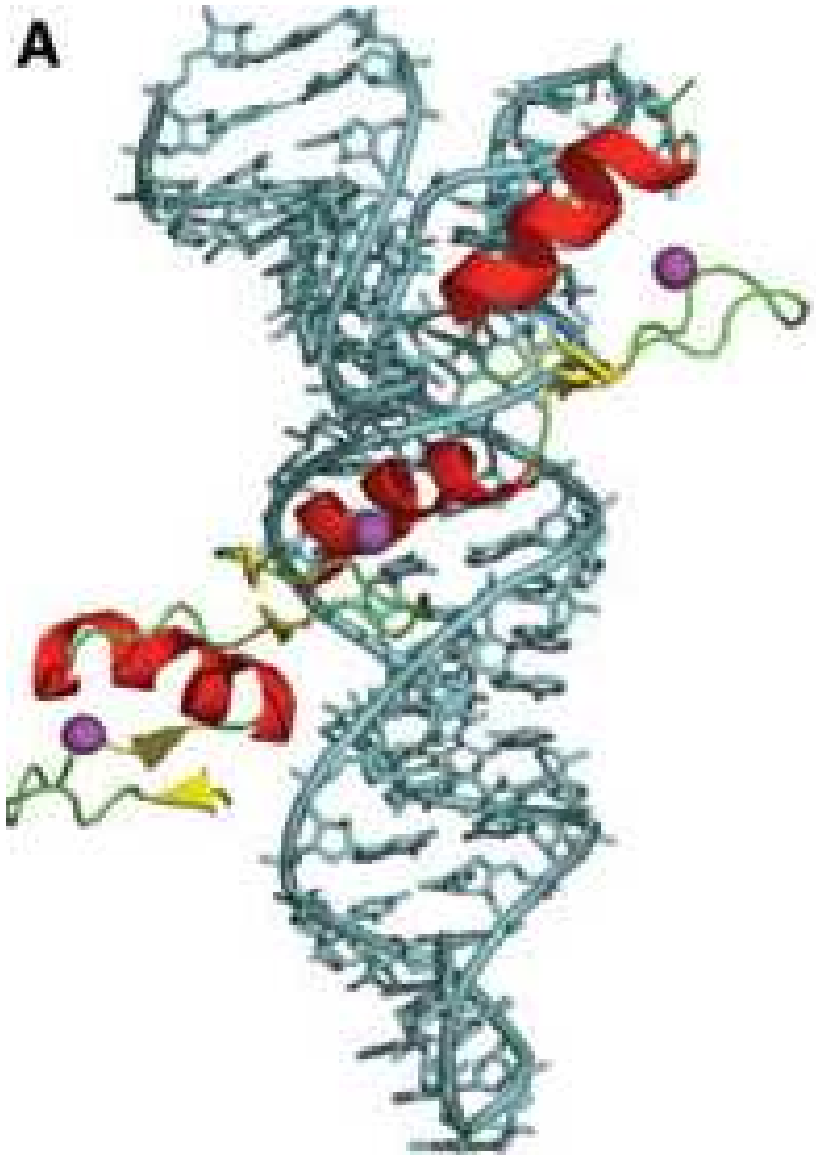
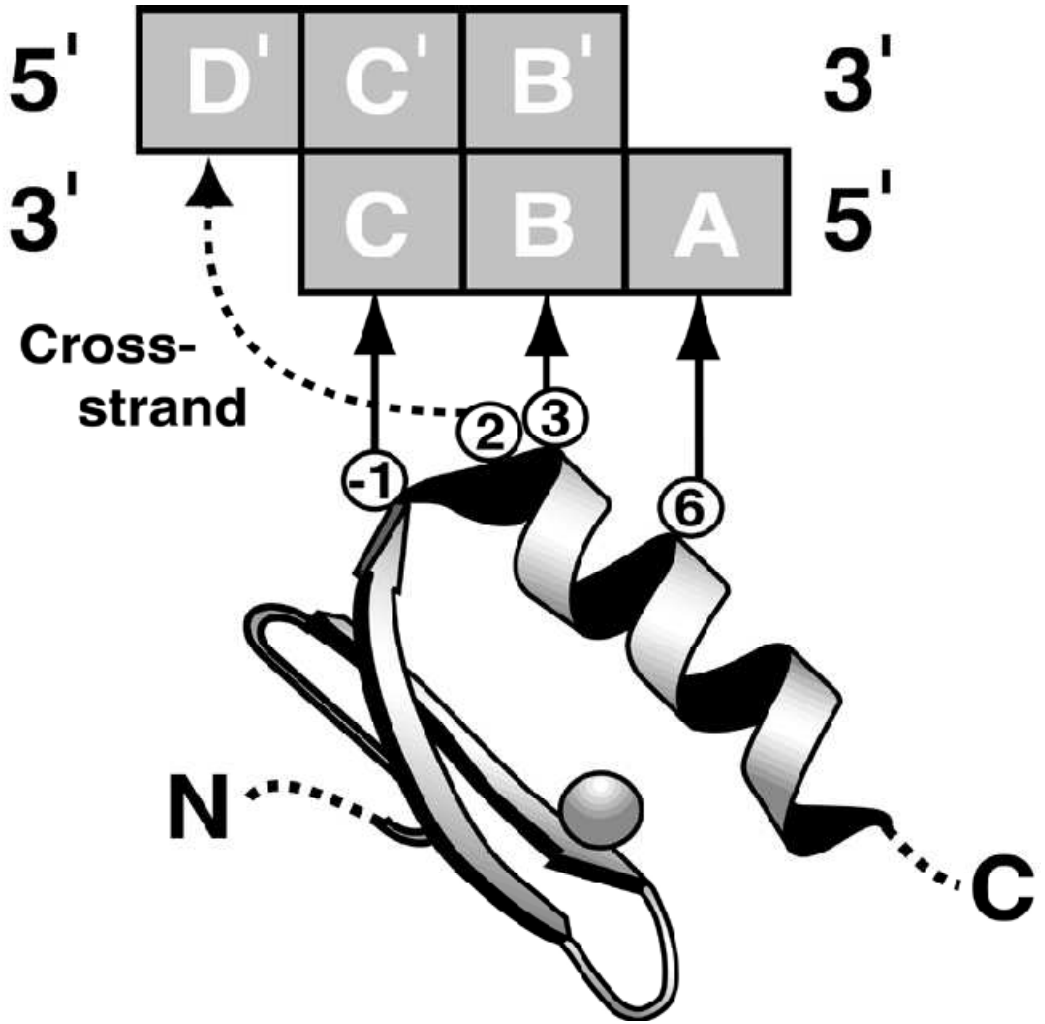
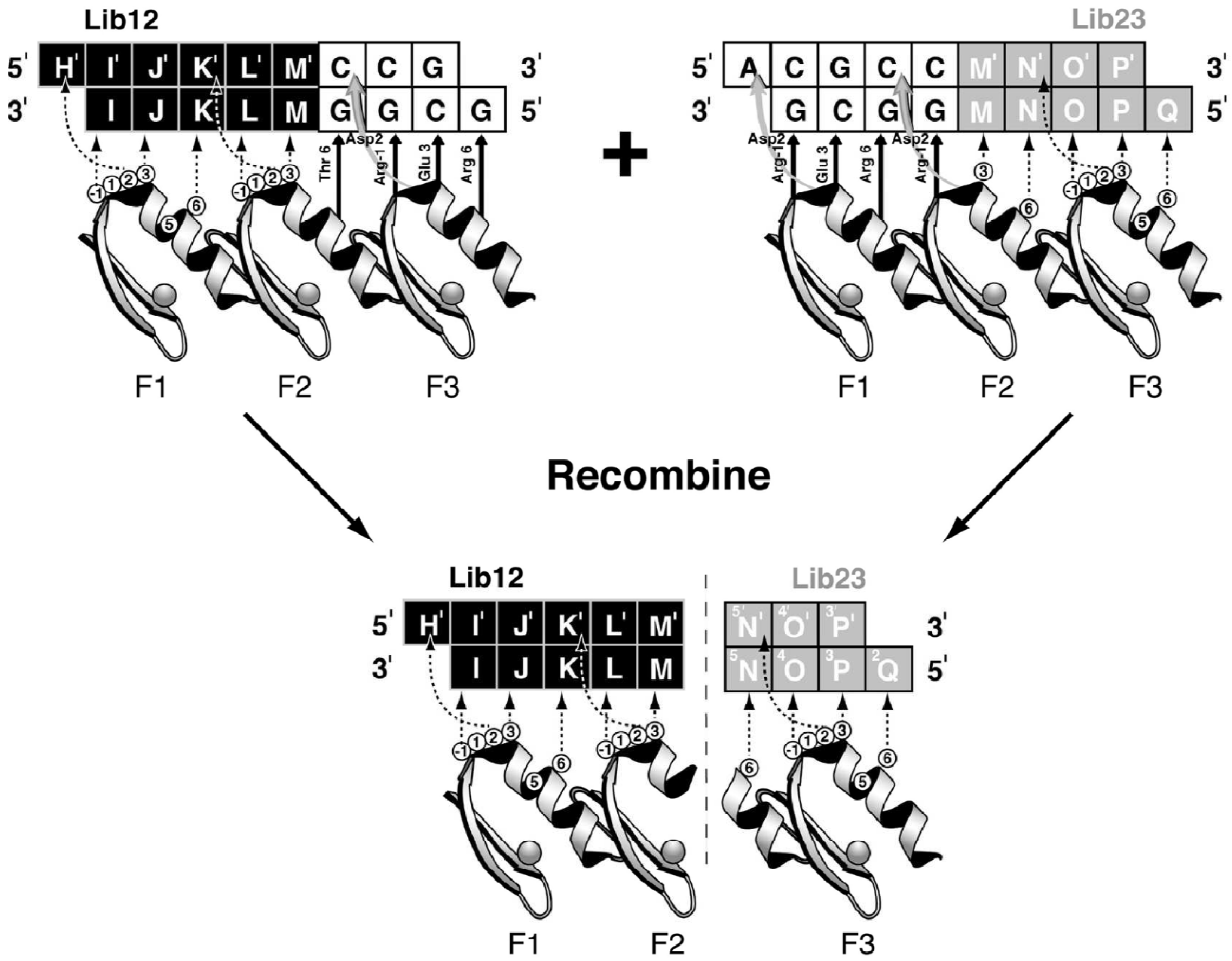
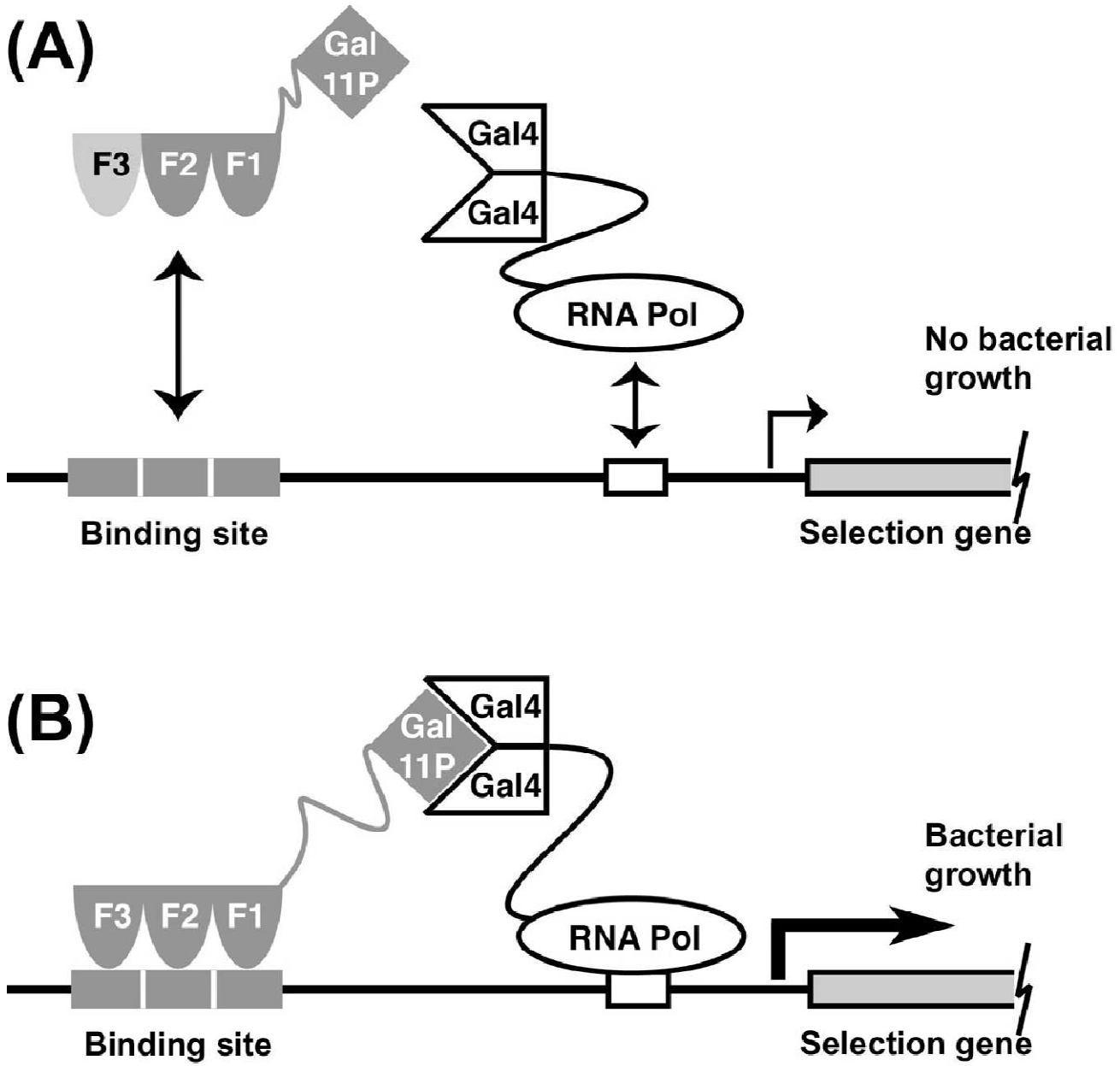
Highly efficient endogenous human gene correction using designed zinc-finger nucleases

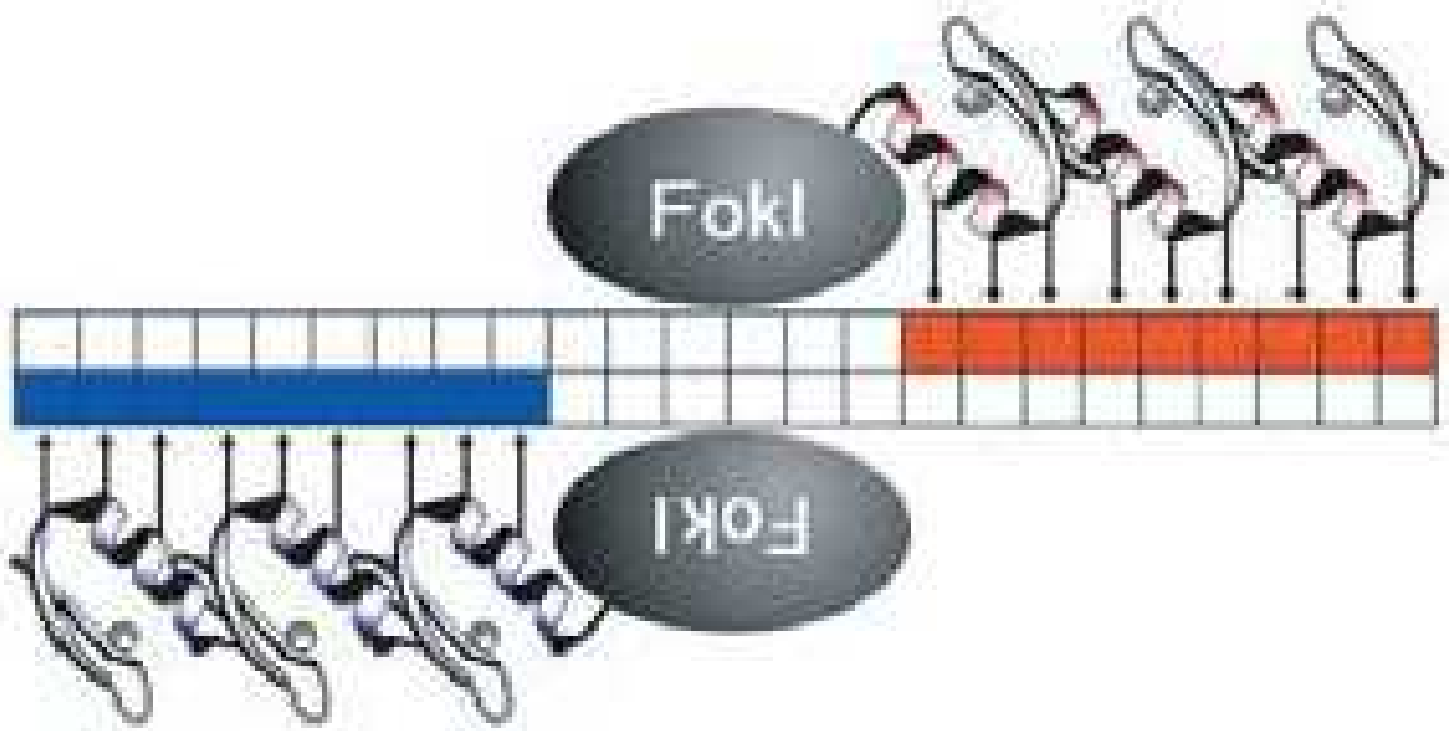
Fyodor D. Urnov[1], Jeffrey C. Miller[1], Ya-Li Lee[1], Christian M. Beausejour[1], Jeremy M. Rock[1], Sheldon Augustus[1], Andrew C. Jamieson[1], Matthew H. Porteus[2], Philip D. Gregory[1] & Michael C. Holmes[1]


A


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


Lib12
5'
3'
H' I' J' K' L' M' C C G
I J K L M G G C G
3'
5'
Asp2
Thr 6
Arg-1
Glu 3
Arg 6
F1
F2
F3
Lib23
A C G C C M' N' O' P'
G C G G M N O P Q
Asp2
Arg-1
Glu 3
Arg 6
Asp2
Arg-1
F1
F2
F3
Recombine
Lib12
H' I' J' K' L' M'
I J K L M
F1
F2
Lib23
N' O' P'
N O P Q
F3


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


FokI
FokI

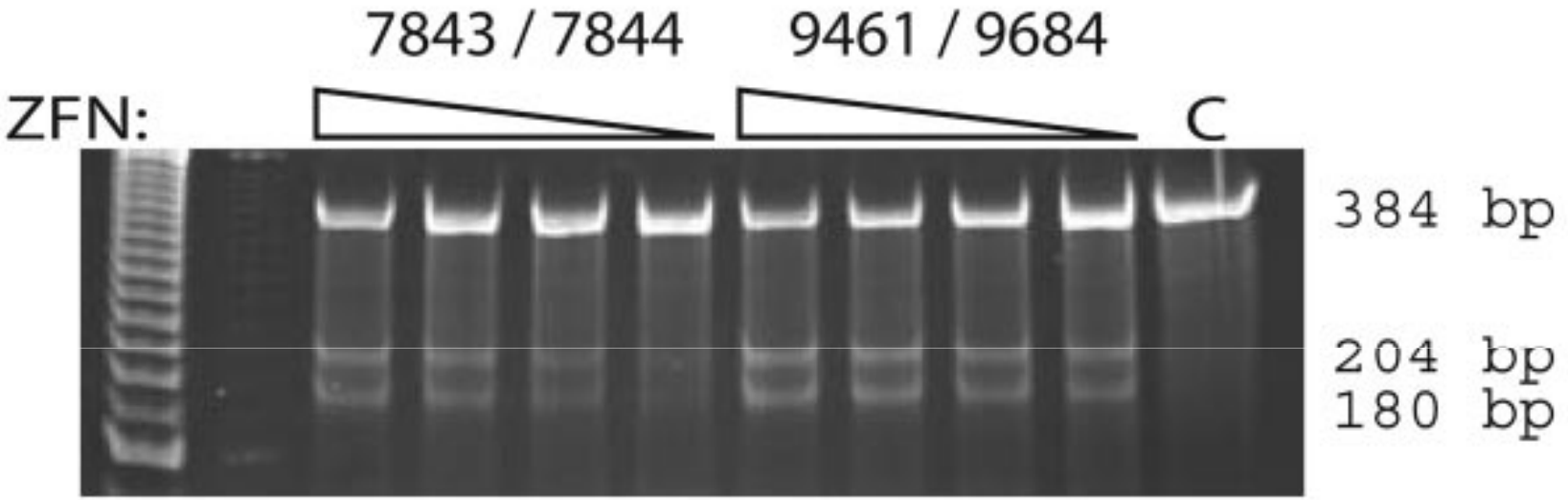
A

B

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

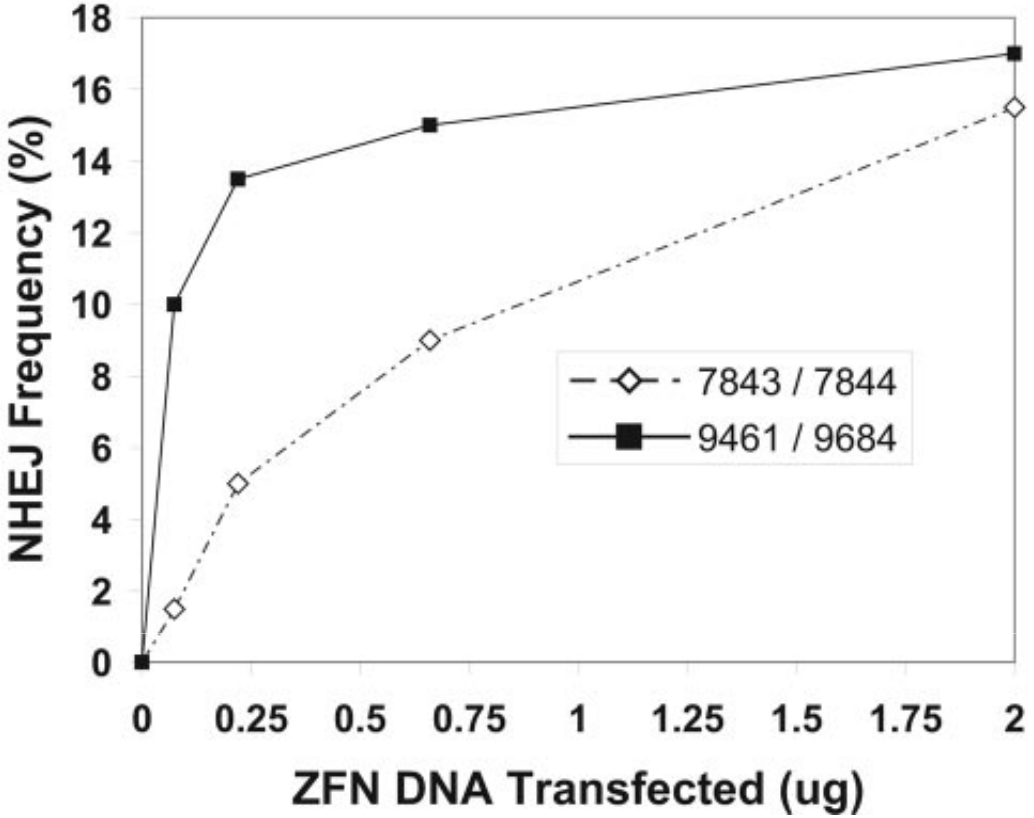
- Section of the DHFR
- gene targeted by ZFNs. TheDNAsequence of the primary binding site for each
- ZFN is boxed. ZFN7843 and ZFN9461 bind the same 12-bp site. ZFN7844 binds
- the 12-bp site AATGCTCAGGTA, whereas ZFN9684 binds the 15-bp site AATGCTCAGGTACTG.
- (*B*) Recognition helix sequences of ZFNs. The sequence of
- the recognition helix from position 1 to 6 (27) is listed below its target
- triplet.

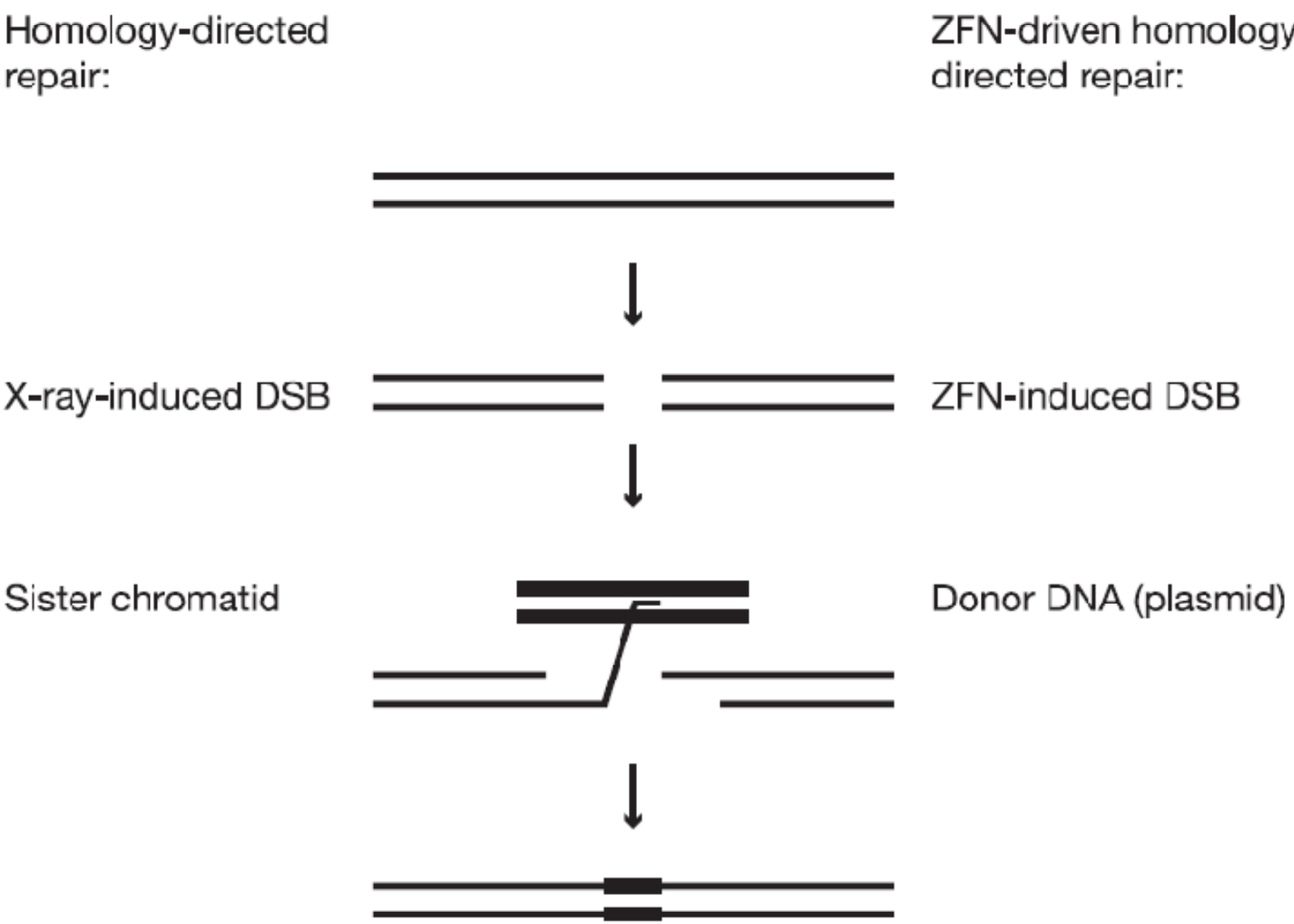
C

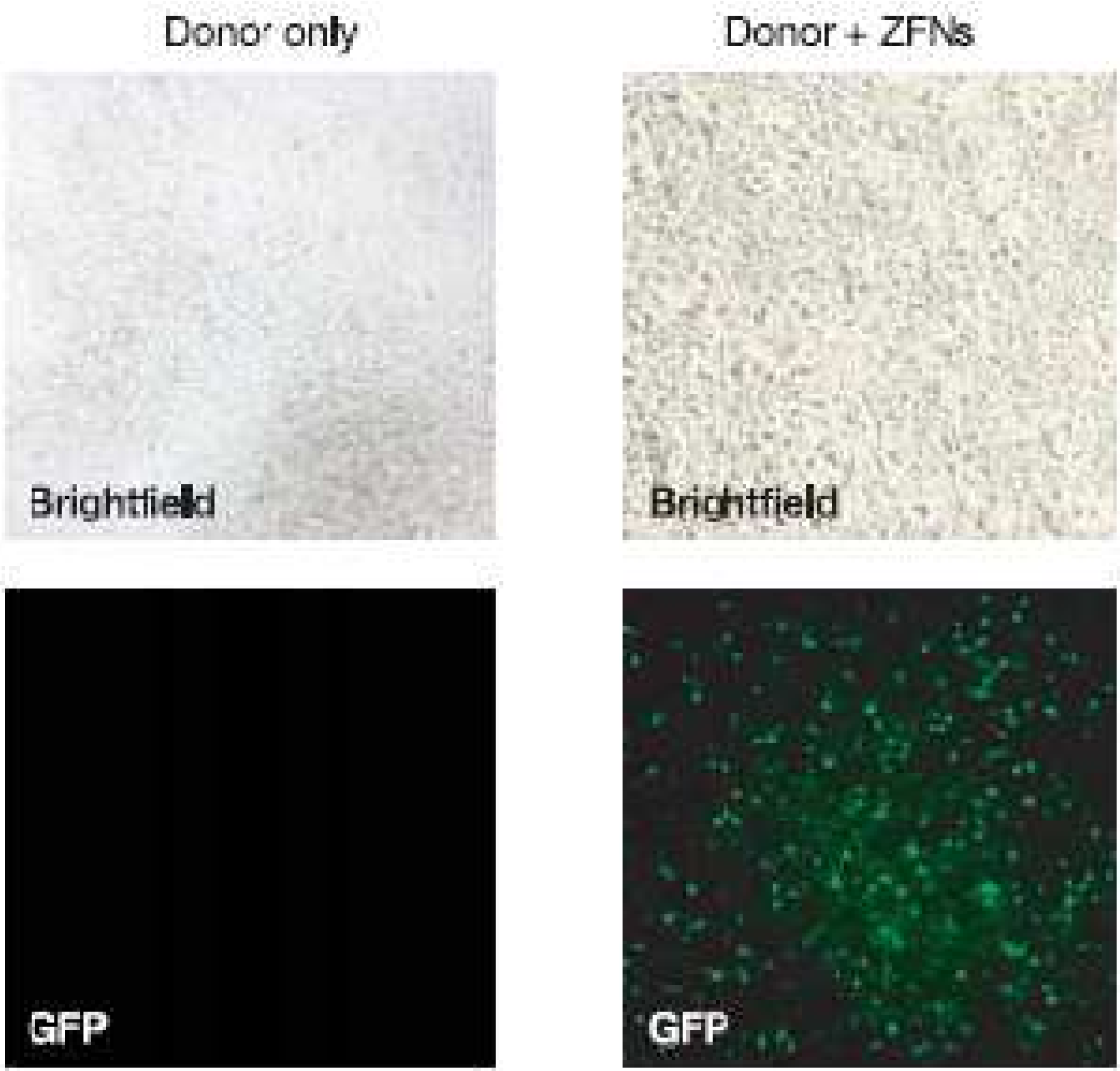


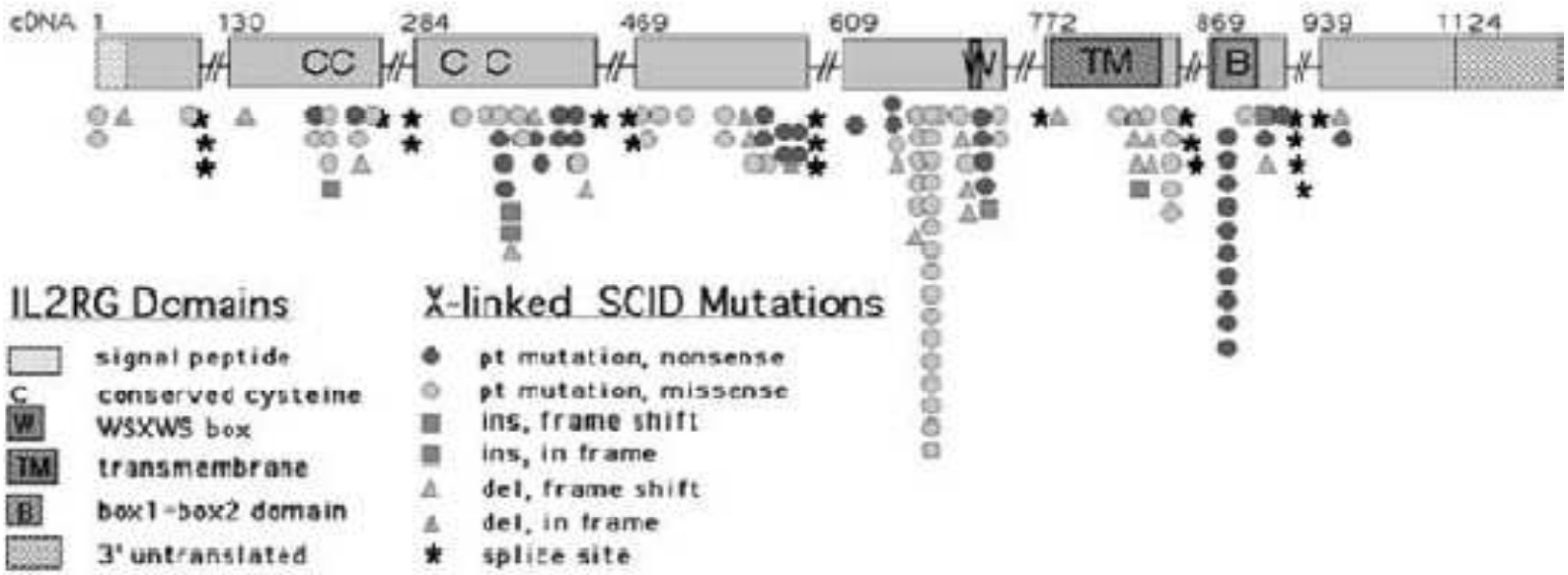
- Comparison
- of ZFN activity. Plasmids encoding each pair of ZFNs (ZFN7843/ZFN7844 and
- ZFN9461/ZFN9684) containing the ELKK FokI variants were delivered in the
- amounts shown to CHO-S cells in suspension culture. The frequency of allelic
- mutation in each pool of treated cells was determined by 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.

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

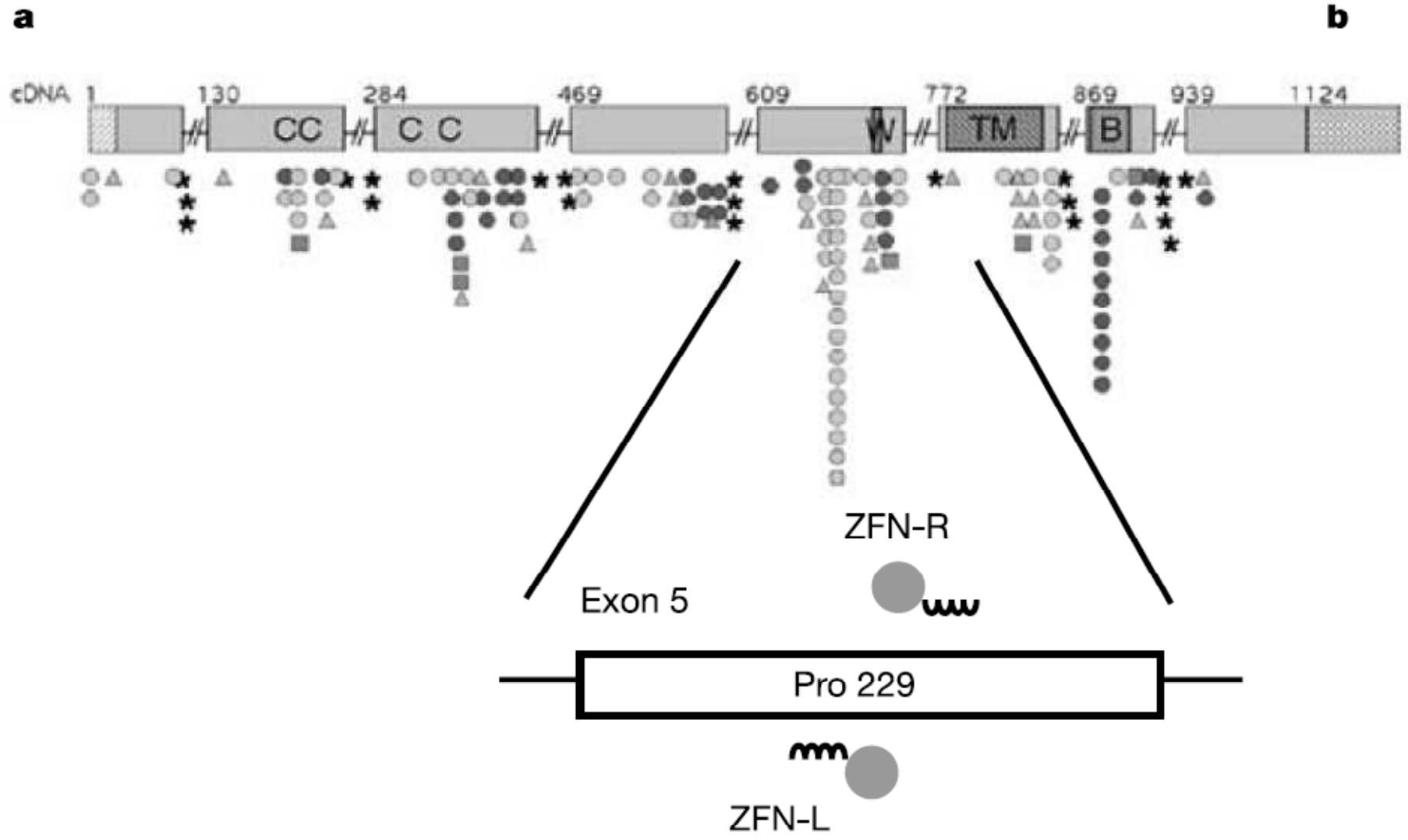

NHEJ Frequency (%)
18
16
14
12
10
8
6
4
2
0
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
ZFN DNA Transfected (ug)
7843 / 7844
9461 / 9684

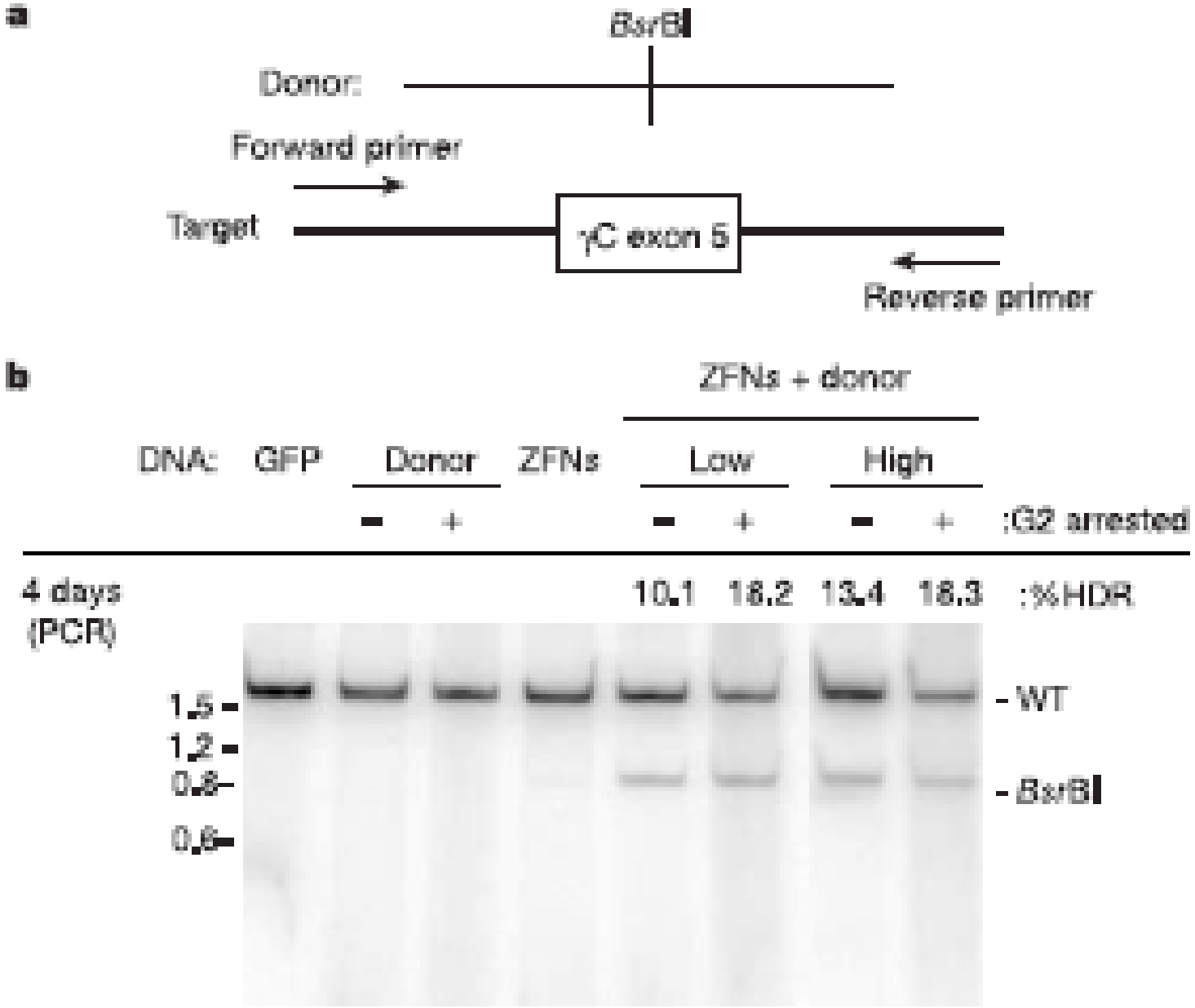

Homology-directed repair:
ZFN-driven homology-directed repair:
X-ray-induced DSB
ZFN-induced DSB
Sister chromatid
Donor DNA (plasmid)

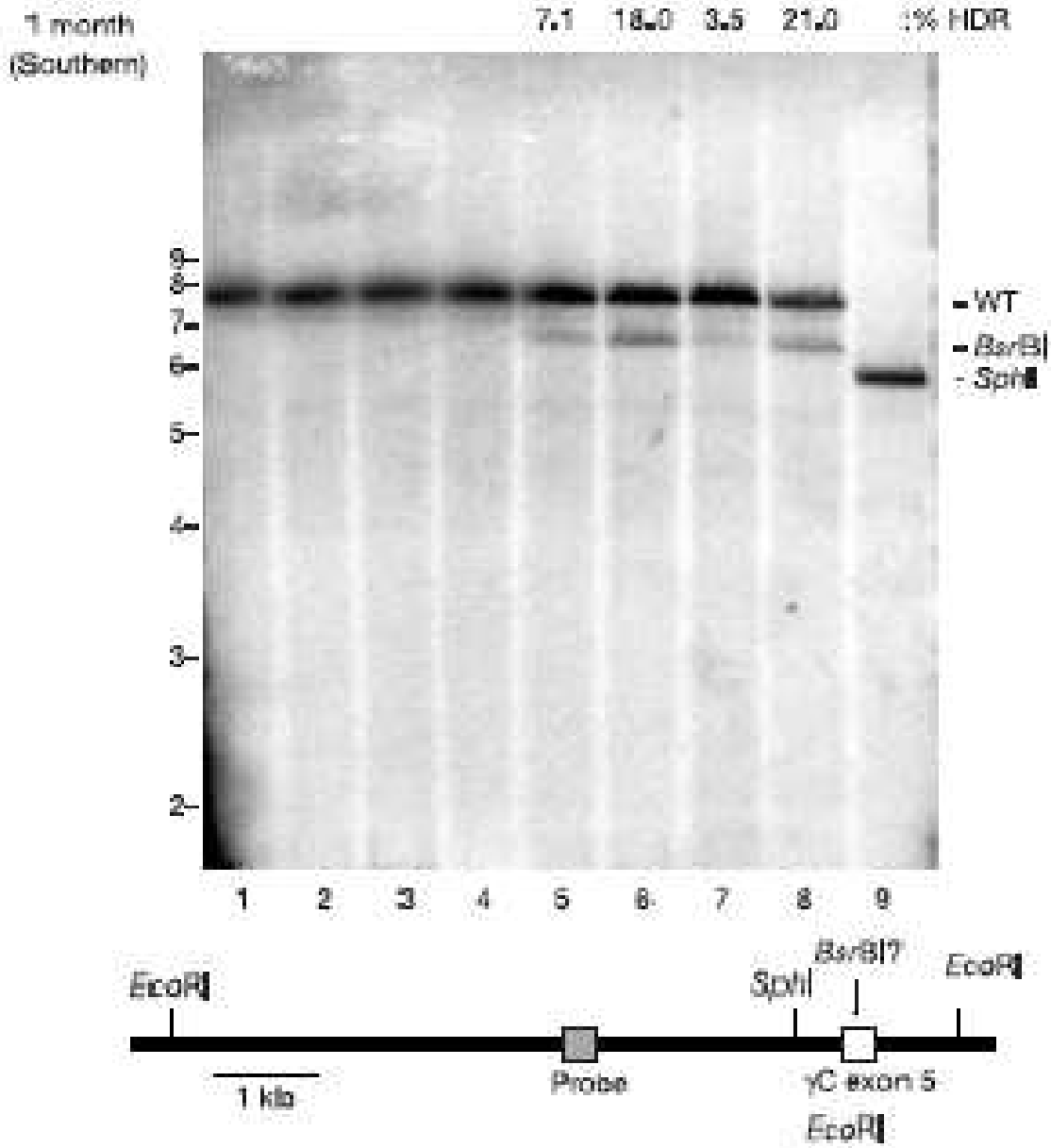

Donor only
Donor + ZFNs
Brightfield
Brightfield
GFP
GFP

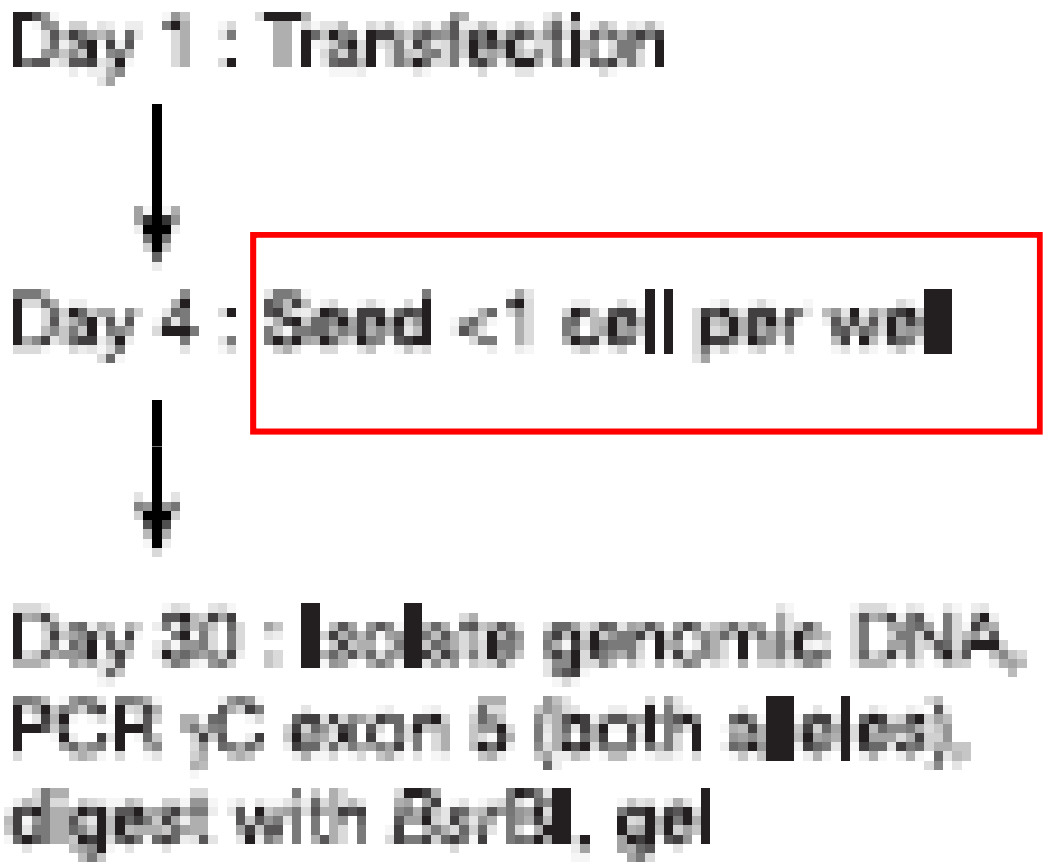


X-linked severe combined immune deficiency (SCID)

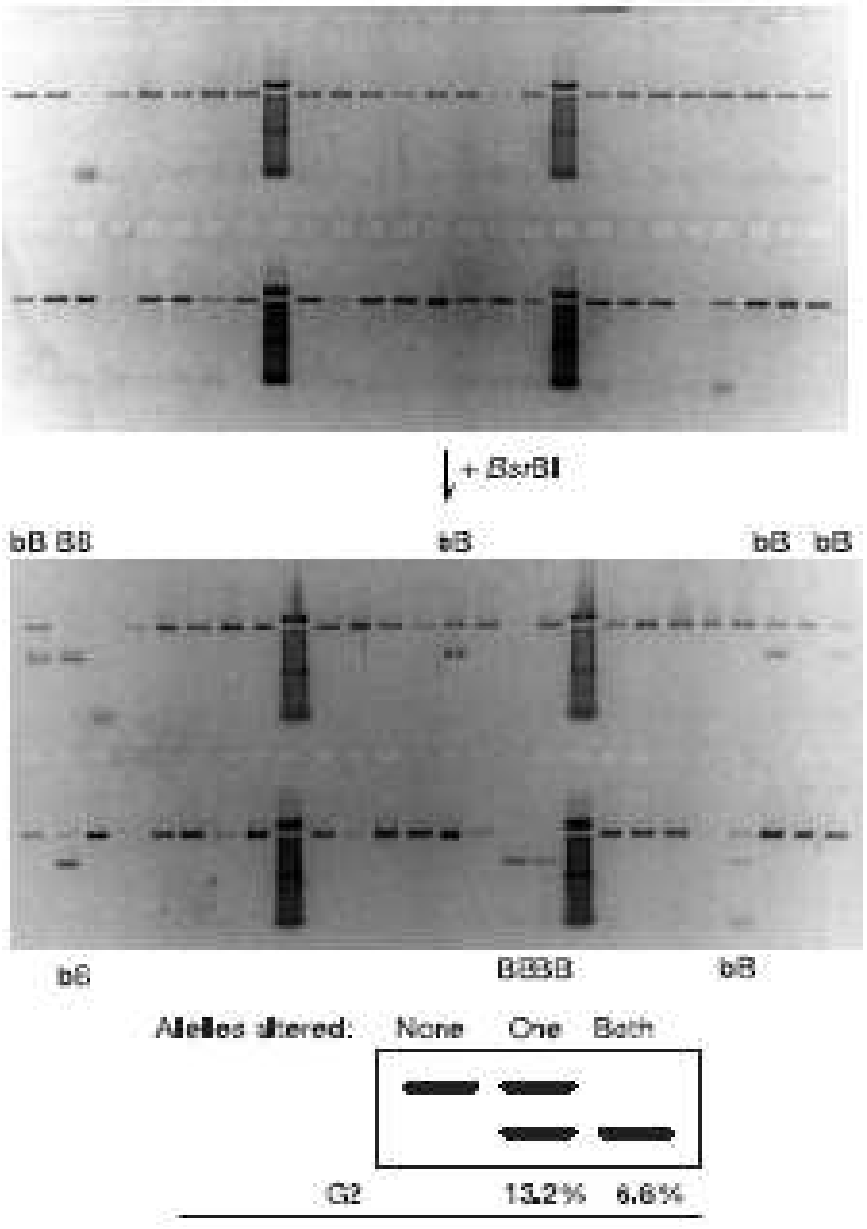

a
b
cDNA
1
130
284
469
609
772
869
939
1124
CC
C C
N
TM
B
ZFN-R
Exon 5
Pro 229
ZFN-L


a
BsrBI
Donor:
Forward primer
Target
γC exon 5
Reverse primer
b
ZFNs + donor
DNA:
GFP
Donor
ZFNs
Low
High
-
+
-
+
-
+
:G2 arrested
4 days
(PCR)
10.1
18.2
13.4
18.3
:%HDR
1.5
1.2
0.8
0.6
WT
BsrBI


1 month
(Southern)
7.1
18.0
3.5
21.0
:% HDR
9
8
7
6
5
4
3
2
WT
BsrBI
SphI
1
2
3
4
5
6
7
8
9
EcoRI
SphI
BsrBI?
EcoRI
1 kb
Probe
γC exon 5
EcoRI


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

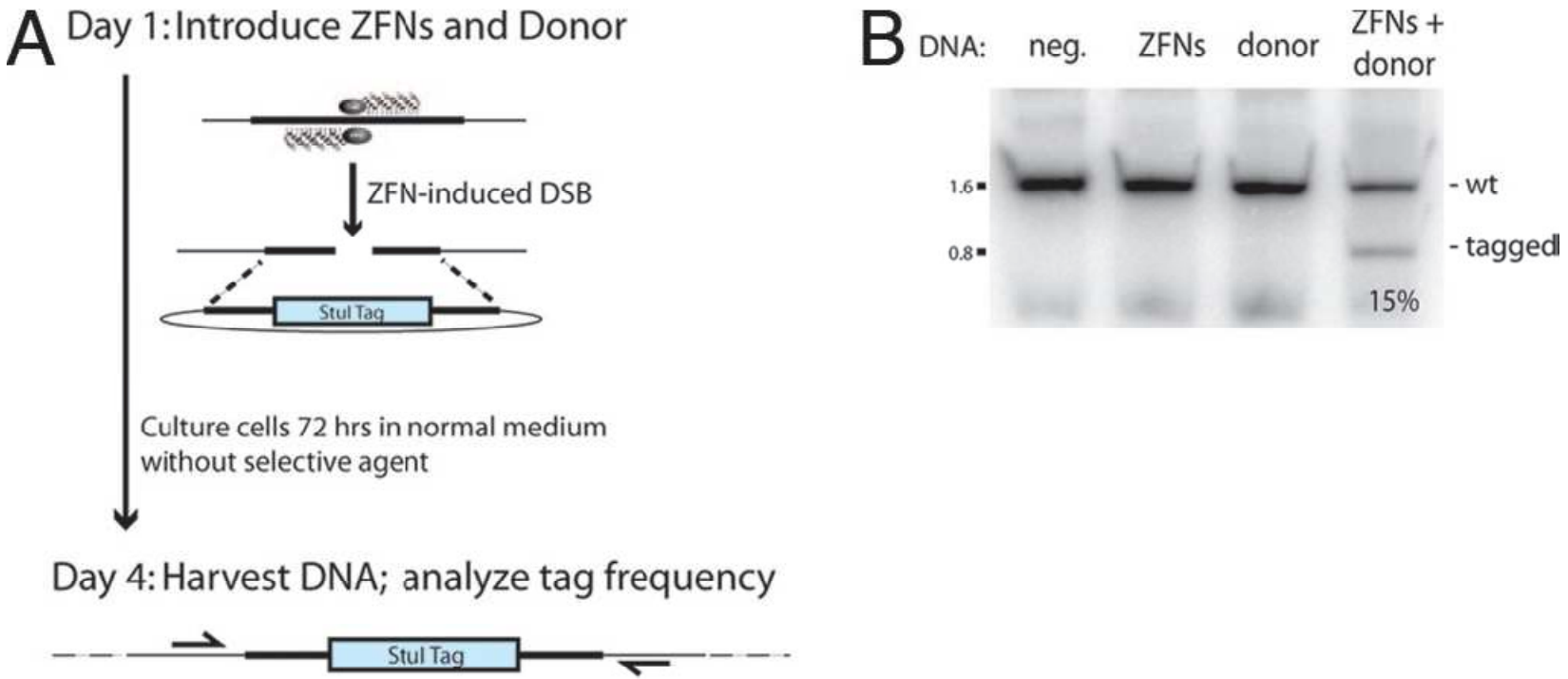
b


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

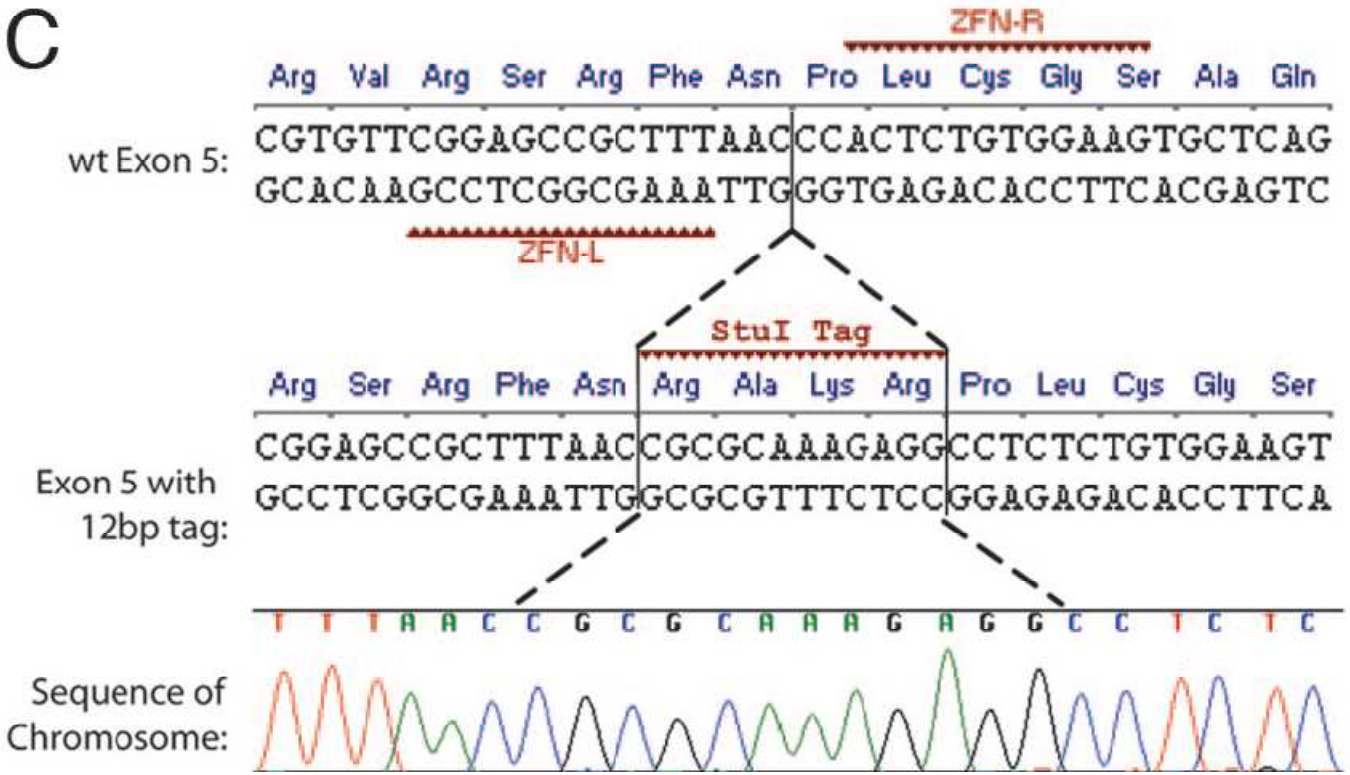
Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases Moehle PNAS 2007

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 donorspecified 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.

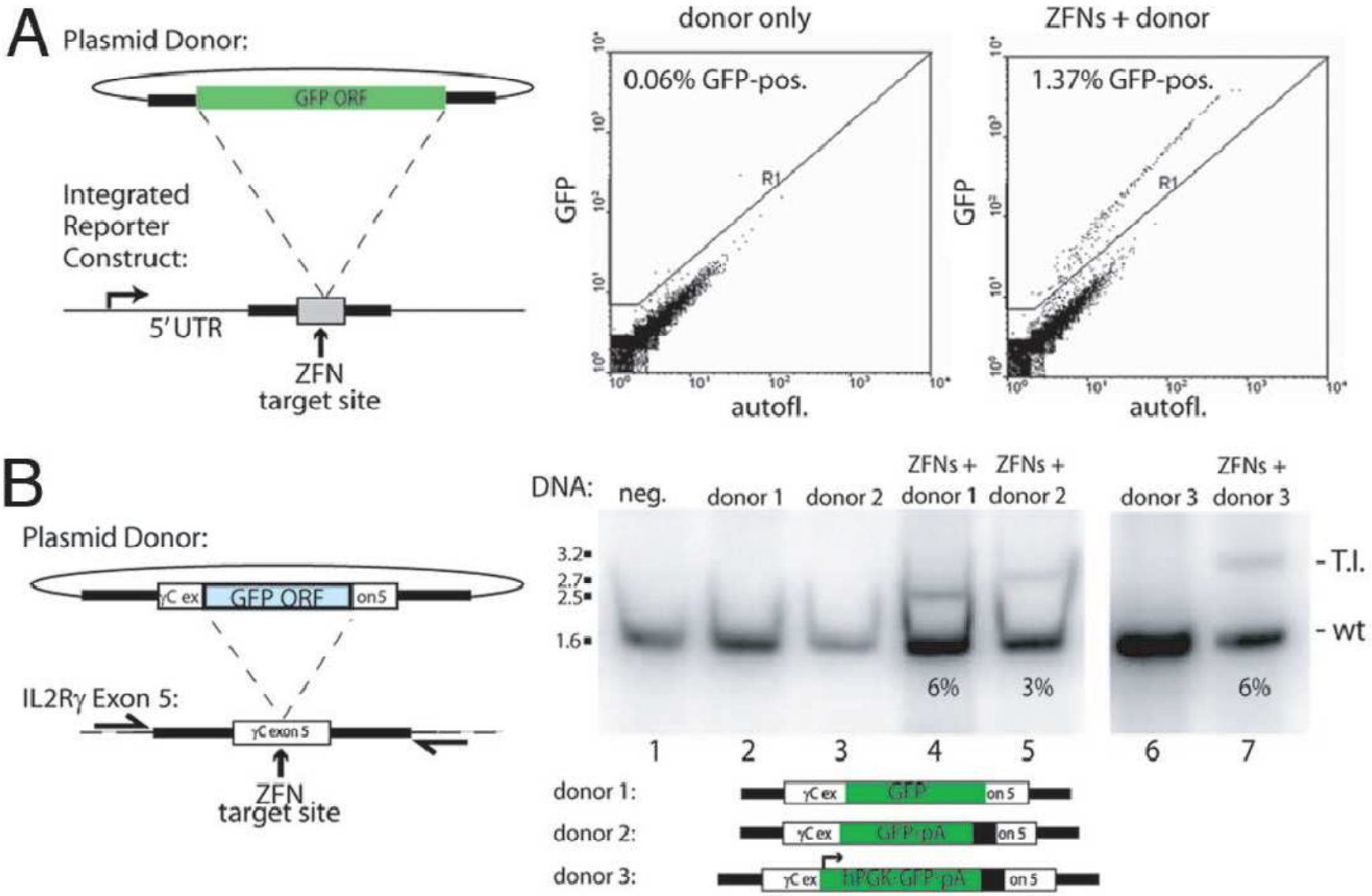
a) the surprising versatility of the specialized polymerase machinery involved in double-strand break repair b) powerful approach to mammalian cell engineering c) possibility of ZFN-driven gene addition therapy for human geneticdisease.


A
Day 1: Introduce ZFNs and Donor
ZFN-induced DSB
StuI Tag
Culture cells 72 hrs in normal medium
without selective agent
Day 4: Harvest DNA; analyze tag frequency
StuI Tag
B
DNA:
neg.
ZFNs
donor
ZFNs +
donor
1.6
0.8
- wt
- tagged
15%

- 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 (16)
- (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. (

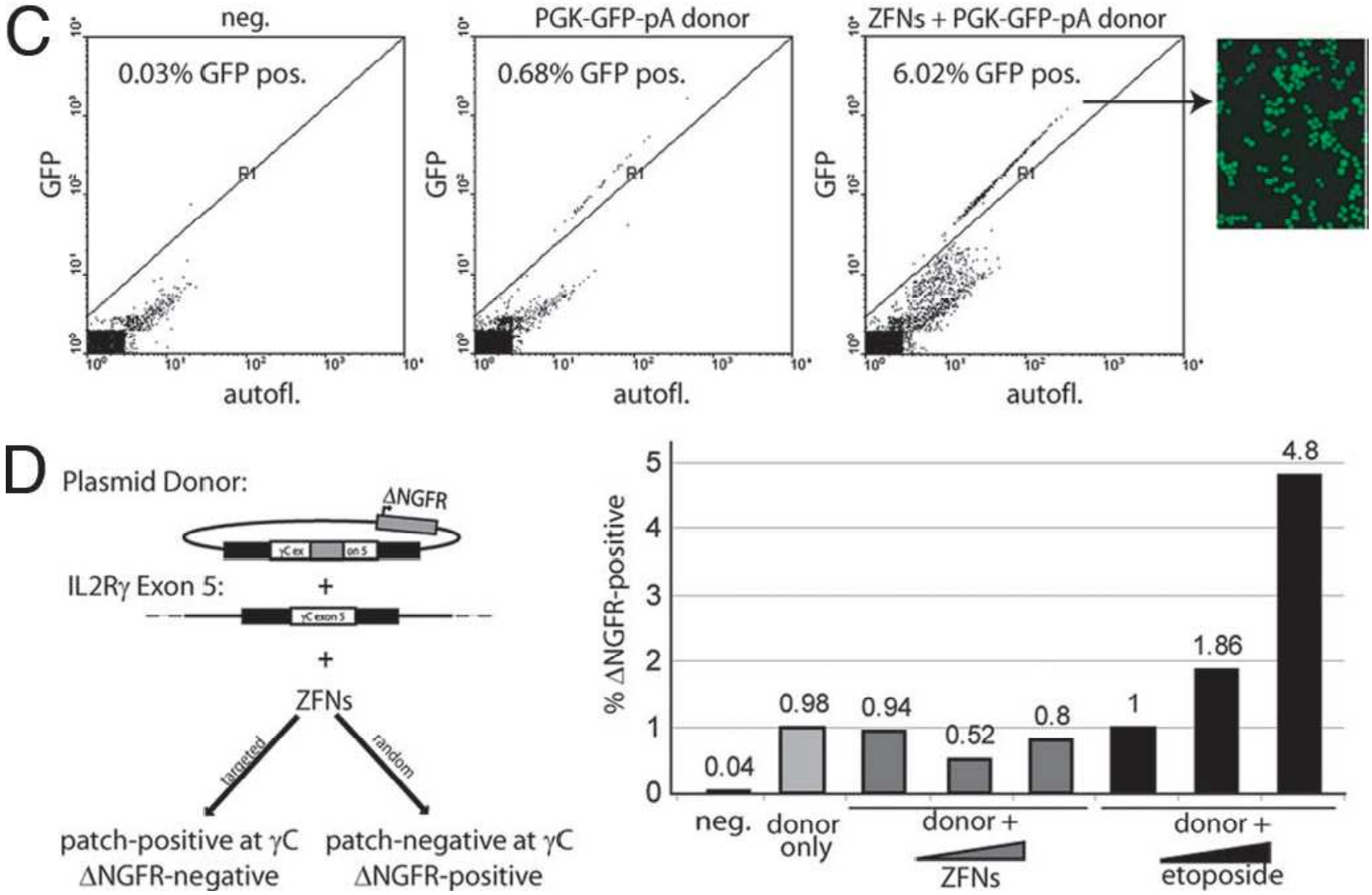

C
ZFN-R
Arg Val Arg Ser Arg Phe Asn Pro Leu Cys Gly Ser Ala Gln
wt Exon 5:
CGTGTTCGGAGCCGCTTTAACCCACTCTGTGGAAGTGCTCAG
GCACAAGCCTCGGCGAAATTGGGTGAGACACCTTCACGAGTC
ZFN-L
StuI Tag
Arg Ser Arg Phe Asn Arg Ala Lys Arg Pro Leu Cys Gly Ser
Exon 5 with
12bp tag:
CGGAGCCGCTTTAACCGCGCAAAGAGGCCTCTCTGTGGAAGT
GCCTCGGCGAAATTGGCGCGTTTCTCCGGAGAGACACCTTCA
Sequence of
Chromosome:

(*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 below.Arepresentative 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.


A
Plasmid Donor:
GFP ORF
Integrated
Reporter
Construct:
5'UTR
ZFN
target site
donor only
0.06% GFP-pos.
R1
GFP
autofl.
ZFNs + donor
1.37% GFP-pos.
R1
GFP
autofl.
B
Plasmid Donor:
γC ex
GFP ORF
on 5
IL2Rγ Exon 5:
γC exon 5
ZFN
target site
DNA:
neg.
donor 1
donor 2
ZFNs + donor 1
ZFNs + donor 2
donor 3
ZFNs + donor 3
3.2
2.7
2.5
1.6
6%
3%
6%
- T.I.
- wt
1
2
3
4
5
6
7
donor 1:
γC ex
GFP
on 5
donor 2:
γC ex
GFP-pA
on 5
donor 3:
γC ex
hPGK-GFP-pA
on 5

- 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.

- (*B*) PCR-based measurements of ZFN-driven integration frequency into the
- IL2R locus in K562 cells. Cells were left untransfected (lane 1) or were
- transfected with an expression cassette for ZFNs that induce a DSB at exon 5
- of IL2R (16) (lane 2), and donor plasmids carrying the indicated inserts
- flanked by 750-bp homology arms, in the absence (lanes 3 and 6) and presence
- (lanes 4, 5, and 7) of the IL2R ZFNs. The donor DNAs tested were as follows:
- a 900-bp GFP ORF (lane 4) or the same ORF followed by a polyA sequence (lane
- 5) and an autonomous expression cassette (human phosphoglycerokinase
- promoter–GFP–polyA; lane 7). Genomic DNA was amplified by using primers
- outside the donor homology arms, and the level of targeted integration was
- determined by PAGE and autoradiography (the integrant-carrying chromosome
- migrates above the wild-type one). The integration frequency is indicated
- for each panel. Note that the autoradiograph for lanes 6 and 7 was
- generated in an experiment distinct from that for lanes 1–5.


C
neg.
0.03% GFP pos.
PGK-GFP-pA donor
0.68% GFP pos.
ZFNs + PGK-GFP-pA donor
6.02% GFP pos.
GFP
autofl.
R1
D
Plasmid Donor:
ΔNGFR
γC ex
on 5
IL2Rγ Exon 5:
γC exon 5
ZFNs
targeted
random
patch-positive at γC
ΔNGFR-negative
patch-negative at γC
ΔNGFR-positive
% ΔNGFR-positive
0
1
2
3
4
5
0.04
0.98
0.94
0.52
0.8
1
1.86
4.8
neg.
donor only
donor +
ZFNs
donor +
etoposide

- (*C*) Functional
- measurement of targeted integration frequency. The percentage of GFPpositive
- cells was measured by FACS in K562 cells transfected with an IL2R
- donor molecule carrying an autonomous GFP expression cassette (*Center*; see
- donor schematic at the bottom *B*), transfected with this donor and IL2R-
- specific ZFNs (*Right*) or untreated control cells (*Left*), and is shown within each
- panel. All measurements were taken 3 weeks after transfection to permit
- decay of expression from the donor episome.Afluorescence micrograph of an
- aliquot of the GFP-positive cells from *Right* is also shown. (*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