



INSERM U643, Nantes

**Les zinc finger nucleases comme outils de modifications
génétiques; l'exemple de génération de rats knock-out.**

Plate-forme IBISA “Rats transgéniques”

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Ignacio Anegon**

**Open Monoclonal
Technology**

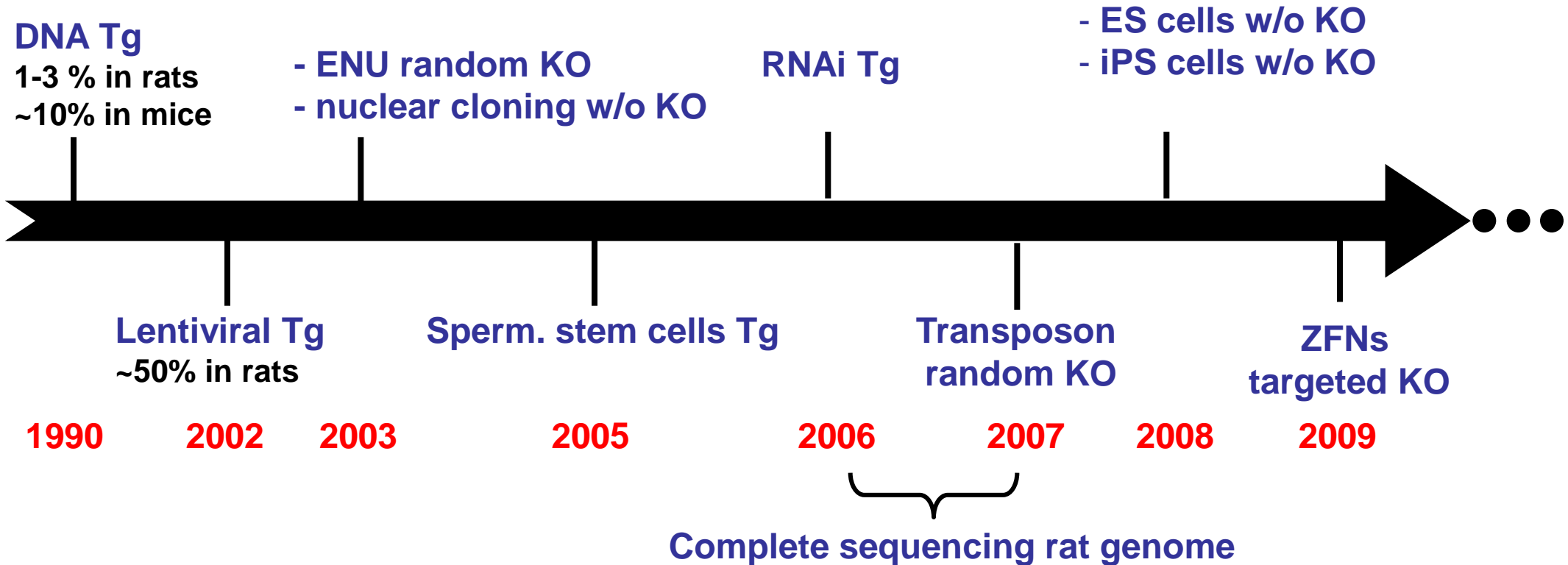


Why transgenic rats ?

- * rats historically used in several fields of research (neurology, cardiovascular, transplantation); data, models and tools available
- * robust physiological models
- * medium size = easier surgery than in mice: multiple sampling of (blood and tissues), anatomical localization of injected substances, ex vivo organ perfusion
- * many inbred, consomic, transgenic and congenic strains
- * certain disease models better reproduced in rats than in mice (hypertension/renin, arthritis/HLA-B27, HIV)

Rats are an important experimental model

Development of rat genome manipulation strategies

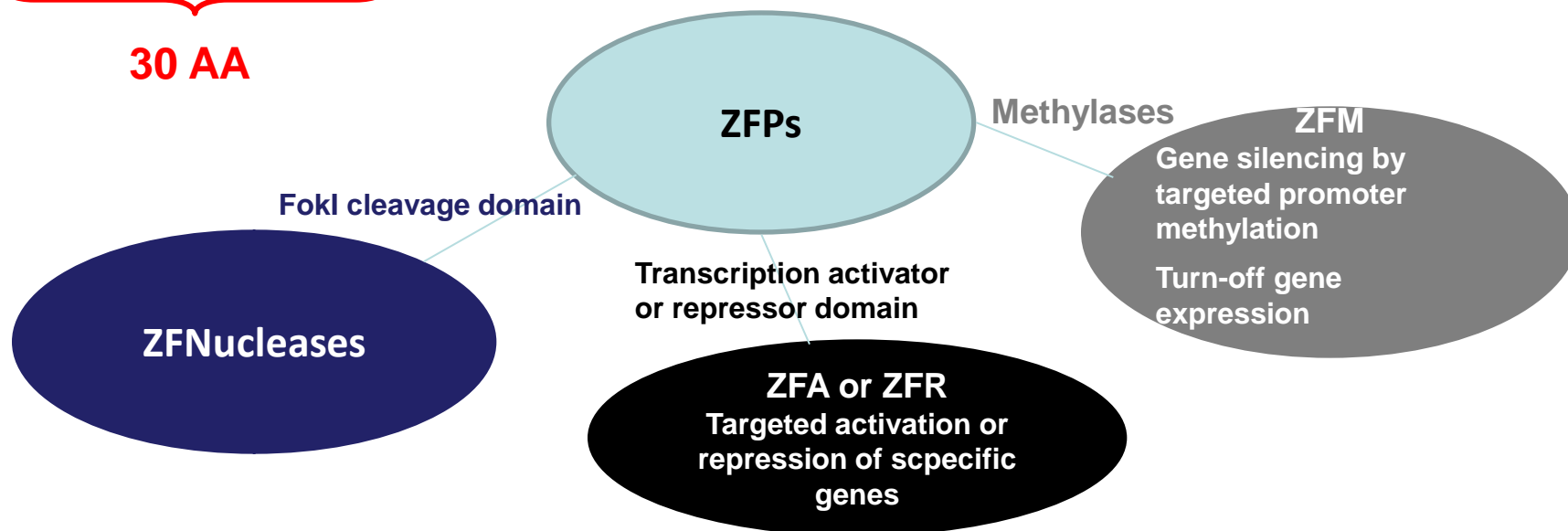
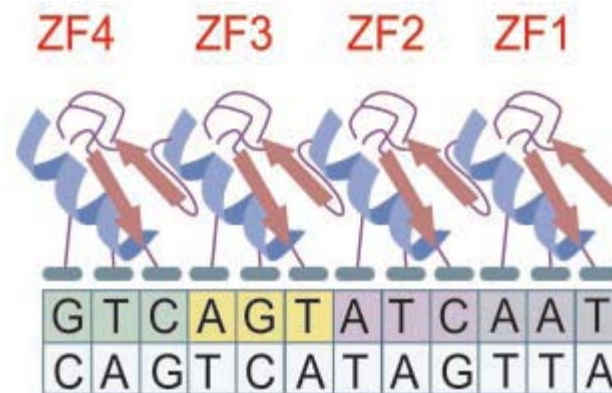
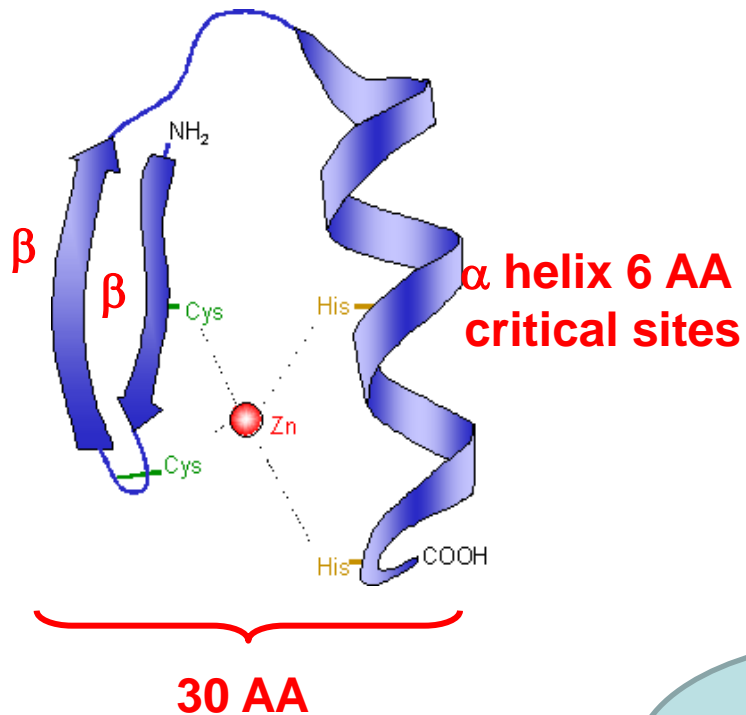


List mutated rats

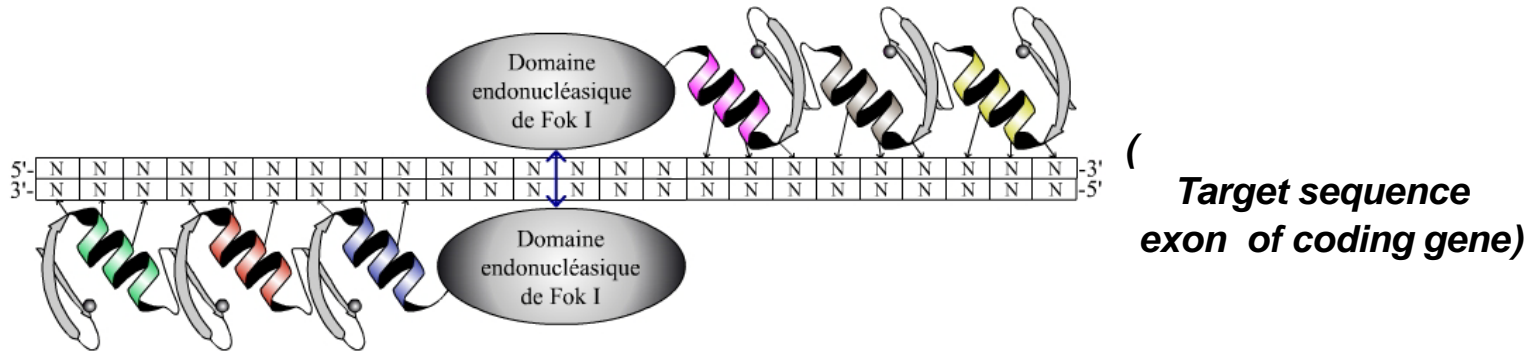
www.ifr26.nantes.inserm.fr/ITERT/transgenese-rat/

Tesson L. et al. Transg. Res. 2005
Aitman T. Nat. Genetics 2008

Zinc Finger Proteins



Zinc Finger Nucleases or chimeric nucleases



Homologous Repair

Non Homologous End - Joining (NHEJ)

(Template)



Homology-directed repair (HDR)
 Precise repair (user-defined)
Targeted Gene Integration

Gene disruption in ~70 % of NHEJ events by out of frame deletions
Targeted Gene Knockout

ZFN-driven Targeted Gene Knockout or Homologous Recombination

Xenopus		Bibikova et al., Mol Cell Biol 2001
Drosophila		Bibikova et al., Genetics 2002
eukaryotic cells	bone marrow (IL2Rγ)	Porteus & Baltimore, Science 2003
	CD4⁺ T cells (CCR5)	Perez et al., Nat. Biotech, 2008
	CHO	Santiago et al., PNAS 2008
	human ES and iPS cells	Hockemeyer et al. Nat. Biotech. 2009
Plants (Arabidopsis)		Lloyd et al., PNAS 2005
C.elegans		Morton et al., PNAS 2006
Zebrafish		Doyon et al., Nat. Biotech 2008
		Meng et al., Nat. Biotech 2008
Rat	one-cell embryos	Geurts et al., Science 2009

**Zinc-finger nucleases: a powerful tool for genetic engineering of animals.
S. Rémy et al. Transgenic Res 2009 in press.**

Methods assembly:

- Direct
- 1+1 context-sensitive (ZFN consortium)
- 2+2 context-sensitive (Sangamo-Sigma)

Pros and cons

- time
- effort
- cost
- control of technology
- academic platform ?



Combinatorial ZFN Assembly of 6bp (2 ZFNs) Modules



The Zinc Finger Consortium

to promote continued research and development of engineered zinc finger technology



<http://www.zincfingers.org/default2.htm>

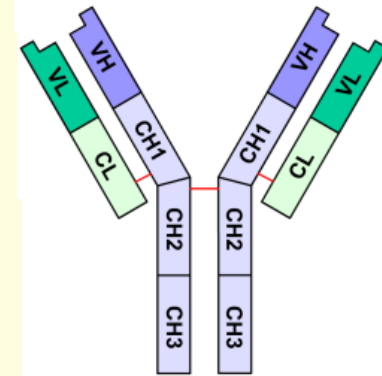
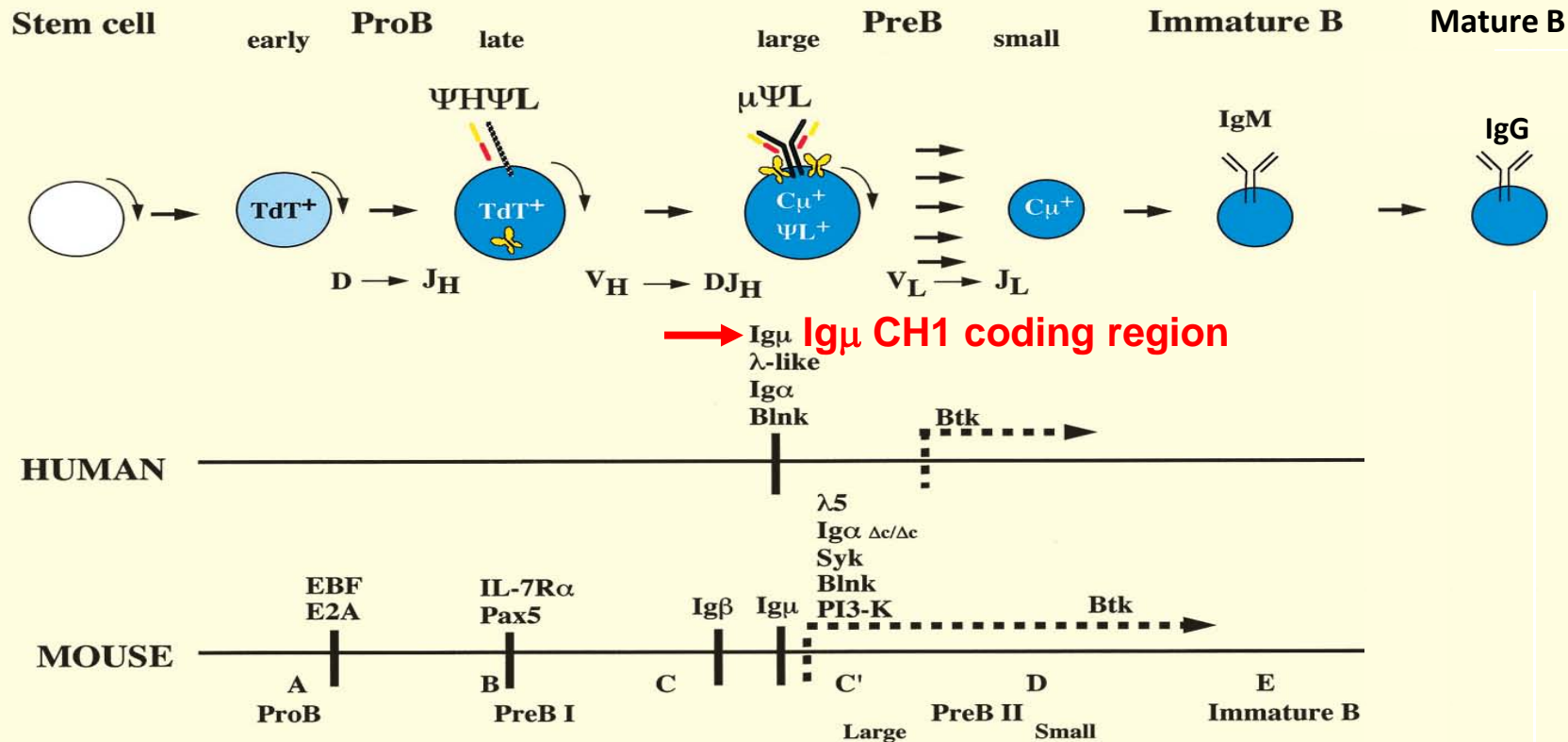
ENTER SITE



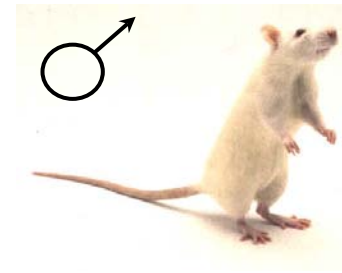
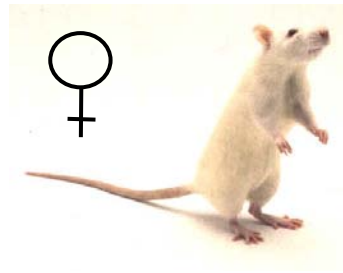
Objective: to generate KO rats for different Ig loci.

Create:

- Rat models for the role of B cells and antibodies in immune-mediated diseases
- Production of human antibodies (Open Monoclonal Technologies)



Targeted Mutagenesis in Rat Embryos



Sprague Dawley



one-cell embryo

ZFN1-ZFN2

- mRNA (capped and polyA)
- DNA plasmid (circular)



2.87 kb

Transfer to
pseudopregnant
females



Extract DNA to look
for ZFN activity



CAGp: CAG promoter

2A: 2A self-splicing sequence

pA: polyA

ZFP-mediated Mutation of Rat IgM

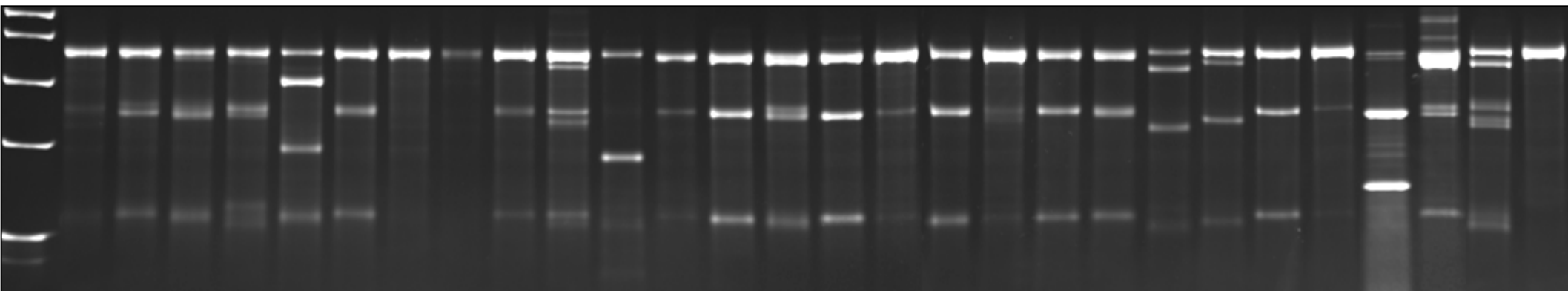
26 mutants

CEL-1 or Surveyor assay (detection of 1 nt mismatch)

Lead ZFNs
in C6 cells

6 7 8 19 46 70 81 107 116 119 122 128 130 147 161 167 174 212 1.1 1.2 4.1 3.3 5.3 9.2 9.1 8.1

Wild-type



10 ng/uL
DNA

2 ng/uL
DNA

0.4 ng/uL
DNA

10 ng/uL 2 ng/uL 10 ng/uL 2 ng/uL
cytoplasm PNI
mRNA

ZFP-mediated Mutation of Rat IgM

Target	Construct	Route	Dose (ng/μl)	Injected / Transferred (%)	New borns (%)	*Mutants (%) **
IgM	Plasmid	PNI	10	609/493 (81)	54 (11.0)	6 (11.1)
IgM	Plasmid	PNI	2	605/468 (77)	82 (17.5)	8 (9.8)
IgM	Plasmid	PNI	0.4	511/423 (77)	62 (14.7)	4 (6.5)
IgM	mRNA	PNI	10	186/104 (55.9)	14 (13.5)	4 (28.6)
IgM	mRNA	PNI	2	230/142 (61.7)	21 (14.8)	4 (19)
IgM	mRNA	PNI	0.4	183/118 (64.5)	19 (16.1)	1 (5.3)
IgM	mRNA	Cytoplasmic	10	272/197 (72)	4 (2)	3 (75)
IgM	mRNA	Cytoplasmic	2	197/134 (68)	17 (12.7)	2 (11.7)

PNI; pronucleus injection

* % of transferred

** % of new borns

Microinjection DNA or mRNA one-cell embryos =2,793

Newborns=273

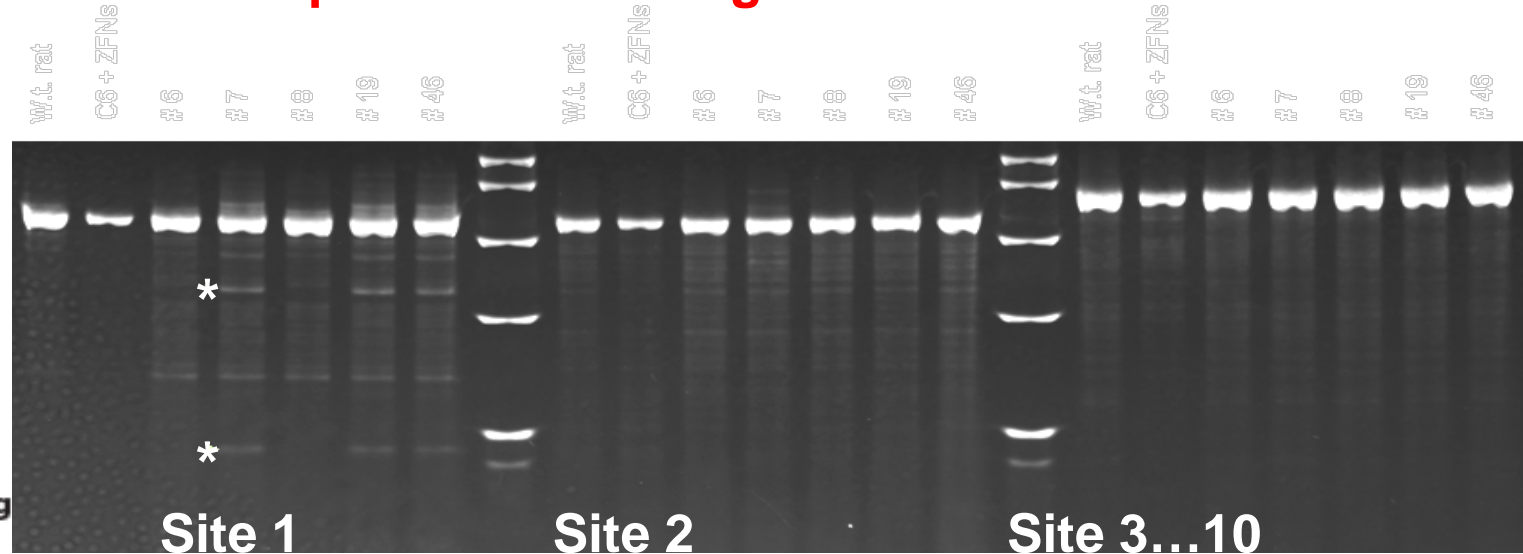
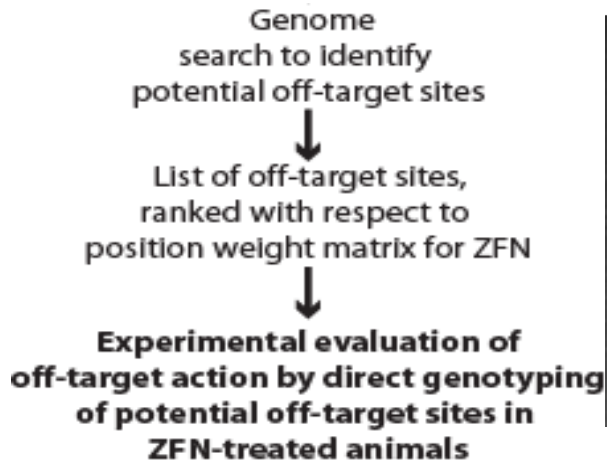
Mutated=32

Analysis of deletions in ZFN C μ KO rats

<u>Rat</u>	<u>wild type sequence</u>	<u>mutant sequence</u>	
wild-type	+	-	
6	+	$\Delta 9$	
7	+	$\Delta 5, \Delta 13, \Delta 15, \Delta 18, \Delta 39$	
8	+	$\Delta 3, \Delta 23$	
19	+	$\Delta 64$	→ homozygous rats derived
46	+	$\Delta 5$	
81	+	$\Delta 3$	
107	+	$\Delta 5$	
119	+	$\Delta 187$	
122	- ○	$\Delta 3, \Delta 4$	
1 .1	+	$\Delta 5$	
1.2	+	$\Delta 39$	
3.3	+	$\Delta 1$	
4.1	+	$\Delta 25$	
8.1	+	$\Delta 29, \Delta 2$	
9.2	+	$\Delta 224, \Delta 123$	

Minimal Off-Target Effects

Screen of 10 predictive off-target sites in 5 mutant founders



- 9/10 potential off-target sites are wild-type in all genome-edited pups.
- For site 1, 3/5 founders reveal ~3% gene disruption (vs. >50% disruption at IgM) due to a SNP found in the outbred SD strain. The gene is not linked to the IgM locus.

Doyon et al.
Nature Biotechnology 2008

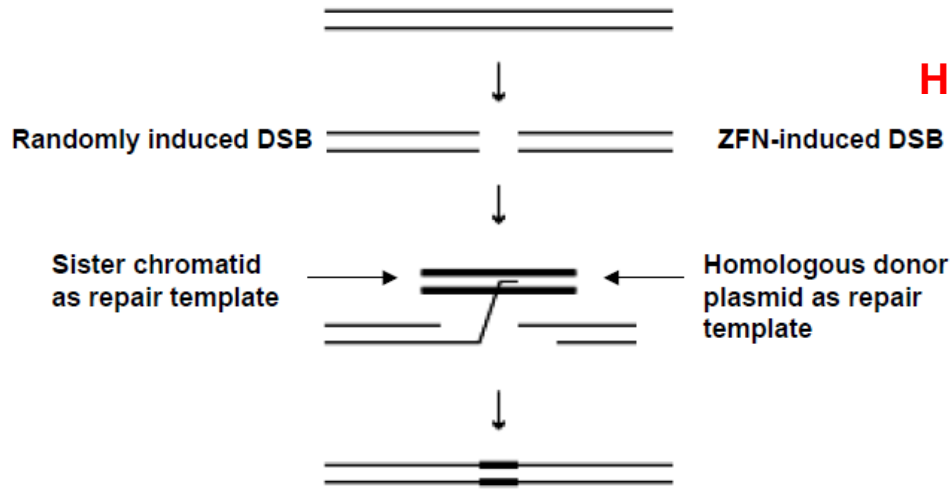
Advantages of ZFN-Mediated Gene KO Technology

- Targeted, rapid, single-step, and permanent KO in rat embryos (inbred and outbred strains)
- Possibility of generating KO in many species with no ES cells
- Also in mice in 1/3 of the time vs. ES cells
- Even of genes non-expressed in eggs
- Minimal off-target effects
- Ig and B cell-deficient rats available for immune models and biotechnology
- Several ZFNs underway

Questions

- How to increase efficiency design of ZFNs ?
- increase efficiency delivery methods ?
- are all genomic sequences equally accessible to ZFNs ?

ZFN-driven Targeted Gene Integration



HR increases during HDR : ~ 1 event in 10^6 cells

HR ~ 1 event in 10^2 cells

Perspective : homologous recombination in zygotes using ZFNs

- Reporter KI, point mutations KI, cDNA KI (expression-permissive sites), conditional alleles

Drosophile

Beumer et al., *PNAS* 2008; Bozas et al., 2009

Rat embryos

in progress

