



XXXVIII
SABATO DELL'ANDROLOGIA

COLLOQUI IN PMA
TRA GINECOLOGI,
BIOLOGI E ANDROLOGI

17 FEBBRAIO 2018
PADERNO DUGNANO

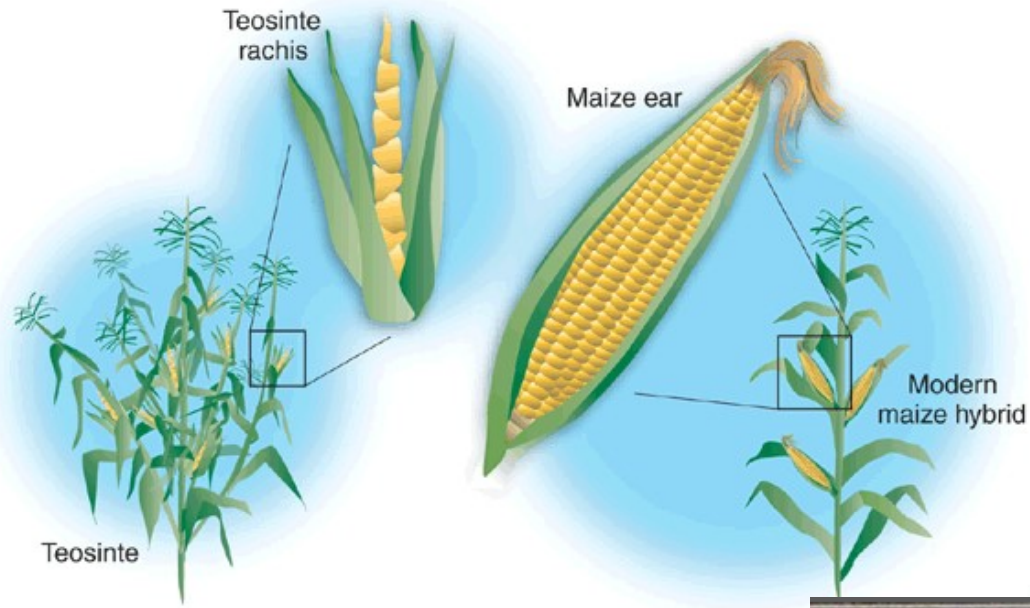
Clinica San Carlo - Via Ospedale, 21
(Auditorium del Nuovo Ospedale)

CON IL PATROCINIO DI S.I.R.U.



Lettura: Le nuove frontiere di ingegneria
genetica in embriologia
A. Paffoni

Ingegneria Genetica



Ingegneria Genetica

Tecnologia del DNA ricombinante

DNA



1953

ENZIMI RESTRIZIONE



Werner Arber

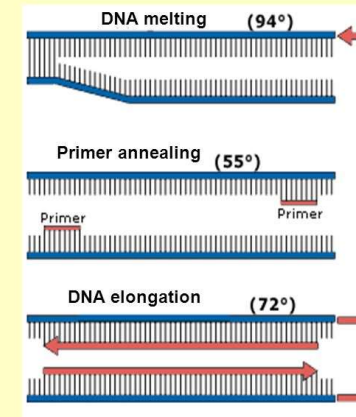
Daniel Nathans

Hamilton O. Smith

The Nobel Prize in Physiology or Medicine 1978 was awarded jointly to Werner Arber, Daniel Nathans and Hamilton O. Smith "for the discovery of restriction enzymes and their application to problems of molecular genetics".

1973

PCR



Nobel Prize in Chemistry 1993,
at age 48
Kary Mullis
(invented PCR in 1983)

1983



Ingegneria Genetica

Tecnologia del DNA ricombinante

APPLICAZIONI PER LA SALUTE:

- . Produzione di farmaci, ormoni, antibiotici, vaccini
- . Correzione di patologie genetiche



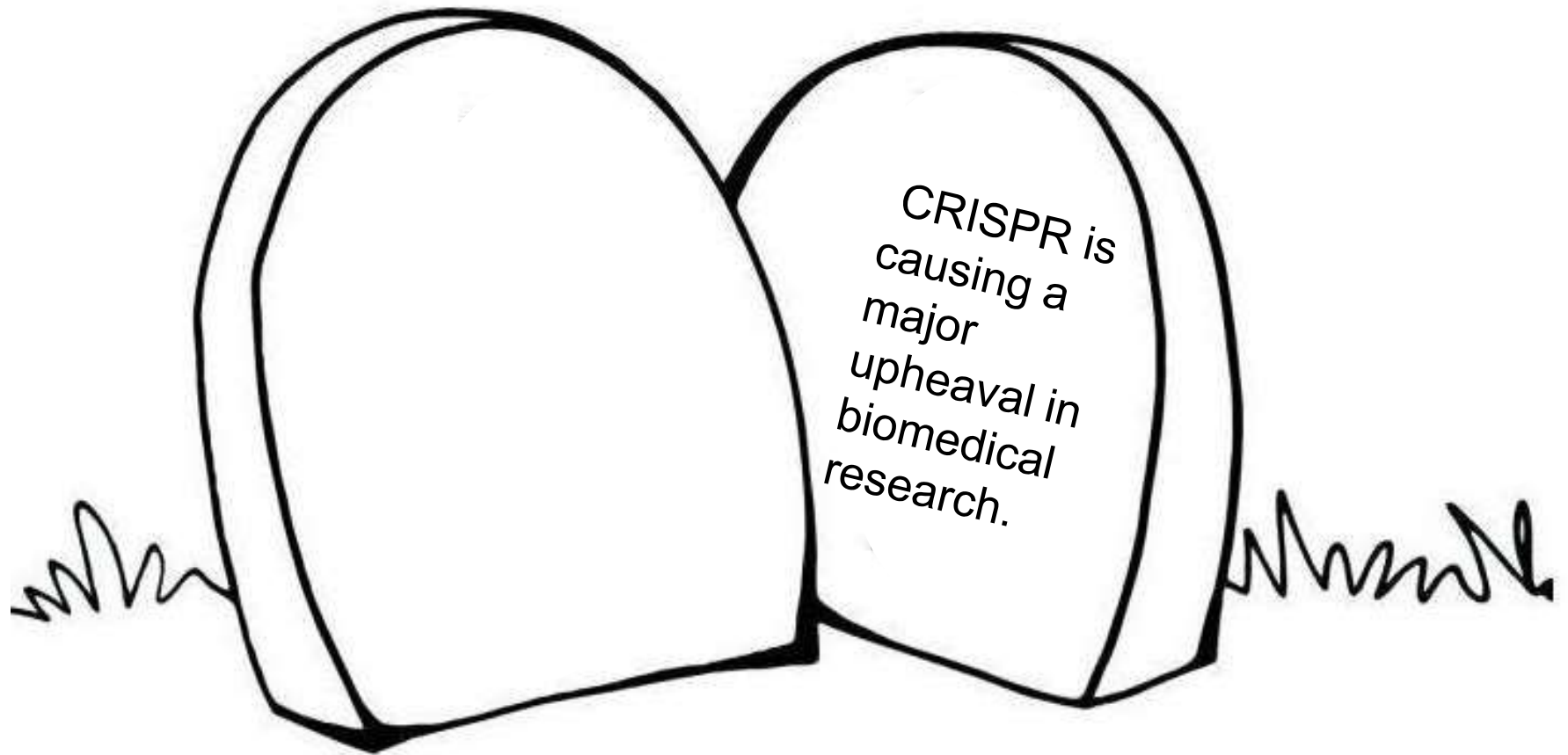
20 | NATURE | VOL 522 | 4 JUNE 2015



CRISPR, THE DISRUPTOR



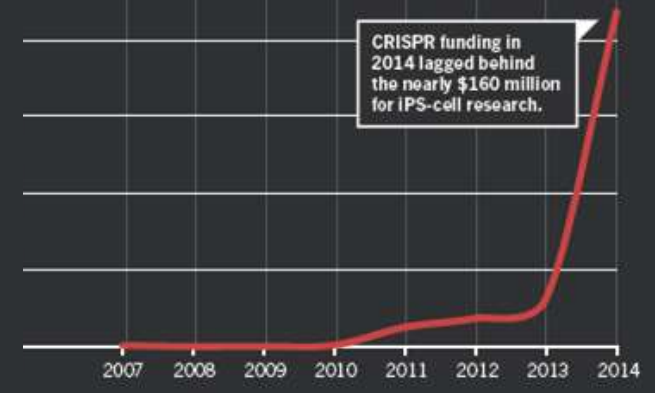
CRISPR-Cas system allows researchers to **quickly change the DNA** of nearly any organism — including humans



THE RISE OF CRISPR

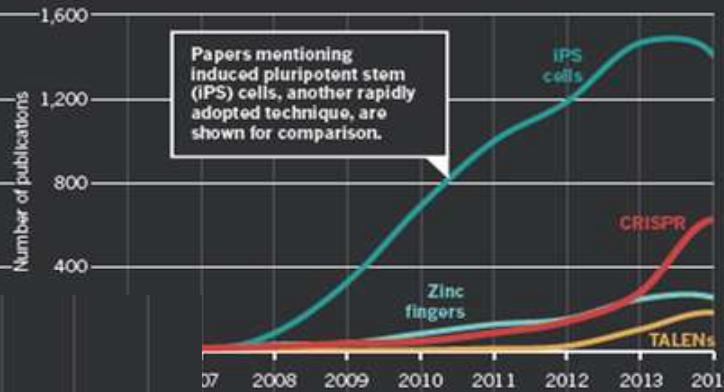
FUNDING

A sharp jump in US National Institutes of Health funding for projects involving CRISPR is a harbinger of future advances.



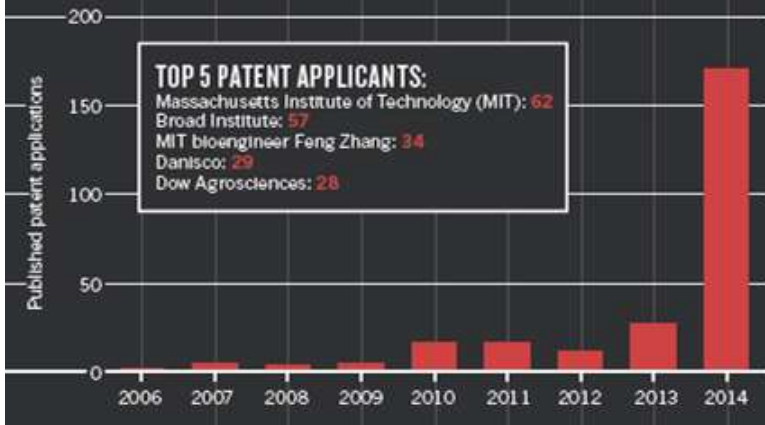
PUBLICATIONS

The number of papers about CRISPR has outstripped the numbers mentioning the gene-editing technologies known as TALENs and zinc fingers.



PATENTS

In 2014, worldwide patent applications that mention CRISPR leapt and a patent battle intensified.



NATURE | VOL 522 | 4 JUNE 2015



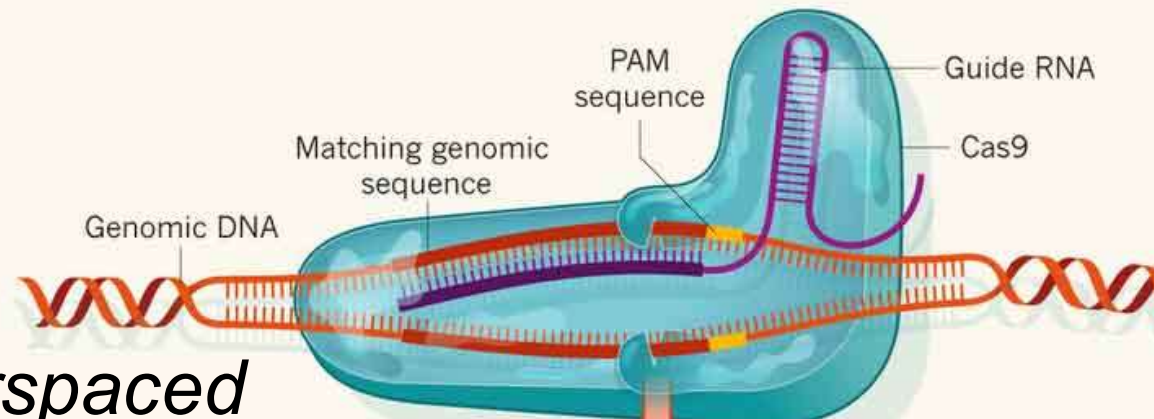
CRISPR- Cas, what?

CRISPR

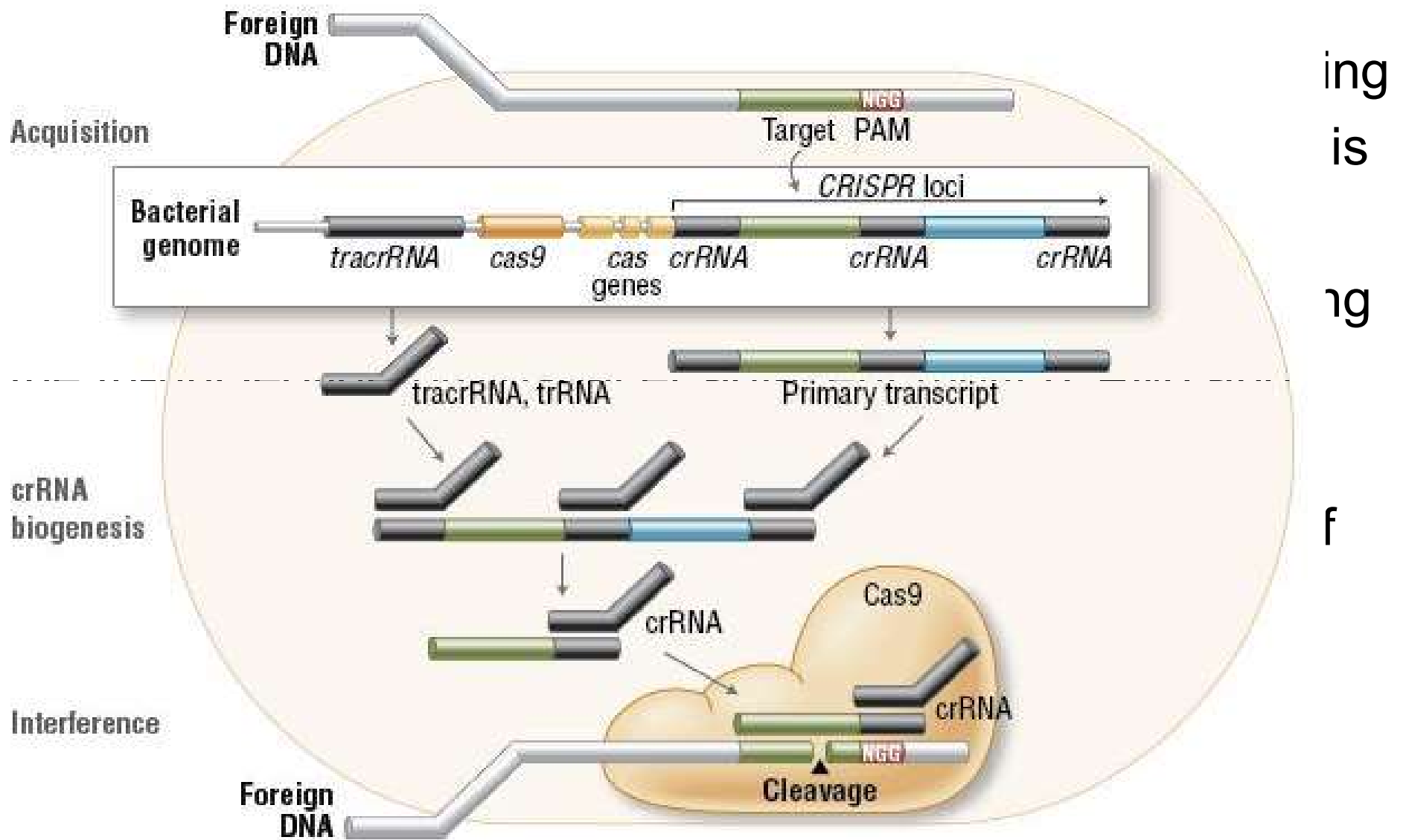
*Clustered Regularly Interspaced
Short Palindromic Repeats*

Cas

CRISPR-associated system



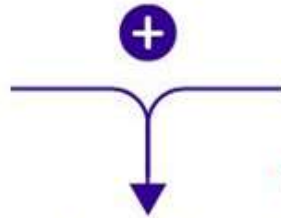
CRISPR-Cas: adaptive immune system





Cas9
An enzyme that cuts DNA

Guide RNA
Directs the Cas9 to the target DNA



Contains a sequence that matches the target DNA

They stick together and seek out the target DNA



Cas9 unzips the target DNA and the guide RNA matches up ...

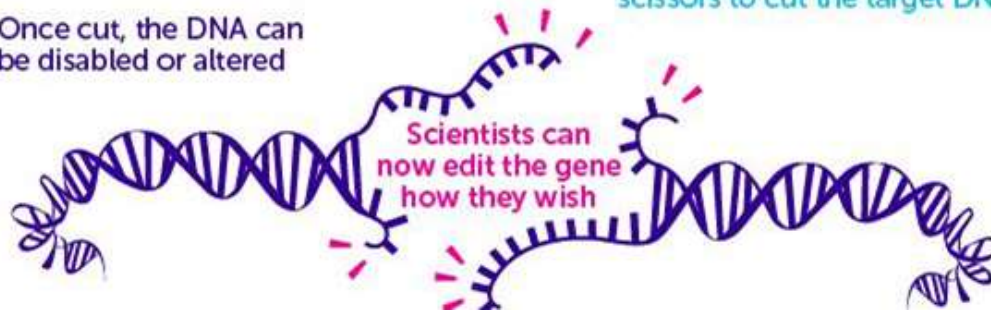
Target DNA



Guide RNA

...if the match is complete the Cas9 then uses molecular scissors to cut the target DNA

Once cut, the DNA can be disabled or altered



Scientists can now edit the gene how they wish

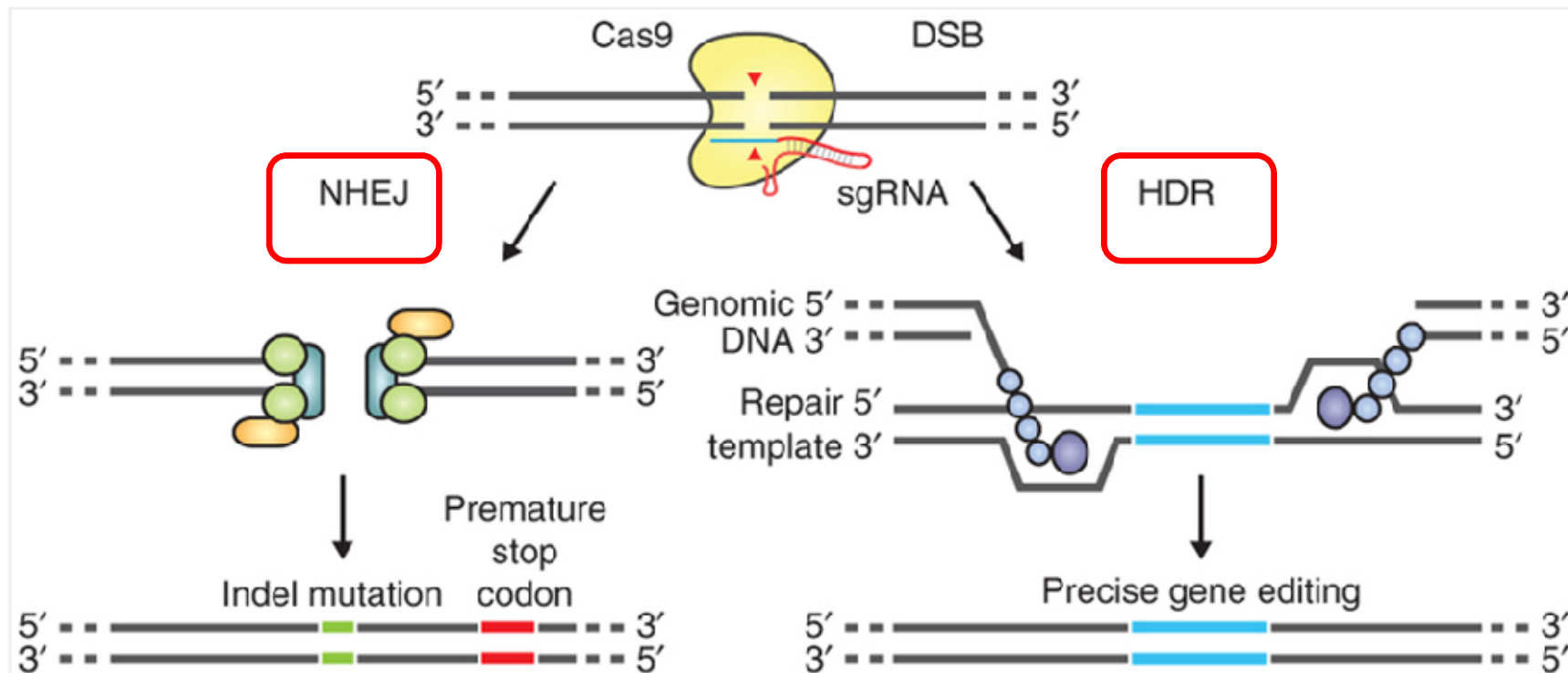


DSB repair promotes genome editing

F Ann Ran, Patrick D Hsu, Jason Wright, Vineeta Agarwala, David A Scott & Feng Zhang

Nature Protocols 8, 2281–2308 (2013) | doi:10.1038/nprot.2013.143

Published online 24 October 2013



DSBs induced by Cas9 (yellow) can be repaired in one of two ways. In the error-prone NHEJ pathway, the ends of a DNA double-strand break are processed by endogenous DNA repair machinery and reconnected, resulting in indel mutations at the site of junction. Indel mutations can result in frameshifts and premature stop codons, resulting in gene knockout. Alternatively, a repair template can be supplied to leverage the HDR pathway, which uses a repair template to fill the gap with high fidelity and precise editing. Single-stranded nucleotides (ssODN) can be used as repair templates in HDR.

A CASO !!!
IL GENE VIENE
INTERROTTO

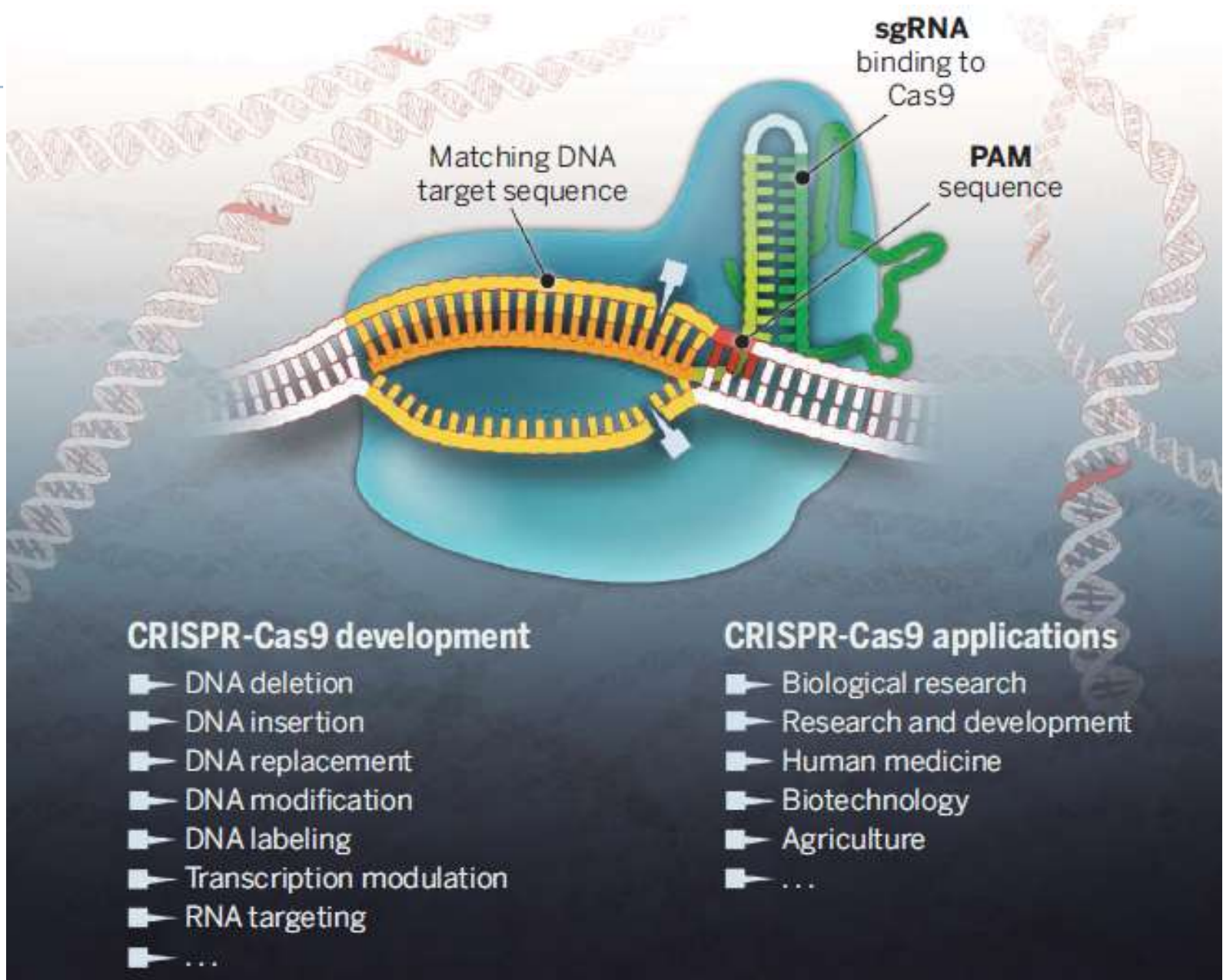
IL GENE VIENE
CORRETTO IN
MODO
SPECIFICO



CRISPR-Cas9

Unlike other gene-editing methods, it is **cheap, quick and easy to use**, and it has swept through labs around the world as a result. Researchers hope to use it to adjust human genes to eliminate diseases, create hardier plants, wipe out pathogens and much more besides





The power of the technology

- ▶ Precise reproduction of tumor-associated mutations (translocations)
- ▶ Analysis of gene function (sgRNA library)
- ▶ Protect plants from disease
- ▶ Correct genetic mutations in inherited disorders
- ▶ Correct genetic mutations responsible for infertility
- ▶ Prevent transmission of genetic mutations

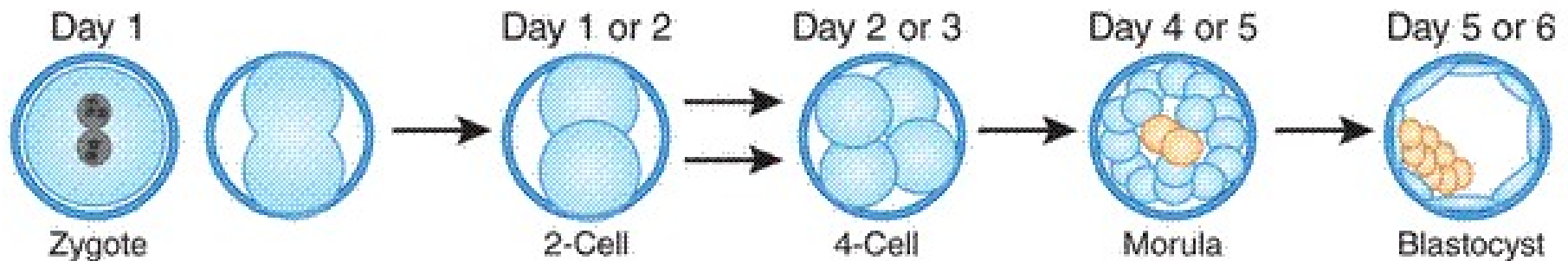
IVF



Germline genome editing

Possible targets

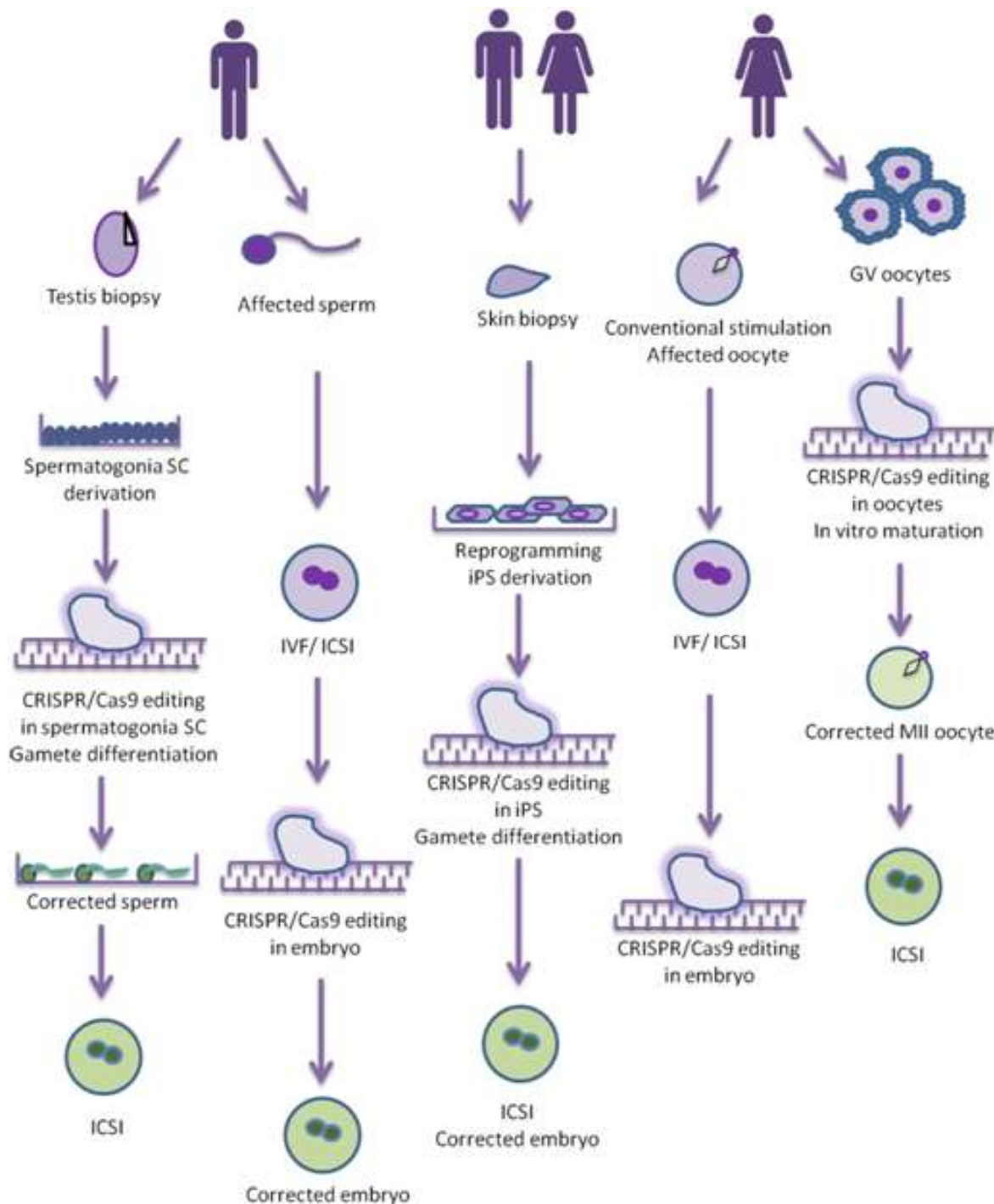
- Gametes
- Zygotes-embryos
- Stem cells (spermatogonial, iPS)



n° cellule

efficacia correzione



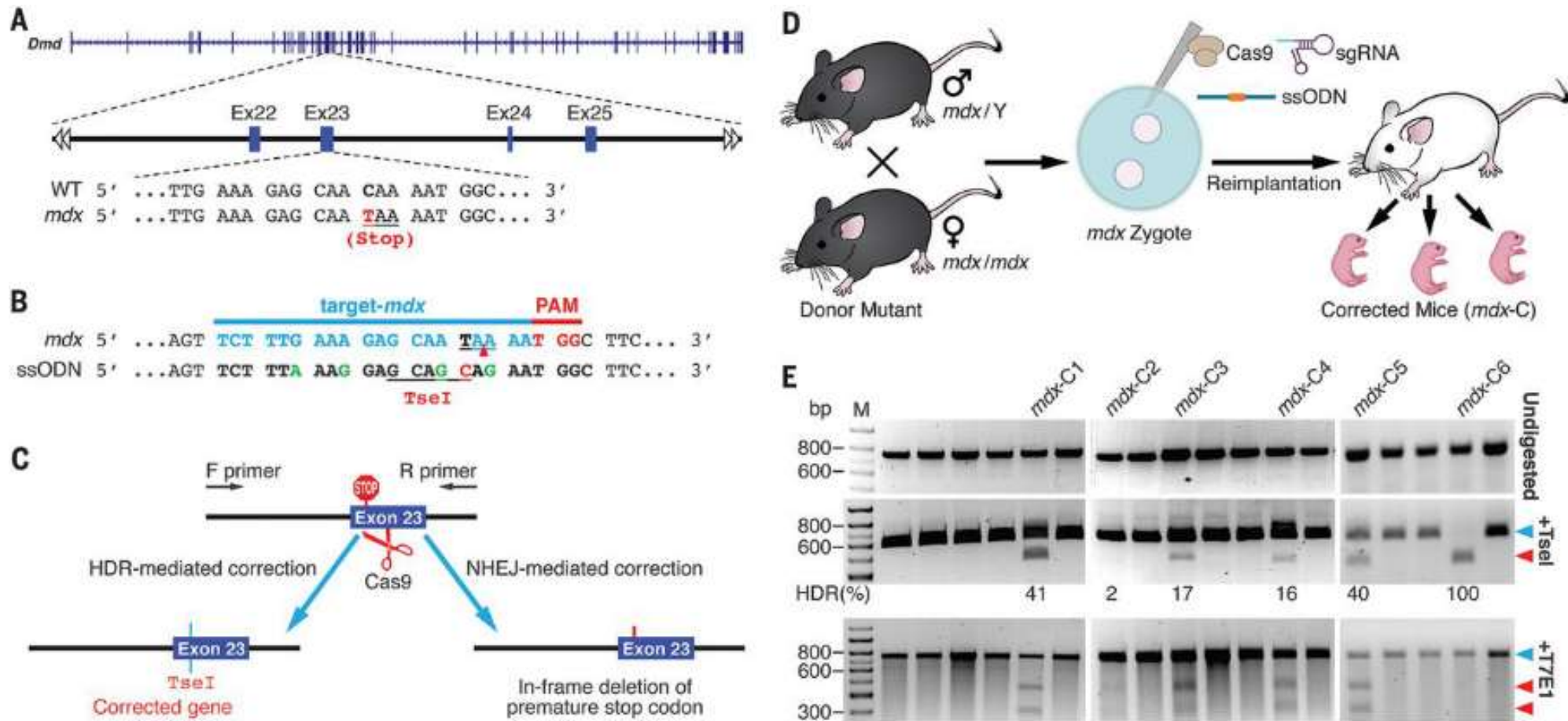


Genome engineering through CRISPR/Cas9 technology in the human germline and pluripotent stem cells

Hum Reprod Update. 2016;22(4):411-419.
doi:10.1093/humupd/dmw005



Correzione patologie genetiche (DMD)



Chengzu Long et al. Science 2014;345:1184-1188



RESEARCH ARTICLE

CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes

Puping Liang, Yanwen Xu, Xiya Zhang, Chenhui Ding, Rui Huang, Zhen Zhang, Jie Lv, Xiaowei Xie, Yuxi Chen, Yujing Li, Ying Sun, Yaofu Bai, Zhou Songyang, Wenbin Ma, Canquan Zhou[✉], Junjiu Huang[✉]

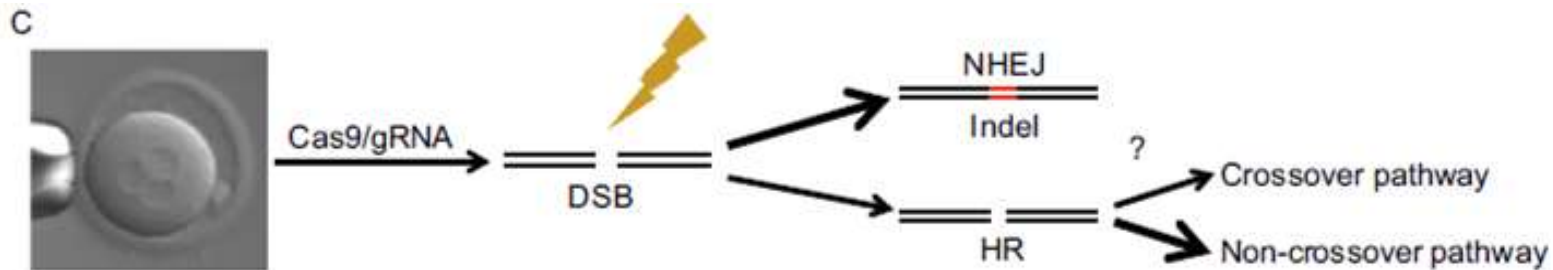
Guangdong Province Key Laboratory of Reproductive Medicine, the First Affiliated Hospital, and Key Laboratory of Gene Engineering of the Ministry of Education, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China

✉ Correspondence: hjunjiu@mail.sysu.edu.cn (J. Huang), zhoucanquan@hotmail.com (C. Zhou)

Received March 30, 2015 Accepted April 1, 2015



EDITING – Efficacy in 3PN human zygotes



A

Targeted editing of the *HBB* gene in human 3PN zygotes by intra-cytoplasmic injection

Group No.	Cas9/gRNA/ssDNA (ng/μL)	Survived /injected	GFP ⁺	PCR-amplified	Cas9-cleaved	Edited with ssDNA	Recombined with HBD
1	100/20/2	10/11	6	6	4	0	1
2	100/20/20	22/29	17	17	7	1	0
3	200/40/200	12/14	12	10*	6	2	2
4	200/40/200	27/32	24	21*	11	1	4
Total	-	71/86 (82.6%)	59	54	28 (51.9%)	4 (14.3%)	7 (25.0%)

Protein Cell 2015, 6(5):363–372



CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes

- ▶ In this study, 3PN zygotes were used to investigate the specificity and fidelity of the CRISPR/Cas9 system.
- ▶ Most of the DSBs generated by Cas9 in 3PN zygotes were also repaired through NHEJ.
- ▶ ssDNA-mediated editing occurred only in 4 embryos (14.3%) and the edited embryos were mosaic, similar to findings in other model systems
- ▶ Endogenous homologous sequences were also used as HDR templates, with an estimated editing efficiency of 25%

Protein Cell 2015, 6(5):363–372



Correction of a pathogenic gene mutation in human embryos

Hong Ma^{1*}, Nuria Marti-Gutierrez^{1*}, Sang-Wook Park^{2*}, Jun Wu^{3*}, Yeonmi Lee¹, Keiichiro Suzuki³, Amy Koski¹, Dongmei Ji¹, Tomonari Hayama¹, Riffat Ahmed¹, Hayley Darby¹, Crystal Van Dyken¹, Ying Li¹, Eunju Kang¹, A.-Reum Park², Daesik Kim⁴, Sang-Tae Kim², Jianhui Gong^{5,6,7,8}, Ying Gu^{5,6,7}, Xun Xu^{5,6,7}, David Battaglia^{1,9}, Sacha A. Krieg⁹, David M. Lee⁹, Diana H. Wu⁹, Don P. Wolf¹, Stephen B. Heitner¹⁰, Juan Carlos Izpisua Belmonte^{3§}, Paula Amato^{1,9§}, Jin-Soo Kim^{2,4§}, Sanjiv Kaul^{10§} & Shoukhrat Mitalipov^{1,10§}

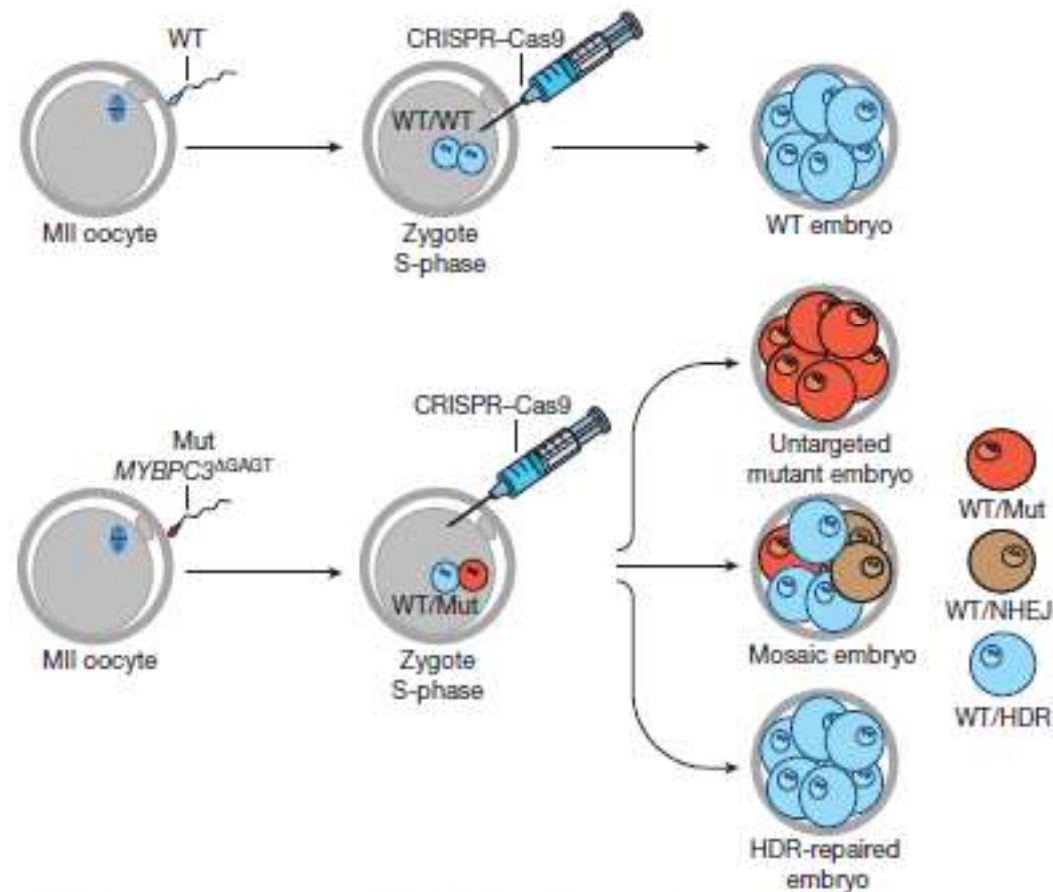


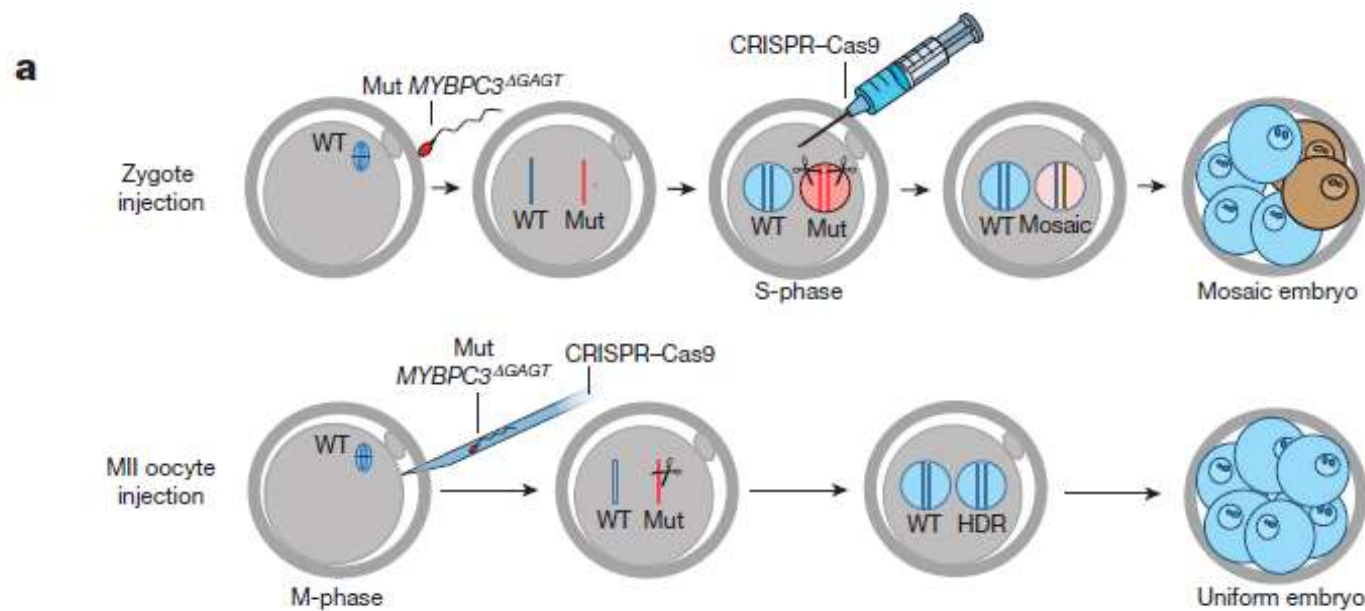
Figure 1 | Gene correction in S-phase-injected human embryos. Schematic of *MYBPC3*^{ΔGAGT} gene targeting by injection of CRISPR-Cas9

Ma, Nature, 2017



Correction of a pathogenic gene mutation in human embryos

Hong Ma^{1*}, Nuria Marti-Gutierrez^{1*}, Sang-Wook Park^{2*}, Jun Wu^{3*}, Yeonmi Lee¹, Keiichiro Suzuki³, Amy Koski Tomonari Hayama¹, Riffat Ahmed¹, Hayley Darby¹, Crystal Van Dyken¹, Ying Li¹, Eunju Kang¹, A.-Reum Park², Sang-Tae Kim², Jianhui Gong^{5,6,7,8}, Ying Gu^{5,6,7}, Xun Xu^{5,6,7}, David Battaglia^{1,9}, Sacha A. Krieg⁹, David M. Lee⁹, Don P. Wolf¹, Stephen B. Heitner¹⁰, Juan Carlos Izpisua Belmonte^{3§}, Paula Amato^{1,9§}, Jin-Soo Kim^{2,4§}, Sanjiv Kai Shoukhrat Mitalipov^{1,10§}



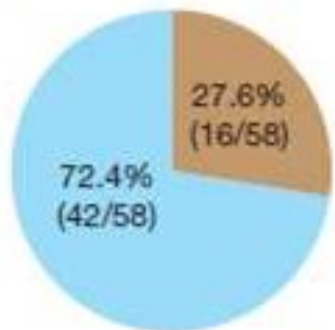
Ma, Nature, 2017



Correction of a pathogenic gene mutation in human embryos

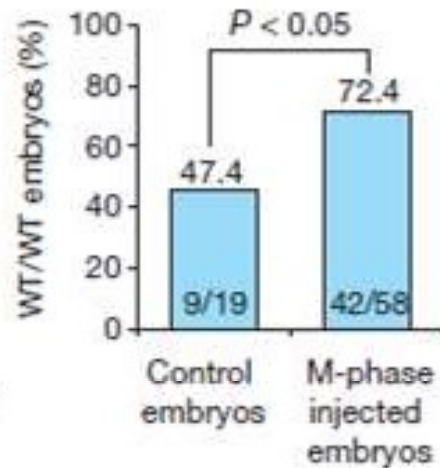
b Targeting outcomes

WT/WT WT/NHEJ



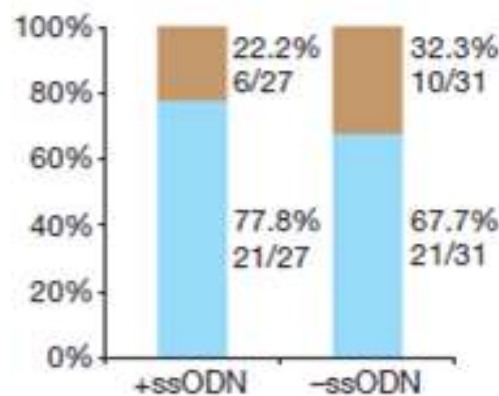
M-phase-injected embryos

c Yield of WT/WT embryos



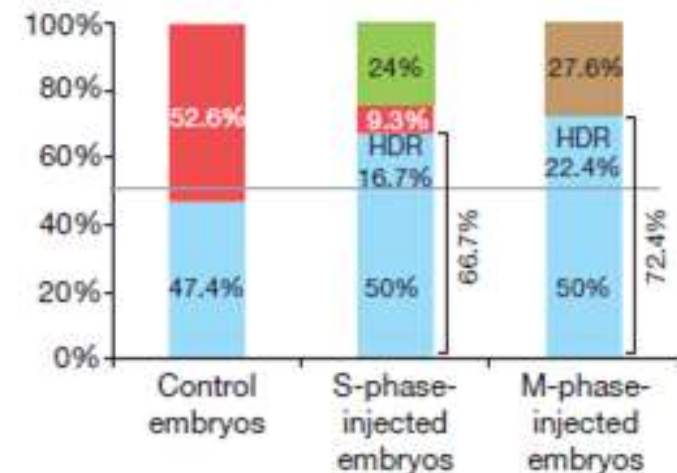
d HDR with and without ssODN

WT/WT WT/NHEJ



e Embryo genotype distribution

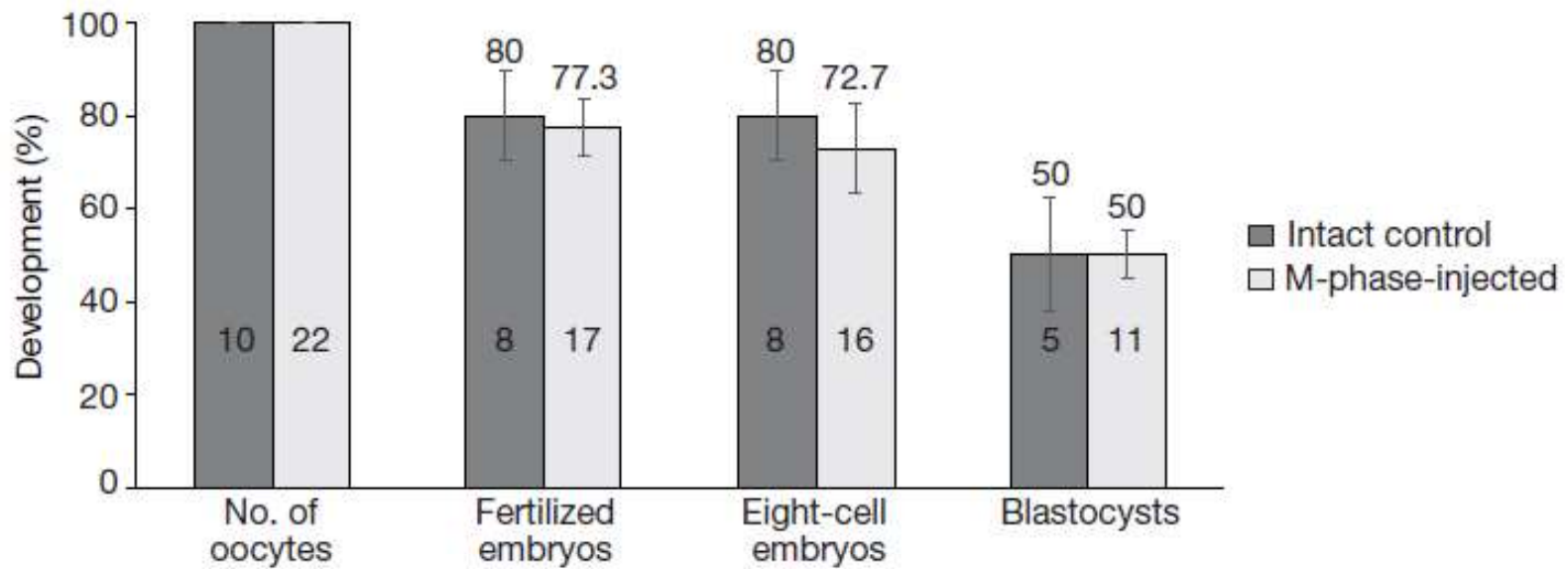
WT/WT WT/Mos WT/Mut WT/NHEJ



Ma, Nature, 2017



a Fertilization and preimplantation development of CRISPR-Cas9-injected oocytes



b



Ma, Nature, 2017



Gene Correction, Human 2PN model

- ▶ CRISPR/CAS9 is effective as a gene-editing tool in human zygotes.
- ▶ Homology-directed repair is frequent, mainly due to the use of homologous wild type maternal sequences.
- ▶ Mosaicism can be reduced modulating the cycle cell stage at which DNA repair was induced

Ma, Nature, 2017



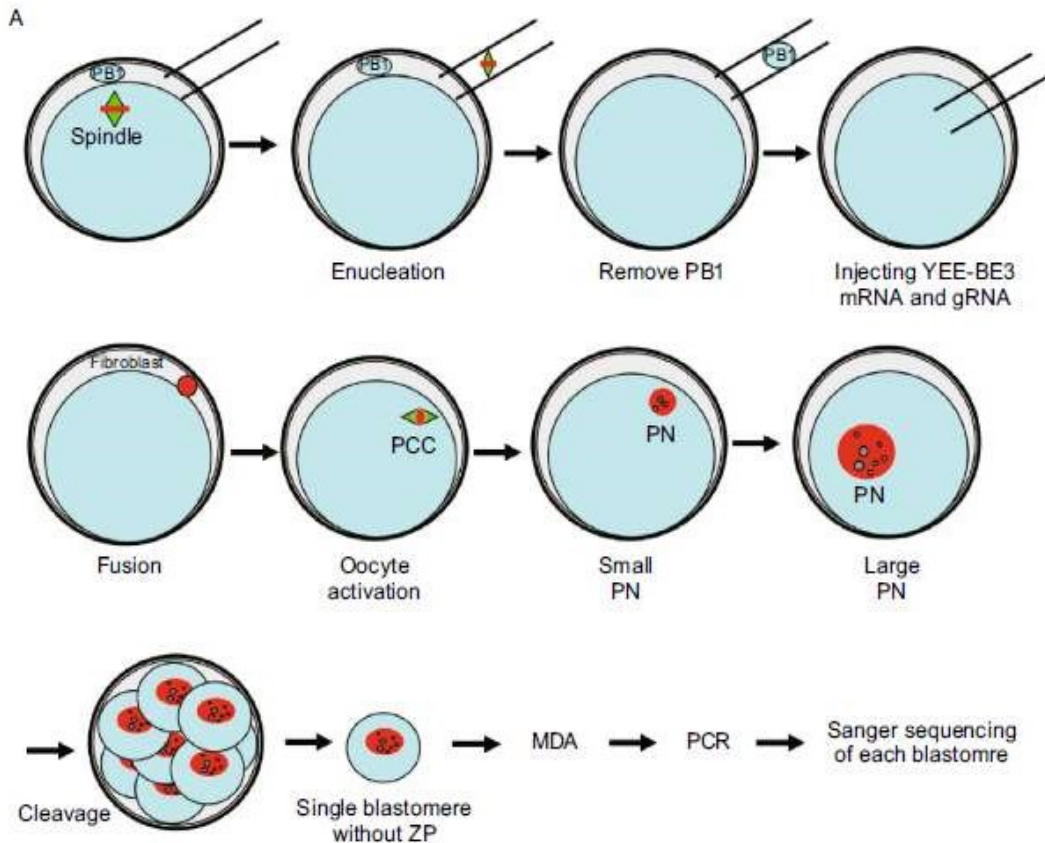
SHORT ARTICLE

Correction of β -thalassemia mutant by base editor in human embryos

Puping Liang^{1,2}, Chenhui Ding², Hongwei Sun¹, Xiaowei Xie¹, Yanwen Xu², Xiya Zhang¹, Ying Sun¹, Yuanyan Xiong¹, Wenbin Ma¹, Yongxiang Liu², Yali Wang², Jianpei Fang³, Dan Liu⁴, Zhou Songyang^{1,2,4}, Canquan Zhou², Junjiu Huang^{1,2}



HBB - Gene correction



- Homozygous mutation disease embryos obtained with nuclear transfer
- Gene correction efficiency over 23%
- In mosaic embryos the % of repaired blastomeres was over 20%

B

Survived embryo No. (Injected embryo No.)	Activated embryo No.	Harvested embryo No.	Total blastomere No.	MDA-amplified blastomere No.	PCR-amplified blastomere No.	<Homozygous> G ₂₄ G ₂₄ /G ₂₄ G ₂₄ blastomere No. (%)	<Heterozygous> A ₂₄ G ₂₄ /G ₂₄ G ₂₄ blastomere No. (%)	<Wild-type> A ₂₄ G ₂₄ /A ₂₄ G ₂₄ blastomere No. (%)
28 (35)	24	20*	73	73	48*	37 (77.1)	3 (6.3)	8 (16.7)

Liang, Prot Cell, 2017



Possible uses of genome editing in embryology

- ▶ Both parents carriers of an Autosomal Recessive Disease (CF, b-tal...)
- ▶ One member affected of an Autosomal Recessive Disease or a chromosomal structural alteration
- ▶ Correction of the affected gene in the germline of the affected member or in embryos
- ▶ Generation of disease models
- ▶ Study of genes involved in pre and post implantation development

PGD!



Safety-Ethical issues

- how to study safety?
- do we need to move to clinical practice? How?
- use of **PGD** to identify modified embryos and **off-target effects**



Off-target effects



The possibility that CRISPR-Cas RGNs might cause additional, unwanted genetic changes has been largely unexplored, so Joung's team set out to investigate the occurrence of "off-target" mutations in human cells expressing CRISPR-Cas RGNs. Since the interaction between the guiding RNA segment and the target DNA relies on only 20 nucleotides, they hypothesized that the RNA might also recognize DNA segments that differed from the target by a few nucleotides



Moving Cas9 Platforms with Reduced Off-Target Effects into the Clinic

CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering

Prashant Mali^{1,4}, John Aach^{1,3}, P Benjamin Stranges¹, Kevin M Esvelt², Mark Moosburner¹, Sriram Kosuri², Luhan Yang³ & George M Church^{1,2}

Mali et al., *Nat Biotechnol.* 2013

Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification

John P Guillinger¹⁻³, David B Thompson¹⁻³ & David R Liu^{1,2}

Guillinger et al., *Nat Biotechnol.* 2014

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} Alexander 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

Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing

Shengdar Q Tsai¹⁻⁴, Nicolas Wyvekens¹⁻³, Cyd Khayter¹⁻³, Jennifer A Foden¹⁻³, Vishal Thapar^{1,2}, Deepak Reyon¹⁻⁴, Mathew J Goodwin¹⁻³, Martin J Aryee^{1,2,4} & J Keith Joung¹⁻⁴

Tsai et al., *Nat Biotechnol.* 2014

Improving CRISPR-Cas nuclease specificity using truncated guide RNAs

Yanfang Fu¹⁻⁵, Jeffrey D Sander¹⁻⁵, Deepak Reyon¹⁻⁴, Vincent M Cascio¹⁻³ & J Keith Joung¹⁻⁴

Fu et al., *Nat Biotechnol.* 2014

A highly specific SpCas9 variant is identified by in vivo screening in yeast.

Casini A¹, Olivieri M¹, Petris G¹, Montagna C¹, Reginato G¹, Maule G¹, Lorenzin F², Prandi D², Romanel A², Demichelis F², Inga A³, Cereseto A¹.

Casini et al., *Nat biotech* 2018

...and additional future improvements to the CRISPR-Cas9 platform



SHUTTERSTOCK

Don't edit the human germ line



“There is a mentality that as long as it works, we don't have to understand how or why it works.”

15



