New methods for gene engineering in animals

Technical Journal Club – Special Series on Laboratory Animal Science

Caihong Zhu

07.06.2016

Overview

I. Definition of “gene engineering”

II. History of gene engineering in animals

III. Recently developed gene engineering methods

IV. Future directions

I. Definition of “gene engineering”

- Gene engineering, gene editing, genetic engineering, genetic modification, etc; Insert, remove, correct or disrupt organisms’ genome using biotechnology;
- Genetically modified organisms (GMO), germline transmissible;
- **Transgenesis – transgenic animals;**
- **Gene targeting – knock-out/knock-in animals.**

X Selective breeding;

X Microbes, worms, flies, fishes, plants;

X Synthetic biology.

II. History of gene engineering in animals

- First animals carrying **experimentally introduced foreign genes** were generated in 1974;
- SV40 gene into mice;
- Germline transmission?

Proc. Nat. Acad. Sci. USA
Vol. 71, No. 4, pp. 1250–1254, April 1974

Simian Virus 40 DNA Sequences in DNA of Healthy Adult Mice Derived from Preimplantation Blastocysts Injected with Viral DNA

(blastocyst microinjection *in vitro*/development/DNA reassociation kinetics of simian virus 40)

RUDOLF JAENISCH* AND BEATRICE MINTZ†

* Armand Hammer Center for Cancer Biology, Salk Institute for Biological Studies, San Diego, California 92112; and † Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111

II. History of gene engineering in animals

- First **transgenic mouse strain** was generated in 1976;
- Moloney leukemia retrovirus (M-MuLV) into mice;
- Germline transmissible.

Proc. Nat. Acad. Sci. USA
Vol. 73, No. 4, pp. 1260–1264, April 1976
Cell Biology

Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus

(*in vitro* infection of 4–8 cell embryos with exogenous Moloney leukemia virus/leukemia/genetic transmission/DNA annealing kinetics/gene amplification)

RUDOLF JAENISCH

Tumor Virology Laboratory, The Salk Institute, P.O. Box 1809, San Diego, California 92112

II. History of gene engineering in animals

- First transgenic mice generated by **microinjection** of recombinant DNA into a pronucleus of fertilized eggs in 1980;
- SV40 and HSVTK into mice;
- Most widely used technical in transgenesis.

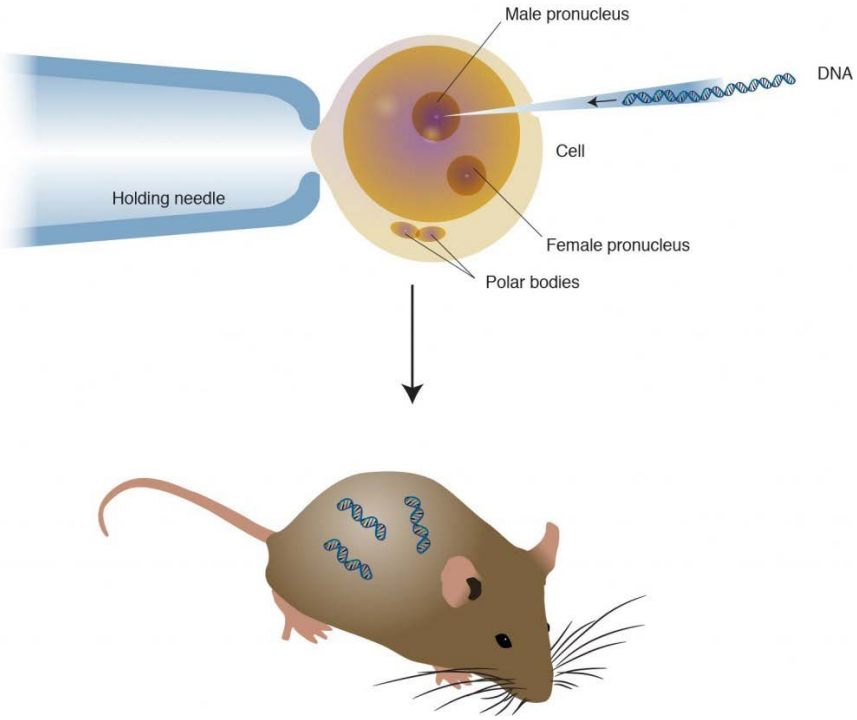
Proc. Natl. Acad. Sci. USA
Vol. 77, No. 12, pp. 7380–7384, December 1980
Genetics

Genetic transformation of mouse purified DNA

(gene transfer/mice)

JON W. GORDON*, GEORGE A. SCANGOS†, DIANE J. FRANK H. RUDDLE*‡

*Department of Biology and ‡Department of Human Genetics, Yale University



II. History of gene engineering in animals

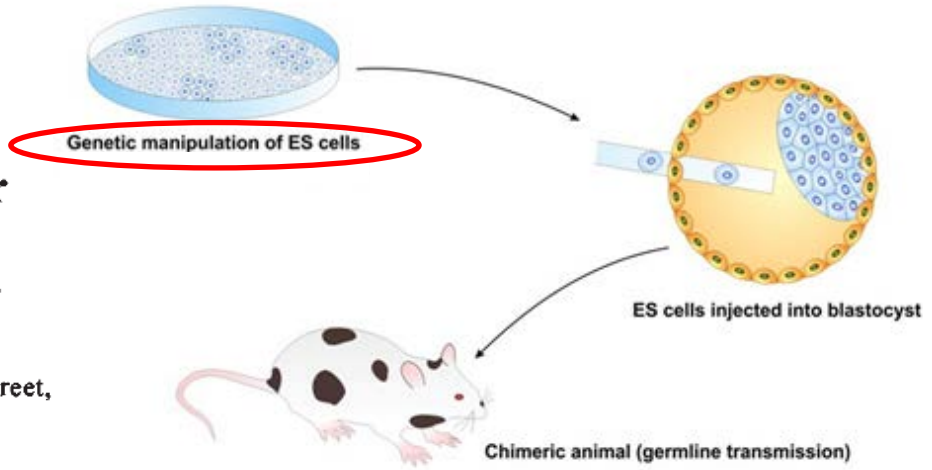
- **Embryonic stem cell**-mediated transgenesis in 1986;
- Transduction or transfection of foreign DNA into ES cells, followed by injection of recombinant ES cells into blastocytes;
- Germline transmission of *neo* gene introduced into ES;
- In vitro genetic manipulation of ES cells.

NATURE VOL. 323 2 OCTOBER 1986

Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector

Elizabeth Robertson, Allan Bradley, Michael Kuehn & Martin Evans

Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK



II. History of gene engineering in animals

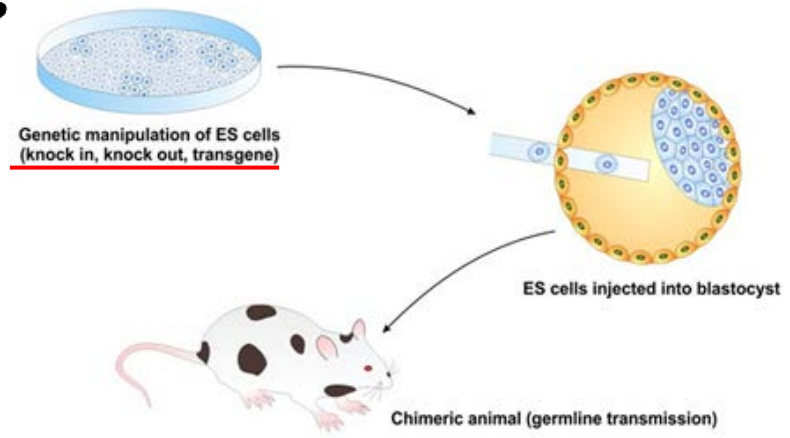
- First **targeted** gene modification in 1987;
- Targeting vector containing homologous arm transfected into ES cells, selection of cell colonies with right homologous recombination, followed by injection of recombinant ES cells into blastocytes;

- Knockout of *HPRT* (hypoxanthine phosphorobosyl transferase) gene in mice.

Cell, Vol. 51, 503–512, November 6, 1987, Copyright © 1987 by Cell Press

Site-Directed Mutagenesis by Gene Targeting in Mouse Embryo-Derived Stem Cells

Kirk R. Thomas and Mario R. Capecchi
Department of Biology
University of Utah
Salt Lake City, Utah 84112



II. History of gene engineering in animals

- First ***Prnp*** gene knockout mice in 1992;
- PrP $aa_{4\text{-}187}$ were replaced by a P_{HSVTK}-*neo* cassette;
- *Prnp*$^{-/-}$ mice develop and behave largely normally, without apparent immunological defects.

NATURE · VOL 356 · 16 APRIL 1992

Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein

Hansruedi Büeler, Marek Fischer, Yolande Lang*, Horst Bluethmann*, Hans-Peter Lipp†, Stephen J. DeArmond‡§, Stanley B. Prusiner§‖, Michel Aguet & Charles Weissmann

Institut für Molekularbiologie I, Universität Zürich, 8093 Zürich, Switzerland
* PRTB, Hoffmann-LaRoche, 4002 Basel, Switzerland
† Anatomisches Institut, Universität Zürich, 8057 Zürich, Switzerland
Departments of ‡ Pathology, § Neurology, and ‖ Biochemistry and Biophysics, University of California, San Francisco, California 94143, USA

The Nobel Prize in Physiology and Medicine 2007

Photo: U. Montan
Mario R. Capecchi
Prize share: 1/3

Photo: U. Montan
Sir Martin J. Evans
Prize share: 1/3

Photo: U. Montan
Oliver Smithies
Prize share: 1/3

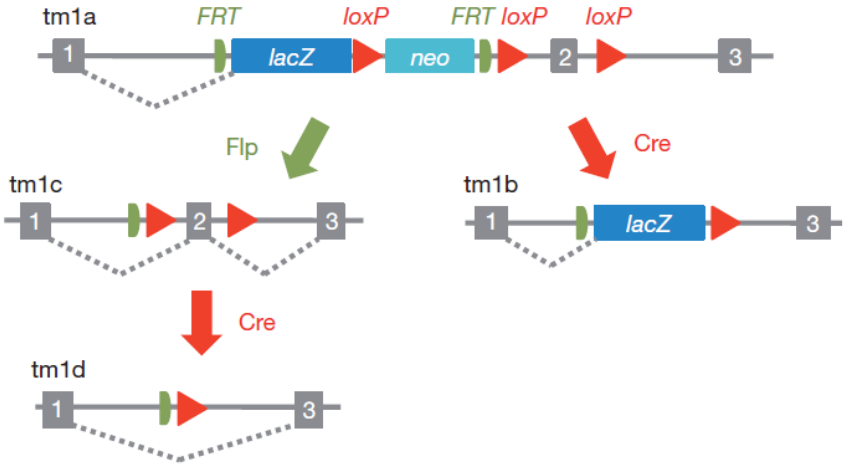
The Nobel Prize in Physiology or Medicine 2007 was awarded jointly to Mario R. Capecchi, Sir Martin J. Evans and Oliver Smithies *"for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells"*.

http://www.nobelprize.org/

International knockout mice consortium (IKMC)

- Knockout Mouse Project (KOMP) – USA
- European Conditional Mouse Mutagenesis Project (EUCOMM) – Europe
- North American Conditional Mouse Mutagenesis Project (NorCOMM) – Canada
- Texas A&M Institute for Genomic Medicine (TIGM)- USA

The members of the IKMC are working together to mutate **ALL** protein-coding genes in C57BL/6**N** mouse embryonic stem (ES) cells



tm1a: knockout first allele
tm1b: lacZ tagged allele
tm1c: conditional allele
tm1d: null allele

Skarnes, Nature, 2011

II. History of gene engineering in animals

- Transgenic **rabbits**, **sheep** and **pigs** were generated in 1985 by microinjection;
- Transgene: MT-hGH: $P_{\text{mouse metallothionein I}}$-human growth hormone;
- Transgenic rabbit and pigs expressed recombinant hGH.

NATURE VOL. 315 20 JUNE 1985

Production of transgenic rabbits, sheep and pigs by microinjection

Robert E. Hammer*, Vernon G. Pursel†, Caird E. Rexroad Jr†, Robert J. Wall†, Douglas J. Bolt†, Karl M. Ebert*, Richard D. Palmiter‡ & Ralph L. Brinster*

* Laboratory of Reproductive Physiology, School of Veterinary Medicine University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA
† Reproduction Laboratory, Agricultural Research Service, USDA, Beltsville, Maryland 20705, USA
‡ Howard Hughes Medical Institute, Department of Biochemistry, University of Washington, Seattle, Washington 98195, USA

II. History of gene engineering in animals

- Functional embryonic stem cells other than mouse (established in 1981) and rat (generated in 2008) have not been established;
- ES cell-mediated gene manipulation in other animas is not possible;
- Targeted gene engineering in other animals?

II. History of gene engineering in animals

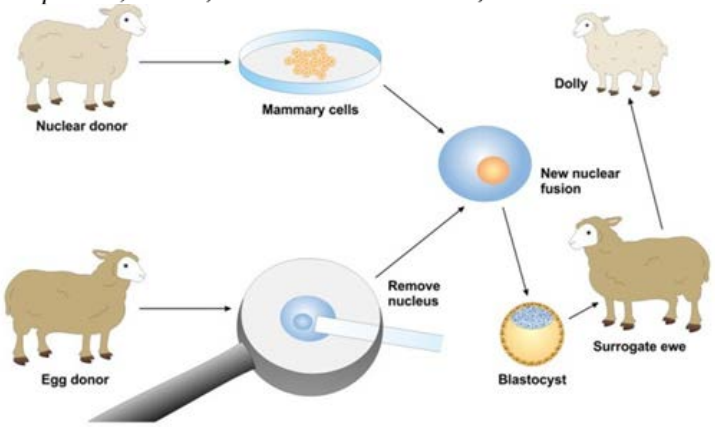
- Cloned animals from somatic cells (adult mammary epithelial cells, fetal fibroblasts) using somatic cell nuclear transfer (SCNT) in 1997;
- Manipulation of somatic cells in vitro, followed by SCNT?

NATURE | VOL 385 | 27 FEBRUARY 1997

Viable offspring derived from fetal and adult mammalian cells

I. Wilmut, A. E. Schnieke*, J. McWhir, A. J. Kind* & K. H. S. Campbell

Roslin Institute (Edinburgh), Roslin, Midlothian EH25 9PS, UK
** PPL Therapeutics, Roslin, Midlothian EH25 9PP, UK*



II. History of gene engineering in animals

- Primary fetal fibroblast co-transfected with *neo* and β-lactoglobulin (BLG)-human coagulation factor IX;
- Neomycin resistant cells were used as donor for SCNT;
- Viable cloned sheep containing transgenes were produced;
- Half animals required for SCNT compared to microinjection.

SCIENCE • VOL. 278 • 19 DECEMBER 1997 • www.sciencemag.org

Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts

Angelika E. Schnieke,* Alexander J. Kind, William A. Ritchie, Karen Mycock, Angela R. Scott, Marjorie Ritchie, Ian Wilmut, Alan Colman, Keith H. S. Campbell†

A. E. Schnieke, A. J. Kind, K. Mycock, A. R. Scott, A. Colman, PPL Therapeutics, Roslin, Midlothian, EH25 9PP, Scotland, UK.
W. A. Ritchie, M. Ritchie, I. Wilmut, K. H. S. Campbell, Roslin Institute, Roslin, Midlothian, EH25 9PS, Scotland, UK.

II. History of gene engineering in animals

- Primary fetal fibroblast transfected with gene targeting vector containing IRES-*neo* and β-lactoglobulin (BLG)-human α1 antitrypsin (AAT), targeting ovine a1(I) procollagen (COL1A1) locus;
- Neomycin resistant cells were screened for correct homologous recombination and used as donor for SCNT;
- Alive cloned sheep were produced, the cloned sheep expressed high level of AAT.

NATURE | VOL 405 | 29 JULY 2000 | www.nature.com

Production of gene-targeted sheep by nuclear transfer from cultured somatic cells

K. J. McCreath , J. Howcroft, K. H. S. Campbell*, A. Colman, A. E. Schnieke & A.J. Kind

PPL Therapeutics Ltd, Roslin, Edinburgh, EH25 9PP, UK
** Present address: University of Nottingham, School of Biological Sciences, Division of Animal Physiology, Loughborough, LE12 5RD, UK*

II. History of gene engineering in animals

- *PRNP* knockout cattle and goats were generated by gene targeting in somatic cells and SCNT in 2007 and 2009

Richt, Nat Biotechnol, 2007
Yu, Mol Reprod Dev, 2009
Zhu, Transgenic Res, 2009

Limitations of conventional gene targeting methodology

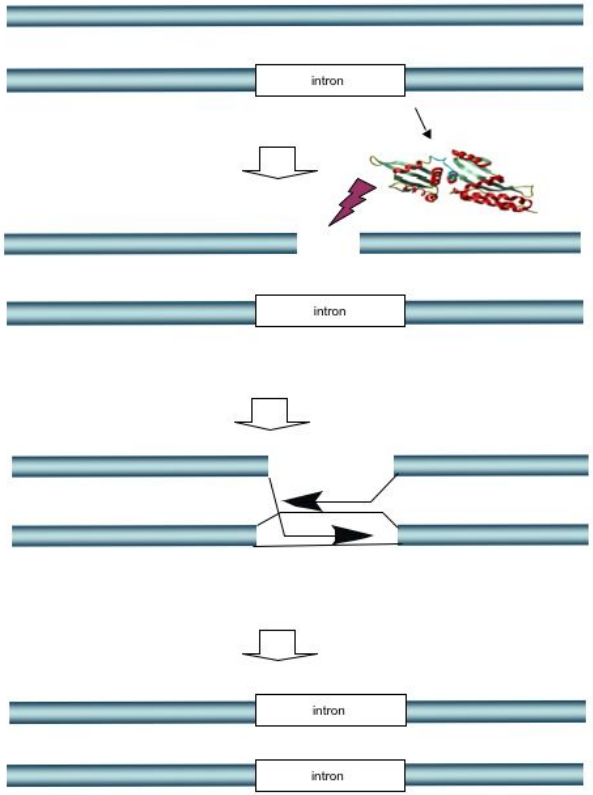
- Low frequency of gene targeting v.s random integration by conventional method (1 in 10^5-10^6 transfected cells or 0.1% in selected clones);

- Vector optimization: positive-negative selection, promoter-less trap, homology length, isogenic vectors, viral vectors – modest effect!!!

- One allele is targeted, time consuming (>1 year) to get KO mice, longer time needed (2-3 years) for large animals.

III. Recently developed gene engineering methods

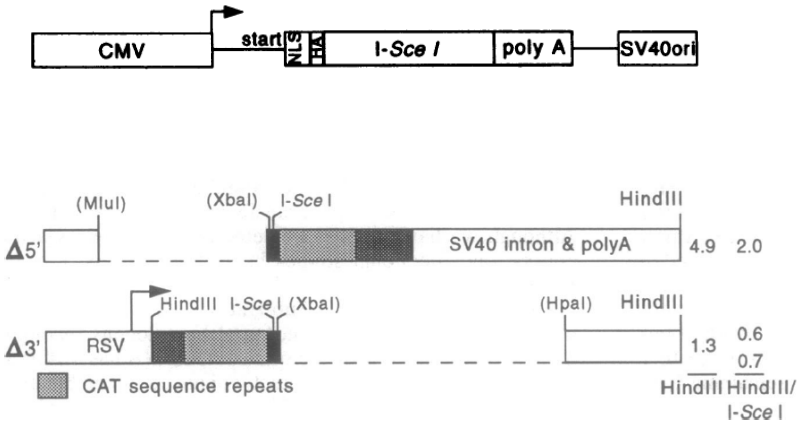
- Meganucleases: intron homing endonuclease, with large recognition site 12-40bp. Meganuclease recognizes the intron-less genes and introduces DSB, followed by inserting their own sequences into the target, resulting duplication.

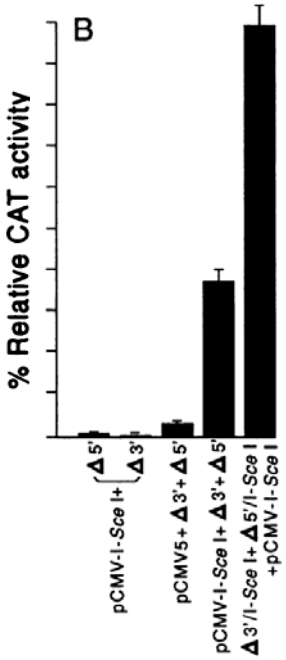


Wikipedia

Meganuclease

- Yeast I-SceI recognizes 18bp site;
- **Double strand break** (DSB) enhances intermolecular recombination efficiency by >1000-fold in mammalian cells;
- **No** recognition site found in human or mouse genome;
- Engineering of meganucleases is **difficult**.





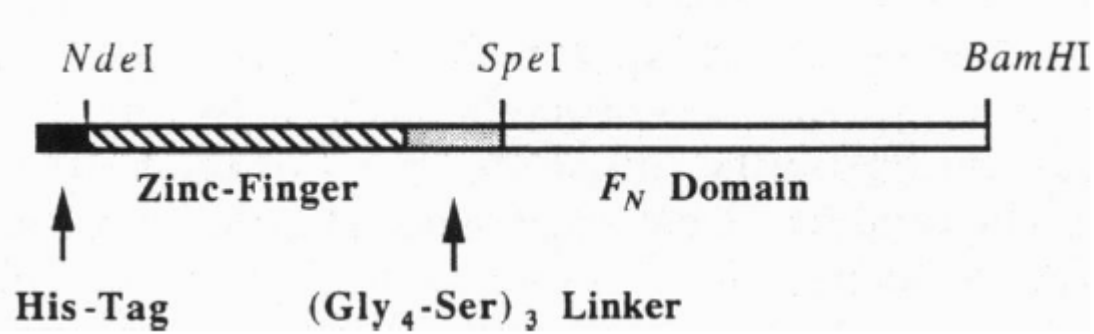
Rouet, PNAS, 1994

III. Recently developed gene engineering methods

- Zinc finger: a small protein structural motif, found in a common transcription factor family in organisms ranging from yeast to human, discovered in 1985. Each finger has 30 aa, folds in ββα configuration, recognizes **triplets** of DNA;

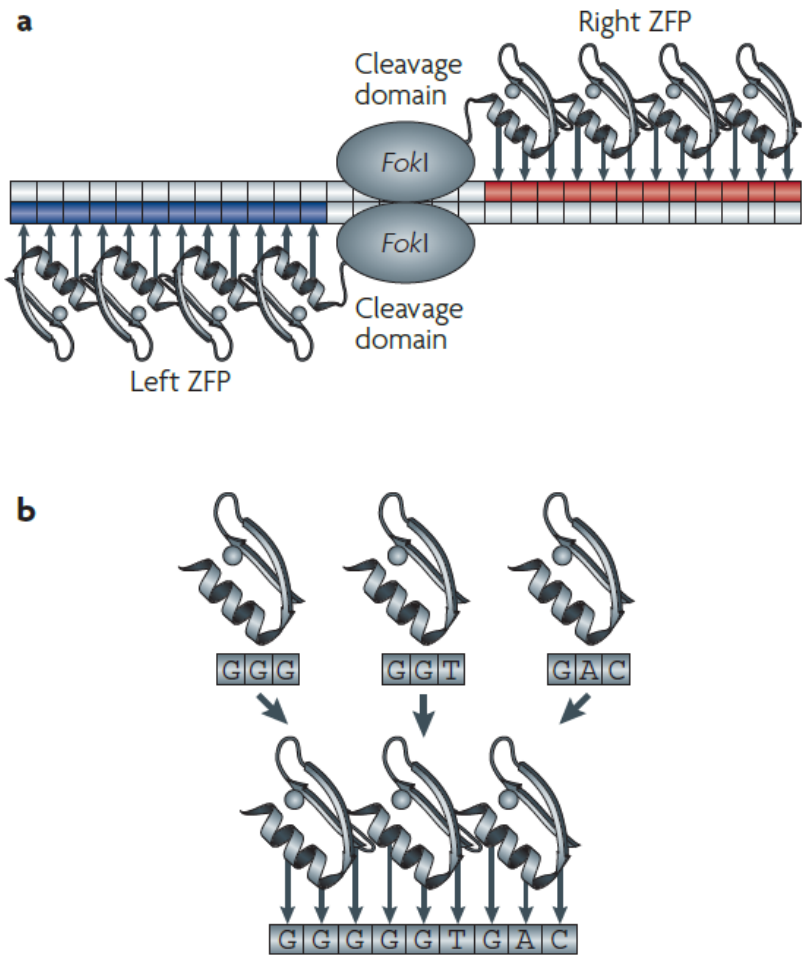
- The specificity of recognition site could be changed by altering the amino acids in zinc fingers;

- Zinc finger nuclease (ZFN): fused 3-6 zinc finger DNA binding domain and nonspecific cleavage domain of FokI endonuclease.



Kim, PNAS, 1996

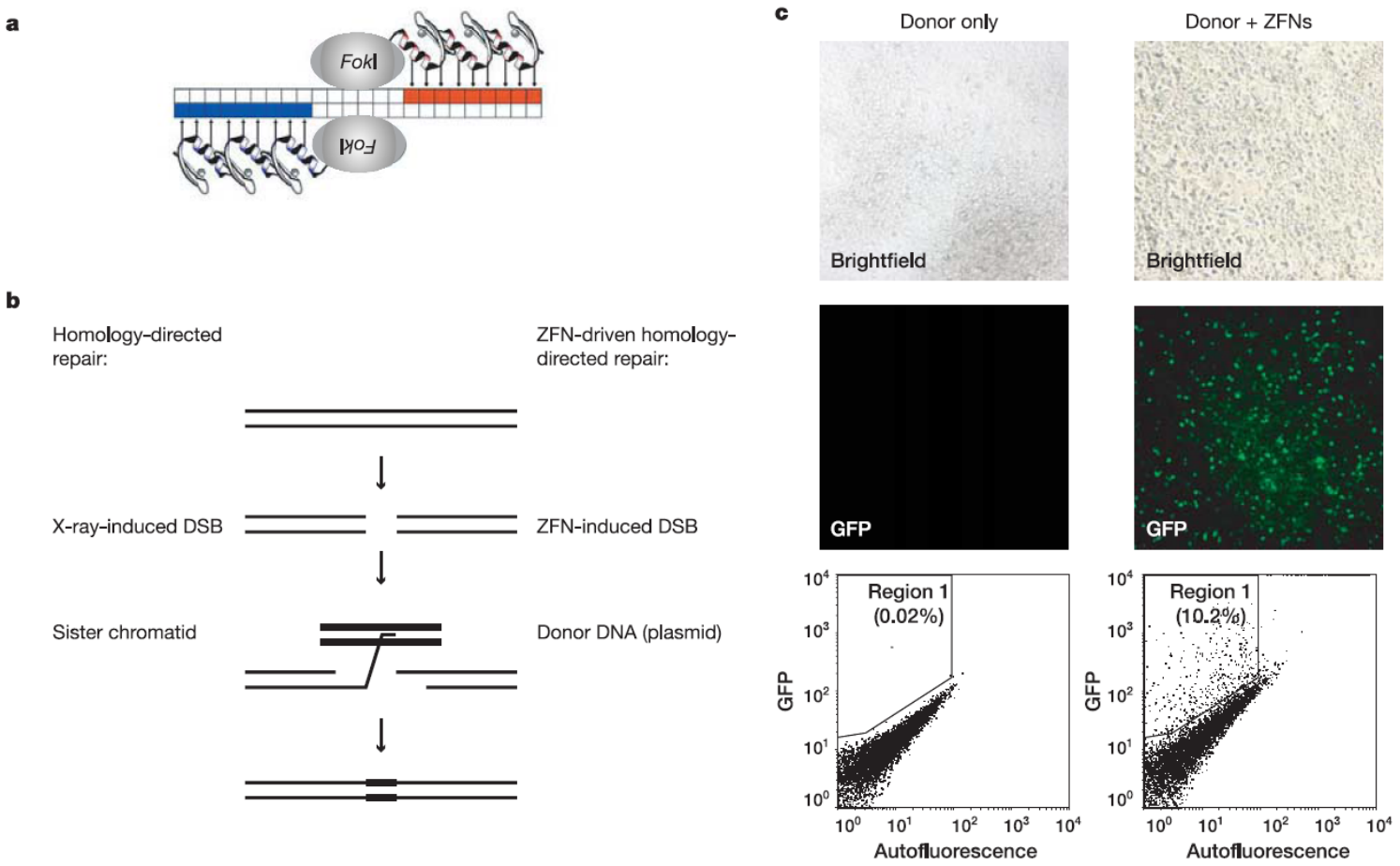
Zinc finger nuclease (ZFN)



Urnov, Nat Rev Genet, 2010

ZFN mediated gene correction

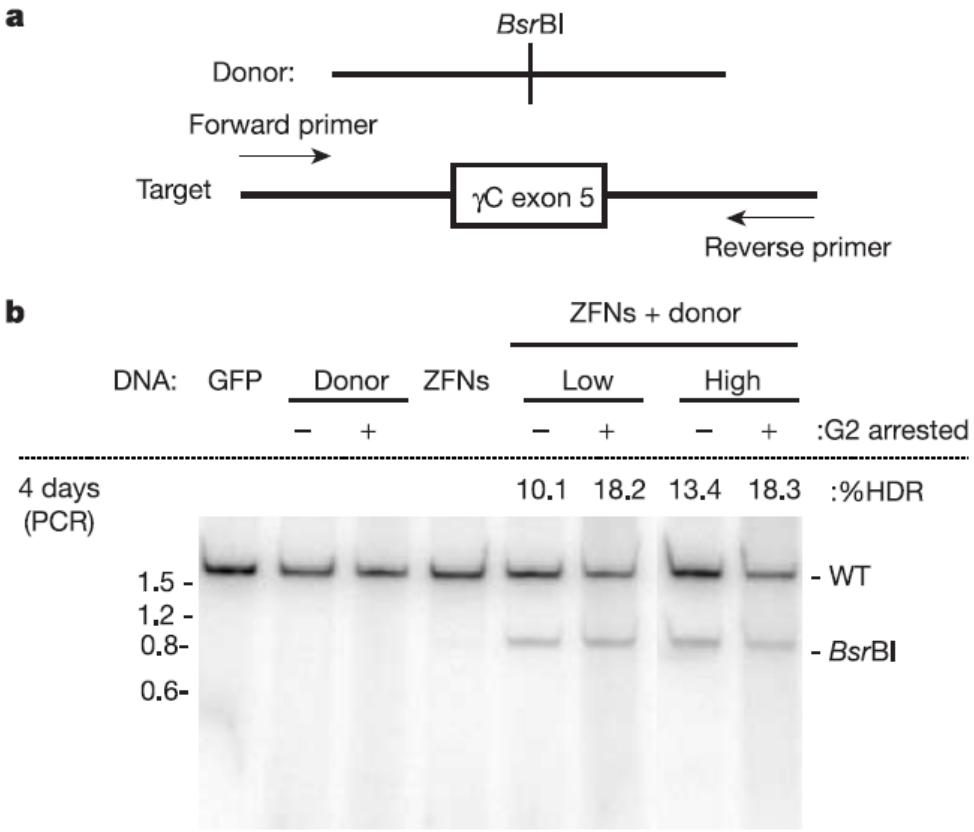
- Mutant GFP integrated in HEK 293 cells;
- ZFN targeting mutant site of the nonfunctional GFP;
- WT GFP donor plasmid.



Urnov, Nature, 2005

ZFN mediated gene correction

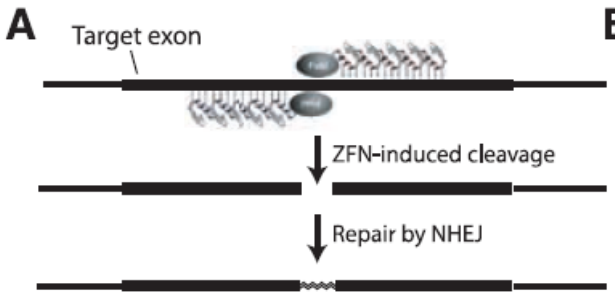
- Introducing a restriction site BsrBI in human endogenous gene *IL2Rγ* (encodes γC) site.

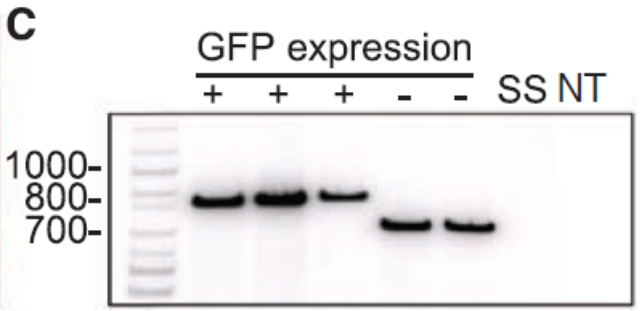


Urnov, Nature, 2005

ZFN mediated gene knockout in animals

- Knockout of GFP or IgM in rats (35/295, 12% mutated; 1 animal biallelic mutations in IgM; germline transmissible)





D

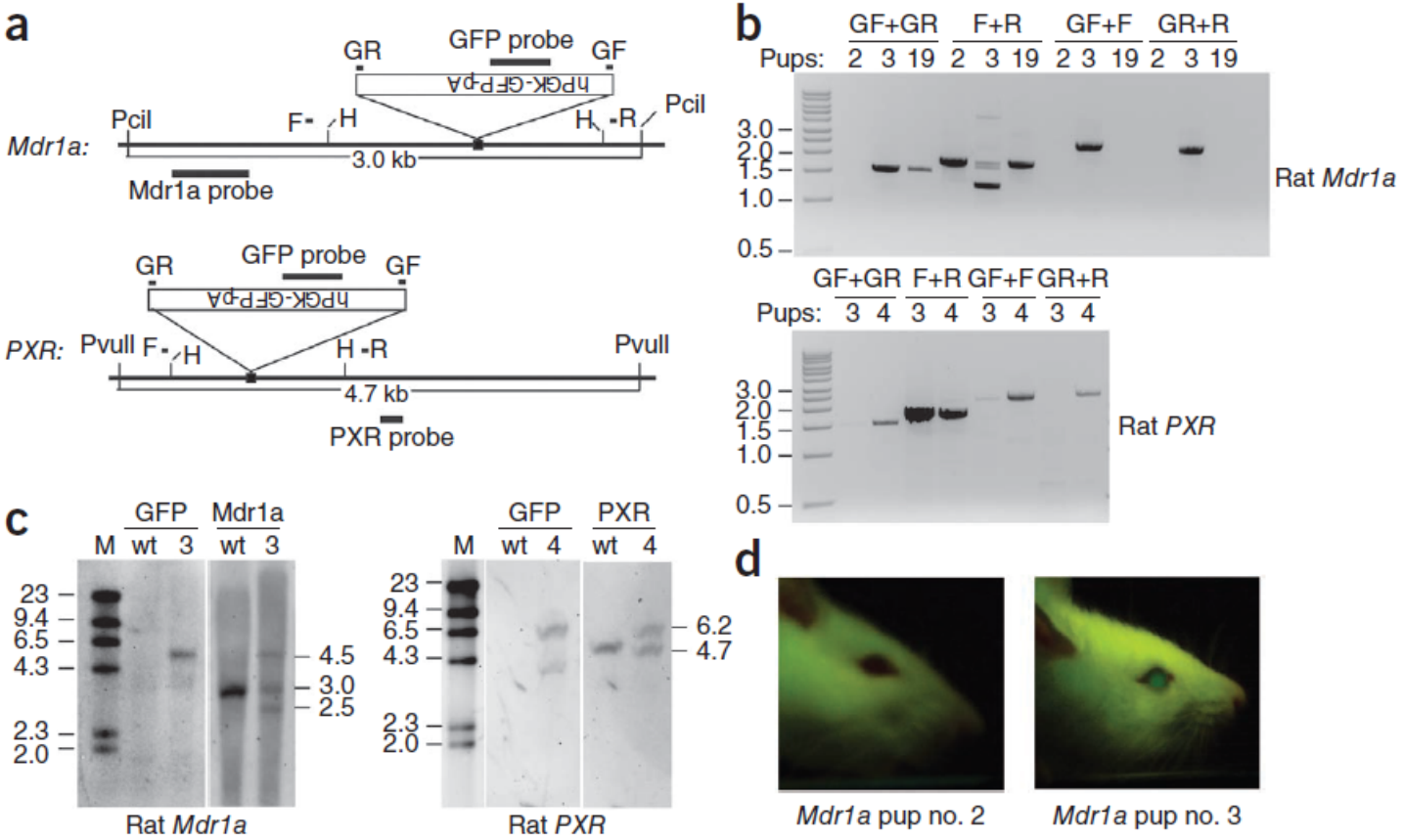
Strain	Target/ Construct	Route/ Dose (ng/ul)	Injected/ Transferred (%)	Founders (%)*	Mutants (%)†	Bred/ F1 inheritance
SS	GFP/mRNA	PNI/1.5	36/25 (69)	5 (20)	2 (40)	1/1
SD	IgM/Plasmid	PNI/10	609/493 (81)	54 (11)	6 (11)	4/3
SD	IgM/Plasmid	PNI/2	605/468 (77)	82 (18)	8 (10)	ND
SD	IgM/Plasmid	PNI/0.4	511/423 (83)	62 (15)	4 (6)	ND
SD	IgM/mRNA	PNI/10	186/104 (56)	14 (13)	4 (29)	ND
SD	IgM/mRNA	PNI/2	230/142 (62)	21 (15)	4 (19)	ND
SD	IgM/mRNA	PNI/0.4	183/118 (64)	19 (16)	1 (5)	ND
SD	IgM/mRNA	IC/10	272/197 (72)	4 (2)	3 (75)	ND
SD	IgM/mRNA	IC/2	197/134 (68)	17 (13)	2 (12)	ND
FHH	Rab38/mRNA	IC/2	91/83 (91)	17 (20)	1 (6)	ND

PNI - pronuclear injection; IC - intracytoplasmic; * - of transferred; † - of liveborn founders; ND - not determined

Geurts, Science, 2009

ZFN mediated gene knockin in animals

- Knockin GFP into mice and rats (HDR: 2.4-8.3%; NHEJ: 11.1-25.3%; germline transmissible)



Cui, Nat Biotechnnol, 2011

ZFN mediated genome editing

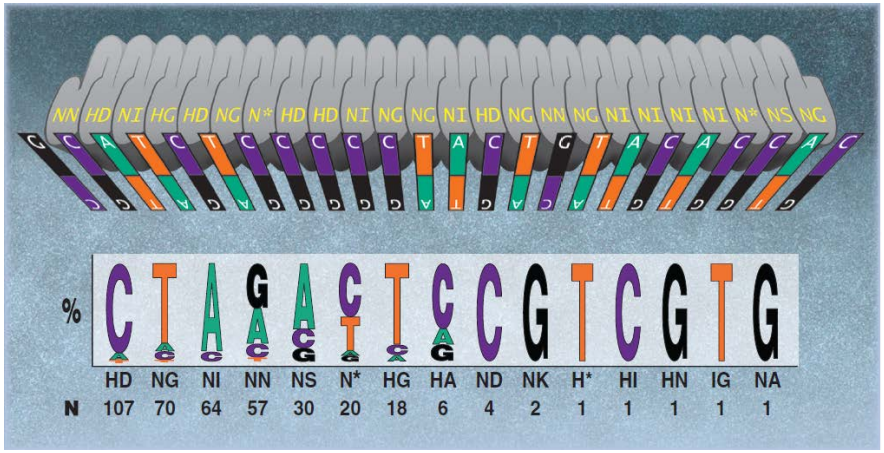
- Zinc finger consortium: www.zincfingers.org;
- Modular assembly method or Oligomerized Pool Engineering (OPEN) method;
- Zinc Finger Targeter program (ZiFiT) to assist to identify potential target site and to design ZFN;
- Zinc Finger database (ZiFDB) to search zinc fingers and zinc finger arrays;
- CompoZr platform from Sigma is commercially available.
- Design efficient and specific ZFN pairs is challenging; off-target etc.

Wright, Nat Protoc, 2006
Sander, Nucleic Acids Res, 2007
Maeder, Molecular Cell, 2008
Sander, Nat Methods, 2011

III. Recently developed gene engineering methods

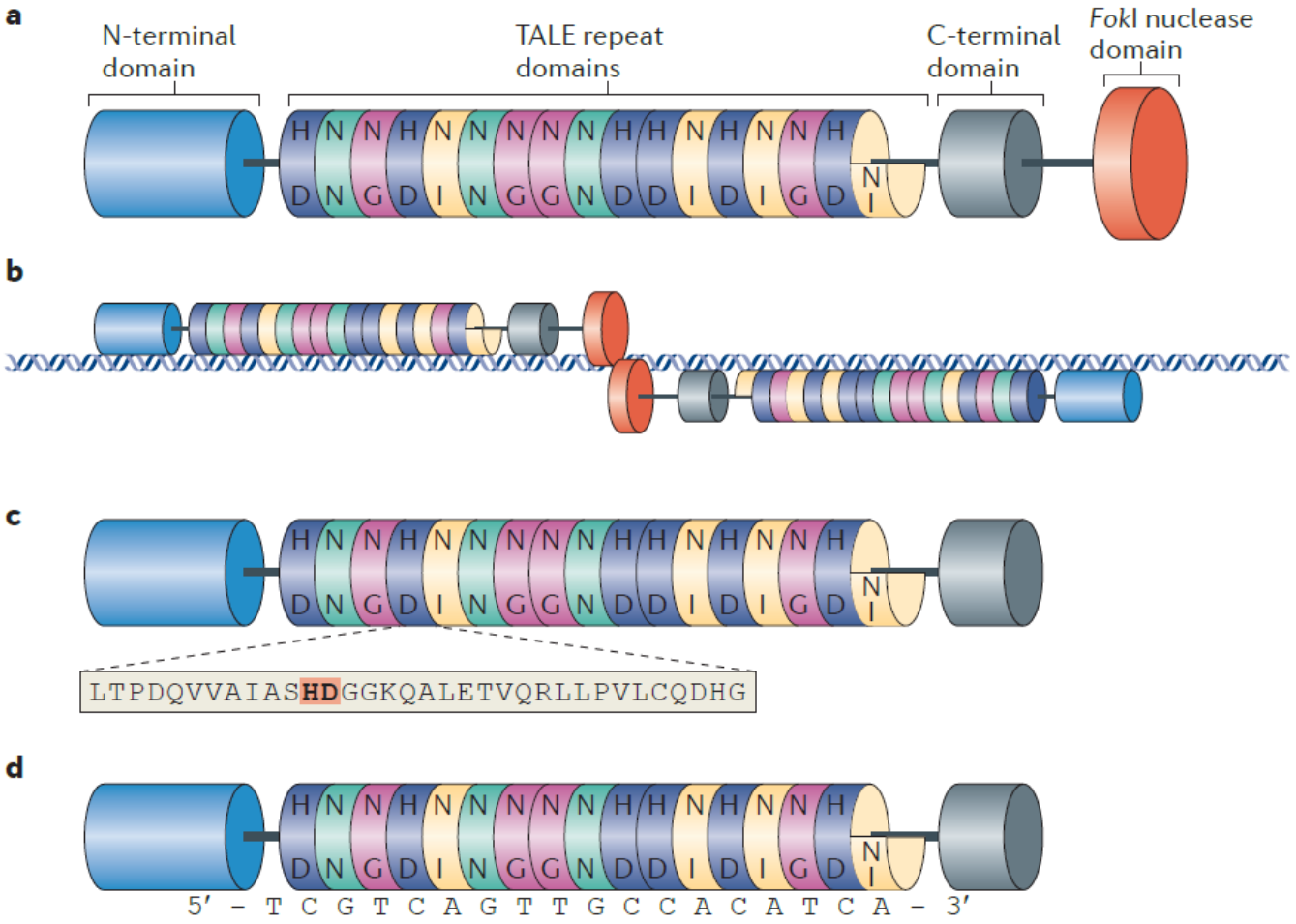
- Transcription activator-like (TAL) effectors: found in plant pathogenic bacteria, contain a central domain with tandem, polymorphic amino acid repeats (13-28 repeats) **independently** specify **single**, contiguous nucleotides in the target DNA. Typically composed of 34 aa per repeat. Position 12 and 13 in the repeats are polymorphic and called repeat-variable di-residue (RVD), bind to specific nucleotide and determine the specificity of TALEs.

- Transcription activator-like effector nuclease (TALEN): TALEs directed FokI nuclease, create site-specific DNA double-strand breaks.



Bogdanove, Science, 2011

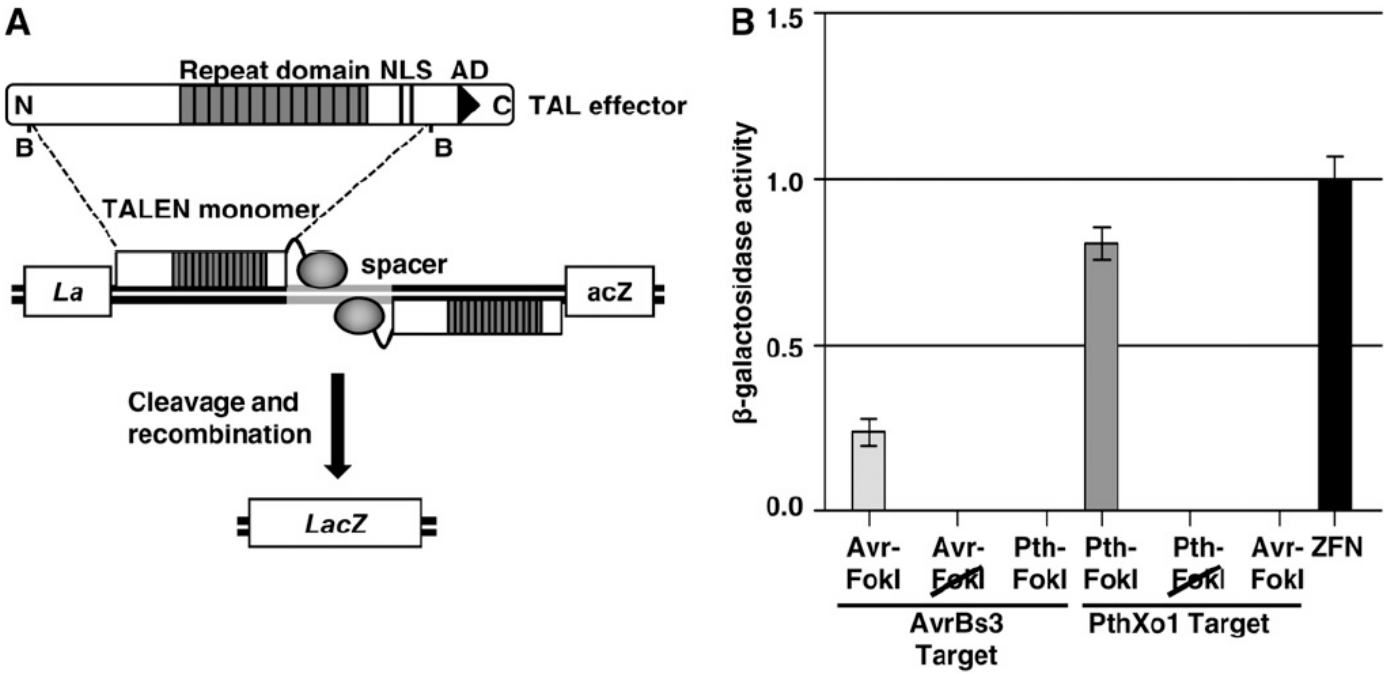
Transcription activator-like effector nuclease (TALEN)



Joung, Nat Rev Mol Cell Biol, 2013

TALEN mediated DNA cleavage and recombination

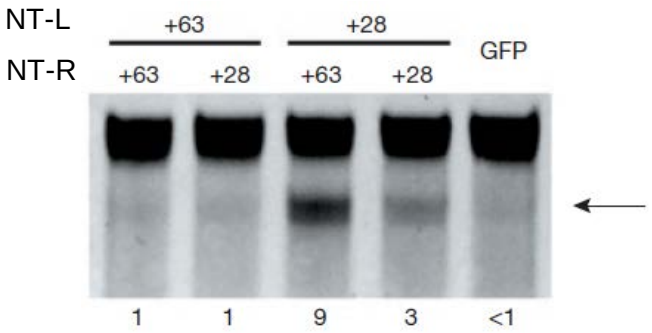
- DNA cleavage and recombination in bacteria



Christian, Genetics, 2010

TALEN mediated gene modification

- Modification of *NTF3* locus in human HEK 293 cells



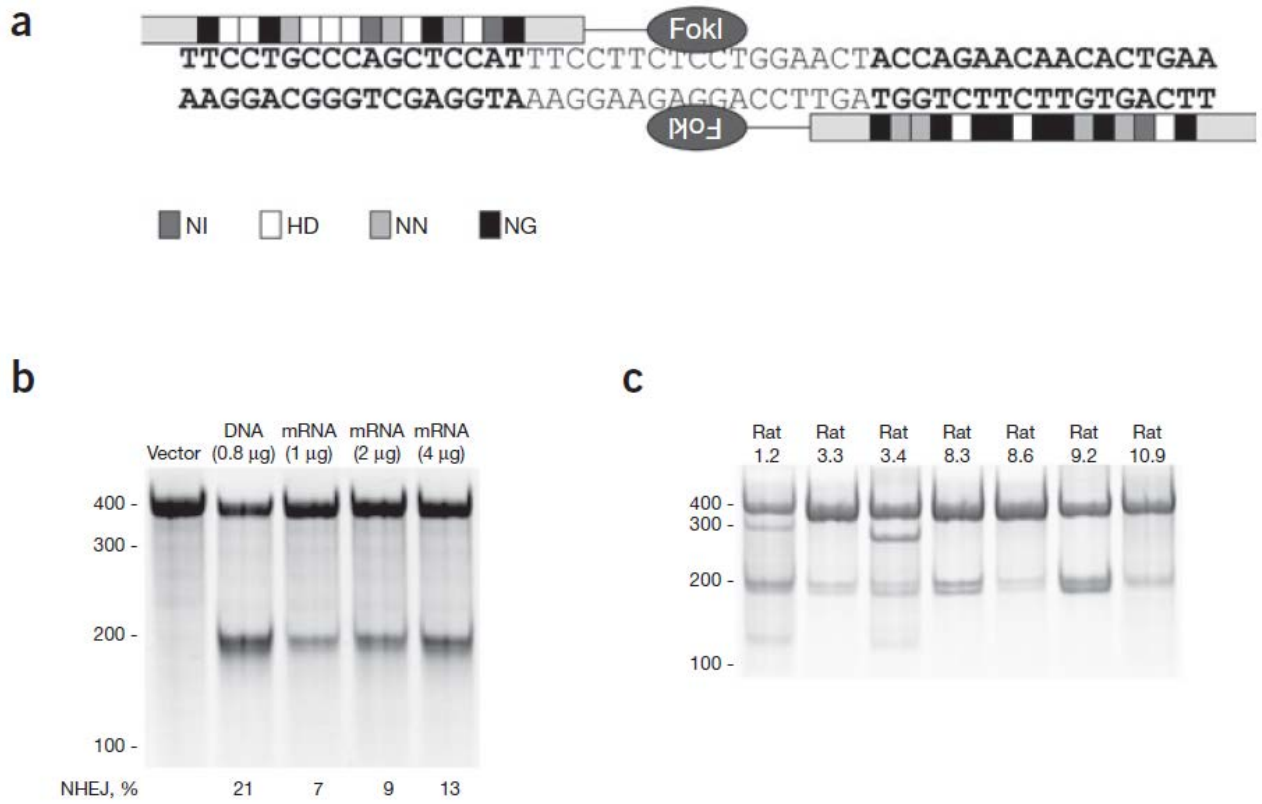

```
CGCGGAGCCATCTGGCCGGGTTGGCTGGTTATAACCGCGCAGATTCTGTTCACGGGACTCAGAGTTGAAGC

CGCGGAGCCATCTGGCCGGGTTGGCTG---ATAACCGCGCAGATTCTGTTCACGGGACTCAGAGTTGAAGC
CGCGGAGCCATCTGGCCGGGTTGGCTG----TAACCGCGCAGATTCTGTTCACGGGACTCAGAGTTGAAGC
CGCGGAGCCATCTGGCCGGGTTGG----TTATAACCGCGCAGATTCTGTTCACGGGACTCAGAGTTGAAGC
CGCGGAGCCATCTGG------------TTATAACCGCGCAGATTCTGTTCACGGGACTCAGAGTTGAAGC
CGCGGAGCCATCTGGCCGGGTTGGC------------GCAGATTCTGTTCACGGGACTCAGAGTTGAAGC
CGCGGAGCCATCTGGCCG------------------CGCAGATTCTGTTCACGGGACTCAGAGTTGAAGC
CGCGGAGCCATCTGGCCGGG------------------------------ACGGGACTCAGAGTTGAAGC
```

Miller, Nat Biotechnol, 2011

TALEN mediated knockout in animals

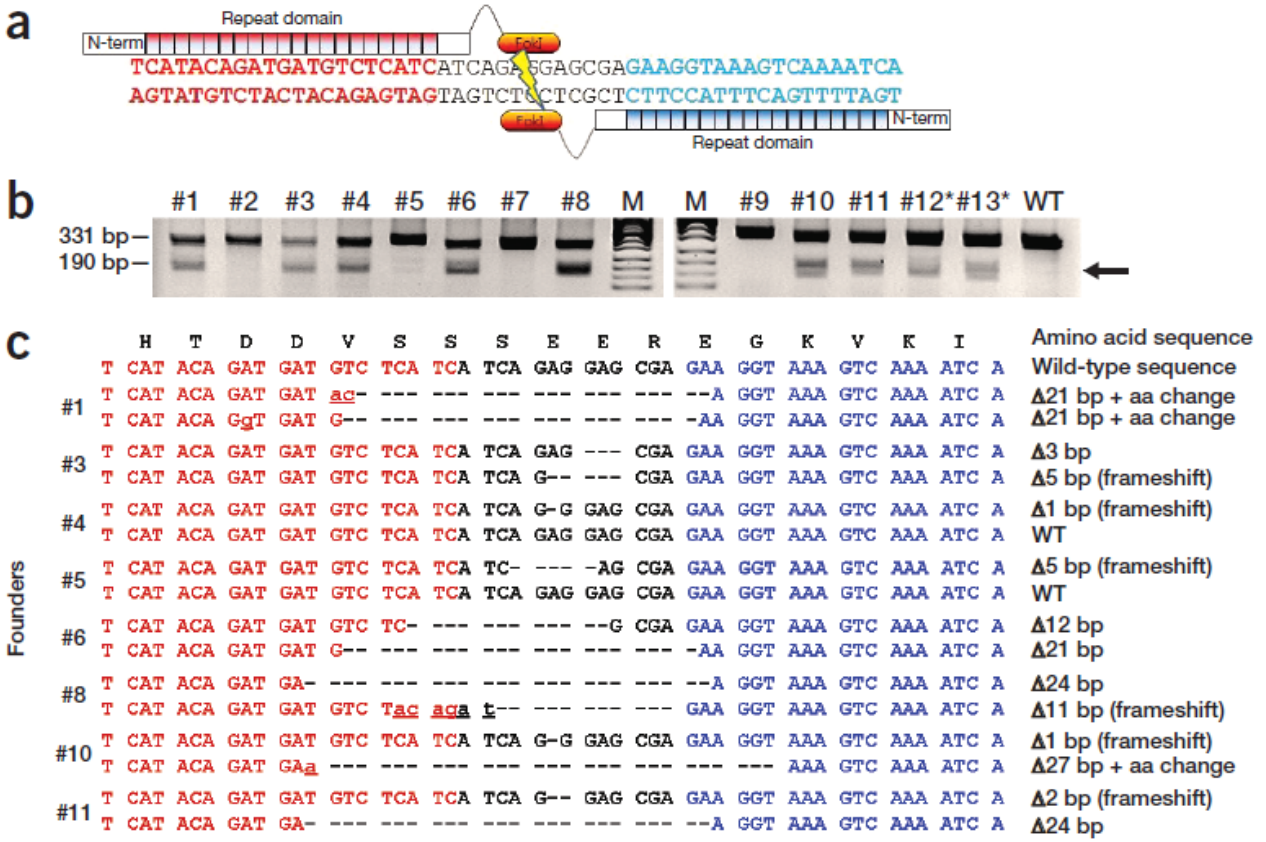
- Knockout of *IgM* in rats (51/88, 58% mutated; 13/52, 25% biallelically mutated in high dose)



Tessen, Nat Biotechnol, 2011

TALEN mediated knockout in animals

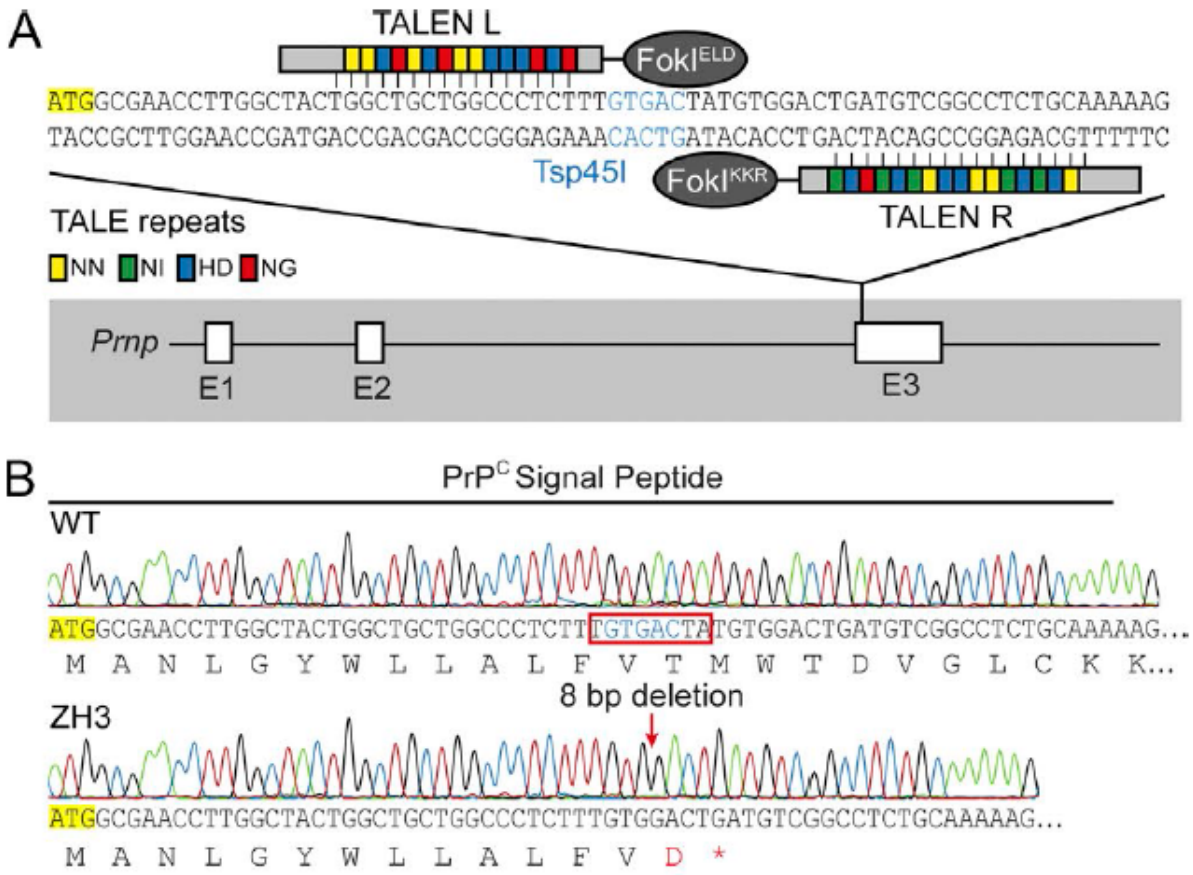
- Knockout of *Pibf1* in mice (29/52, 55.8% mutated; 7/29, 24.1% biallelically mutated, dose-dependent)



Sung, Nat Biotechnol, 2013

TALEN mediated knockout in animals

- Knockout of *Prnp* in C57BL/6J mice (ZH3)



Nuvolone, JEM, 2016

TALEN mediated genome editing

- A 102aa domain derived from naturally occurring TALE required for DNA binding at N-terminal
- **A series of 34aa TALE repeat domain with different RVDs and a 20aa half-repeat at C-terminal**
- A 63aa domain derived from naturally occurring TALE required for DNA binding at C-terminal
- ZiFiT program to assist to identify potential target site and to design TALEN: zifit.parterners.org;
- TALE-NT platform to design TALEN: https://tale-nt.cac.cornell.edu;
- Design efficient and specific TALEN pairs is laborious; off-target etc.

Cermak, Nucleic Acids Res, 2011
Reyon, Nat Biotechnol, 2012
Sanjana, Nat Protoc, 2012
Doyle, Nucleic Acid Res, 2012

III. Recently developed gene engineering methods

- CRISPR: clustered regularly interspaced short palindromic repeats, derived from type II adaptive immune system of many bacterial and archaea.

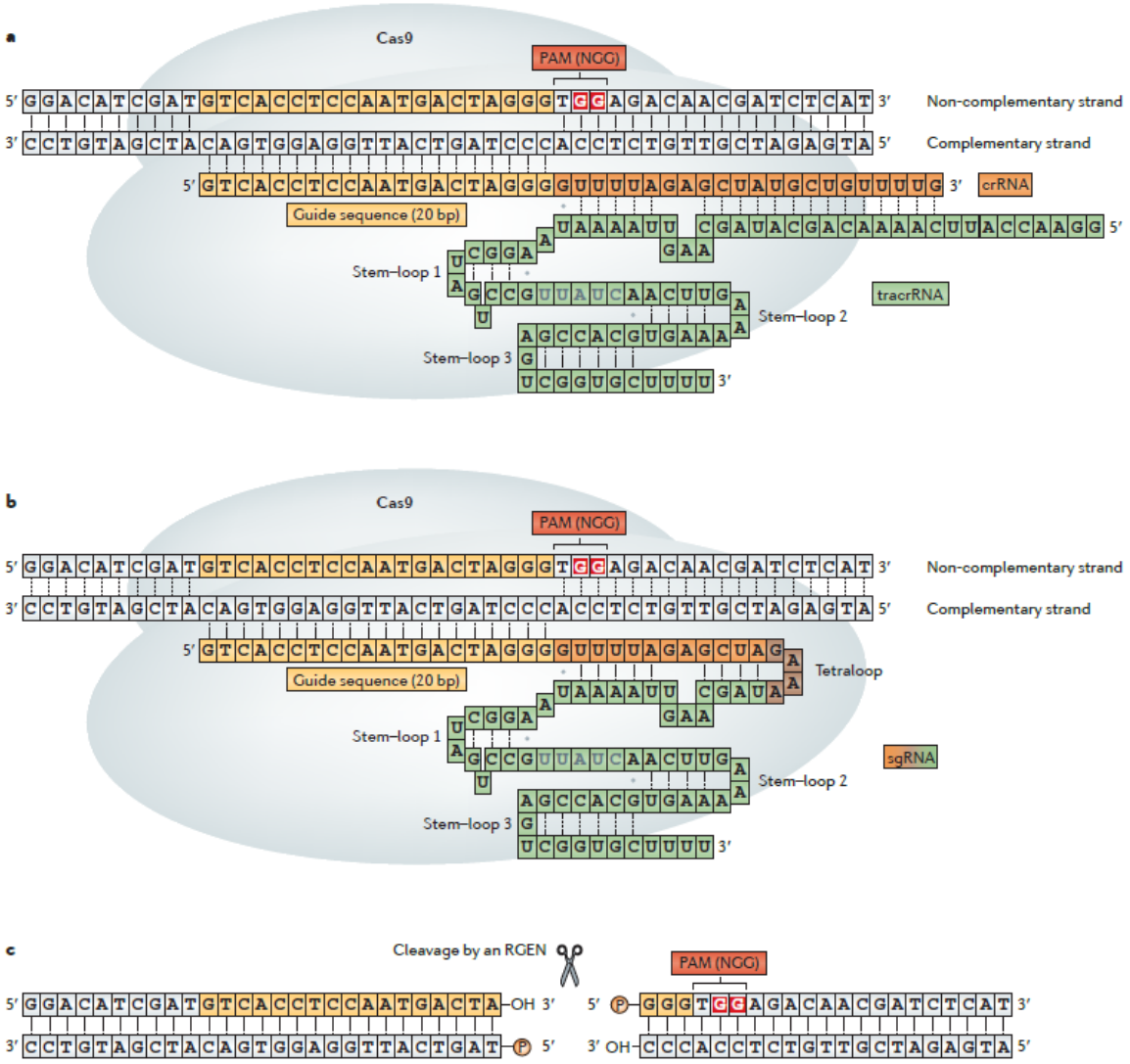
- Three components:

  - Cas9: CRISPR associated protein 9 from *Streptococcus pyogenes*

  - crRNA: CRISPR RNA

  - tracrRNA: trans-activating CRISPR RNA

RNA guided engineered nuclease (RGEN)



Kim, Nat Rev Genet, 2014

CRISPR-Cas9 mediated genome engineering

SCIENCE VOL 339 15 FEBRUARY 2013

Multiplex Genome Engineering Using CRISPR/Cas Systems

Le Cong,[1,2]* F. Ann Ran,[1,4]* David Cox,[1,3] Shuailiang Lin,[1,5] Robert Barretto,[6] Naomi Habib,[1] Patrick D. Hsu,[1,4] Xuebing Wu,[7] Wenyan Jiang,[8] Luciano A. Marraffini,[8] Feng Zhang[1]†

SCIENCE VOL 339 15 FEBRUARY 2013

RNA-Guided Human Genome Engineering via Cas9

Prashant Mali,[1]* Luhan Yang,[1,3]* Kevin M. Esvelt,[2] John Aach,[1] Marc Guell,[1] James E. DiCarlo,[1] Julie E. Norville,[1] George M. Church[1,2]†

NATURE BIOTECHNOLOGY VOLUME 31 NUMBER 3 MARCH 2013

Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease

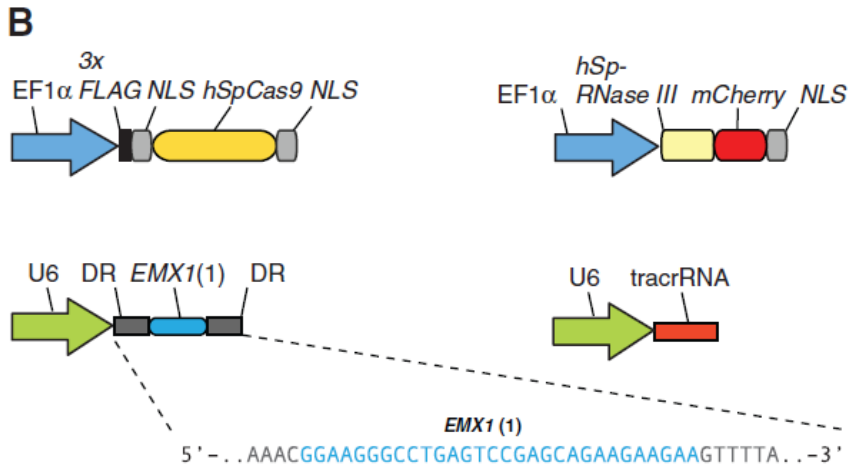
Seung Woo Cho[1–3], Sojung Kim[1–3], Jong Min Kim[1,2] & Jin-Soo Kim[1,2]

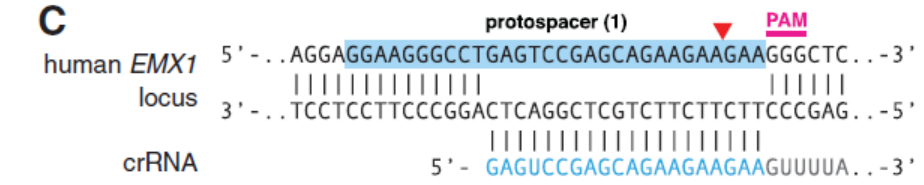
eLife 2013;2:e00471.

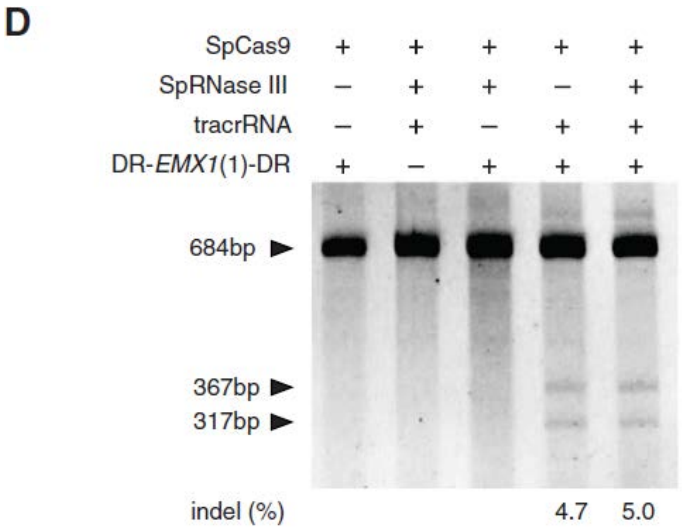
RNA-programmed genome editing in human cells

Martin Jinek[1,2], Alexandra East[2], Aaron Cheng[2], Steven Lin[1,2], Enbo Ma[2], Jennifer Doudna[1,2,3,4]*

CRISPR-Cas9 mediated genome engineering



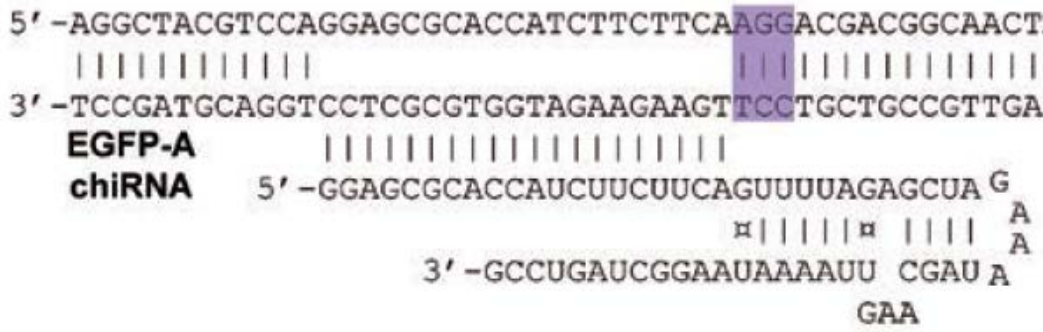


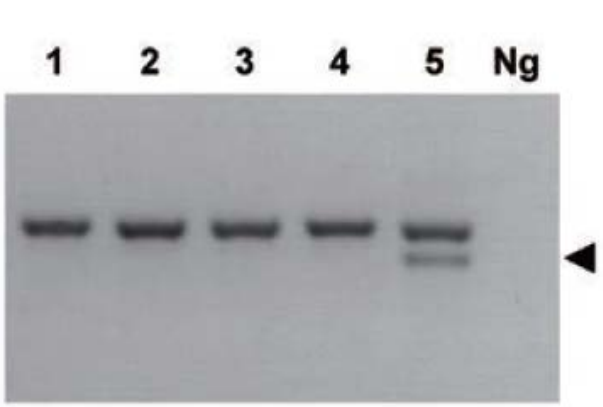


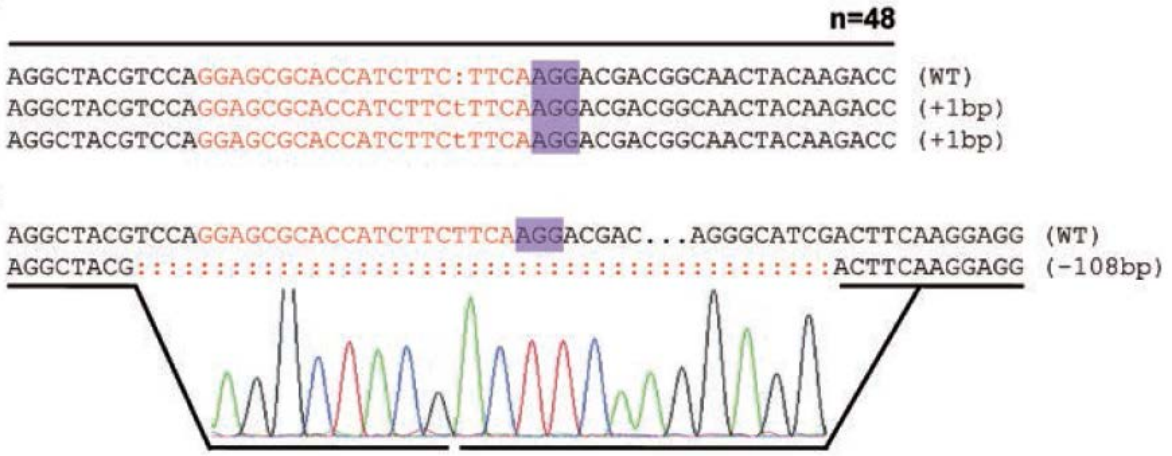
Cong, Science, 2013

CRISPR-Cas9 mediated knockout in animals

- Knockout of EGFP in Pouf5-IRES-EGFP or CAG-EGFP transgenic mice (1/5, 1/7 contained mutated GFP)

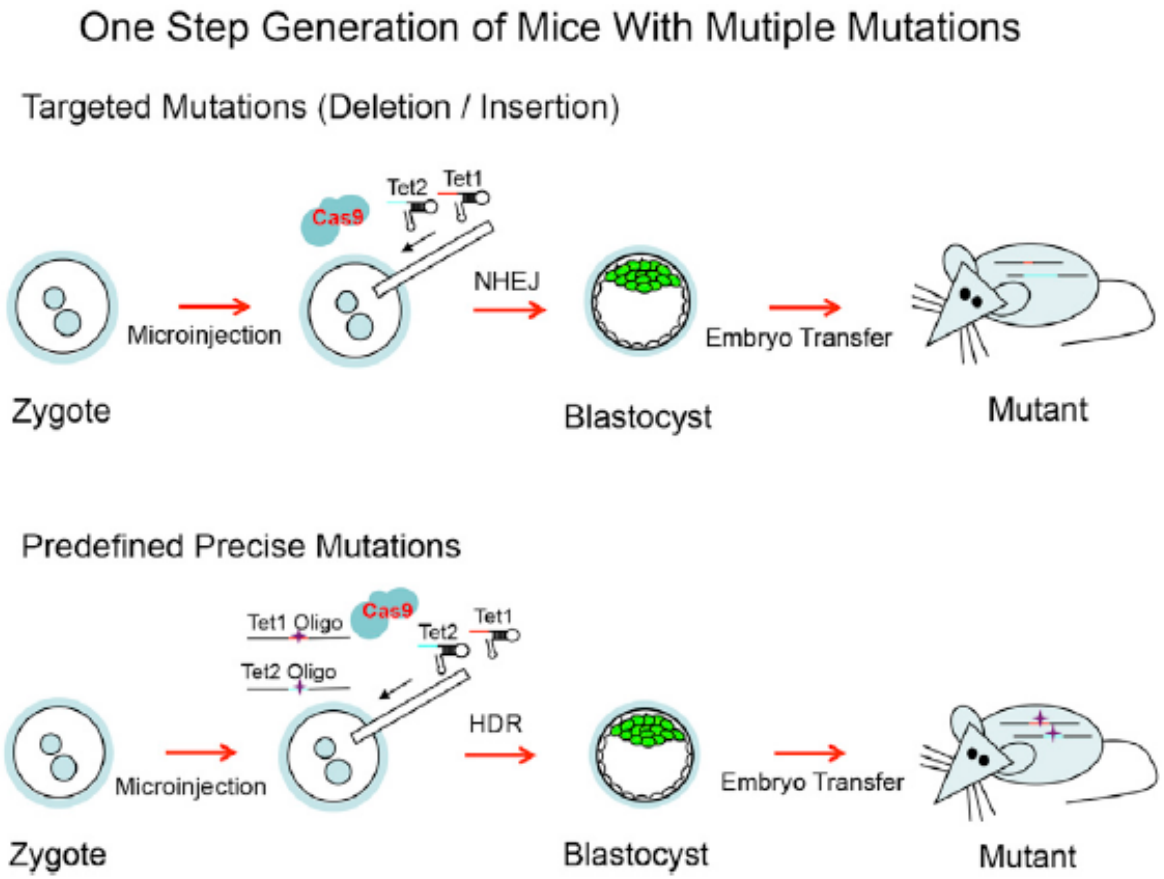






Shen, Cell Res, 2013

CRISPR-Cas9 mediated knockout in animals



Wang, Cell, 2013

CRISPR-Cas9 mediated knockout in animals

- CRISPR-Cas9 mediated DSB, followed by NHEJ

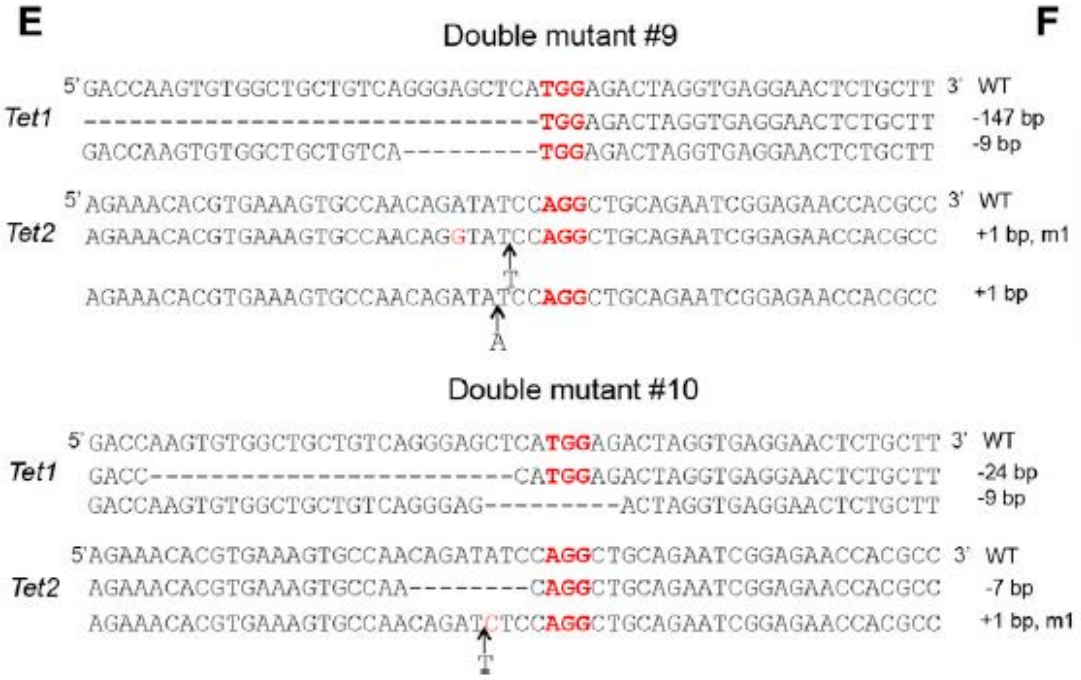


Table 3. CRISPR/Cas-Mediated Double-Gene Targeting in BDF2 Mice

Gene	Cas9/sgRNA (ng/μl)	Blastocyst/Injected Zygotes	Transferred Embryos (Recipients)	Newborns (Dead)	Mutant Alleles per Mouse/Total Mice Tested[a]				
					4	3	2	1	0
Tet1 + *Tet2*	100 / 50	194/229	144(7)	31(8)	22/28	4/28	1/28	1/28	0/28
	20 / 20	92/109	75(5)	19(3)	11/19	1/19	2/19	3/19	2/19

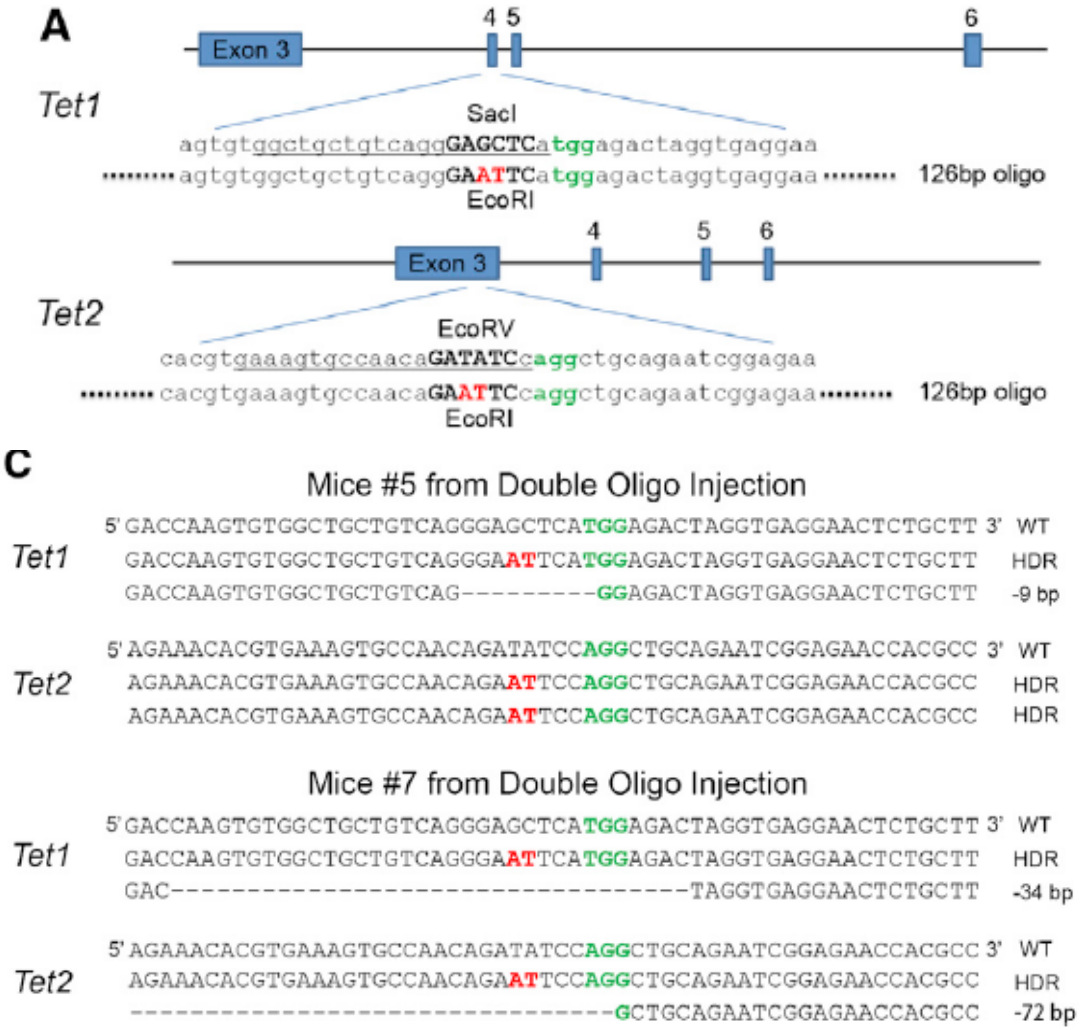
Cas9 mRNA and sgRNAs targeting *Tet1* and *Tet2* were coinjected into fertilized eggs. The blastocysts derived from the injected embryos were transplanted into foster mothers and newborn pups were obtained and genotyped. The number of total alleles mutated in each mouse is listed from 0 to 4 for *Tet1* and *Tet2*. The number of mice containing each specific number of mutated alleles is shown in relation to the number of total mice screened in each experiment.

[a]Some of the pups were cannibalized.

Wang, Cell, 2013

CRISPR-Cas9 mediated knockout in animals

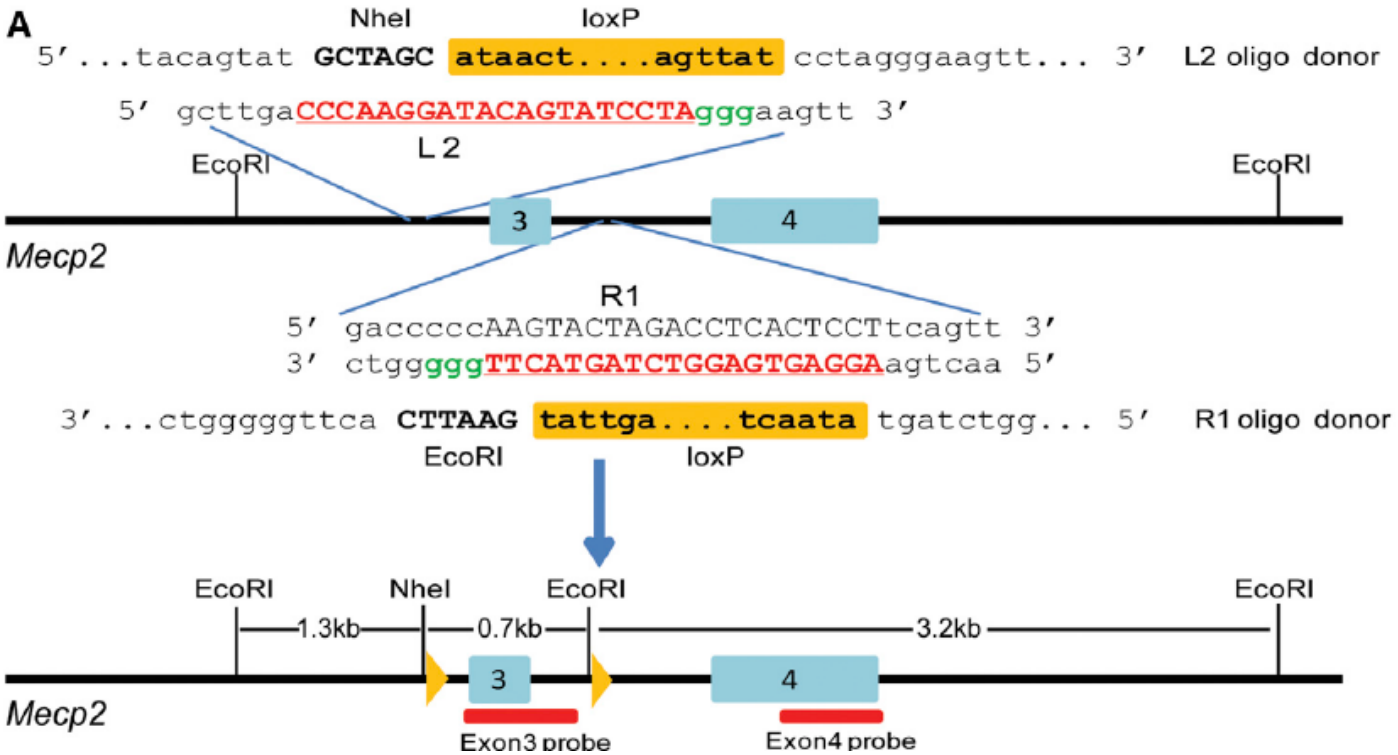
- CRISPR-Cas9 mediated DSB, followed by HDR (4/14 have Tet1 HDR, 7/14 have Tet2 HDR, 1 has Tet1 and Tet2 HDR)



Wang, Cell, 2013

CRISPR-Cas9 mediated knockin in animals

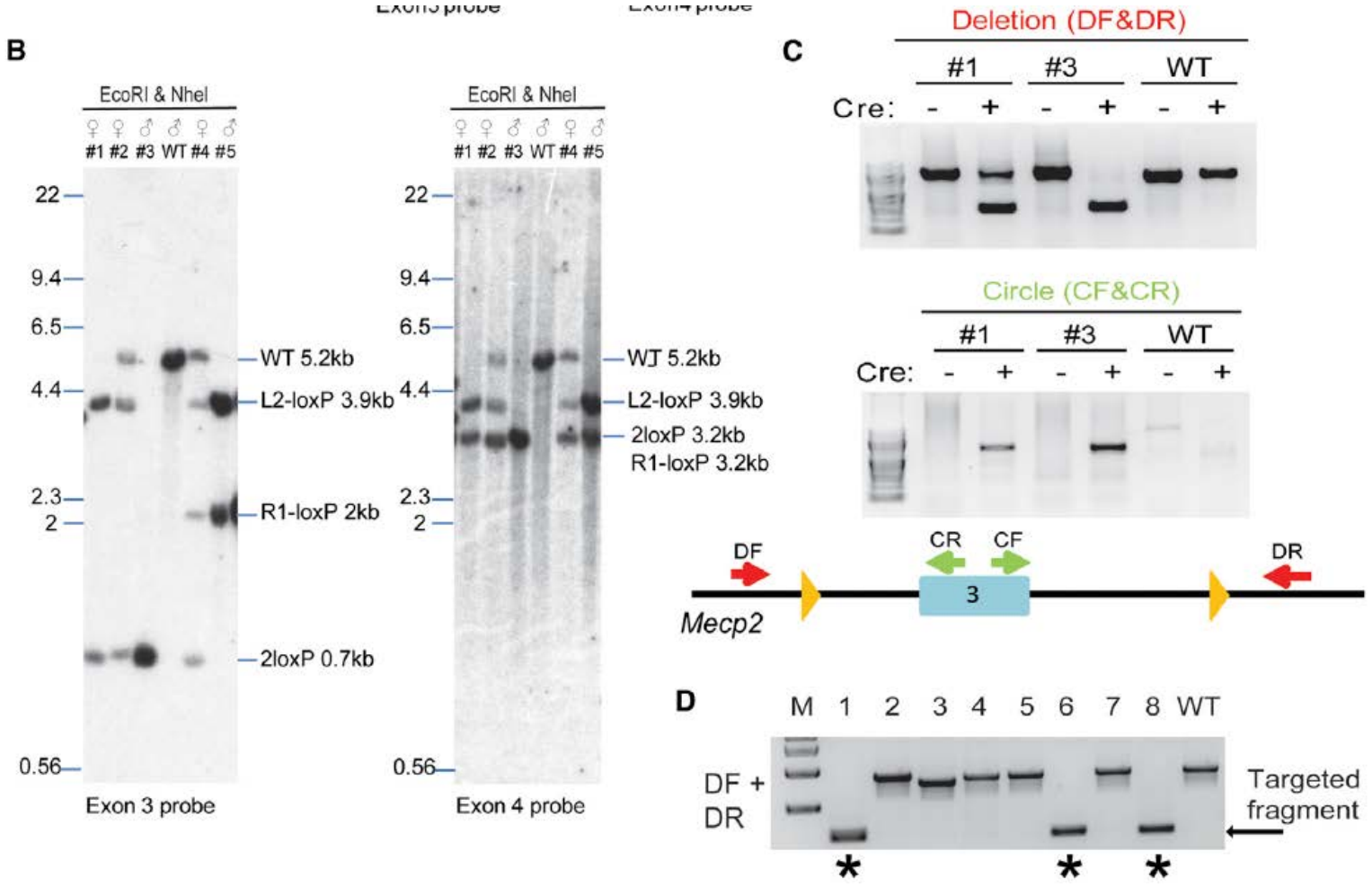
- CRISPR-Cas9 mediated KI of LoxP sites



Yang, Cell, 2013

CRISPR-Cas9 mediated knockin in animals

- CRISPR-Cas9 mediated KI



Yang, Cell, 2013

CRISPR-Cas9 mediated knockin in animals

Table 2. Conditional *Mecp2* Mutant Mice

Donor	Blastocyst/Injected Zygotes	Transferred Embryos (Recipients)	Sex	Pre- and Postnatal Mice with loxP/Total				
				Total[a]	L2[b]	R1[c]	Two loxP in Two Alleles	Two loxP in One Allele
Mecp2-L2 + Mecp2-R1	367/451	360(18)	Male	28/60	26/60	12/60	2[d]/60	8/60
			Female	21/38	19/38	13/38	3/38	8/38
			Total	49/98	45/98	25/98	5/98	16/98

Cas9 mRNA, sgRNAs targeting *Mecp2*-L2 and *Mecp2*-R1, and single-stranded DNA oligos were injected into fertilized eggs. The blastocysts derived from the injected embryos were transplanted into foster mothers and pre- and postnatal mice were genotyped.

[a]Total mice containing loxP site integration in the genome.

[b]Mice containing loxP site integrated at L2 site.

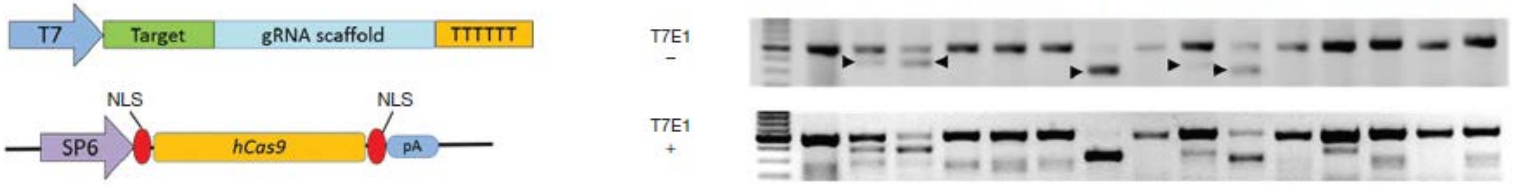
[c]Mice containing loxP site integrated at R1 site.

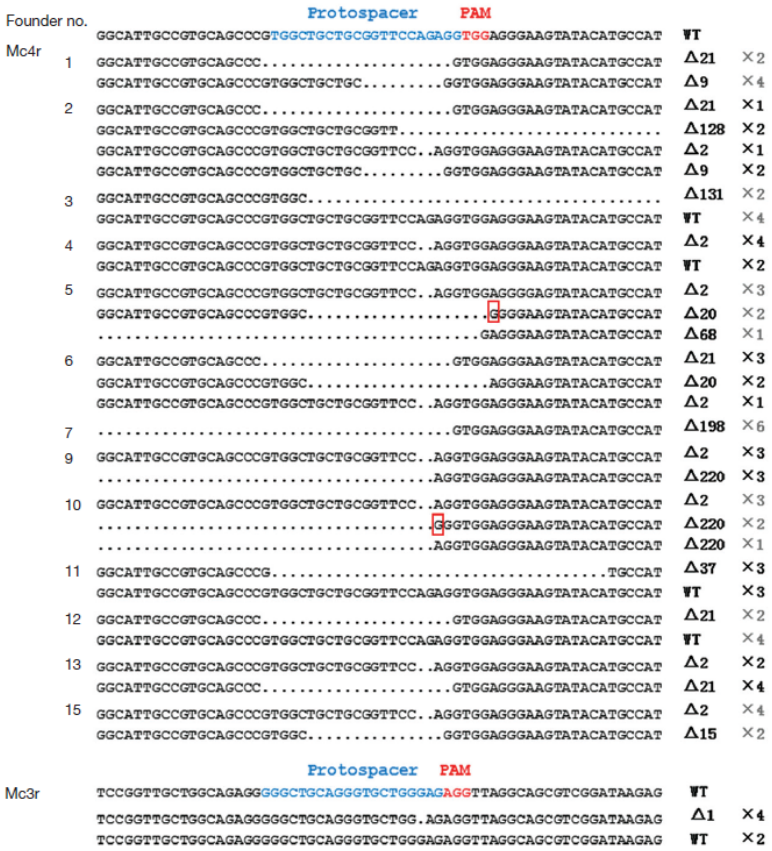
[d]These male mice were mosaic.

Yang, Cell, 2013

CRISPR-Cas9 mediated knockout in animals

- CRISPR-Cas9 mediated knockout in mice and rats





D. Li, Nat Biotechnol, 2013

CRISPR-Cas9 mediated knockout in animals

- CRISPR-Cas9 mediated knockout in mice and rats

Table 1 Generation of knockout mice and rats via the CRISPR-Cas system

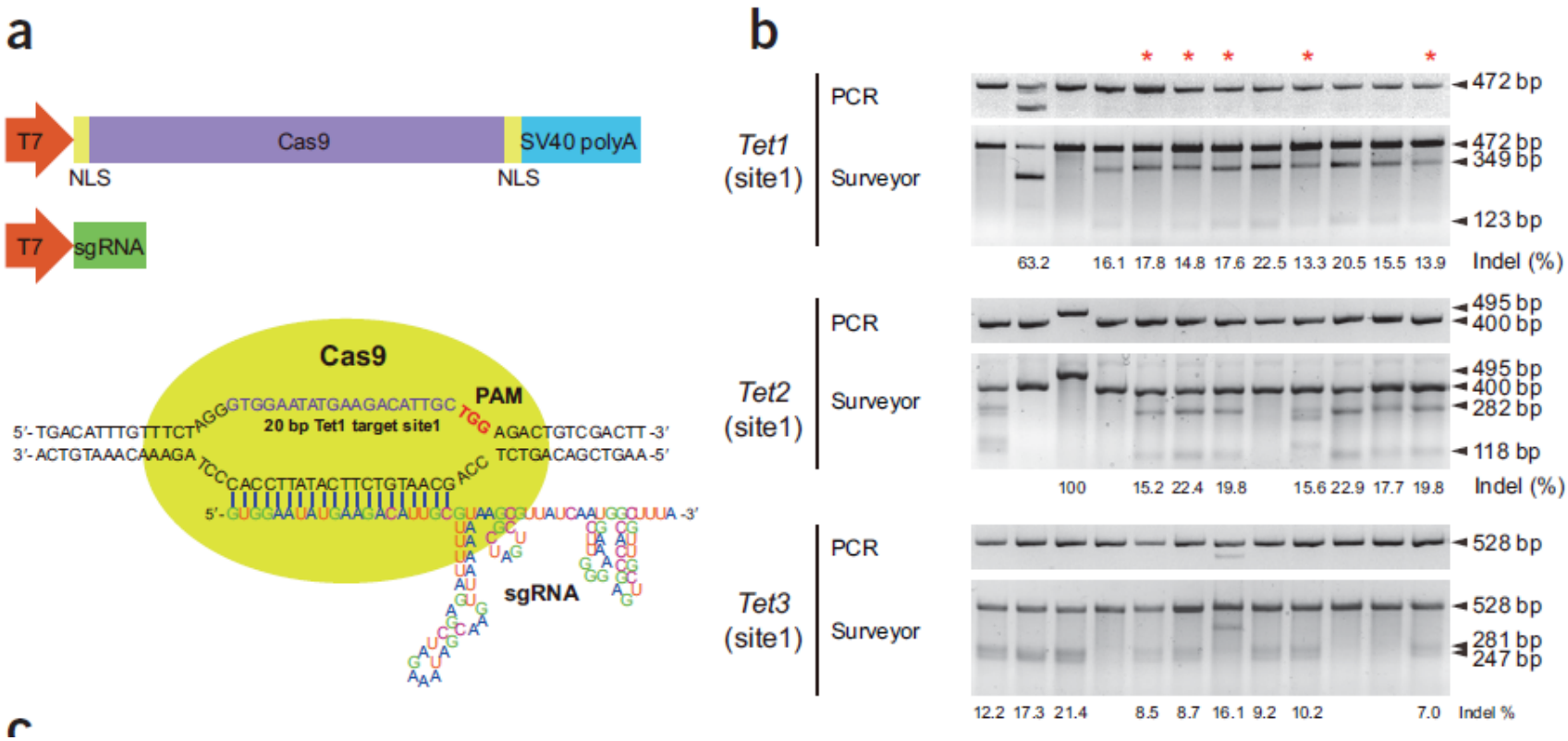
Gene	Strain	Dose (ng/μl)	Number injected/ transferred (%)	Total number of newborns (%)	Mutant number	Mutation rate	
						As a percentage of total number of newborns	As a percentage of total number of injected embryos
DNA							
Th	FVB/N	1	125/50 (40)	10 (8)	0	0	0
Th	FVB/N	2.5	350/75 (21)	11 (3)	1	9	0.3
RNA							
Th	B6	25/12.5	120/100 (83)	9 (7.5)	8	90	6.7
Rheb	B6	25/12.5	115/45 (40)	4 (3.5)	3	75	2.6
Uhrf2	FVB/N	25/12.5	105/33 (31)	12 (11.5)	11	92	10.5
Co-injection							
Mc4r	Sprague-Dawley	25/12.5 + 12.5	122/68 (56)	15 (12)	13	87	10.6
Mc3r	Sprague-Dawley				1	7	0.8

sgRNA:Cas9 system for each target gene was injected into fertilized eggs. DNA was injected into male pronuclei and RNA was injected into the cytoplasm of mouse or rat zygotes which were then transferred into pseudopregnant females. Mutations of the newborns were confirmed by sequencing after weaning.

D. Li, Nat Biotechnol, 2013

CRISPR-Cas9 mediated knockout in animals

- CRISPR-Cas9 mediated knockout in rats



W. Li, Nat Biotechnol, 2013

CRISPR-Cas9 mediated knockout in animals

- CRISPR-Cas9 mediated knockout in rats

Table 1 Multiple gene disruption in rats by means of CRISPR-Cas systems

Injected sgRNAs	Injected embryos	Transferred embryos	Newborns (% of transferred)	Assayed rats	Mutated rats (%)						
					Single (%)			Double (%)			Triple (%)
					Tet1	*Tet2*	*Tet3*	*Tet1/Tet2*	*Tet1/Tet3*	*Tet2/Tet3*	*Tet1/Tet2/Tet3*
sg*Tet3*-1/ sg*Tet3*-2	130	100 (76.9)	42 (42)	18	–	–	18 (100)	–	–	–	–
sg*Tet1*-1/ sg*Tet2*-1	140	105 (75.0)	30 (28.6)	24	16 (67.7)	23 (95.8)	–	15 (62.5)	–	–	–
sg*Tet1*-2/ sg*Tet2*-2	120	80 (66.7)	22 (31.2)	20	16 (80.0)	19 (95.0)	–	15 (75.0)	–	–	–
sg*Tet1*-1/ sg*Tet2*-1/ sg*Tet3*-1	90	70 (77.8)	22 (31.4)	22	15 (68.2)	20 (90.9)	16 (72.7)	15 (68.2)	13 (59.1)	16 (72.7)	13 (59.1)

W. Li, Nat Biotechnol, 2013

CRISPR-Cas9 mediated knockout in animals

- CRISPR-Cas9 mediated knockout in monkeys

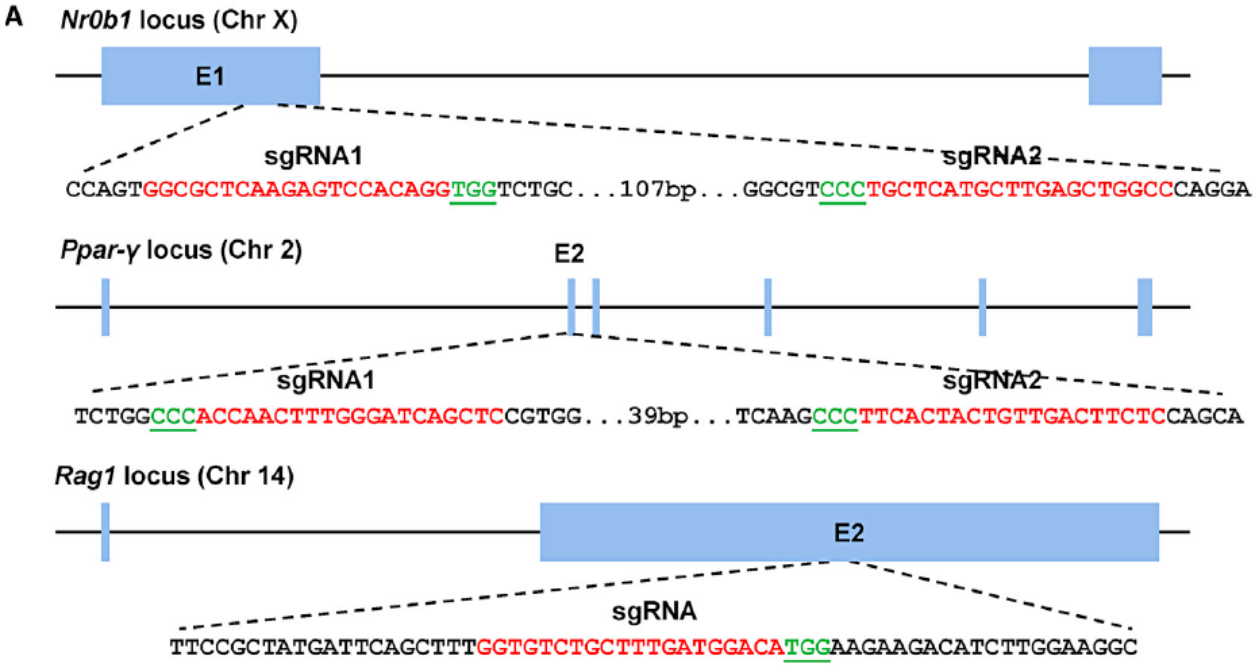


Table 1. Summary of Embryo Microinjection of Cas9 mRNA and sgRNAs

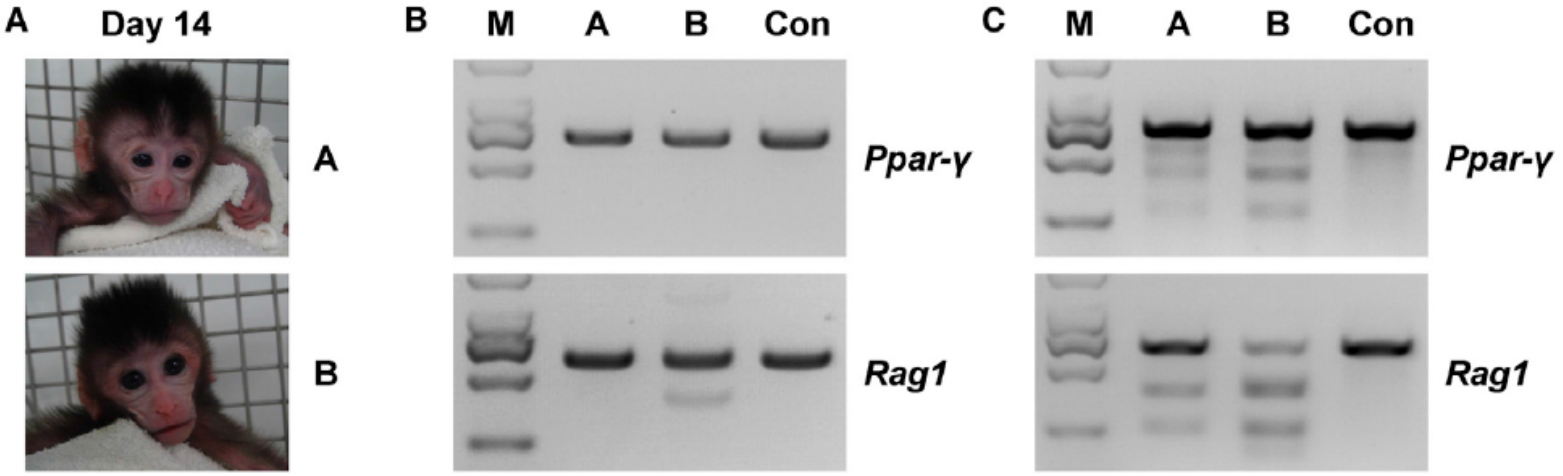
MII Oocyte	Injected Embryos	Embryos for ET	Pregnancies /Surrogates	Single Pregnancy	Multiple Pregnancy	Fetuses
198	186	83	34.5% (10/29)	4[a]	3 twins, 3 triplets	19

[a]One miscarried 36 days after embryo transfer.

Niu, Cell, 2014

CRISPR-Cas9 mediated knockout in animals

- CRISPR-Cas9 mediated knockout in monkeys



D

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Ppar-γ ACTCCTTTGACATCAAGCCCTTCACTACTGTTGACTTCTCCAGCATTTCTGCTCCAC  (WT)
  A    ACTCCTTTGACATCAAGCCCTTCACTACTGTTGACTTCTCCAGCATTTCTGCTCCAC  (+1,2/23)
                             ^a
  B    ACTCCTTTGACATCAAGCCCTTCACTACTGTTGACTTCTCCAGCATTTCTGCTCCAC  (+1,6/23)
                             ^a

 Rag1  CGCTATGATTCAGCTTTGGTGTCTGCTTTGATGGACATGGAAGAAGACATCTTGGAA  (WT)
  A    CGCTATGATTCAGCTTTGGTGTCTGCTTTGATGG::::::AAGAAGACATCTTGGAA  (-6,3/20)
  B    CGCTATGATTCAGCTTTGGTGTCTGCTTT::::GACATGGAAGAAGACATCTTGGAA  (-4,2/18)
       CGCTATGATTCAGCTTTGGTGTCTGCTTT:::::::::::::::::::::::::::  (-203,2/18)
       CGCTATGATTCAGCTTTGGTGTCTGCTTTGATGGACATGGAAGAAGACATCTTGGAA  (+6,7/18)
                                    ^gaagaa
```

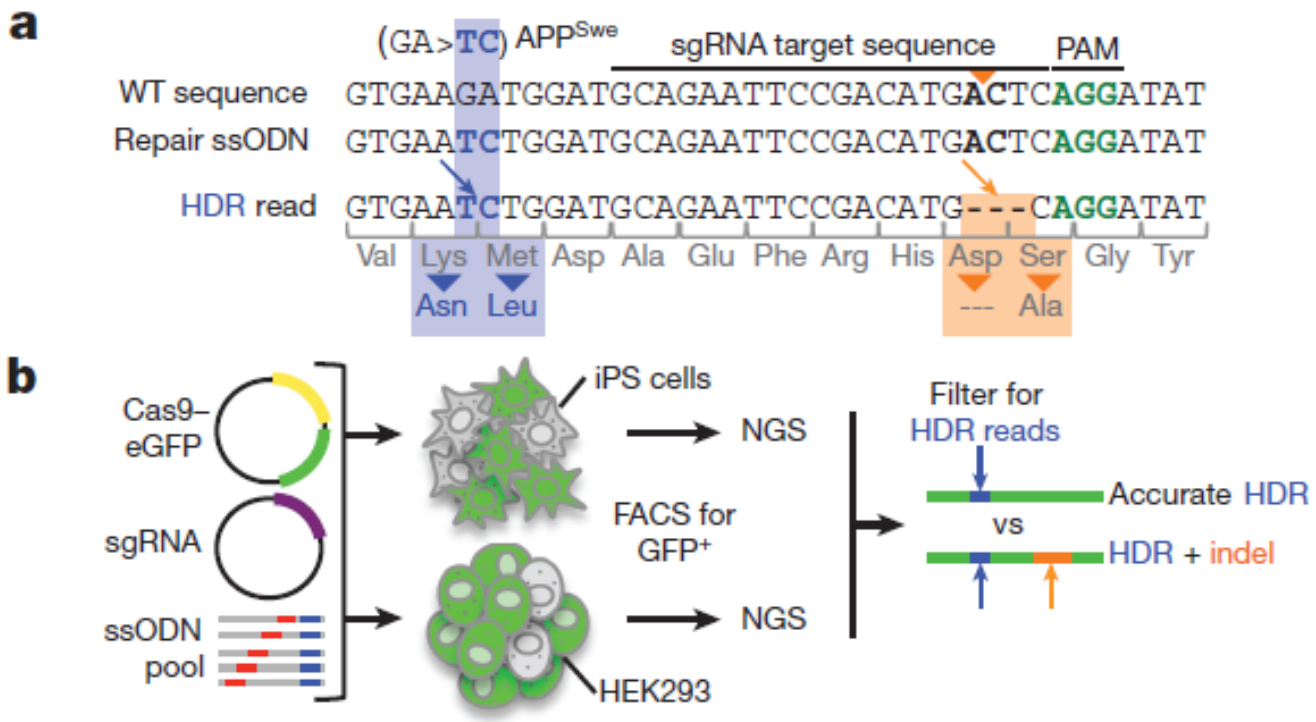
Niu, Cell, 2014

CRISPR-Cas9 mediated genome editing

- Simple to design the guide RNA to target almost any gene;
- Highly efficient in NHEJ, resulting in knockout animals;
- Biallelic mutation – no need to breed heterozygosity to homozygosity;
- Multiple mutation – simultaneously mutate >1 genes;
- HDR is less efficient, and often associated with NHEJ!

Improvement of CRISPR-Cas9 mediated HDR

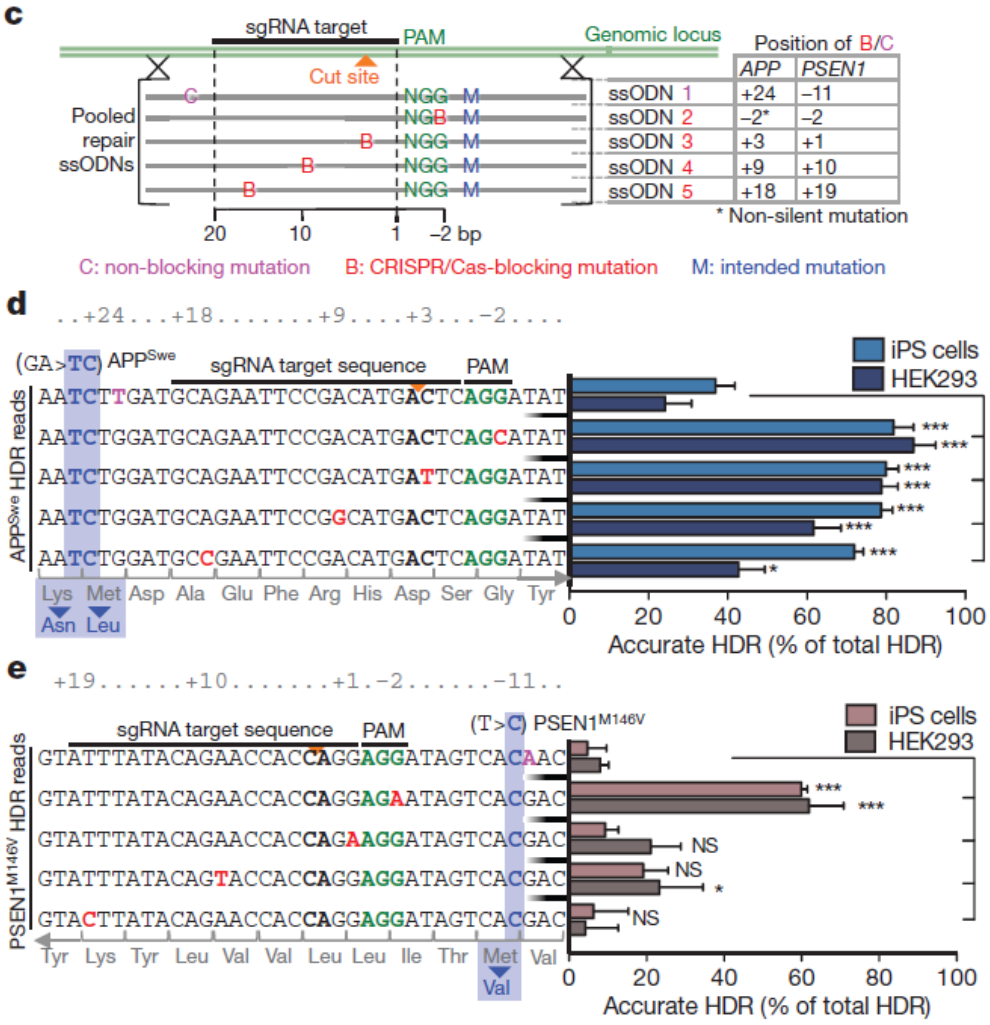
- HDR followed by NHEJ



Paquet, Nature, 2016

Improvement of CRISPR-Cas9 mediated HDR

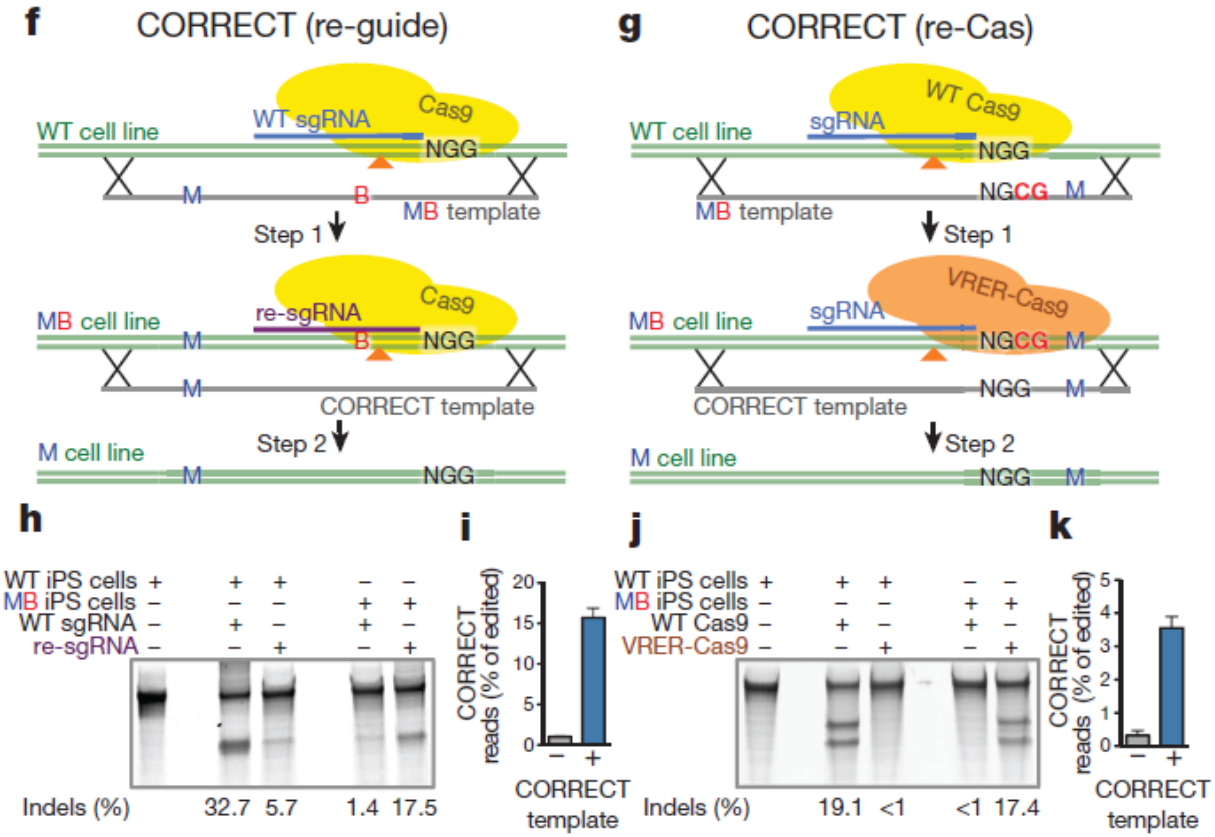
- Introduce a blocking mutation at sgRNA or PAM sites of ssDNA increase the accurate HDR



Paquet, Nature, 2016

Improvement of CRISPR-Cas9 mediated HDR

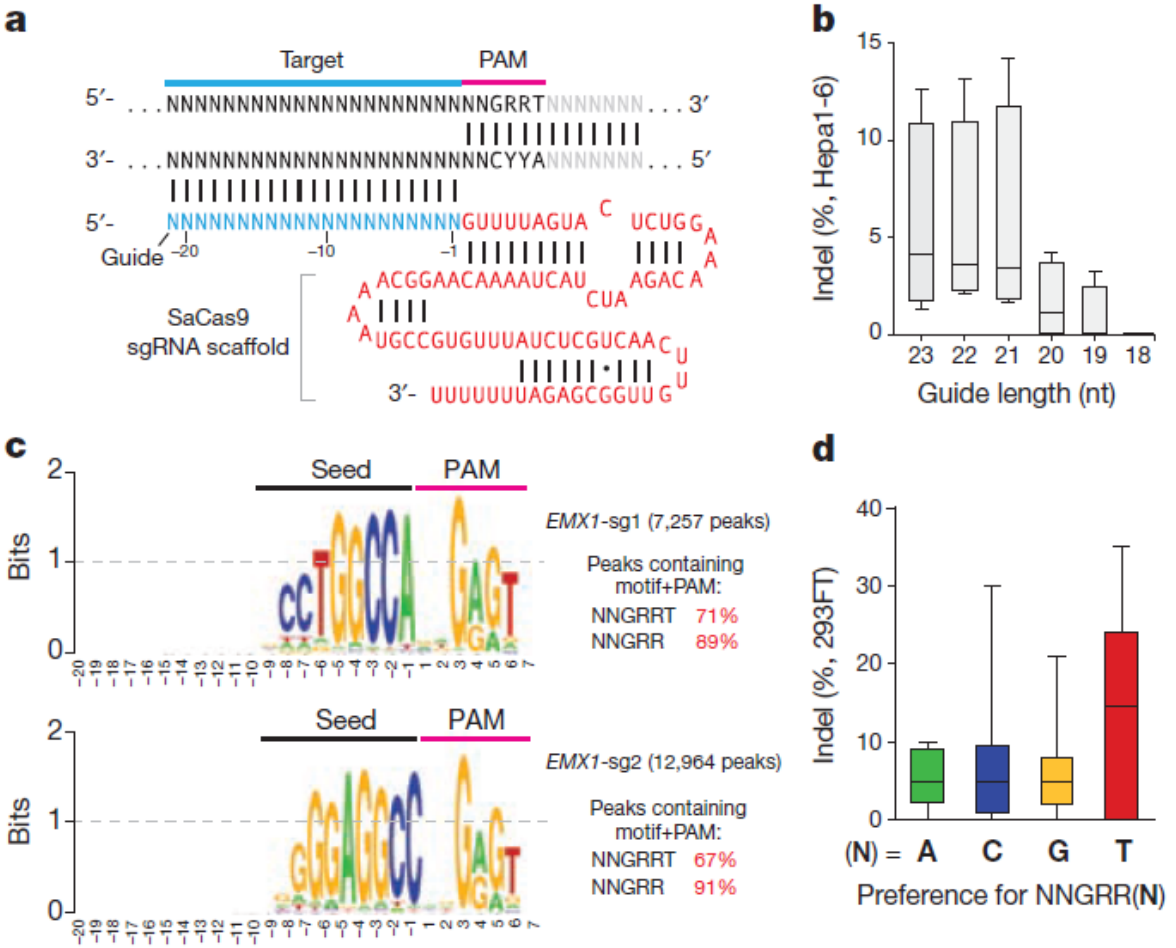
- Correct the blocking mutation by consecutive re-guide or re-Cas



Paquet, Nature, 2016

SpCas9 substitutes

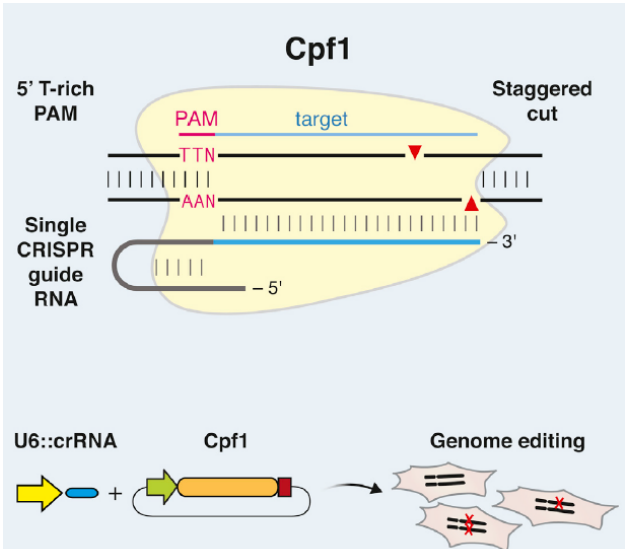
- *Staphylococcus aureus* (SaCas9) - 1kb smaller in size, viral vector
- PAM: NNGRRT

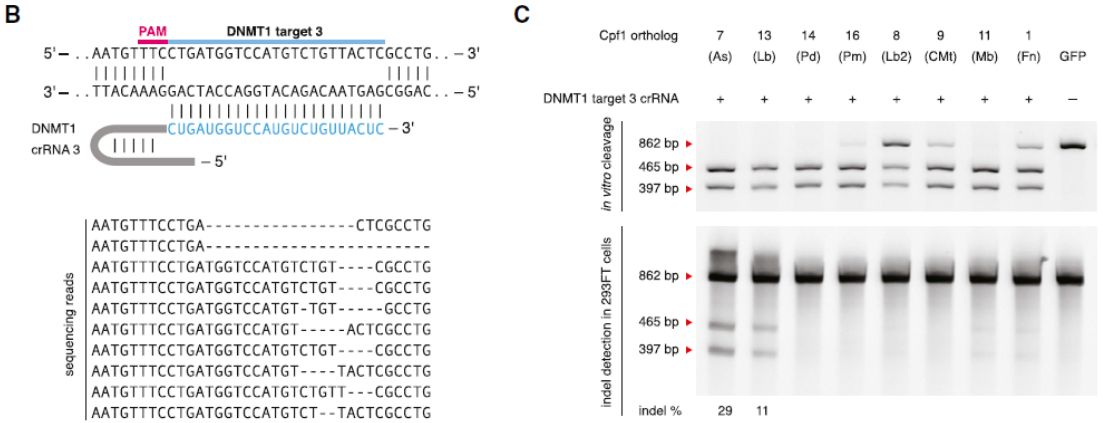


Ran, Nature, 2015

SpCas9 substitutes

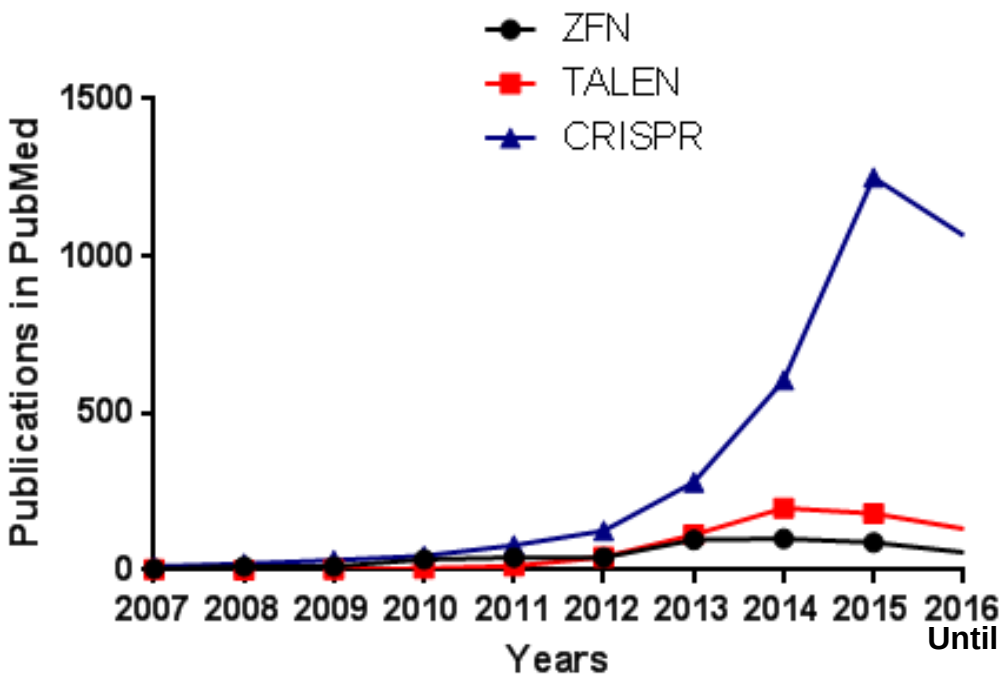
- *Acidaminococcus* (As) and *Lachnospiraceae* (Lb) Cpf1, need only crRNA; PAM: NTT; staggered DNA cleavage.





Zetsche, Cell, 2015

Impact of ZFN, TALEN and CRISPR



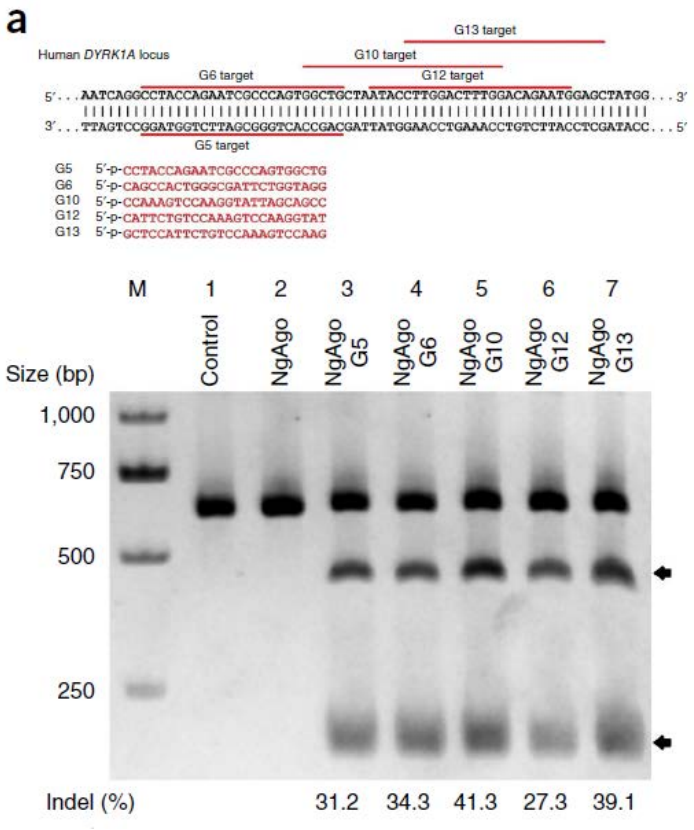
Until 07.06.2016

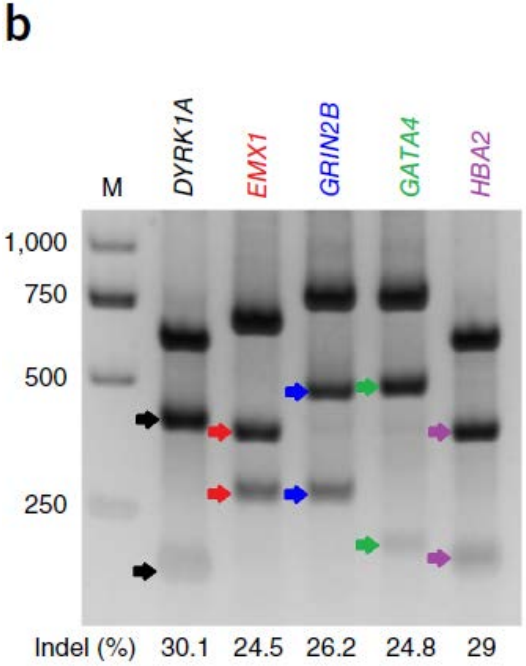
III. Recently developed gene engineering methods

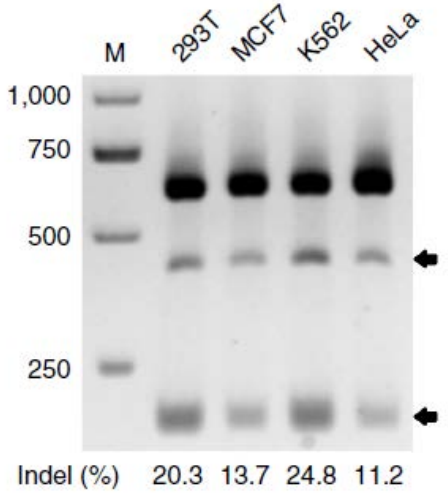
- Argonaute: a family of endonuclease that use 5’ phosphotylated short single strand nucleic acids (RNA or DNA) as guide to cleave target; human Ago2 in RNA-induced silencing complex (RISC);
- No specific preference of secondary structure of guide to bind Ago;
- Argonaute is preserved in evolution and exist in virtually all organisms;
- *Natronobacterium gregoryi* Argonaute (NgAgo), show some similarities to *Thermus thermophilus* Argonaute (TtAgo) or *Pyrococcus furiosus* Argonaute (PfAgo) which are known to bind 5’-p-ssDNA at >65°C, and cleave target in vitro;
- DNA guided genome editing.

NgAgo mediated genome engineering

- NgAgo mediated NHEJ



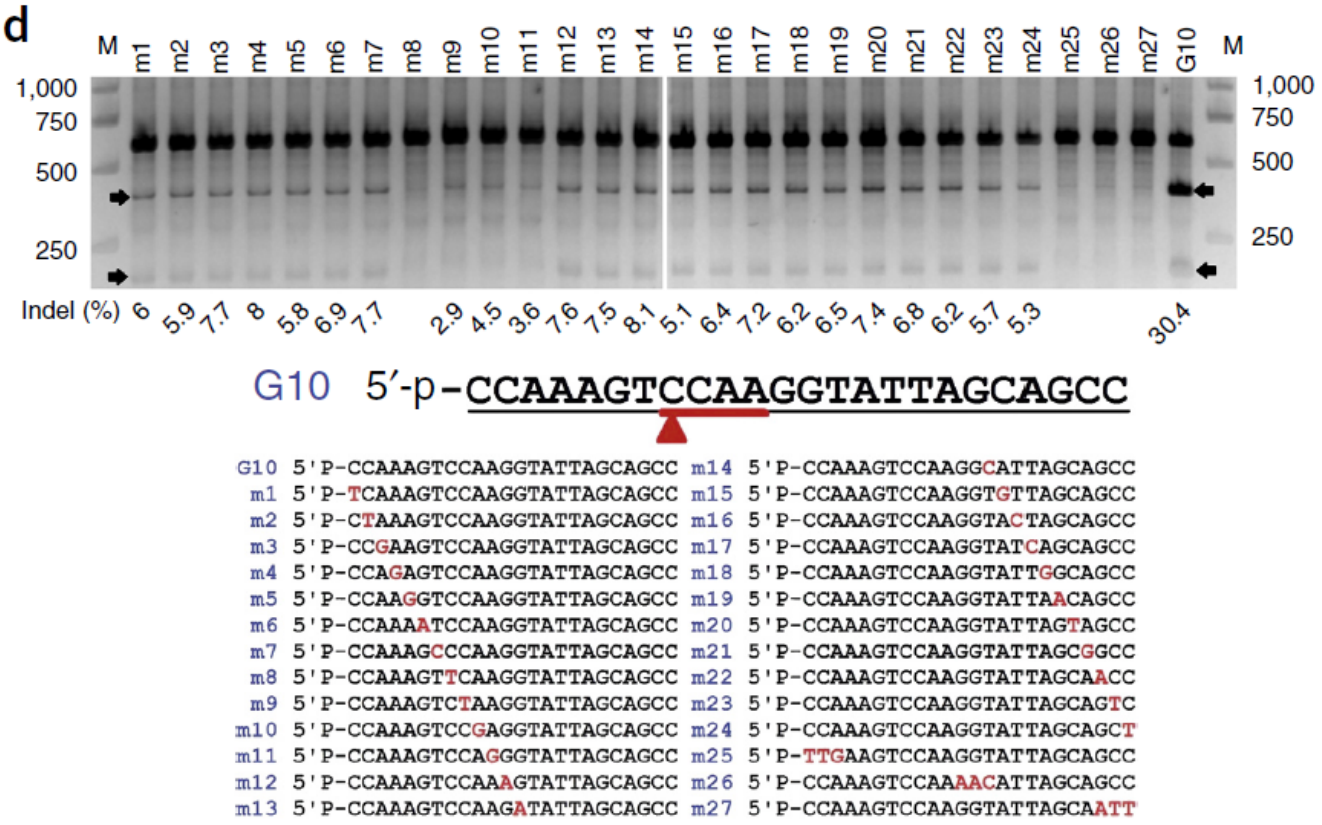




Gao, Nat Biotechnol, 2016

NgAgo mediated genome engineering

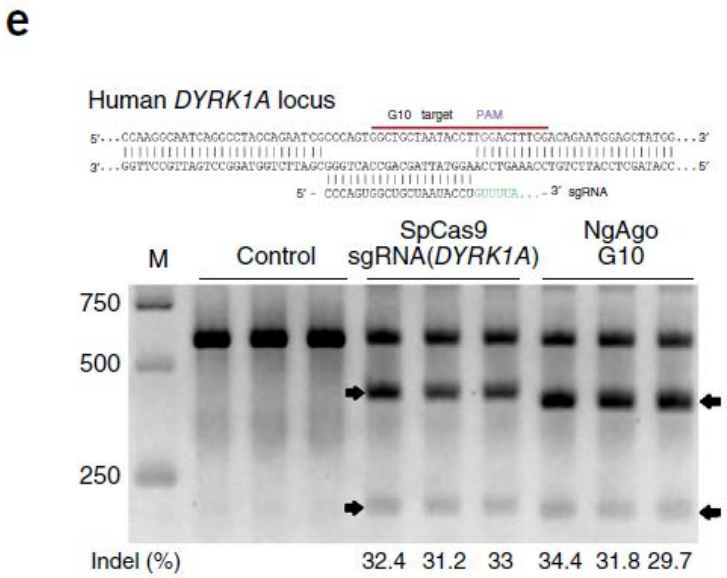
- High sensitivity to gDNA mutation – high specificity

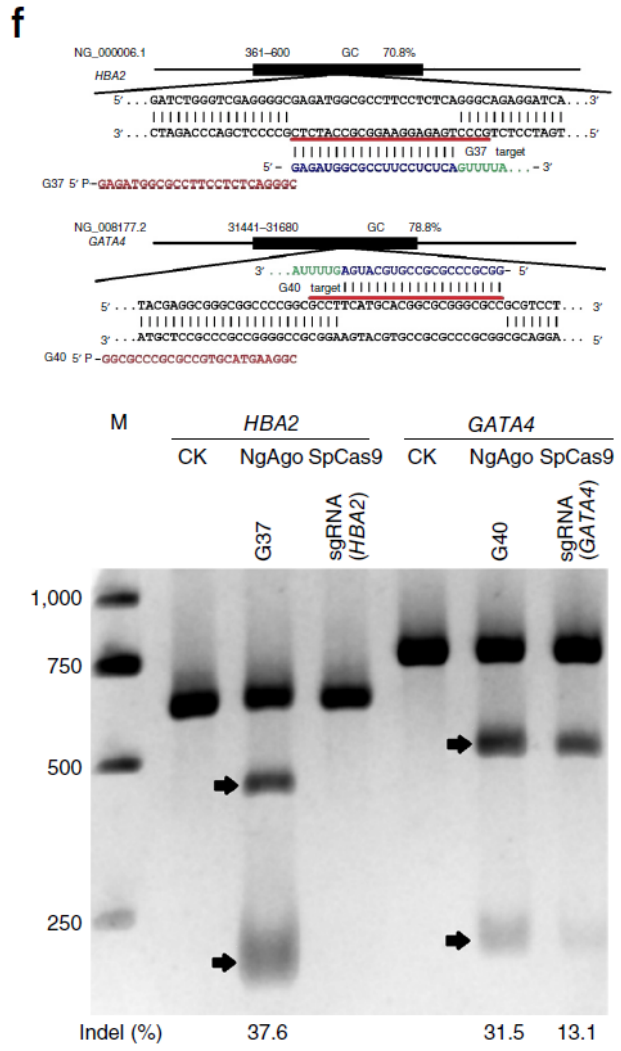


Gao, Nat Biotechnol, 2016

NgAgo mediated genome engineering

- High efficiency– comparable to SpCas9, better performance in GC-rich areas

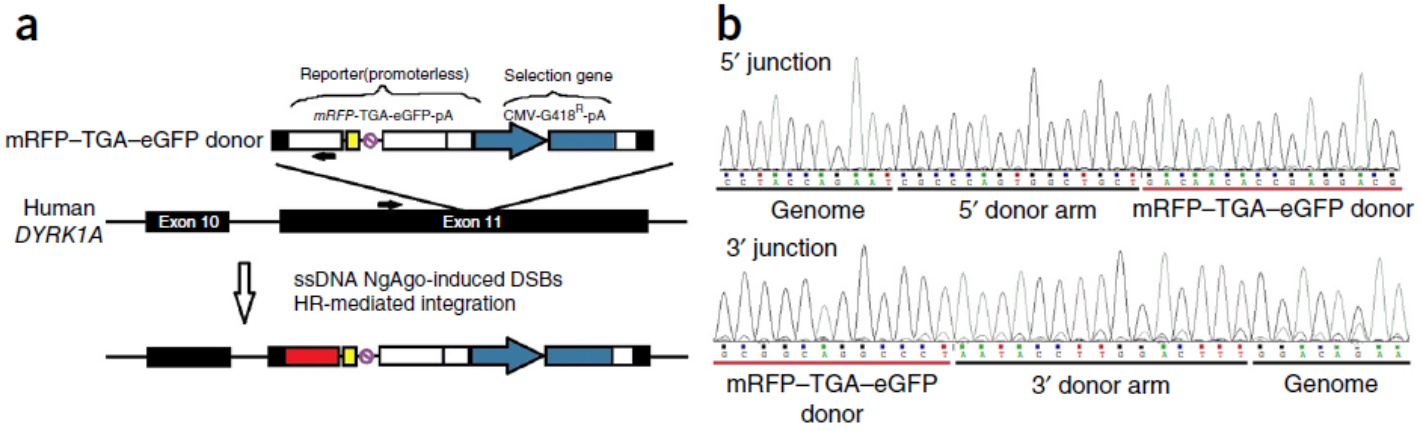


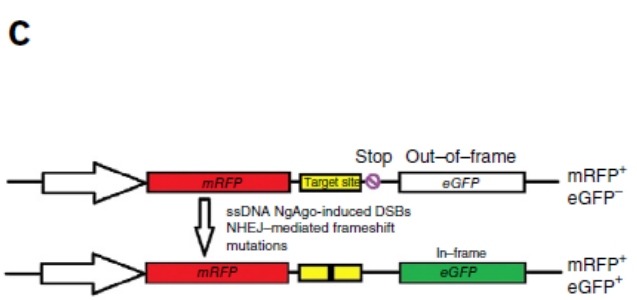


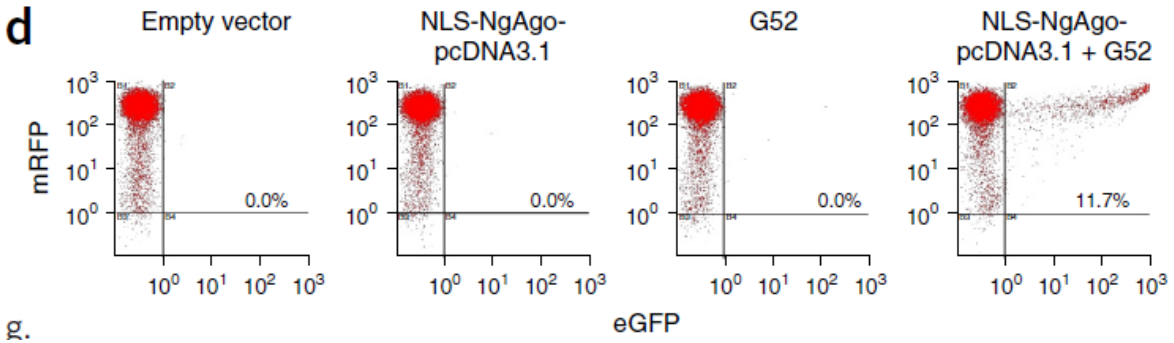
Gao, Nat Biotechnol, 2016

NgAgo mediated genome engineering

- NgAgo mediated HDR







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Gao, Nat Biotechnol, 2016

IV. Future directions

- ES cell based gene targeting is routinely used for gene knockout in mice, IKMC supplies targeted ES cell clones;

- Genome editing methods are useful for precise genome modification (point mutation) for gene therapy, disease modeling etc in mice and other animals, such as non-human primates;

- New genome editors identified in nature for more efficient and precise genome editing.

Thank you!