UAB COMPREHENSIVE ARTHRITIS, MUSCULOSKELETAL, BONE, AND AUTOIMMUNITY CENTER

Knowledge that will change your world

Sponsored by
Rheumatic Diseases Core Center (P30-AR048311) &
Comprehensive Arthritis, Musculoskeletal, Bone and Autoimmunity Center (CAMBAC)

Please come to the **Research in Progress Seminar Series**

WONDERFUL WORLD OF TECHNOLOGY

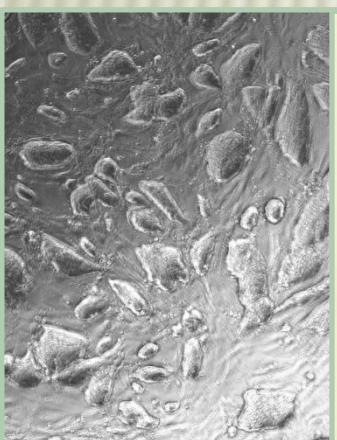
event featuring

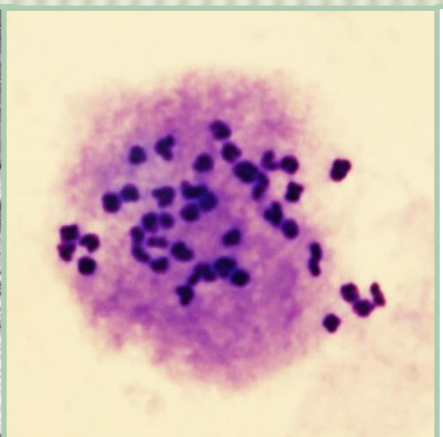
Thomas M. Ryan, PhD
Associate Professor,
Biochemistry & Molecular Genetics

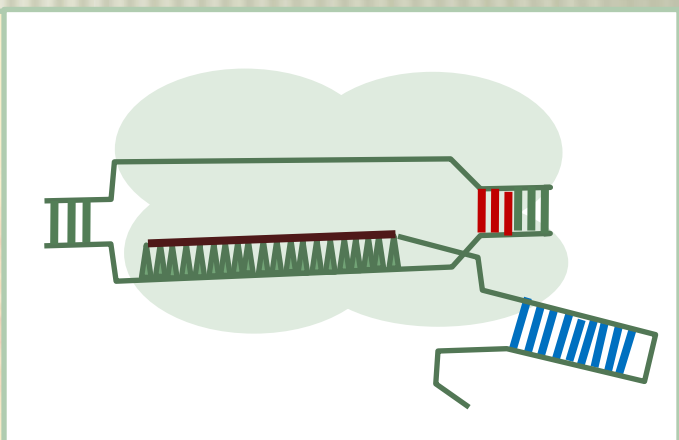
The Wonderful World of Gene Editing Using CRISPR/Cas9

Thursday, Feb 5, 2015
12:00 – 1:00 PM
SHEL 515, 1825 University Blvd.

Raw data welcome!
You provide the science, and we'll provide the food.







The Wonderful World of Gene Editing Using CRISPR/Cas9

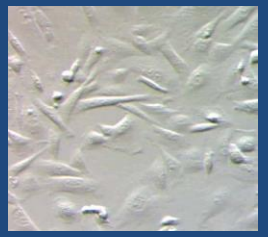
2/5/2015

Thomas M. Ryan, PhD
Biochemistry and Molecular Genetics

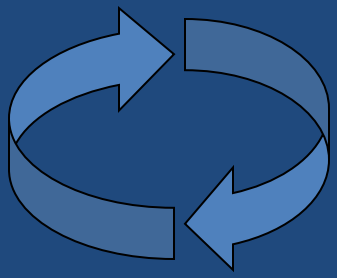
Regenerative Medicine

Patient

Somatic Cell Biopsy



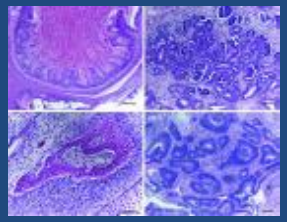
Derive Isogenic Pluripotent Stem Cells



Repair DNA lesion

Corrected Stem Cells

In vitro differentiation



Transplant back into patient

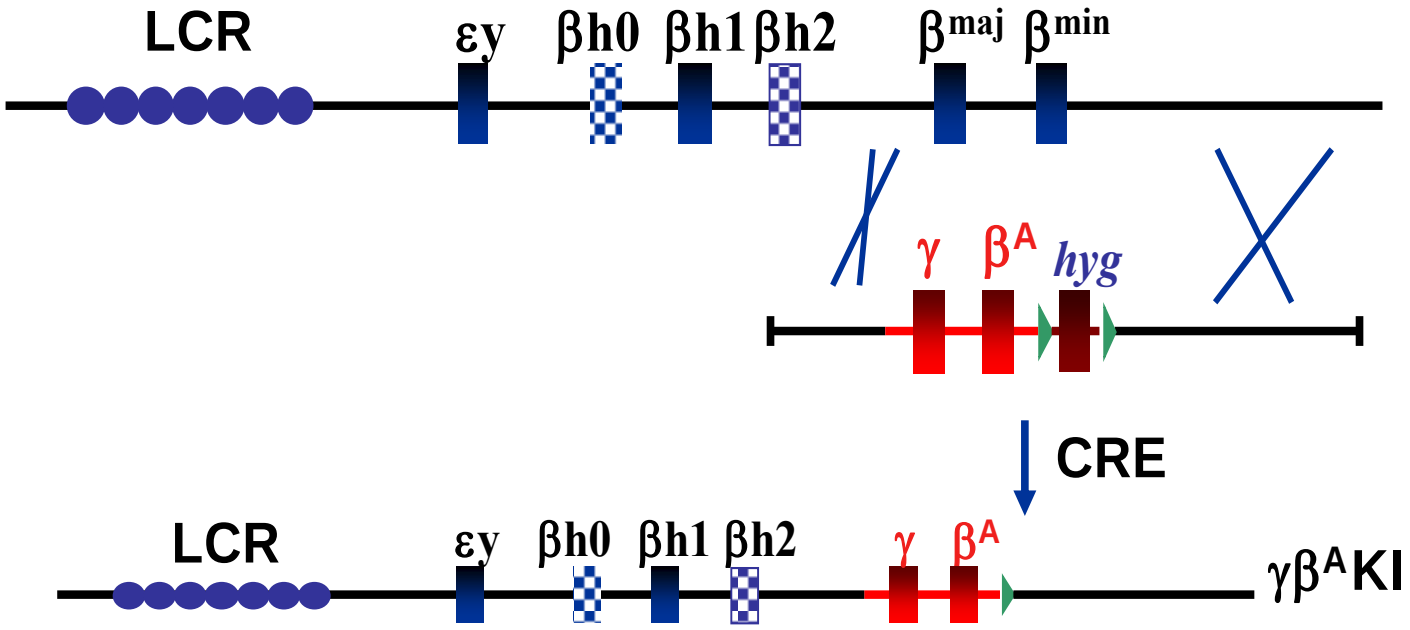
Gene Correction by Homologous Recombination in Pluripotent Stem Cells

Mouse: Homologous recombination (HR) methodology in murine ES cells is relatively straight forward. Gene targeting (knockouts, knockins, etc.) using targeting constructs with 5' and 3' homology regions flanking a selectable marker have been used to modify the mouse genome for over 25 years.

Human: Gene correction by HR has proven much more difficult in human ES/iPS cells. Their slower growth and lower plating efficiencies have resulted in only a handful of genes to be targeted by standard techniques.

Newer gene correction methods with higher efficiencies are needed.

Humanized Hb Mouse Model: Human γβ^A Globin Knock-In



Mario Capecchi, Martin Evans, and Oliver Smithies were awarded the Noble Prize for Physiology and Medicine in 2007 for this "Gene Targeting" technique.

Double strand breaks (DSB) in the DNA can stimulate the amount of homologous and non-homologous recombination

DNA double strand breaks can be repaired by two different mechanisms:

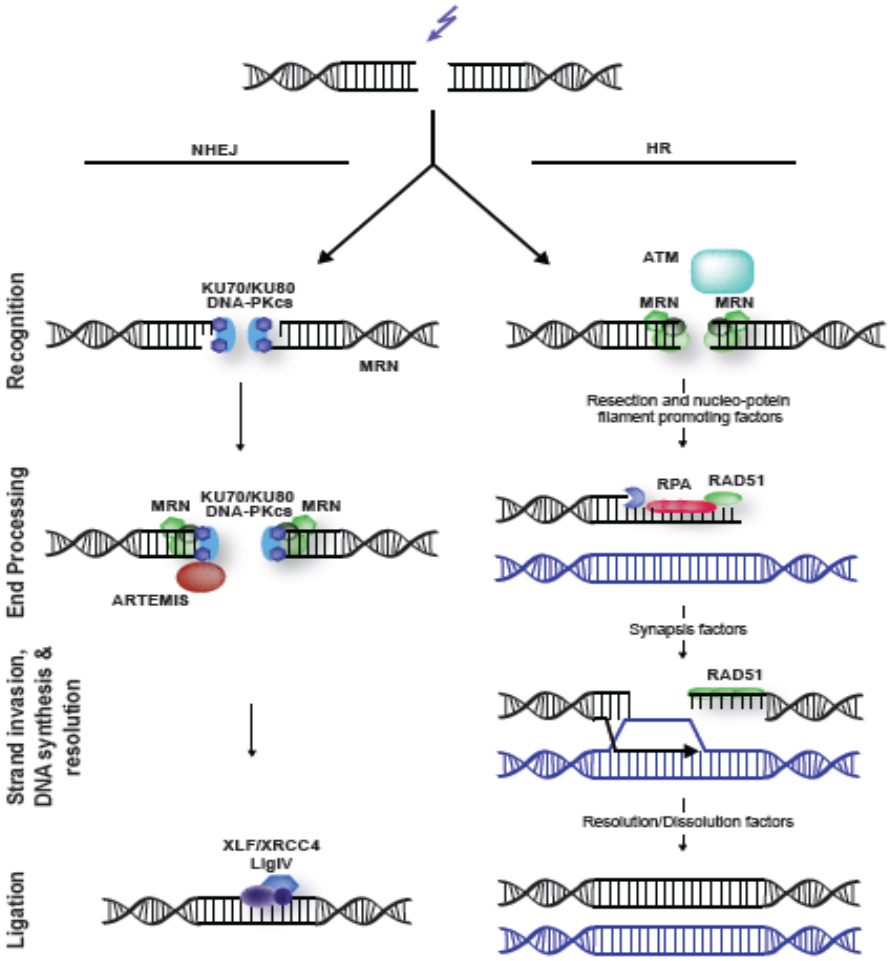
1. **Non-homologous end joining (NHEJ)**
2. **Homology directed repair (HDR)**

Non-Homologous End Joining (NHEJ)
Versus
Homology Directed Repair (HDR)

Gene editing systems can be used to mutate or repair genes.

Synthetic endonuclease systems such as ZFNs, TALENS or CRISPR/Cas 9 can efficiently generate site specific double strand breaks in the DNA.

The DNA can be repaired in one of two ways: Non-Homologous End Joining or Homology Directed Repair.



Lans et al. Epigenetics & Chromatin 2012

Synthetic DNA Nucleases can Generate Site Specific Double Strand Breaks

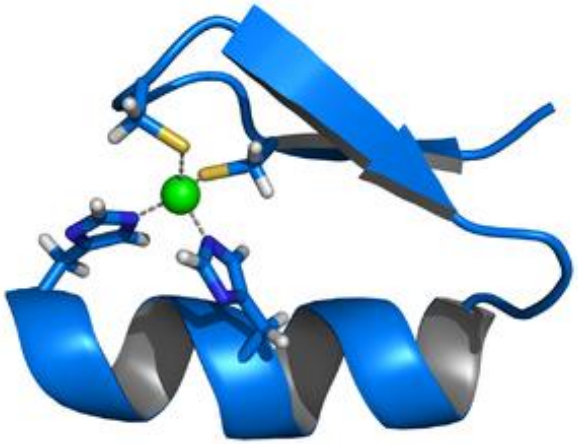
Synthetic DNA Binding Proteins Fused to a Type IIS Restriction Endonuclease (usually FokI)

Two main types:

ZFNs: Zinc Finger Nucleases

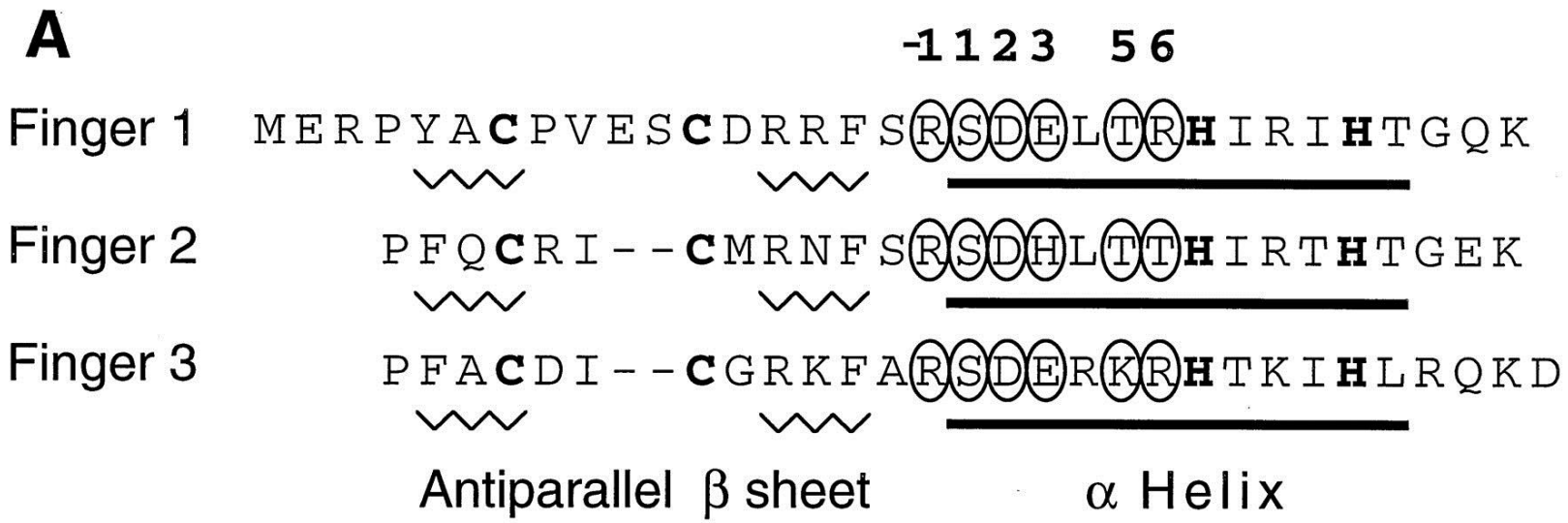
TALENs: Transcription Activator-Like Effector Nucleases

Zinc Finger Proteins



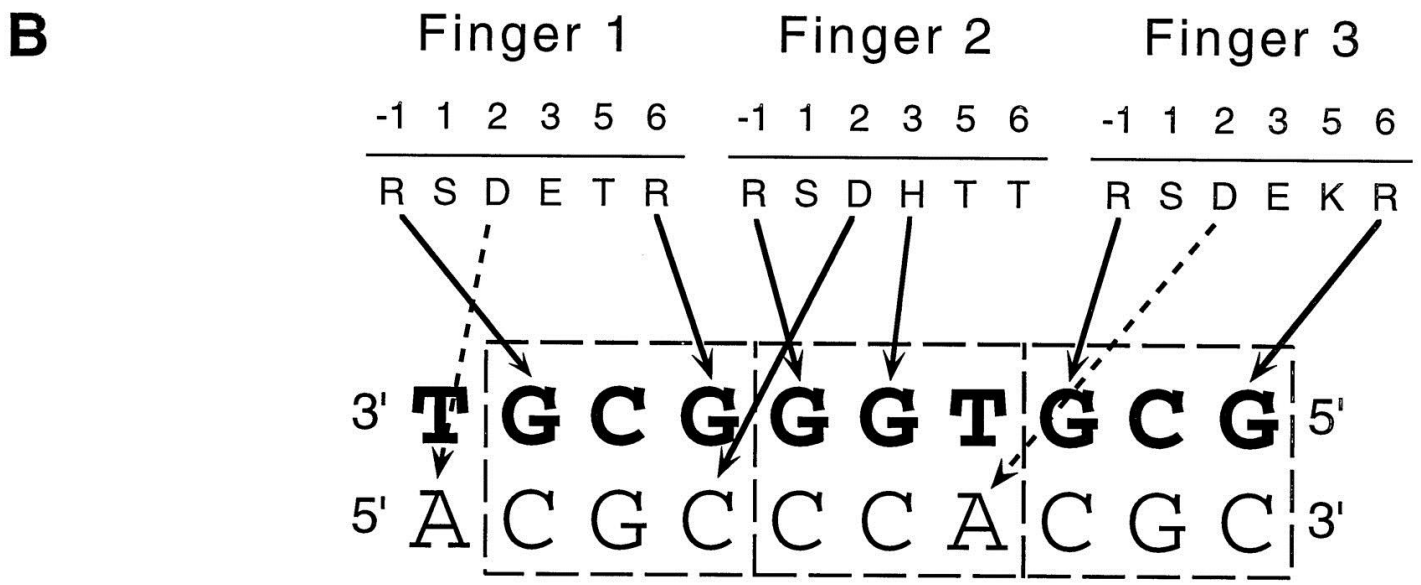
- **Each finger recognizes several DNA bases**
- **Bind in the major groove of DNA**
- **Libraries of zinc fingers have been constructed that recognize each DNA triplet**
- **Combining 4 zinc fingers together enables the recognition and binding to a 12 base pair sequence.**

Important Contact Amino Acids in the Zif268 Alpha Helix



H A Greisman, and C O Pabo Science 1997;275:657-661

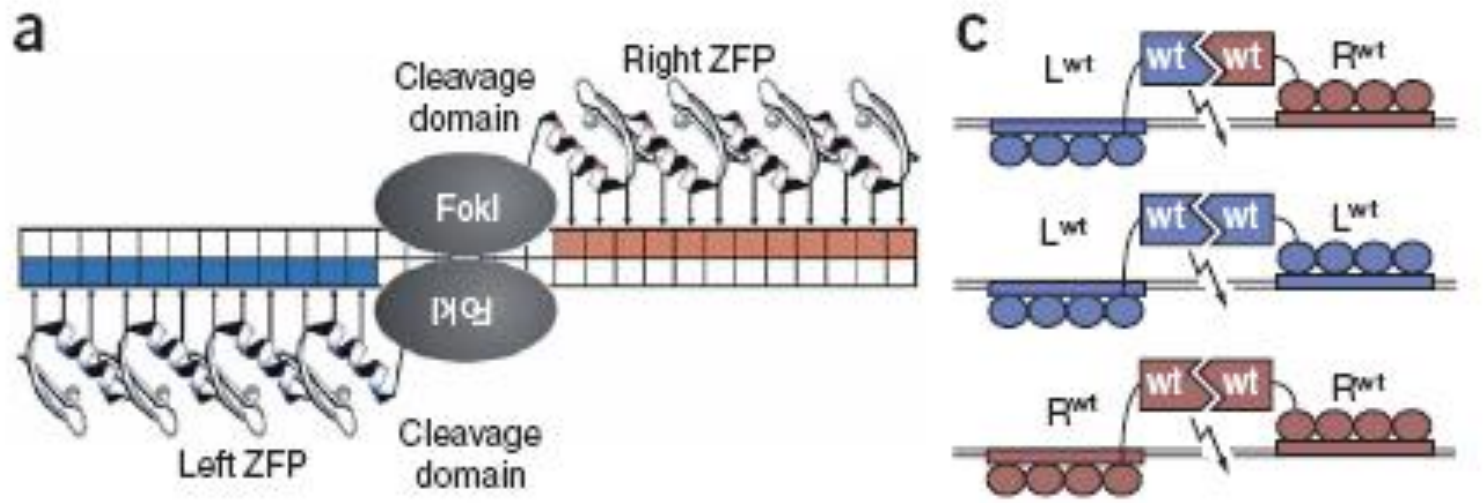
Inter and Intra Finger Amino Acid Contacts in Zif268



H A Greisman, and C O Pabo Science 1997;275:657-661

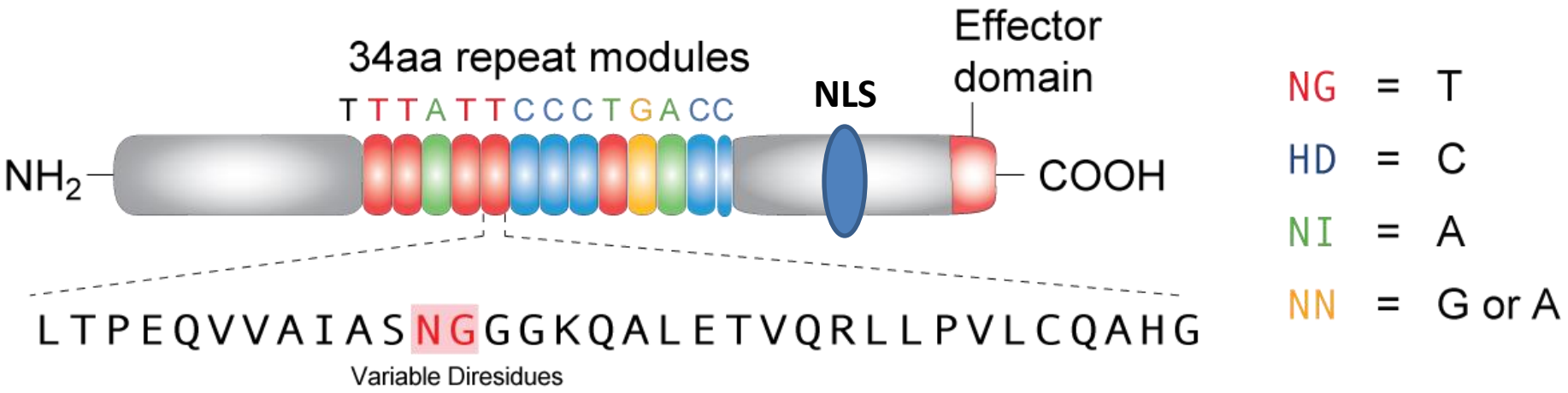
Zinc Finger Nuclease

VOLUME 25 NUMBER 7 JULY 2007 NATURE BIOTECHNOLOGY



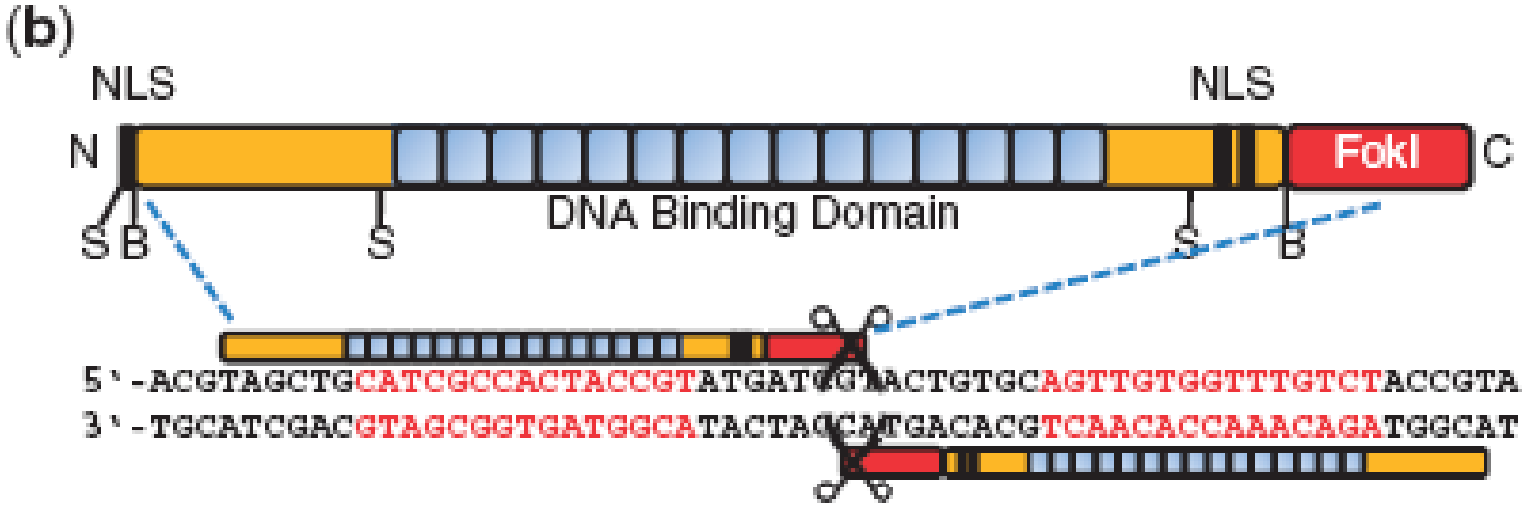
- **Catalytic domain of the FokI restriction endonuclease is fused to the zinc finger DNA binding domains**
- **FokI works as a homodimer—ZFNs are used in pairs (Left and Right)**
- **FokI homodimer cleaves the DNA making a double strand break (DSB)**
- **Non-Homologous End Joining (NHEJ) DNA repair machinery will fix the DSB, but this repair is not exact--generates mutations**
- **Undesired "off target" DNA mutation can occur at sites that bind only the left or right ZFN**

Transcription Activator-Like (TAL) Effectors



- **Transcription Activator-Like (TAL) effectors are a new class of specific DNA binding proteins**
- **Produced by pathogenic *Xanthomonas* bacteria (plant pathogen)**
- **Normal function is to induce gene expression in the host plant**
- **DNA binding domain is a repeating 34 amino acid sequence that varies at positions 12 and 13**
- **This dipeptide is termed the repeat-variable di-residue (RVD)**
- **Each RVD recognizes a single DNA base**

TALENs--Transcription Activator-Like Effector Nucleases



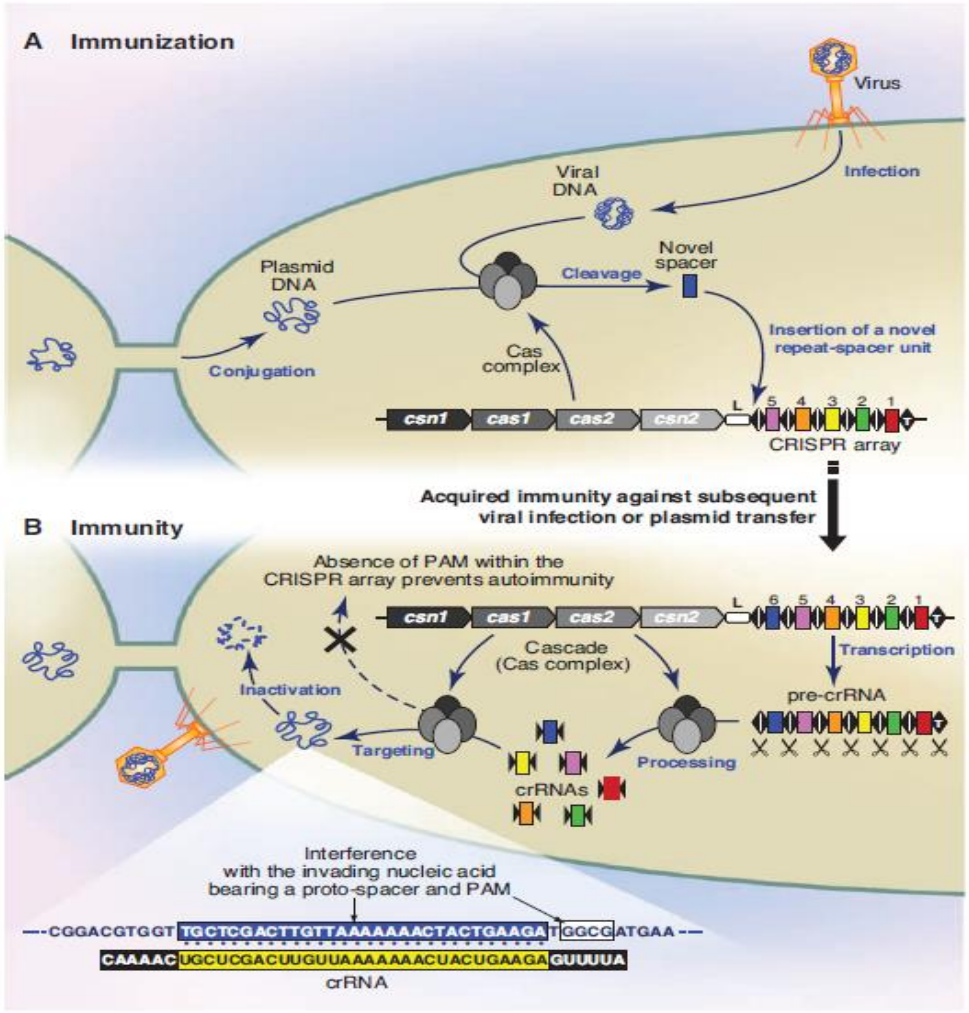
Nucleic Acids Research, 2011, 1–11
doi:10.1093/nar/gkr218

- **TALEN pairs can be designed to specifically make DSBs in DNA**
- **DNA DSBs can be repaired by NHEJ generating mutations**
- **DSBs can be repaired by homology directed repair (HDR) by providing short pieces of donor DNA that share homology with the cut sequence**

CRISPR/Cas

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)
CRISPR-associated (Cas)

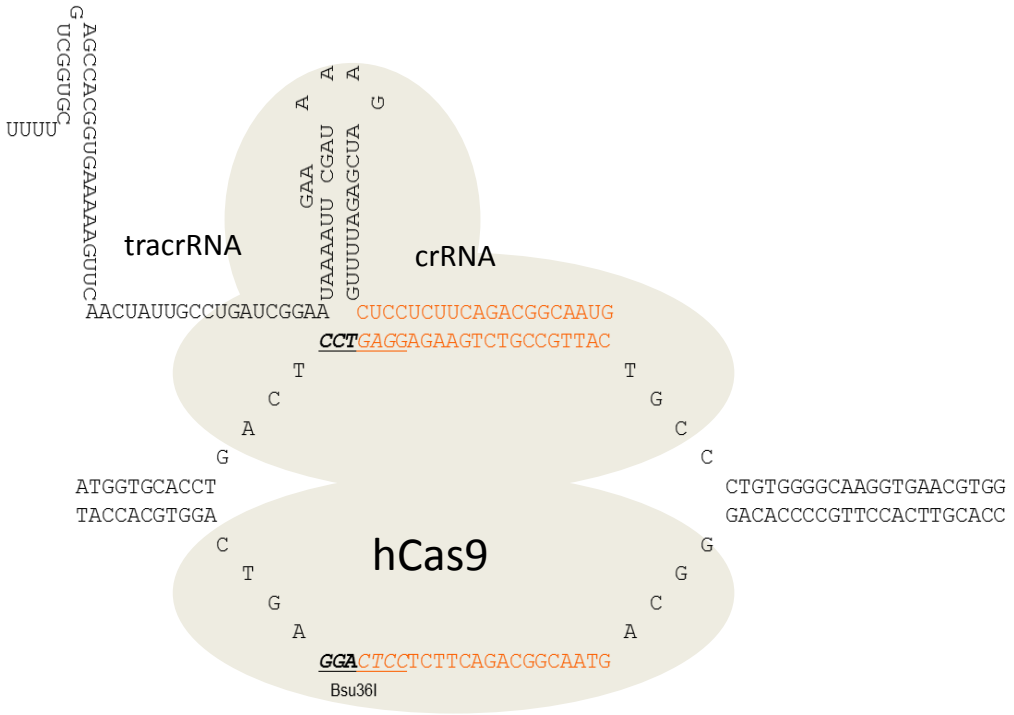
- **Bacterial and archaeal adaptive immune system**
- **CRISPR locus contains several endonucleolytic proteins and palindromic repeats**
- **Foreign nucleic acids containing a proto-spacer adjacent motif (PAM) are cut into small fragments and incorporated into the bacterial genome between the spacer repeats**
- **Subsequent challenge by the same species triggers the expression of the locus**
- **Pre-crRNA is transcribed that is processed to individual crRNAs which can bind to their complementary foreign DNA.**
- **A trans acting CRISPR RNA (tracrRNA) helps assemble the Cas nuclease with the crRNA annealed to the DNA.**
- **A Cas nuclease cuts the foreign DNA before the PAM.**



Horvath and Barrangou Science 2010

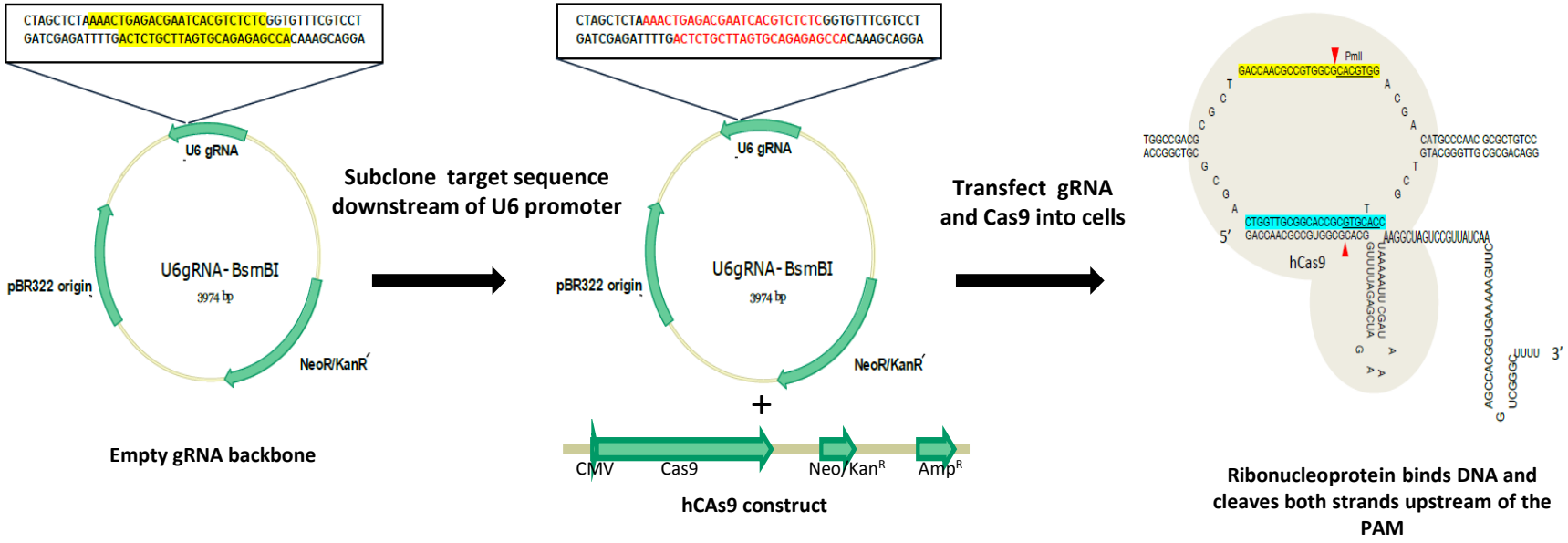
CRISPR/Cas System

- Representative structure formed at the genomic site when CRISPR/Cas binds the DNA
- dsDNA is separated and strands are bound by different domains of the complex
- gRNA anneals to complementary sequence and each DNA strand is cleaved upstream of the PAM

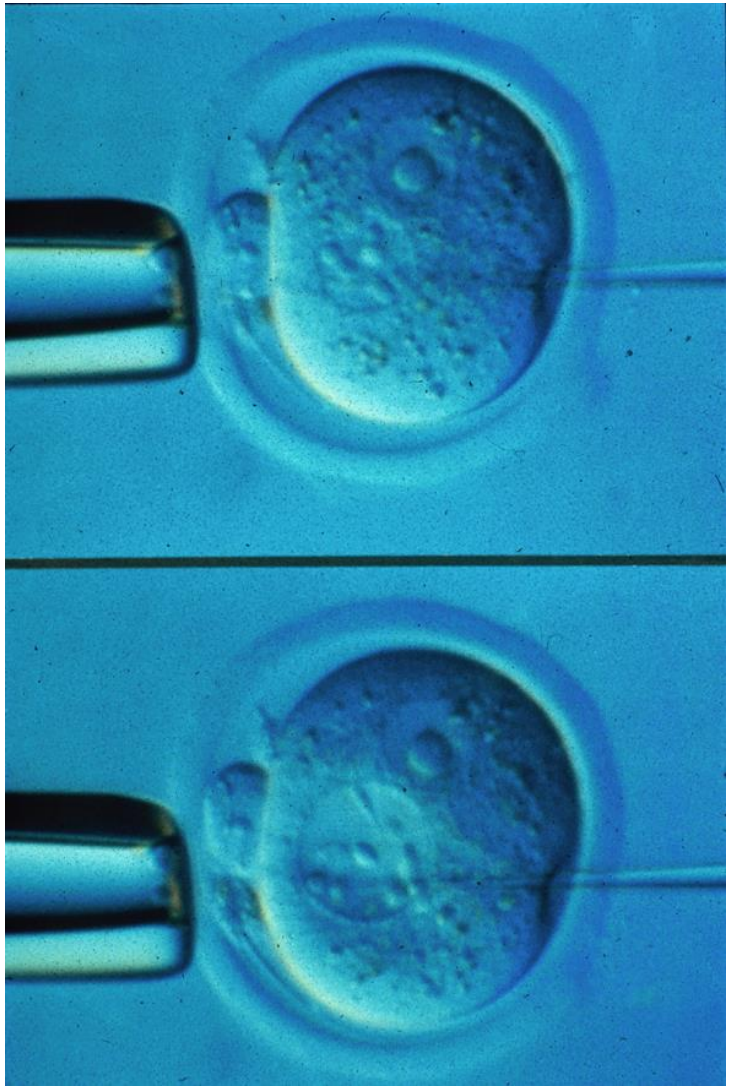


Adapted from Mali et al. Science 2013

sgRNA Production



Generation of Transgenic/Knockout Mice by Gene Editing

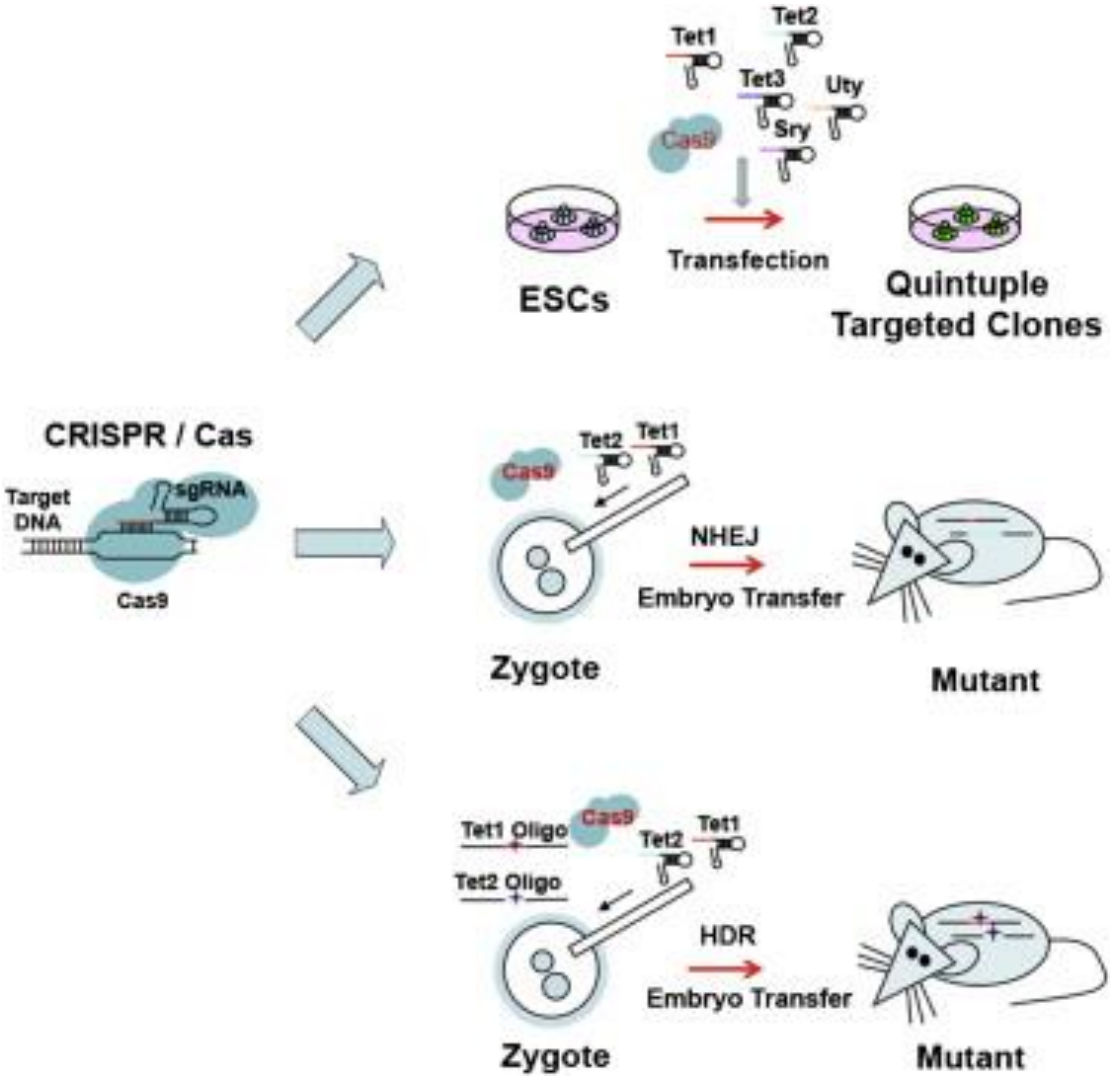


Injection of mRNAs that translate into ZFN or TALEN pairs into fertilized mouse embryos.

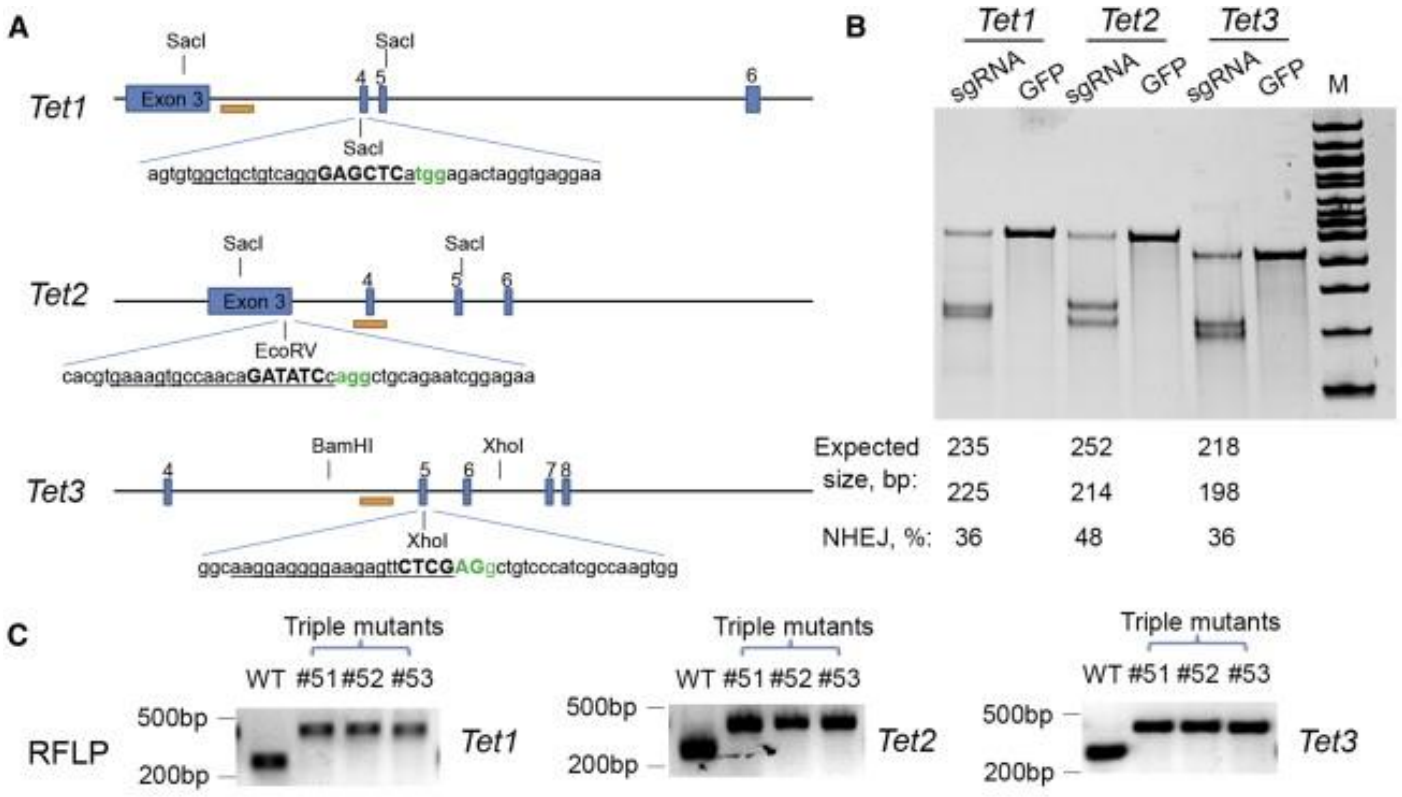
Injection of sgRNAs and mRNA for Cas9 into fertilized mouse embryos.

One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering

Haoyi Wang[6], Hui Yang[6], Chikdu S. Shivalila[6], Meelad M. Dawlaty, Albert W. Cheng, Feng Zhang, Rudolf Jaenisch
[6]These authors contributed equally to this work
DOI: http://dx.doi.org/10.1016/j.cell.2013.04.025



Tet Gene Mutation in ES Cells Using CRISPR/Cas9



Surveyor Assay (Cel I) used in B to test CRISPR/Cas9 efficiency.

Restriction Fragment Length Polymorphism (RFLP) assay used in C to analyze triple mutants.

DOI: http://dx.doi.org/10.1016/j.cell.2013.04.025

Tet Gene Mutation in ES Cells Using CRISPR/Cas9

Table 1. CRISPR/Cas-Mediated Gene Targeting in V6.5 ES Cells

	Mutant Alleles per Clone / Total Clones Tested						
Gene	6	5	4	3	2	1	0
Tet1	N/A				27/48	4/48	17/48
Tet2					37/48	2/48	9/48
Tet3					32/48	3/48	13/48
Tet1+ Tet2 + Tet3	20/96	16/96	2/96	2/96	1/96	0/96	55/96

Plasmids encoding Cas9 and sgRNAs targeting *Tet1*, *Tet2*, and *Tet3* were transfected separately (single targeting) or in a pool (triple targeting) into ES cells. The number of total alleles mutated in each ES cell clone is listed from 0 to 2 for single-targeting experiment, and 0 to 6 for triple-targeting experiment. The number of clones containing each specific number of mutated alleles is shown in relation to the total number of clones screened in each experiment. See also Table S1.

DOI: http://dx.doi.org/10.1016/j.cell.2013.04.025

Tet Gene Mutation In Mouse Embryos Using CRISPR/Cas9

Table 2. CRISPR/Cas-Mediated Single-Gene Targeting in BDF2 Mice

Gene	Cas9/sg RNA (ng/μl)	Blastocysts/Injected Zygotes	Transferred Embryos (Recipients)	Newborns (Dead)	Mutant Alleles per Mouse/Total Mice Tested[a]		
					2	1	0
Tet1	200/20	38/50	19 (1)	2 (0)	2/2	0/2	0/2
	100/20	50/60	25 (1)	3 (0)	2/3	0/3	1/3
	50/20	40/50	40 (2)	8 (3)	4/7	2/7	1/7
	100/50	167/198	60 (3)	12 (2)	9/11	1/11	1/11
Tet2	100/50	176/203	108 (5)	22 (3)	19/20	0/20	1/20
Tet3	100/50	85/112	64 (4)	15 (13)	9/13	2/13	2/13

Cas9 mRNA and sgRNAs targeting *Tet1*, *Tet2*, or *Tet3* were injected into fertilized eggs. The blastocysts derived from 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 2. The number of mice containing each specific number of mutated alleles is shown in relation to the total number of mice screened in each experiment. See also Table S2.

[a]Some of the pups were cannibalized.

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

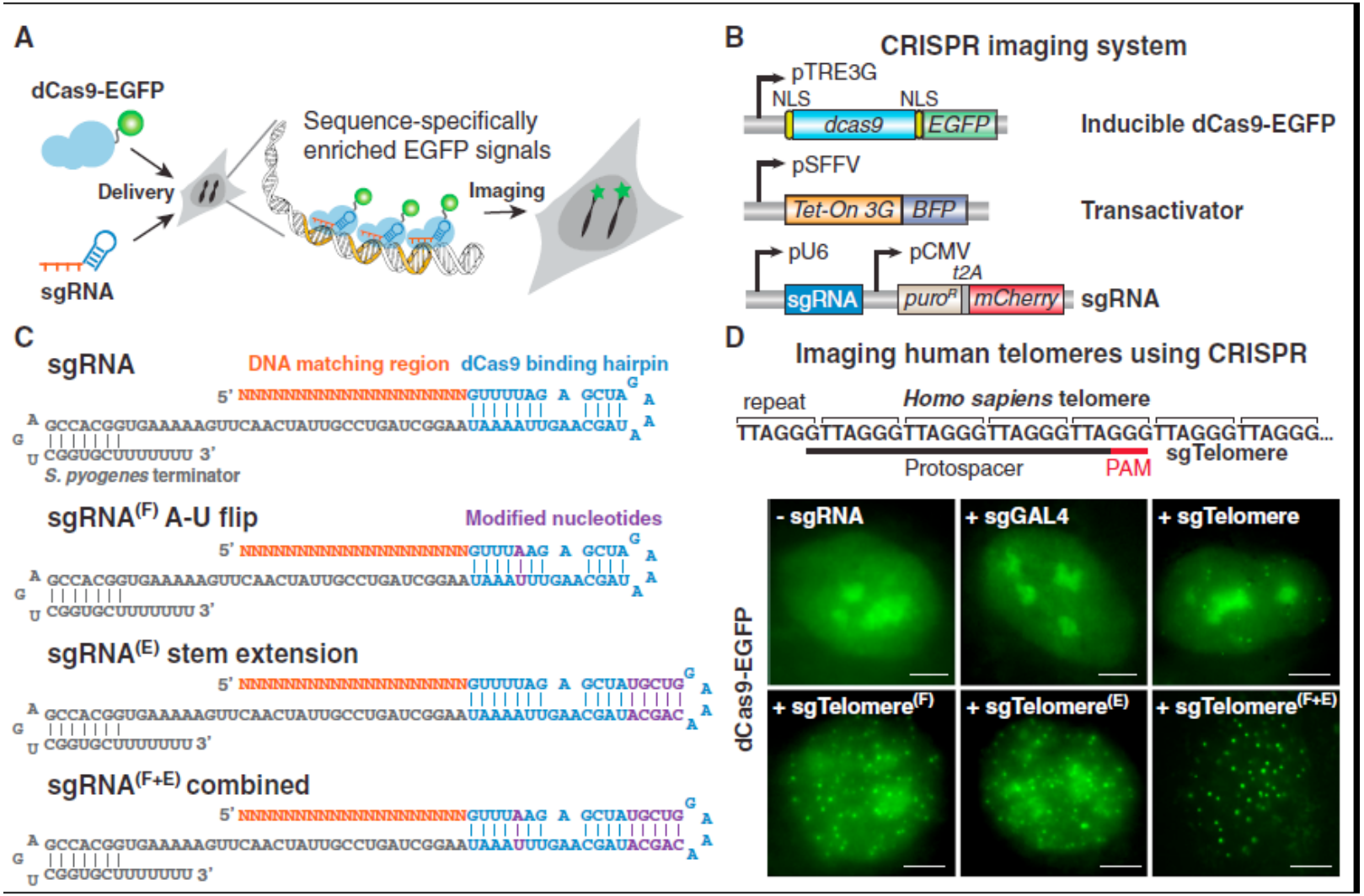
Gene	Cas9/sgRNA (ng/μl)	Blastocyst/Injected Zygotes	Transferred Embryos (Recipients)	Newborns (Dead)	Mutant Alleles per Mouse/Total Mice Tested[a]				
					4	3	2	1	0
Tet1 + *Tet2*	100 / 50	194/229	144(7)	31(8)	22/28	4/28	1/28	1/28	0/28
	20 / 20	92/109	75(5)	19(3)	11/19	1/19	2/19	3/19	2/19

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.

DOI: http://dx.doi.org/10.1016/j.cell.2013.04.025

Optimizing sgRNA and Cas9 Interaction: sgRNA with Flip and Extension (F&E)

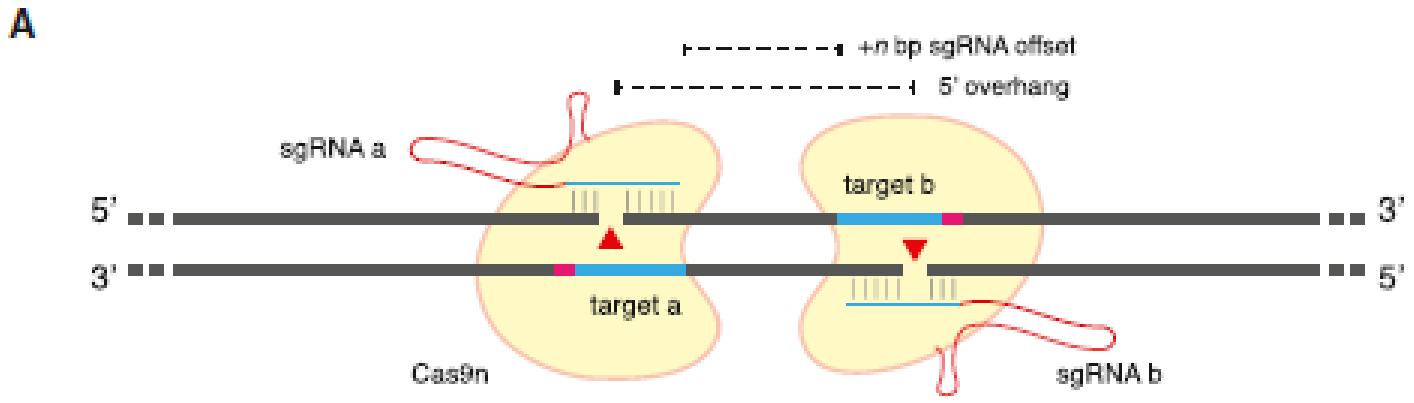


Cell 155, 1479–1491, December 19, 2013

Double Nicking By Mutant Cas9 and a Pair of sgRNAs

Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity

F. Ann Ran,[1,2,3,4,5,11] Patrick D. Hsu,[1,2,3,4,5,11] Chie-Yu Lin,[1,2,3,4,6] Jonathan S. Gootenberg,[1,2,3,4] Silvana Konermann,[1,2,3,4] Alexandro E. Trevino,[1] David A. Scott,[1,2,3,4] Azusa Inoue,[7,8,9,10] Shogo Matoba,[7,8,9,10] Yi Zhang,[7,8,9,10] and Feng Zhang[1,2,3,4,*]

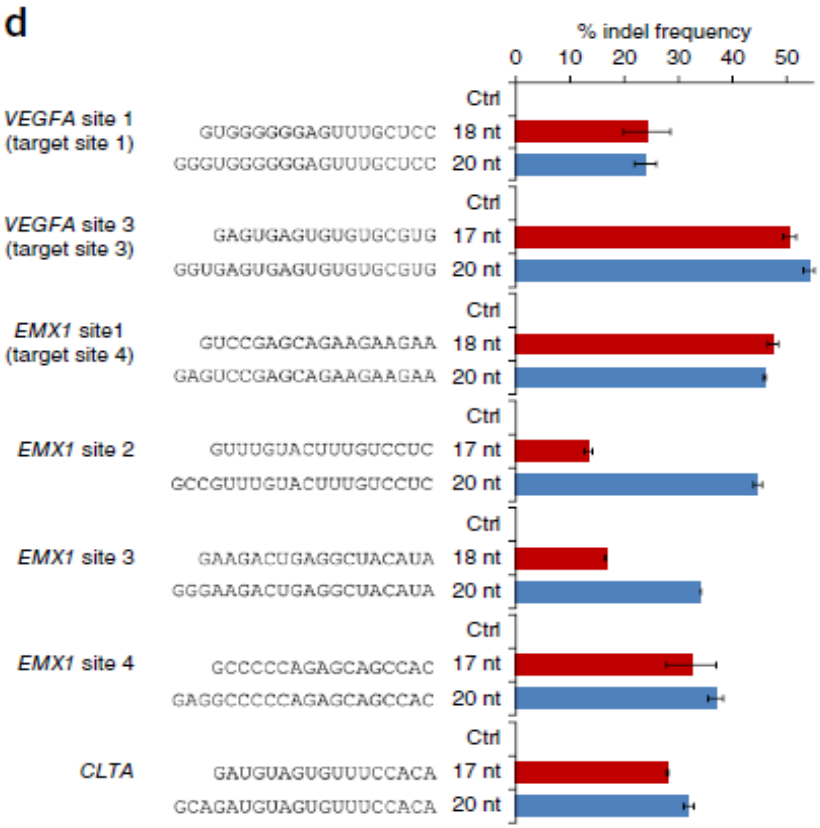


Ran et al. Cell 2013

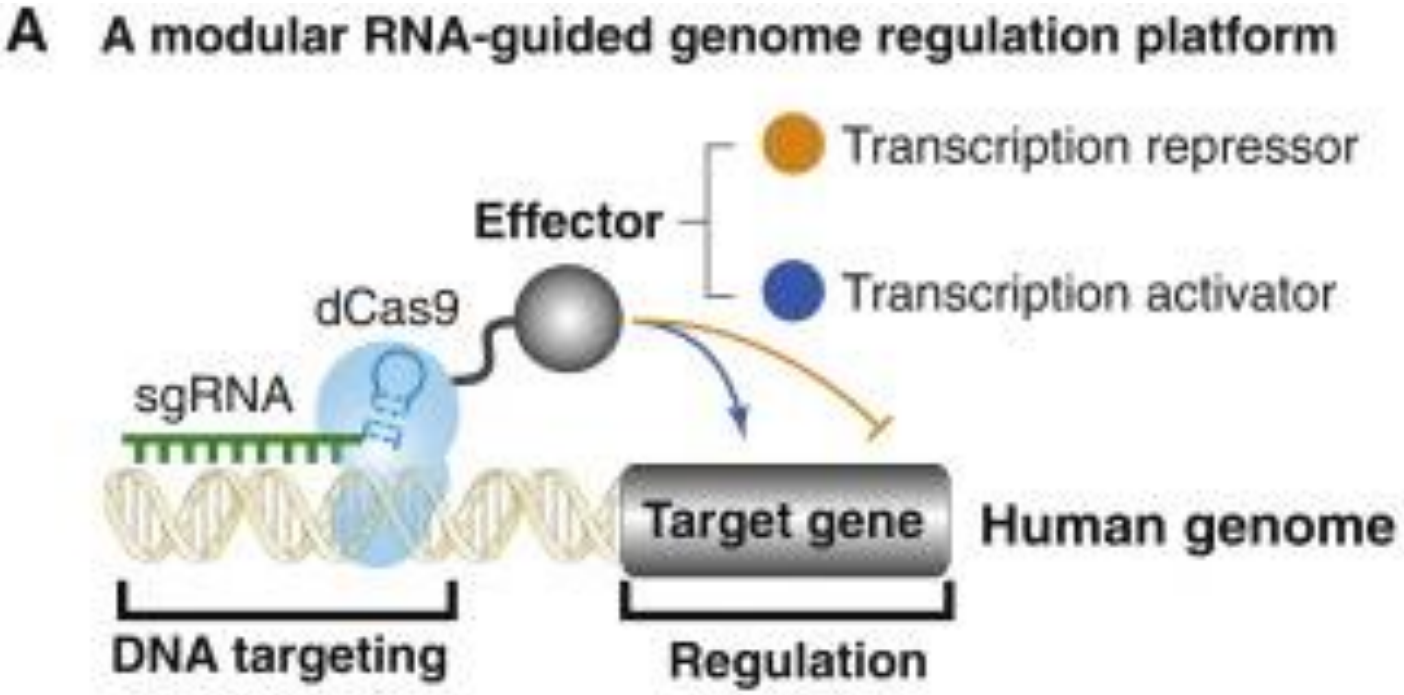
Truncated gRNAs (tru-gRNAs)

Improving CRISPR-Cas nuclease specificity using truncated guide RNAs
Yanfang Fu, Jeffry D Sander, Deepak Reyon, Vincent M Cascio & J Keith Joung
Nature Biotechnology **32**: 279-284 (2014)

- "We hypothesized that off-target effects of RGNs might be minimized by decreasing the length of the gRNA-DNA interface… we reasoned that shorter gRNAs might be more sensitive to mismatches and thus more specific. "
- 17 or 18 bp gRNAs which are hypothesized to be as specific as full length sgRNAs.

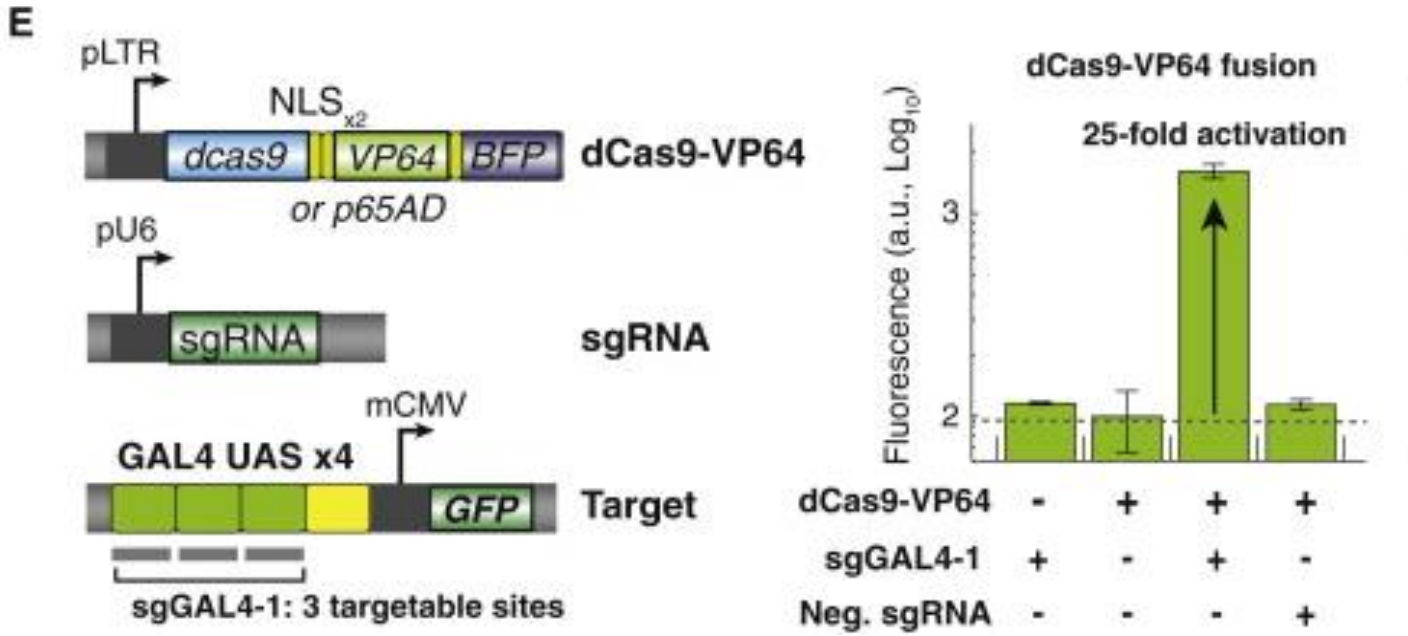


Controlling Gene Expression with CRISPR/dCas9



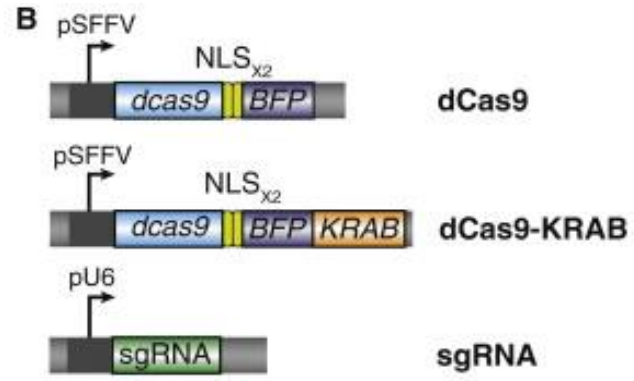
Cell, Volume 154, Issue 2, 2013, 442 - 451

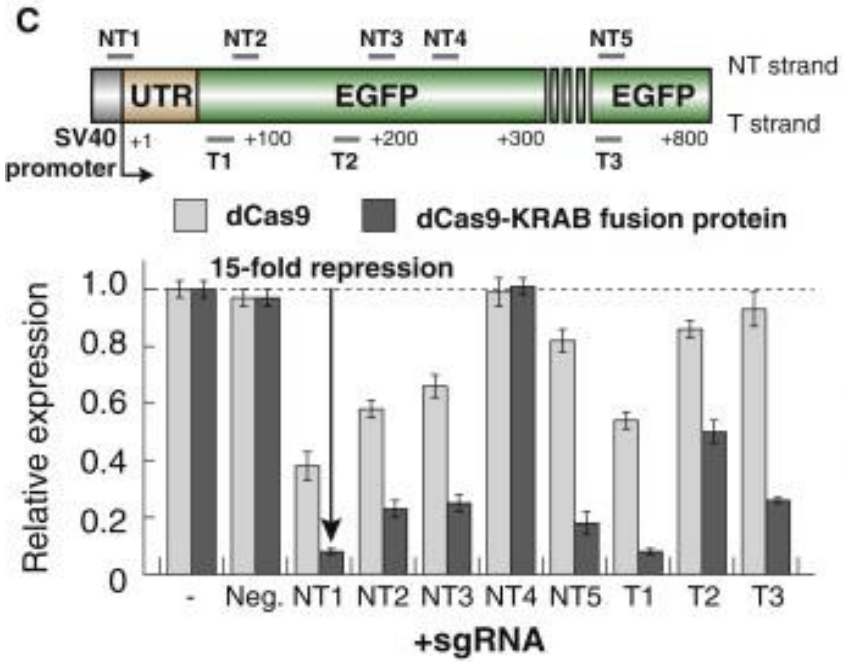
Activating Gene Expression with CRISPR/dCas9-VP64

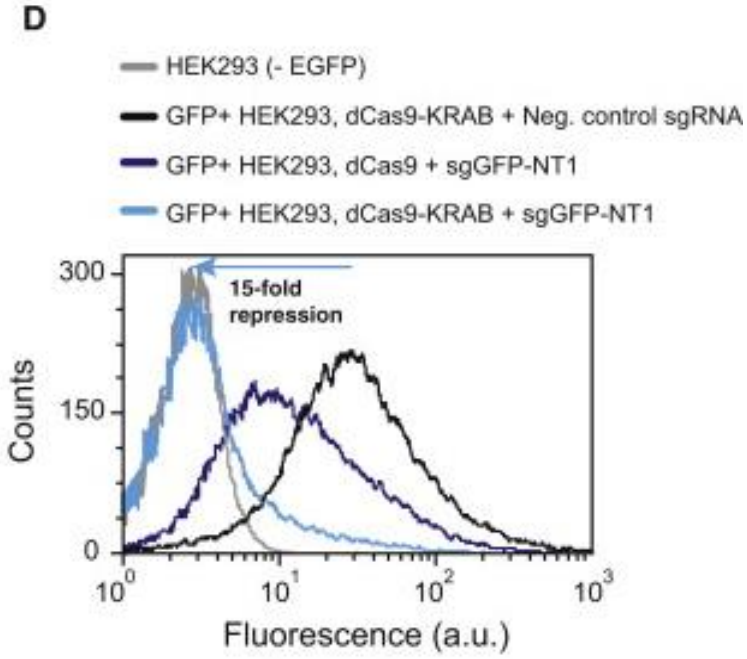


Cell, Volume 154, Issue 2, 2013, 442 - 451

Repressing Gene Expression with dCas9-KRAB



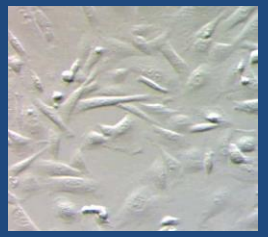




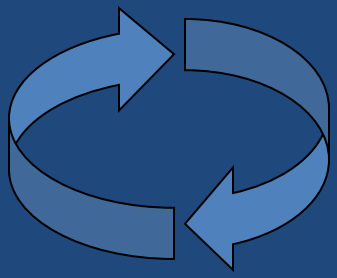
Regenerative Medicine

Patient

Somatic Cell Biopsy



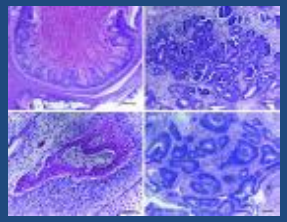
Derive Isogenic Pluripotent Stem Cells



Repair DNA lesion

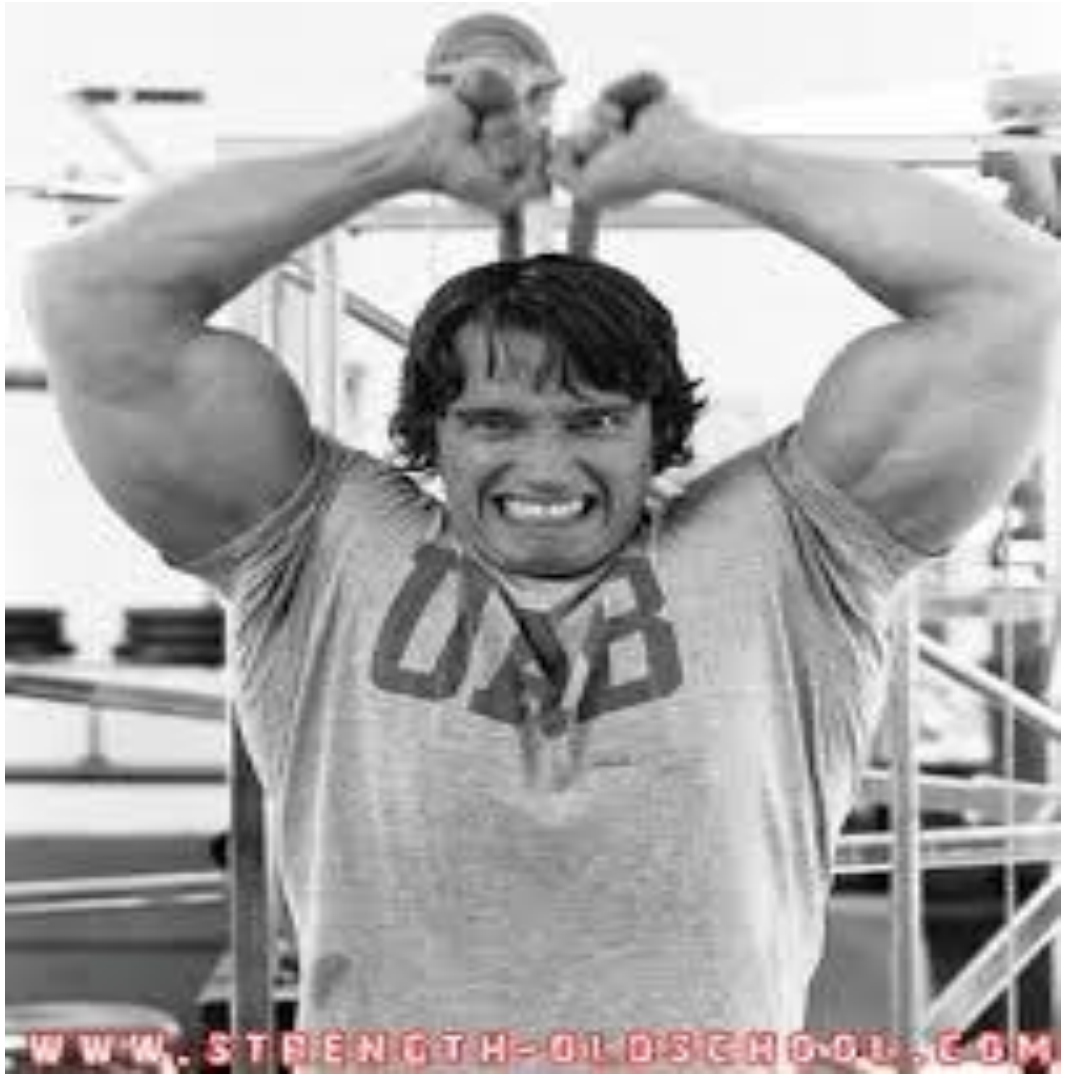
Corrected Stem Cells

In vitro differentiation



Transplant back into patient

GO BLAZERS!!!



Acknowledgements

Ryan Lab

(Former)

Yongliang Huo

Sean McConnell

Rui Yang

Tingting Zhang

Jin-Xiang Ren

(Current)

Shanrun Liu

Suean Fontenard

Jonathan Lockhart

Michael Berlett

NIH NHLBI

Cooley's Anemia FOUNDATION
Leading the Fight Against Thalassemia

UNICO National

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM