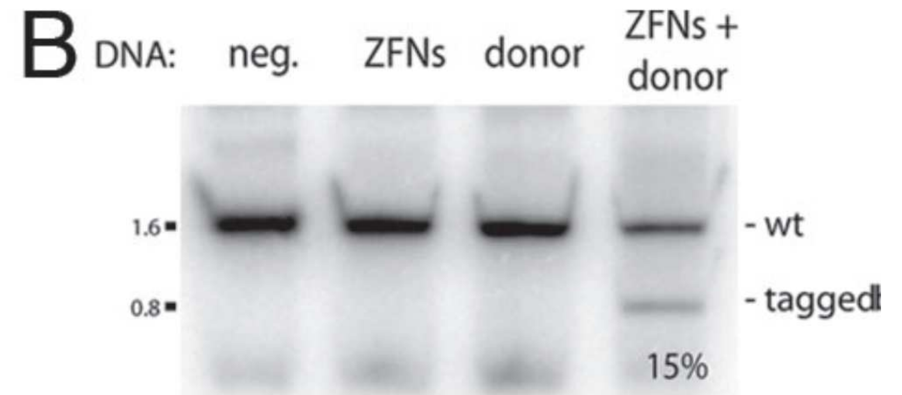
Powerful approach to mammalian cell engineering

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



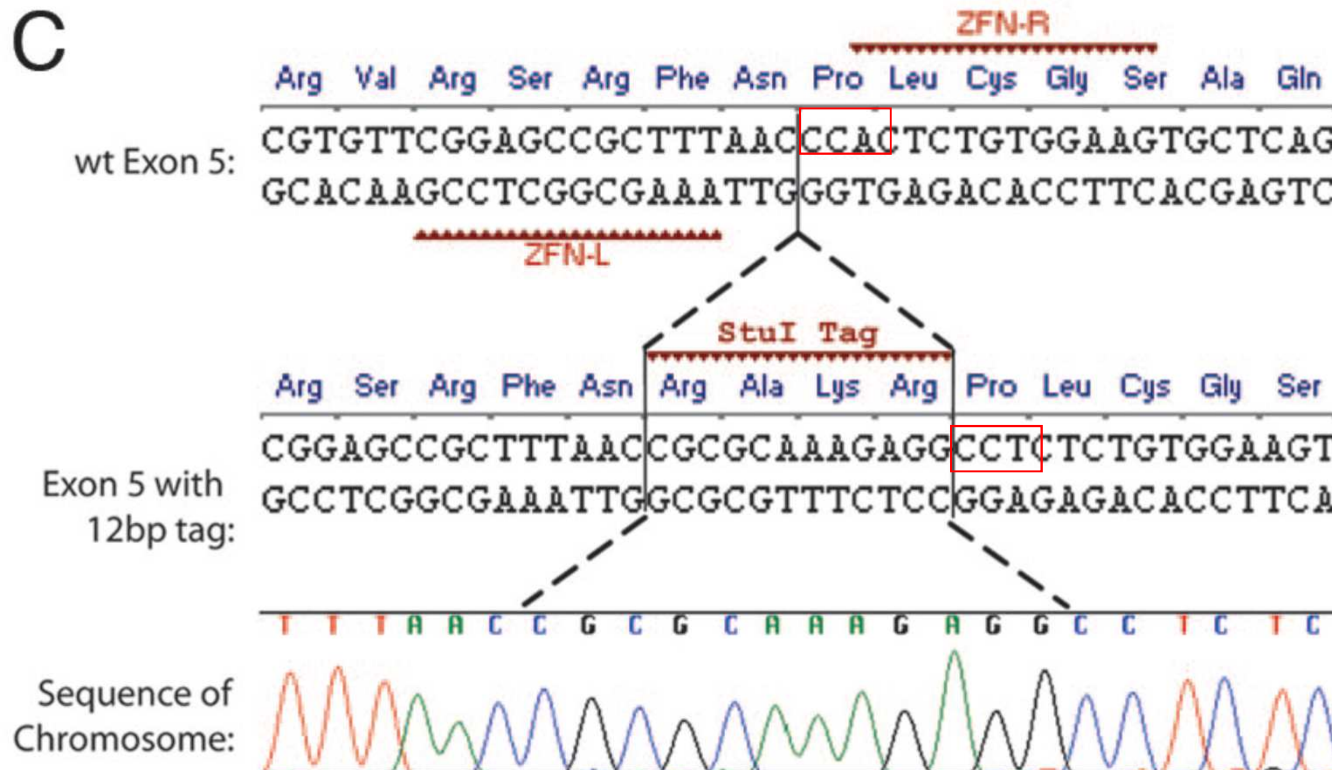


PCR products were digested with StuI

A ZFN-induced DSB leads to efficient, homology-based tag transfer into a native chromosomal locus.

- (*A*) Experimental outline and a schematic of the process whereby a ZFN-induced DSB is repaired by using an extrachromosomal donor as a template.
- (*B*) PCR-based measurements of ZFN-driven tag integration frequency into the IL2R locus in K562 cells. Cells were left untransfected (first lane, ‘‘neg.’’ for negative control) or were transfected with an expression cassette for ZFNs that induce a DSB at exon 5 of IL2R (second lane), and donor plasmids carrying a 12-bp tag flanked by 750-bp homology arms, in the absence (third lane) and presence (fourth lane) of the IL2R ZFNs.
- Genomic DNA was extracted 72 h later. The IL2R locus was amplified by 20 cycles of PCR in the presence of radiolabeled dNTPs by using primers that hybridize to the chromosome outside of the donor homology arms, and the PCR products were digested with StuI, resolved by 10% PAGE, and autoradiographed. The percentage of StuI-sensitive DNA is indicated below the fourth lane.

Sequence analysis of ZFN-driven insertion

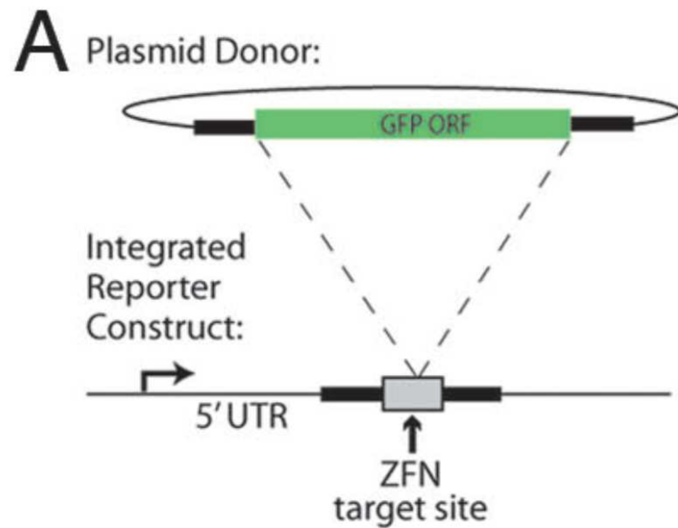


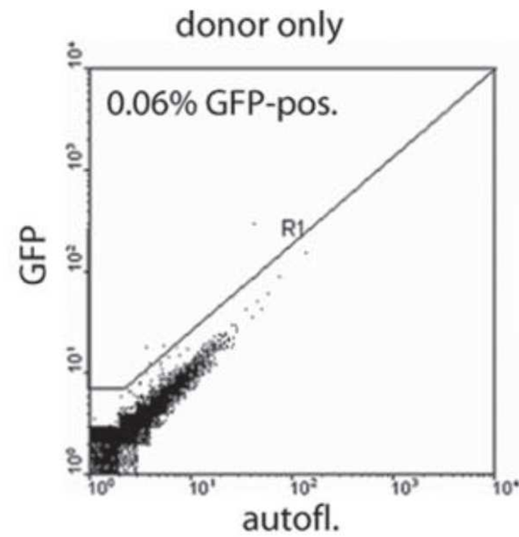
(*C*) Sequence analysis of ZFN-edited chromatids.

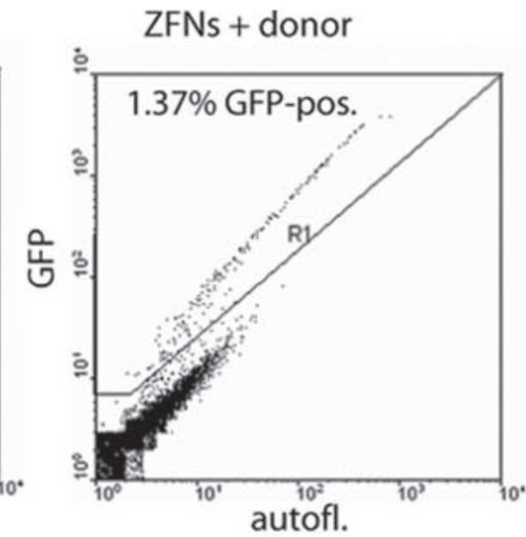
The primary DNA sequence, and the amino acid sequence it encodes, of exon 5 of the human IL2R gene, along with the target sites of the designed ZFNs, are indicated. The central portion of the donor sequence, along with the tag, is shown.

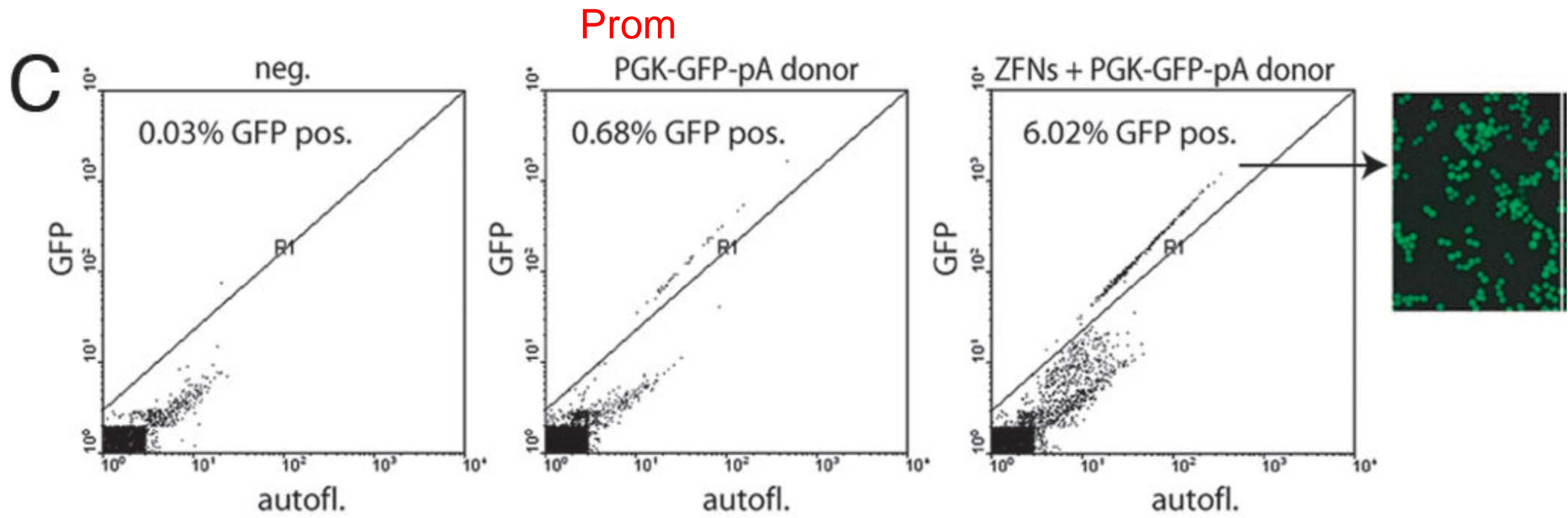
A representative chromatogram of theDNAsequence of one of the chromatids obtained from sample 4 (in *B*) is provided, showing the chromosomal sequence to be altered precisely in the manner specified by the donor, i.e., by copy-pasting of codons for four new amino acids in-frame with the endogenous ORF. Note that an additional silent SNP (Pro229 CCA3CCT), introduced for cloning purposes, is also transferred from the donor.

ZFN-driven targeted integration of large DNA sequences



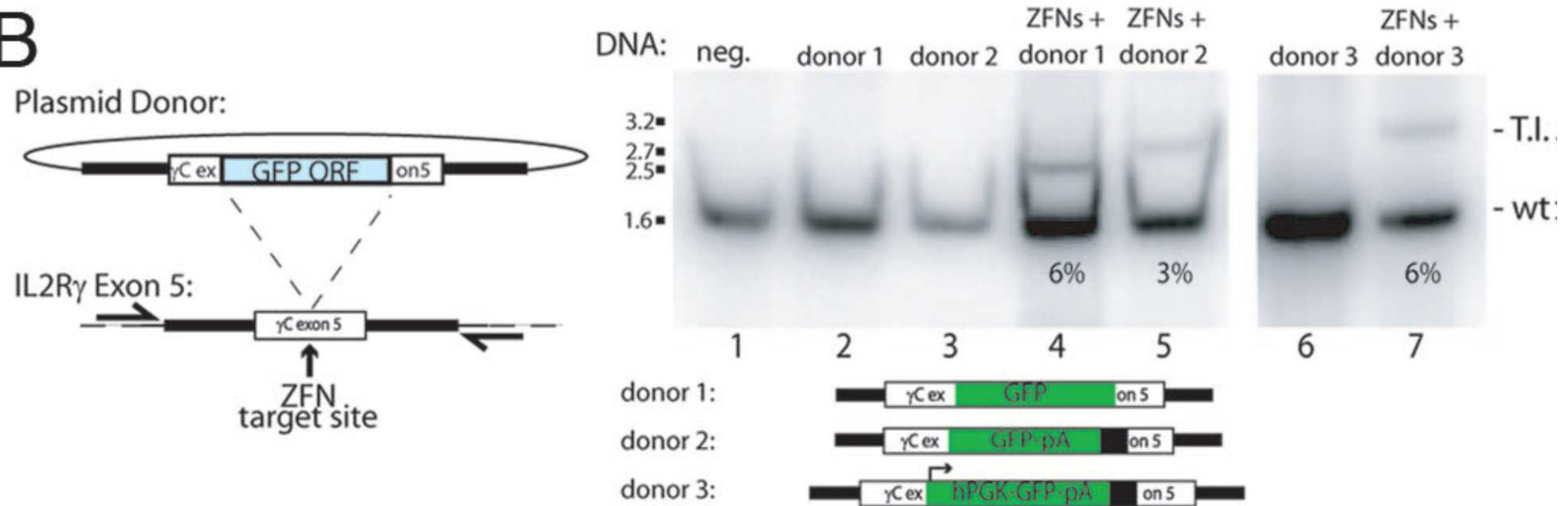




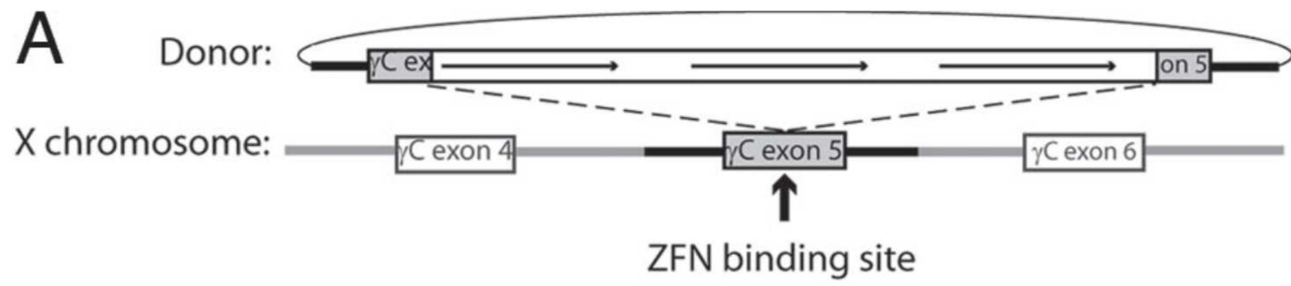


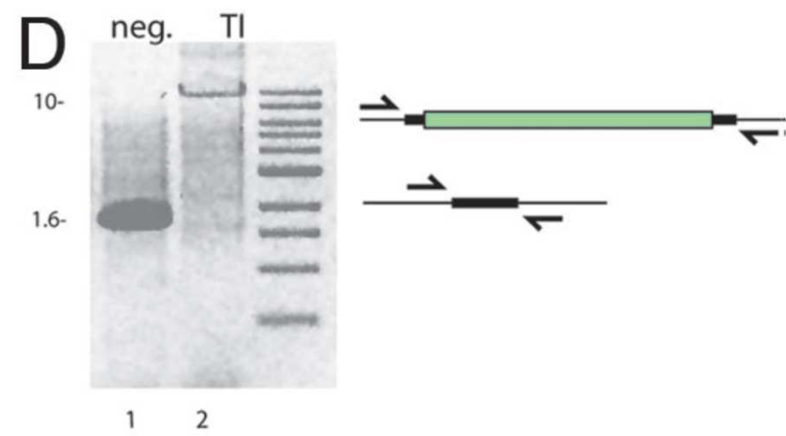
Prom
C
neg.
0.03% GFP pos.
PGK-GFP-pA donor
0.68% GFP pos.
ZFNs + PGK-GFP-pA donor
6.02% GFP pos.
R1
GFP
autofl.

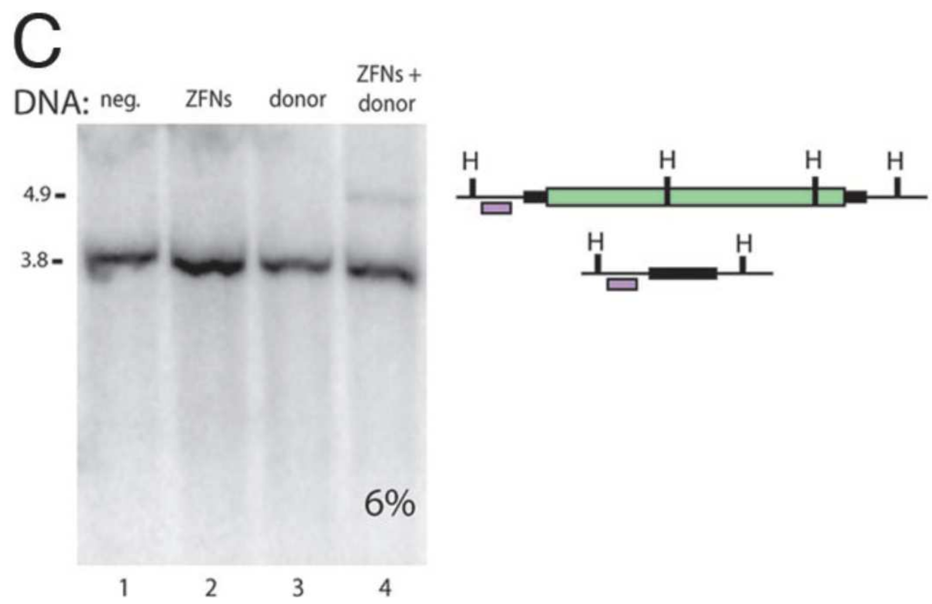
ZFN-driven targeted integration of a series of progressively larger DNA sequences

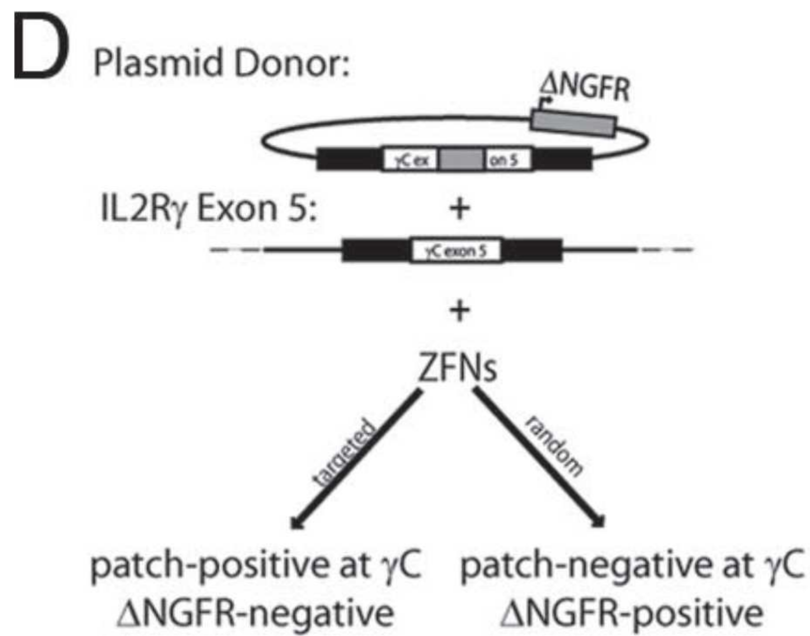


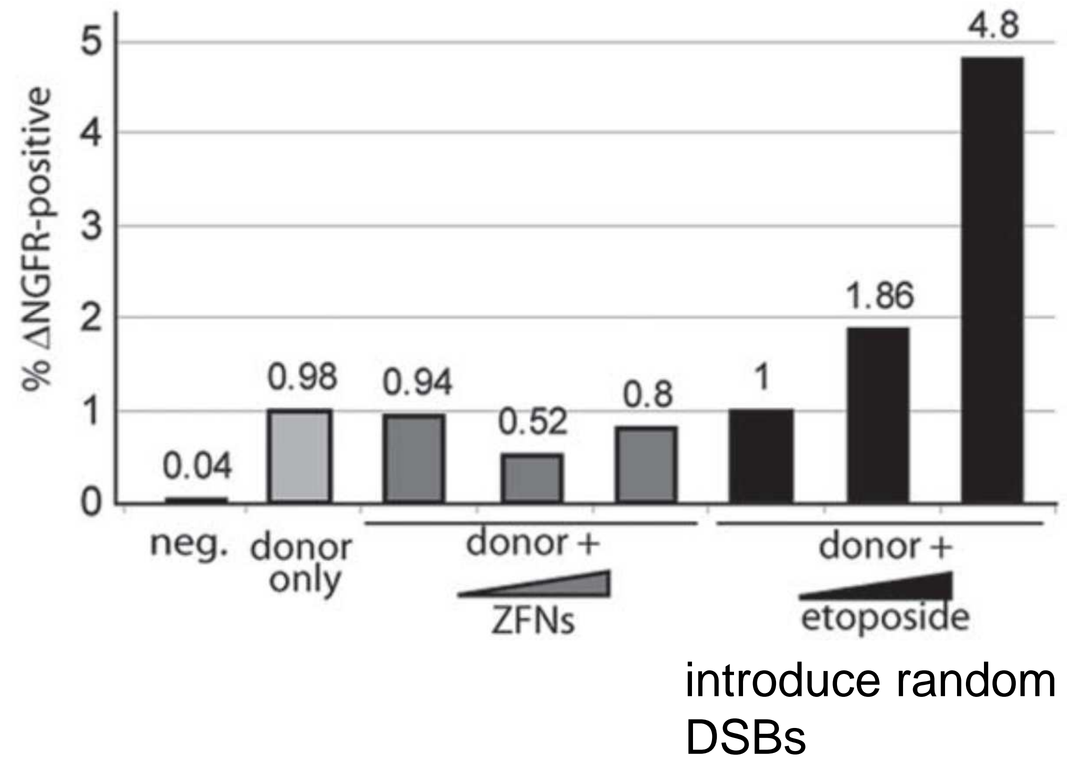
- ZFN-driven targeted integration of a series of progressively larger DNA sequences into an endogenous locus. (*A Left*) A schematic of a chromosomal reporter construct in HEK293 cells that contains the recognition site for two ZFNs (gray box) and a donor molecule that carries the GFP ORF (green rectangle) flanked by homology arms. The percentage of GFP-positive HEK293 cells was measured by FACS (*Center* and *Right*) and is indicated in each panel.


A
Donor:
γC ex
on 5
X chromosome:
γC exon 4
γC exon 5
γC exon 6
ZFN binding site


D
neg.
TI
10-
1.6-
1
2


C
DNA:
neg.
ZFNs
donor
ZFNs +
donor
4.9
3.8
6%
1
2
3
4
H
H
H
H
H
H





introduce random DSBs

NGFR= autonomous expression cassette for a cell surface marker

- (*D*) FACS-based measurement of the rate of plasmid DNA random integration.
- (*Left*) The plasmid donor construct (a tag-interrupted homology stretch flanked by an autonomous expression cassette for a cell surface marker, NGFR).
- Cell phenotypes expected from a targeted (lower left) or random (lower right) integration event are shown.
- (*Right*) FACS data from an experiment in which K562 cells were treated with only the donor molecule, the donor molecule together with the ZFN expression cassette, or the donor molecule and an increasing concentration of etoposide. The percentage of cells positive for the NGFR marker (as measured by FACS after sufficient cell passaging to allow for donor DNA decay) in each sample is indicated.
- Moehle *et al*. PNAS **February 27, 2007** vol. 104 no