CRISPR-Cas9 genome engineering

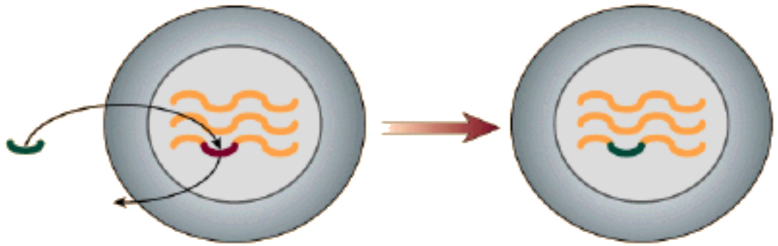
BIOL 426/626
Approaches to Molecular Biology

Reading

- Horii T, Arai Y, Yamazaki M, Morita S, Kimura M, Itoh M, Abe Y, Hatada I. Validation of microinjection methods for generating knockout mice by CRISPR/Cas-mediated genome engineering. Sci Rep. 2014 4:4513
- Knott GJ, Doudna JA. CRISPR-Cas guides the future of genetic engineering. Science. 2018 361:866 (recommended review)

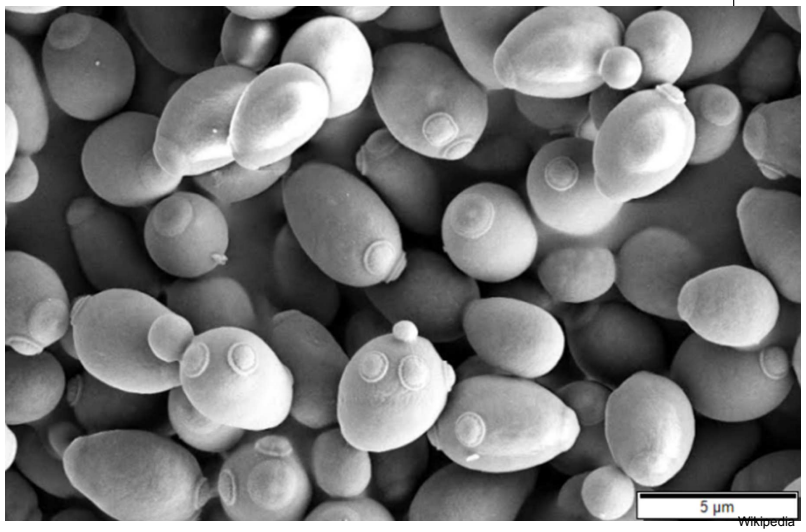
Holy grail of molecular genetics…

Gene replacement



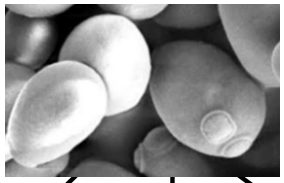
http://www.macalester.edu

Brewer's yeast (*Saccharomyces cerevisiae*)



Wikipedia

Brewer's yeast (*Saccharomyces cerevisiae*)



Wine (7000 BC, China)

Beer (4000 BC, Sumeria)

Genetics (1935, Copenhagen)

Biotechnology

Molecular clones of yeast genes

Yeast genes complemented bacterial mutations

Proc. Natl. Acad. Sci. USA
Vol. 74, No. 12, pp. 5255–5259, December 1977
Biochemistry

Production of a functional eukaryotic enzyme in *Escherichia coli*: Cloning and expression of the yeast structural gene for imidazole-glycerolphosphate dehydratase (*his*3)

(gene expression/cloned yeast genes/gene selection/yeast *his*3)

KEVIN STRUHL AND RONALD W. DAVIS

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

Communicated by I. R. Lehman, August 15, 1977

Transformation of yeast

Clone yeast genes could be inserted into yeast chromosomes

Proc. Natl. Acad. Sci. USA
Vol. 75, No. 4, pp. 1929–1933, April 1978
Genetics

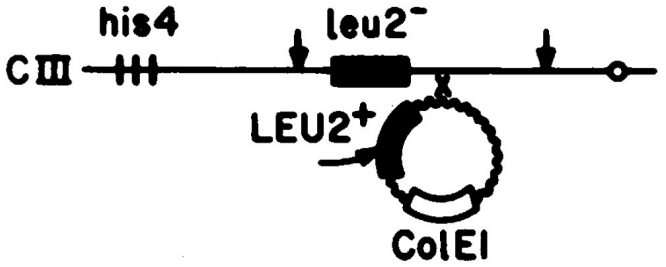
Transformation of yeast

(gene exchange/hybrid plasmid/integration)

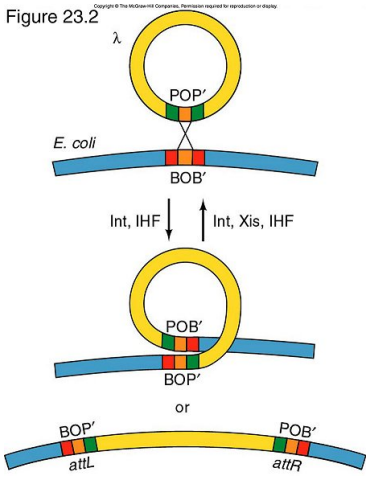
ALBERT HINNEN, JAMES B. HICKS, AND GERALD R. FINK

Department of Botany, Genetics and Development, Cornell University, Ithaca, New York 14853

Communicated by Adrian M. Srb, January 25, 1978

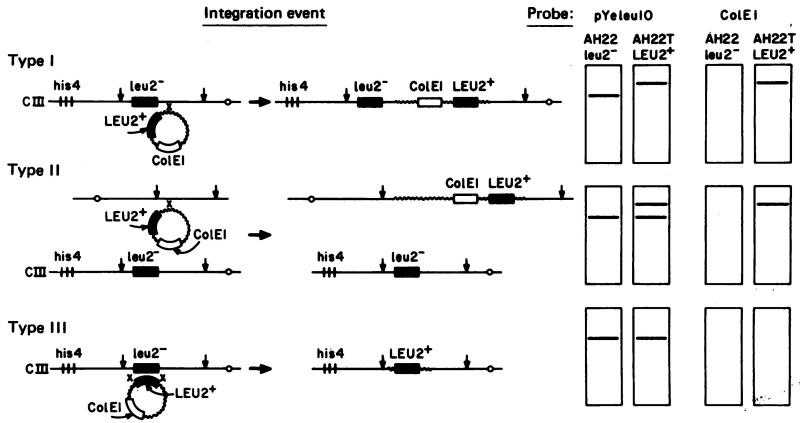


Similar to λ phage integration

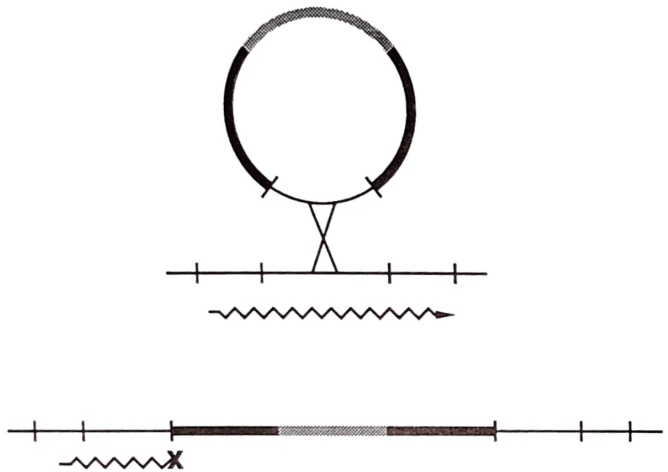


http://biology.kenyon.edu

Three types of yeast transformants...

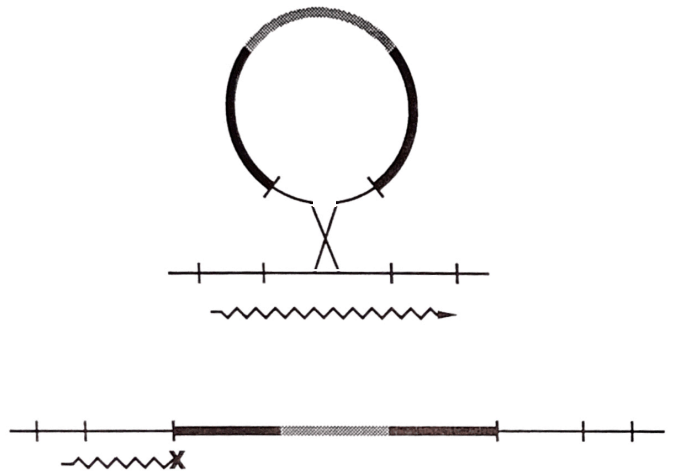


Integration by homologous recombination



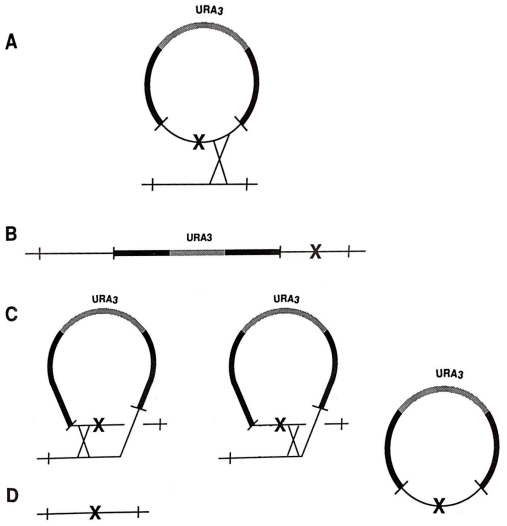
Rothstein Meth Enzymol. 1992 194:281

Directing integration by a double-strand break



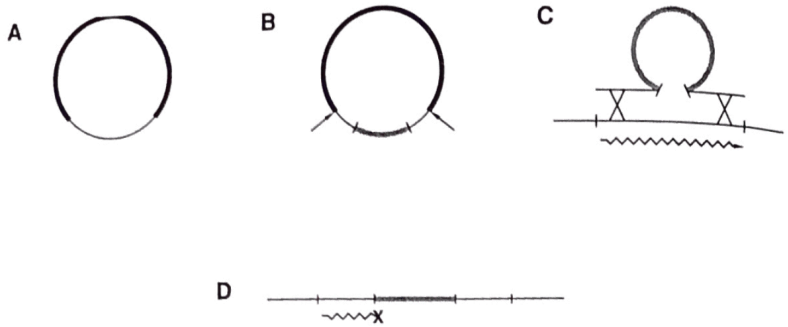
Rothstein Meth Enzymol. 1992 194:281

Excision can leave a mutation in the target gene



Rothstein Meth Enzymol. 1992 194:281

Insertional disruption in one step



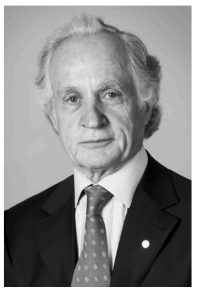
Rothstein Meth Enzymol. 1992 194:281

A yeast model for genome engineering

- Genes can be introduced into a target genome
- They can be directed to a single site
  - Requires a double-strand DNA break
  - This stimulates homologous recombination
- Regions of the target chromosome can be replaced
  - Can be done by two-step integration/excision
  - With a positive selection for the result, one-step disruption is possible
- But, homologous recombination in higher eukaryotes is much less active
  - Something needed to be done to increase the efficiency of site-specific recombination into the genome

Gene knockouts in mammalian cells

The Nobel Prize in Physiology or Medicine 2007



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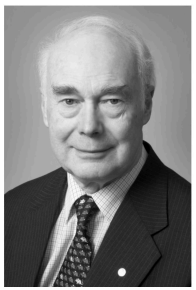
Mario R. Capecchi

Prize share: 1/3



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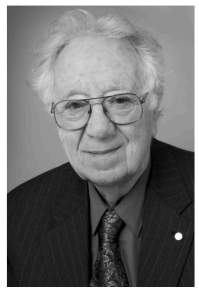
Sir Martin J. Evans

Prize share: 1/3



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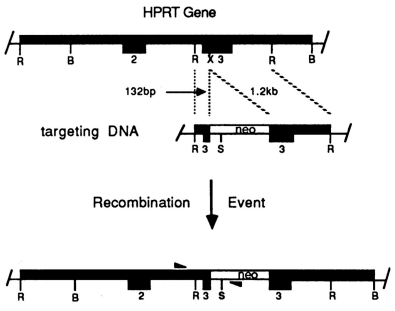
Oliver Smithies

Prize share: 1/3

https://www.nobelprize.org/

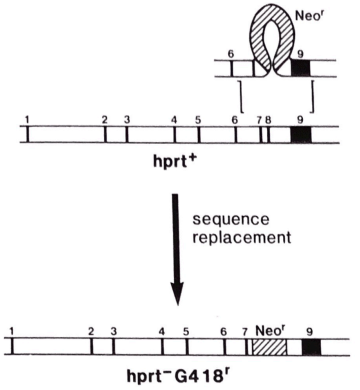
Inserting neomycin resistance gene into mouse *hprt* gene

Oliver Smithies lab



Doetschman et al. Proc Natl Acad Sci U S A. 1988 85:8583

Mario Cappechi's lab



Thomas &Capecchi. Cell. 1987 51:503

HPRT = hypoxanthine-guanine phosphoribosyltransferase

How did this procedure work?

- Double positive selection
  - Selected for resistance to G-418 (target of the neoR resistance gene); cells that expressed the neoR gene survived
  - Performed a second selection for resistance to 6-thioguanine (a suicide compound that must be phosphorylated by HGPRT to be used in DNA synthesis); cells in which HGPRT was mutated survived
- The double selection allowed identification of transfectants carrying the inserted gene at frequencies of about 1 in 100,000 cells
- Could this procedure work without a double selection?
- How would that work to mutate a gene without a negative selection against its expression?

Lesson of double strand brakes

- The yeast work showed a targeted double-strand break greatly increased the frequency of homologous recombination
- How could that be achieved in mammalian species?

Introduction of site specific cleavage sites

- Use of extremely rarely cutting endonuclease
  - SceI: endonuclease from yeast mitochondrial intron

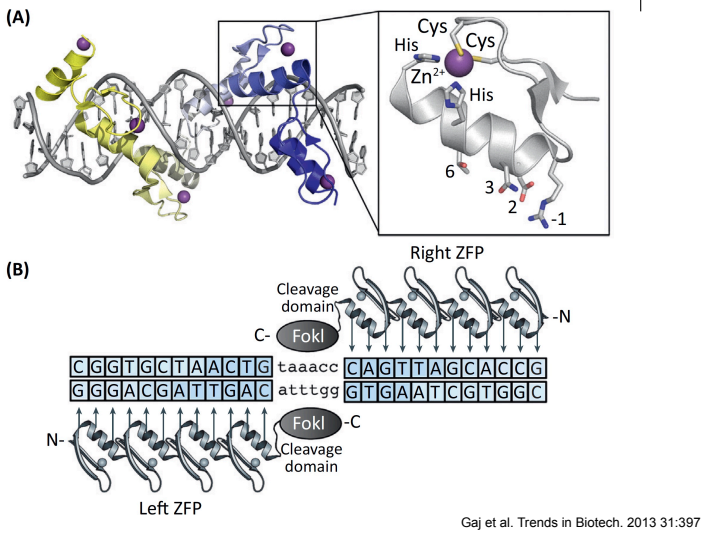
5′...TAGGGATAACAGGGTAAT...3′
3′...ATCCCTATTGTCCCATTA...5′

- Use of site-specific recombination systems
  - Cre-Lox (bacteriophage P1)
  - Flp-Frt (yeast 2μ circle endogenous plasmid)
- Problems?
  - Severe limits on where sites can be located
  - Need to generate strains with targets in required location
- These systems are used in special circumstances but not for mutagenesis

Creating designer site-specific endonuclease

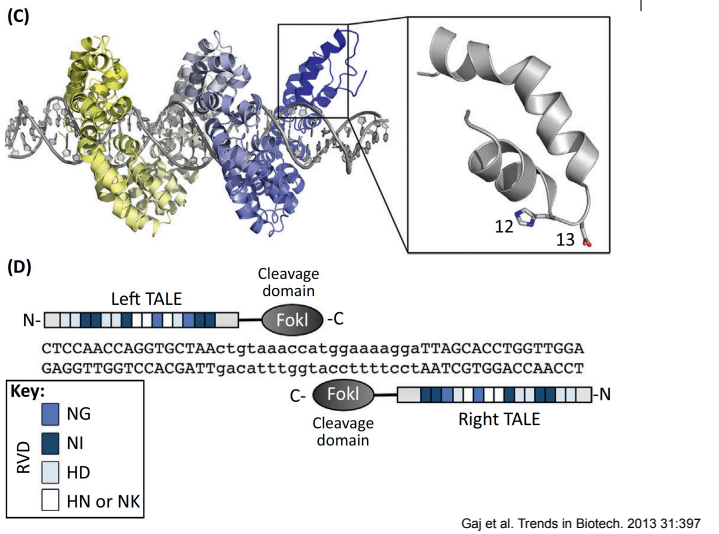
- Zinc finger nucleases (ZFN)
- Transcription activator-like effector nuclease (TALEN)
- Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR Associated Protein 9 (Cas9)

Zinc-finger nuclease mechanism



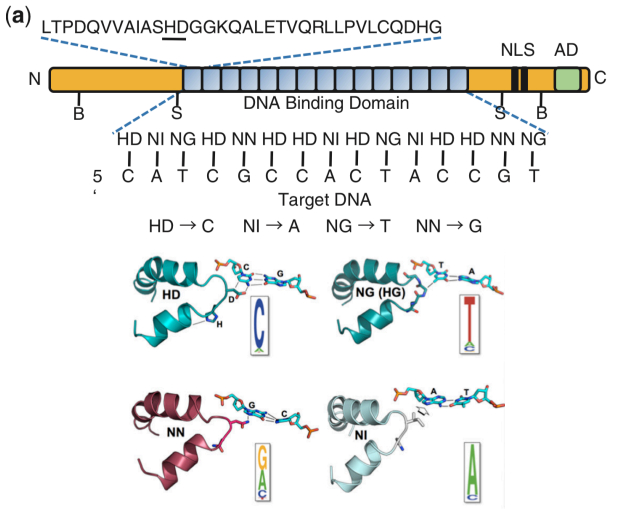
Gaj et al. Trends in Biotech. 2013 31:397

Transcription activator-like effector nuclease (TALEN) mechanism



Gaj et al. Trends in Biotech. 2013 31:397

Organization of TAL Proteins



Mak et al. Curr Opin Struct Biol. 2013 23:93

Cermak et al. Nucleic Acids Res. 2011 39:e82

Pros and Cons about these nucleases

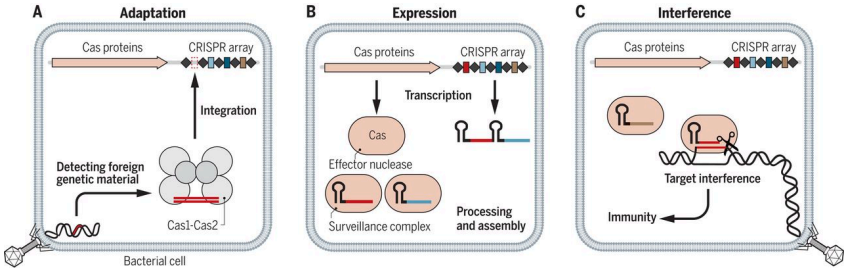
Pro

- Nucleases can be specifically designed for a target sequence
- High specificity recognition (little or no off-site targeting)
- Generate a double-strand cut appropriate for stimulating recombination

Cons

- Time consuming to generate nucleases
- Expensive
- Single use

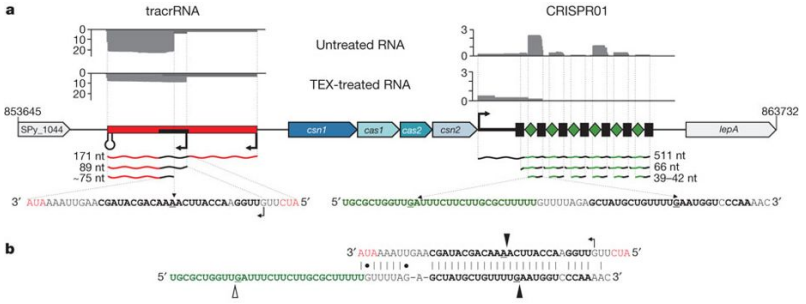
CRISPR-Cas in bacteria



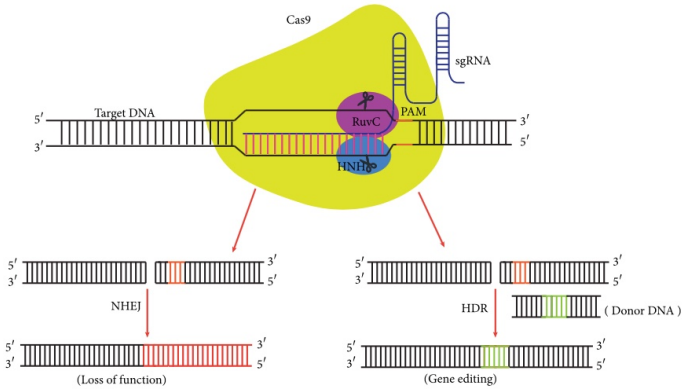
Knott & Doudna Science 2018 361:866

Structure of *Streptococcus pyogenes* CRISPR region

- Two types of small RNAs are encoded:
  - CRISPR RNA (crRNA)
  - Transactivating CRISPR RNA (tracrRNA)
- These pair in the CRISPR-Cas complex
- They can be combined into a single guide RNA (sgRNA)



Model of a CRISPR-Cas9 complex



PAM = protospacer adjacent motif (= 5'-NGG-3' for Cas9)

sgRNA = single guide RNA

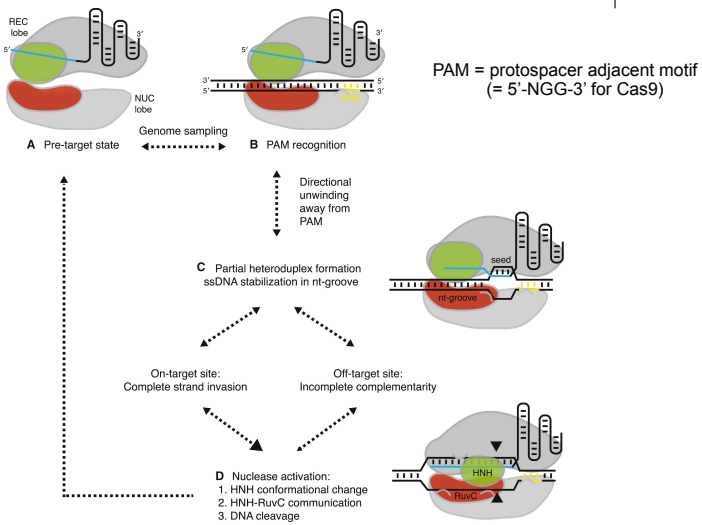
HNH & RuvC = single strand nucleases

HDR = homology-directed repair

NHEJ = non-homologous end joining

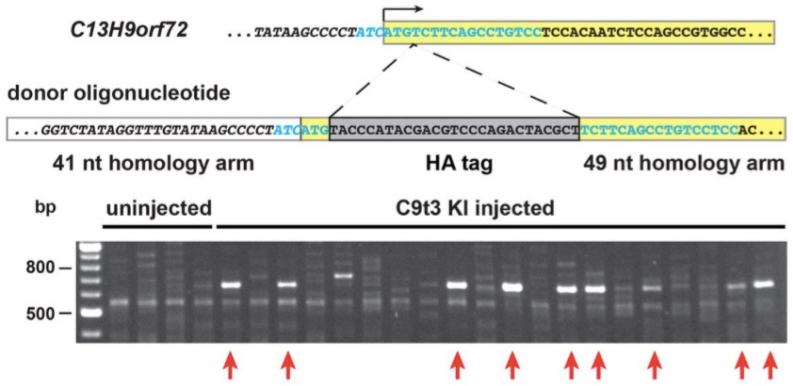
Lone et al. Genet Res Int. 2018 2018:3797214

Mechanism of CRISPR-Cas DNA cleavage

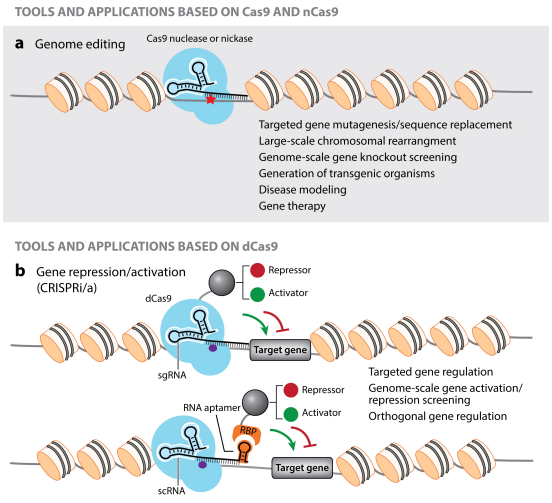


CRISPR is very efficient

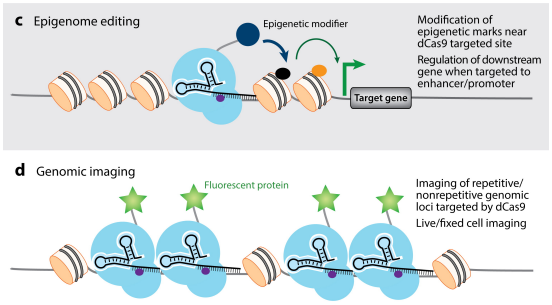
- Between 3 and 50% of unselected pups carry the CRISPR induced mutation
- Analysis of 20 unselected pups, with 45% mutated



Uses of CRISPR-Cas



More uses of CRISPR-Cas



Wang H, et al. 2016.
Annu. Rev. Biochem. 85:227–64

Reading for next time:

- Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell. 2013 154:442

Figure 1

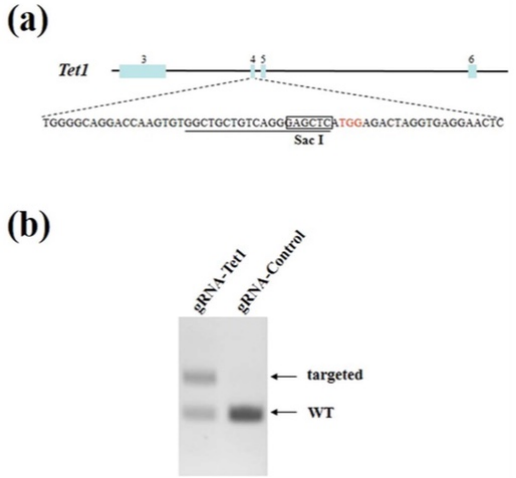


Figure 2

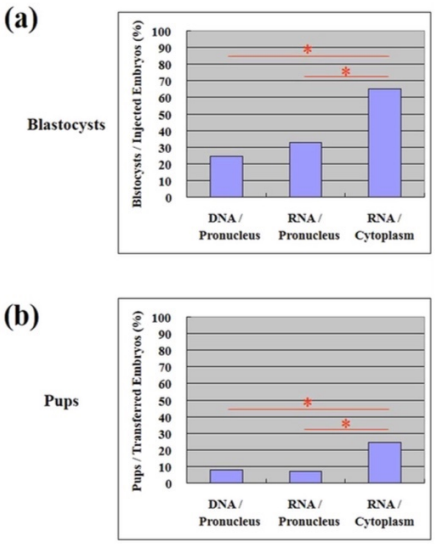


Table 2 & 3

Table 1 | *In vitro* development of CRISPR/Cas introduced embryos using three microinjection methods

gRNA	microinjection method	survived/injected	blastocyst/survived
Tet1 Ex4	DNA/Pronucleus	41/52 (78.8%)	10/41 (24.4%)
	RNA/Pronucleus	52/61 (85.2%)	17/52 (32.7%)
	RNA/Cytoplasm	46/63 (73.0%)	30/46 (65.2%)

DNAs or RNAs of hCas9 and gRNA were mixed and injected into mouse zygotes by three methods: (1) injection of DNA into the pronucleus, (2) injection of RNA into the pronucleus, and (3) injection of RNA into the cytoplasm.

Table 2 | Efficiency of CRISPR/Cas-mediated gene targeting using three microinjection methods

gRNA	microinjection method	survived/injected	pups/transferred	KO/pups	homo/pups	wild:hetero:homo
Tet1 Ex4	DNA/Pronucleus	62/82 (75.6%)	5/62 (8.1%)	4/5 (80.0%)	1/5 (20.0%)	1:3:1
	RNA/Pronucleus	72/81 (88.9%)	5/72 (6.9%)	5/5 (100%)	5/5 (100%)	0:0:5
	RNA/Cytoplasm	55/72 (76.4%)	9/37 (24.3%)	9/9 (100%)	8/9 (88.9%)	0:1:8
Tet1 Ex7	DNA/Pronucleus	41/54 (75.9%)	8/41 (19.5%)	2/8 (25.0%)	1/8 (12.5%)	6:1:1
	RNA/Pronucleus	46/56 (82.1%)	15/46 (32.6%)	7/15 (46.7%)	3/15 (20.0%)	8:4:3
	RNA/Cytoplasm	31/46 (67.4%)	19/31 (61.3%)	10/19 (52.6%)	4/19 (21.1%)	9:6:4

DNAs or RNAs of hCas9 and gRNA were mixed and injected into mouse zygotes by three methods: (1) injection of DNA into the pronucleus, (2) injection of RNA into the pronucleus, and (3) injection of RNA into the cytoplasm. The injected eggs were transferred into pseudopregnant females. The mutations were identified by digestion of PCR amplified fragment containing target with a restriction enzyme at the hCas9 cleavage sites followed by agarose gel electrophoresis.

Figure 3

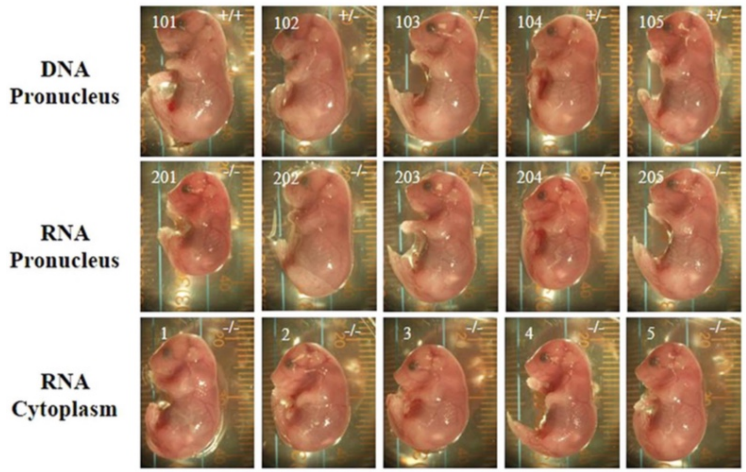


Figure 4

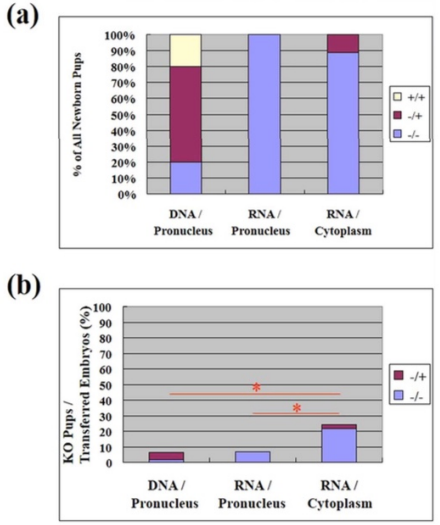


Figure 5