DELTA BIOTECHNOLOGY

Applications of CRISPR/CAS Technology in Biomedical Sciences

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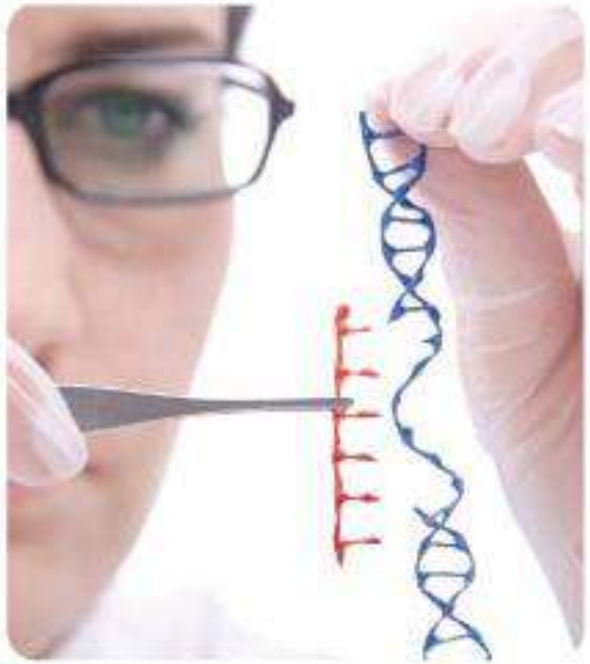


Discovery of CRISPR

TGAAAATGGGAGGGAGTTCTACCGCAGAGGCGGGGGAACTCCAAGTGATATCCATCATCGCATCCAGTGCGCC (1,451)
(1,452) CGGTTTATCCCCGCTGATGCGGGGAACACCAGCGTCAGGCGTGAAATCTCACCGTCGTTGC (1,512)
(1,513) CGGTTTATCCCTGCTGGCGCGGGGAACTCTCGGTTCAGGCGTTGCAAACCTGGCTACCGGG (1,573)
(1,574) CGGTTTATCCCCGCTAACGCGGGGAACTCGTAGTCCATCATTCCACCTATGTCTGAACTCC (1,634)
(1,635) CGGTTTATCCCCGCTGGCGCGGGGAACTCG (1,664)

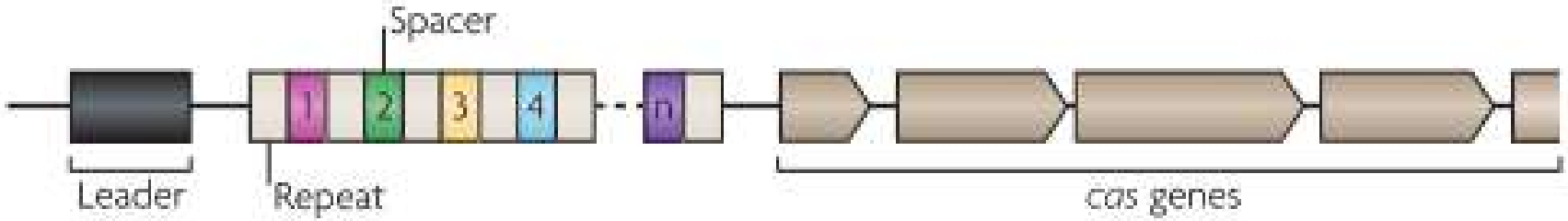
consensus: CGGTTTATCCCCGCT$^{GG}_{AA}$CGCGGGGAACTC

FIG. 5. Comparison of direct-repeat sequences consisting of 61 base pairs in the 3′-end flanking region of *iap*. The 29 highly conserved nucleotides, which contain a dyad symmetry of 14 base pairs (underlined), are shown at the bottom. Homologous nucleotides found in at least two DNA segments are shown in boldface type. The second translational termination codon is boxed. The nucleotide numbers are in parentheses.

"The biological significance of these sequences is not known."

- Similar patterns were found in many other species of bacteria.

- By 2002, this pattern had a name, CRISPR (clustered regularly interspaced short palindromic repeats).
- Nearby Cas (CRISPR-associated) genes were also described.



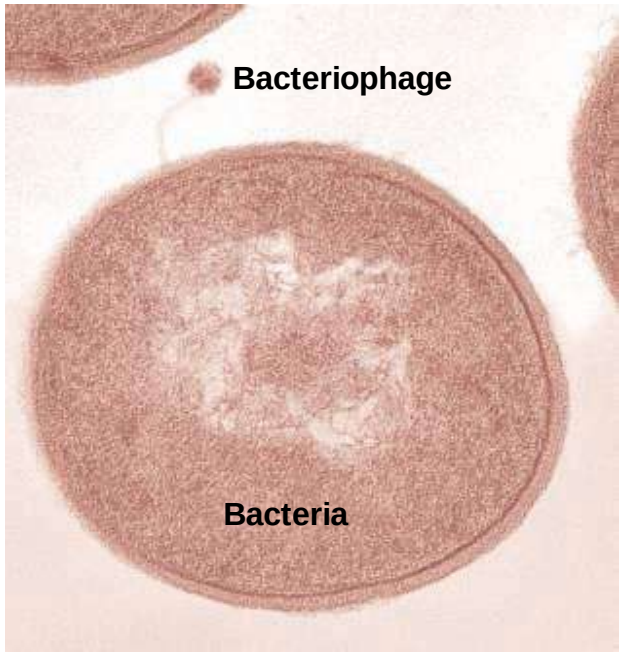
But CRISPR/Cas was still a curiosity, with no known function.

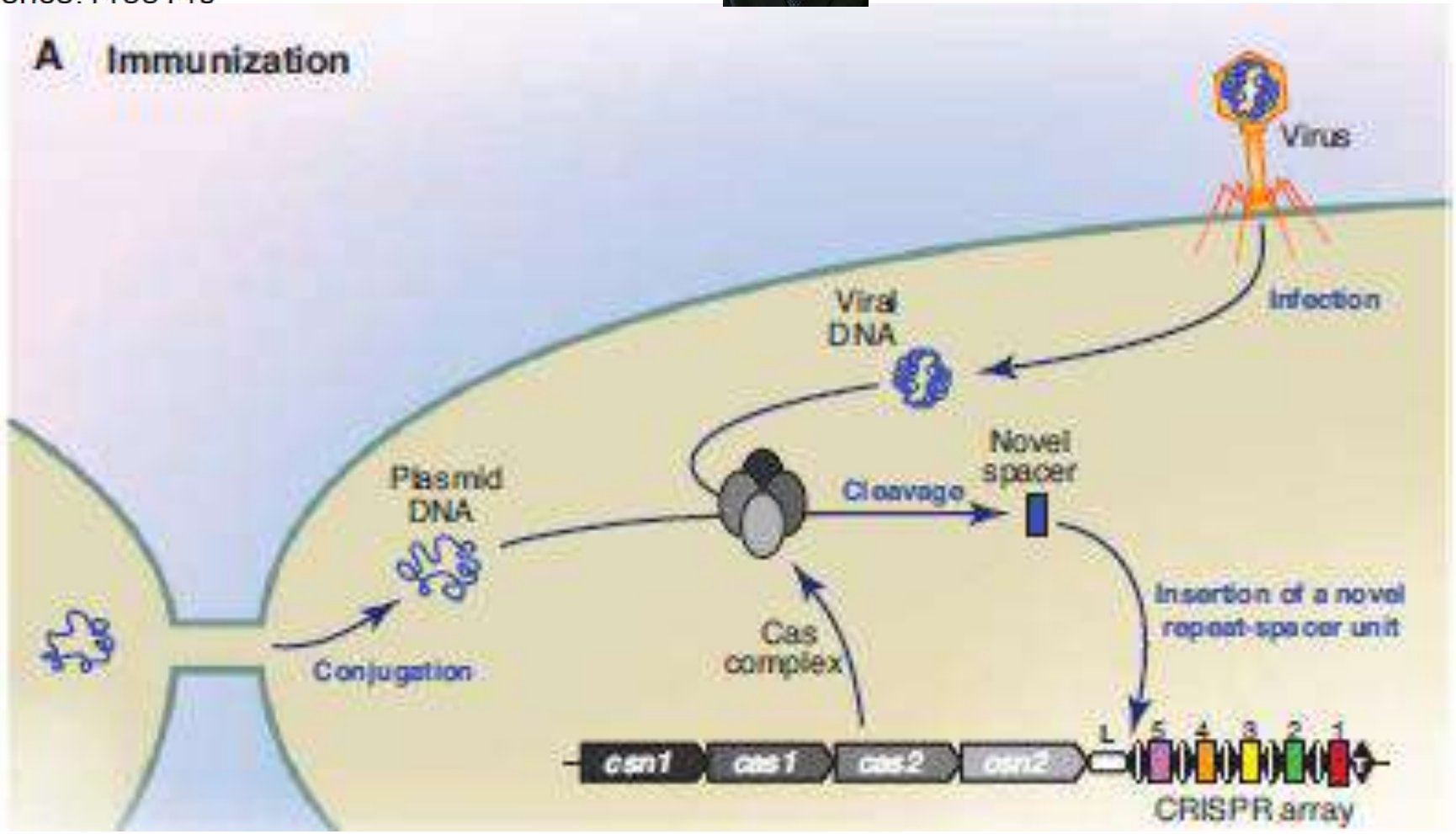
Houtman et al., FASEB, Horizons in Bioscience
Marraffini & Sontheimer Nat. Rev. Genet, 2010

CRISPR/CAS, the immune system of bacteria

Science
AAAS

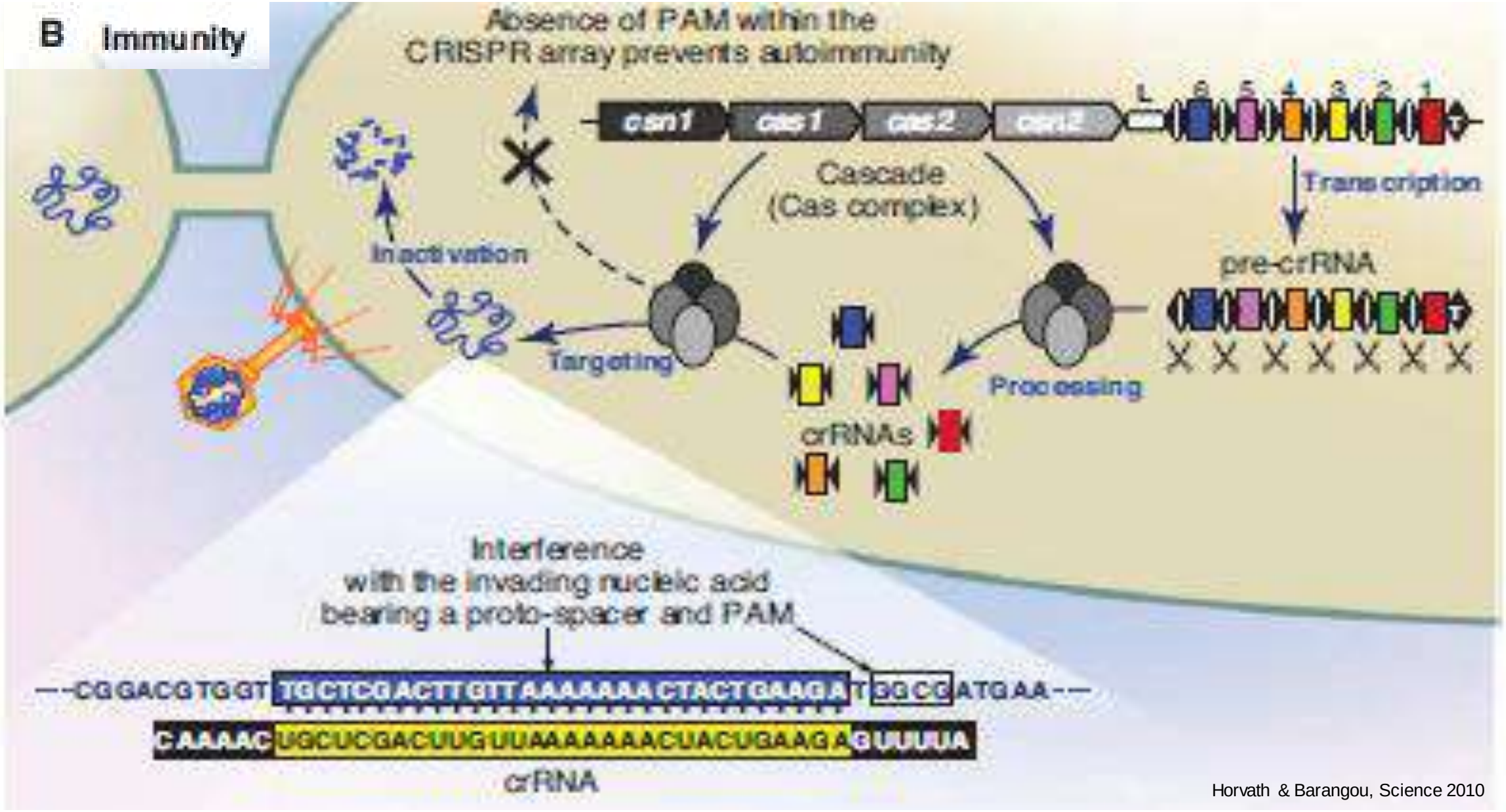
CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes
Rodolphe Barrangou, *et al.*
Science **315**, 1709 (2007);
DOI: 10.1126/science.1138140





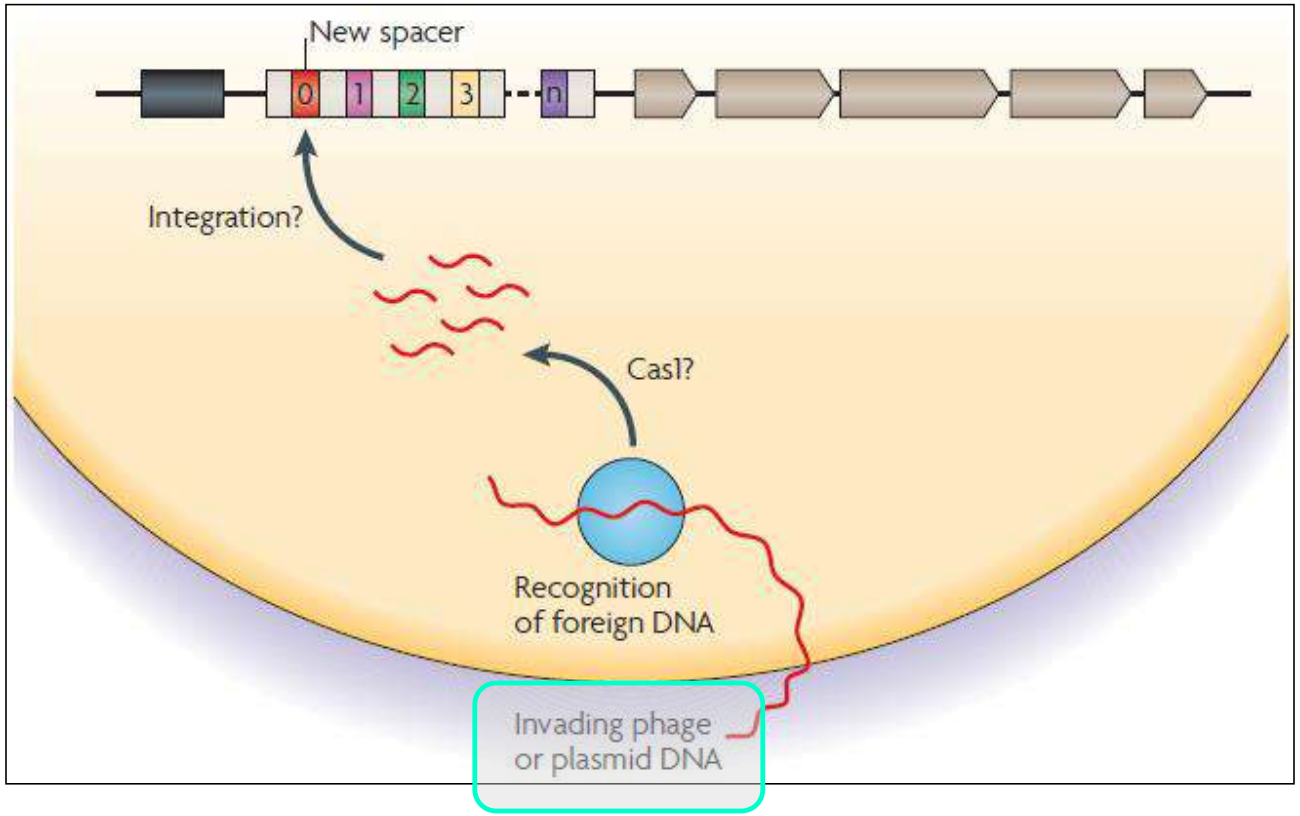
Horvath & Barangou, Science 2010

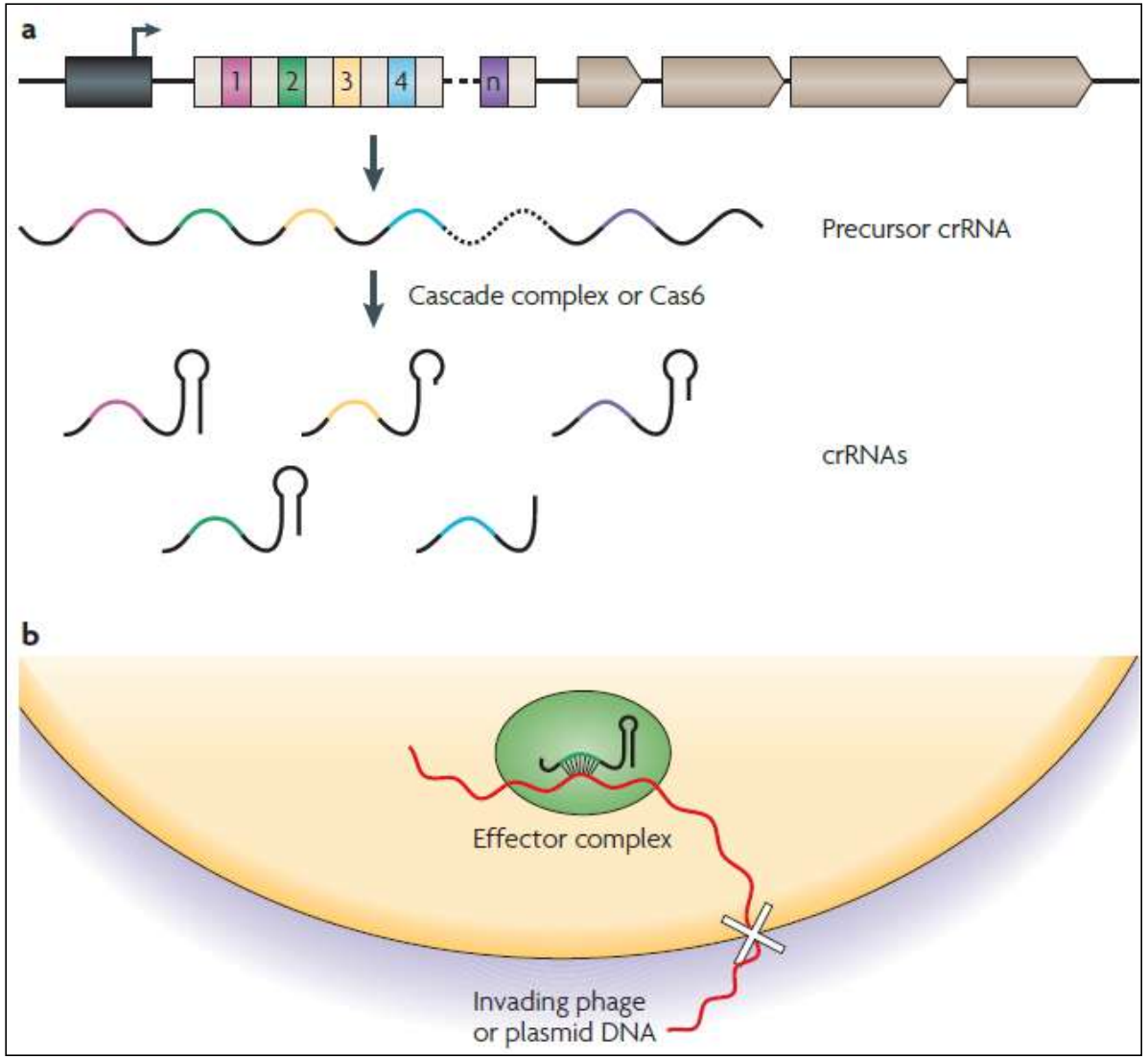
CRISPR/CAS, the immune system of bacteria and archaea



Horvath & Barangou, Science 2010

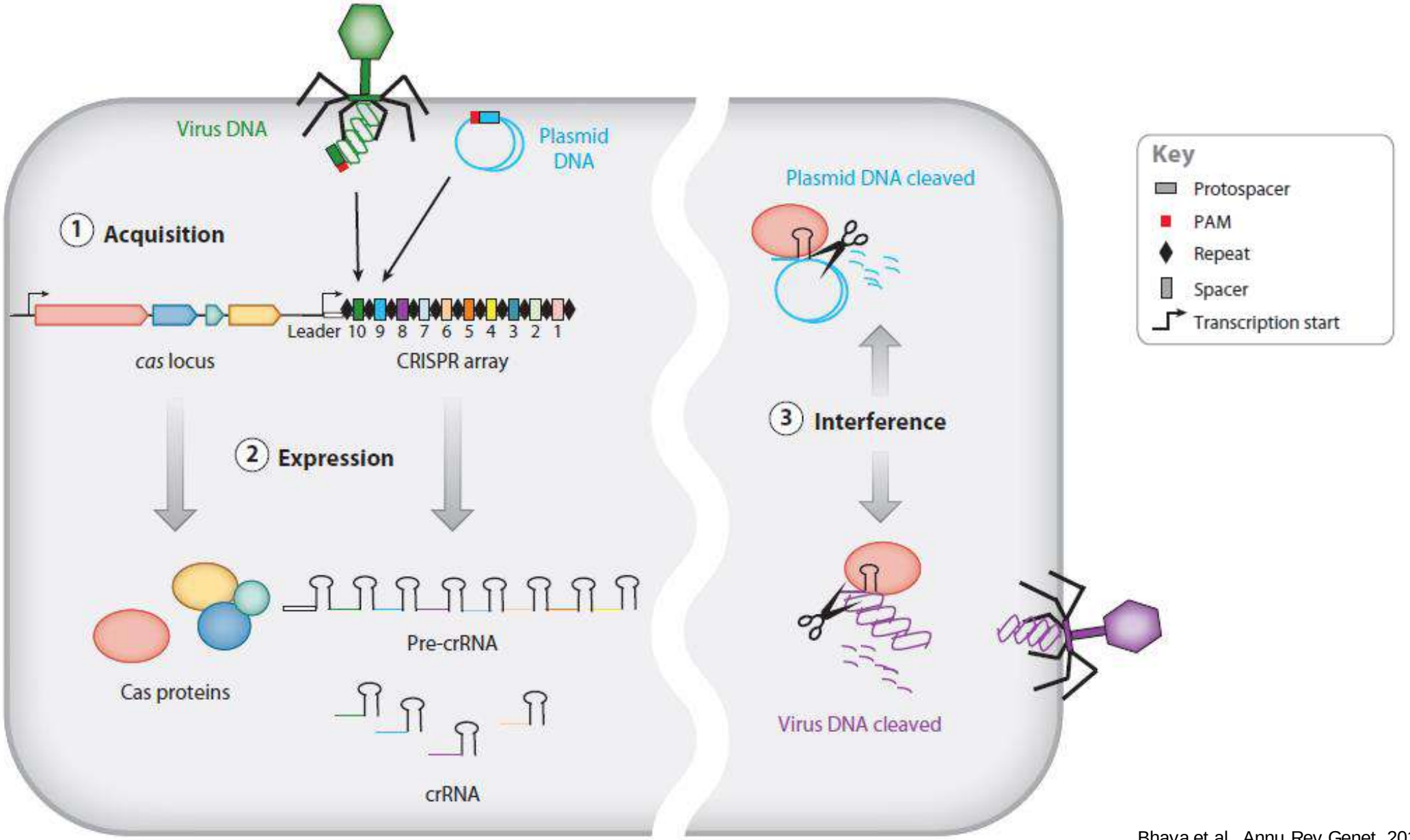
CRISPR/CAS, the immune system of bacteria and archaea





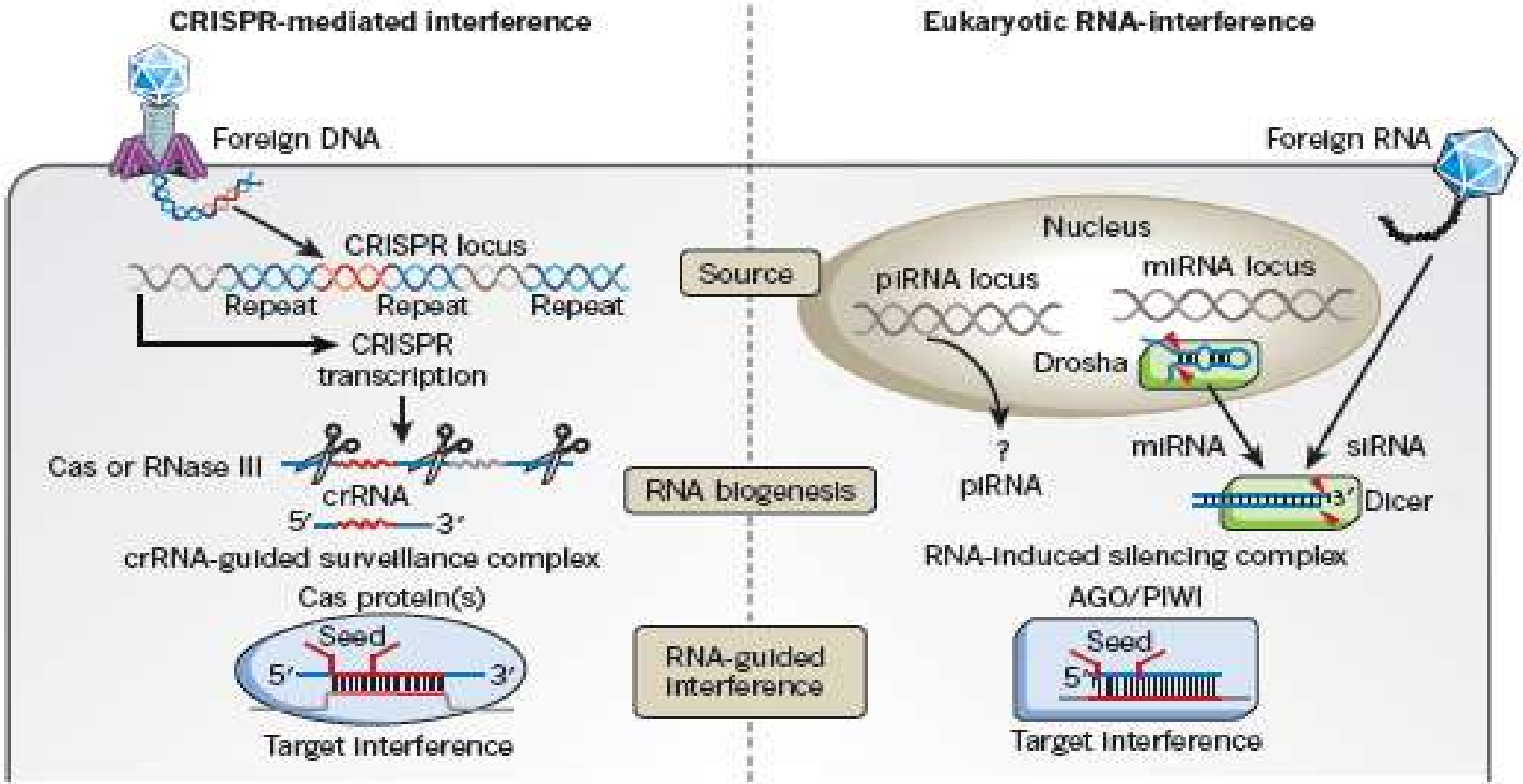
Marraffini & Sontheimer Nat. Rev. Genet, 2010

3 phases of CRISPR/CAS adaptive immune system



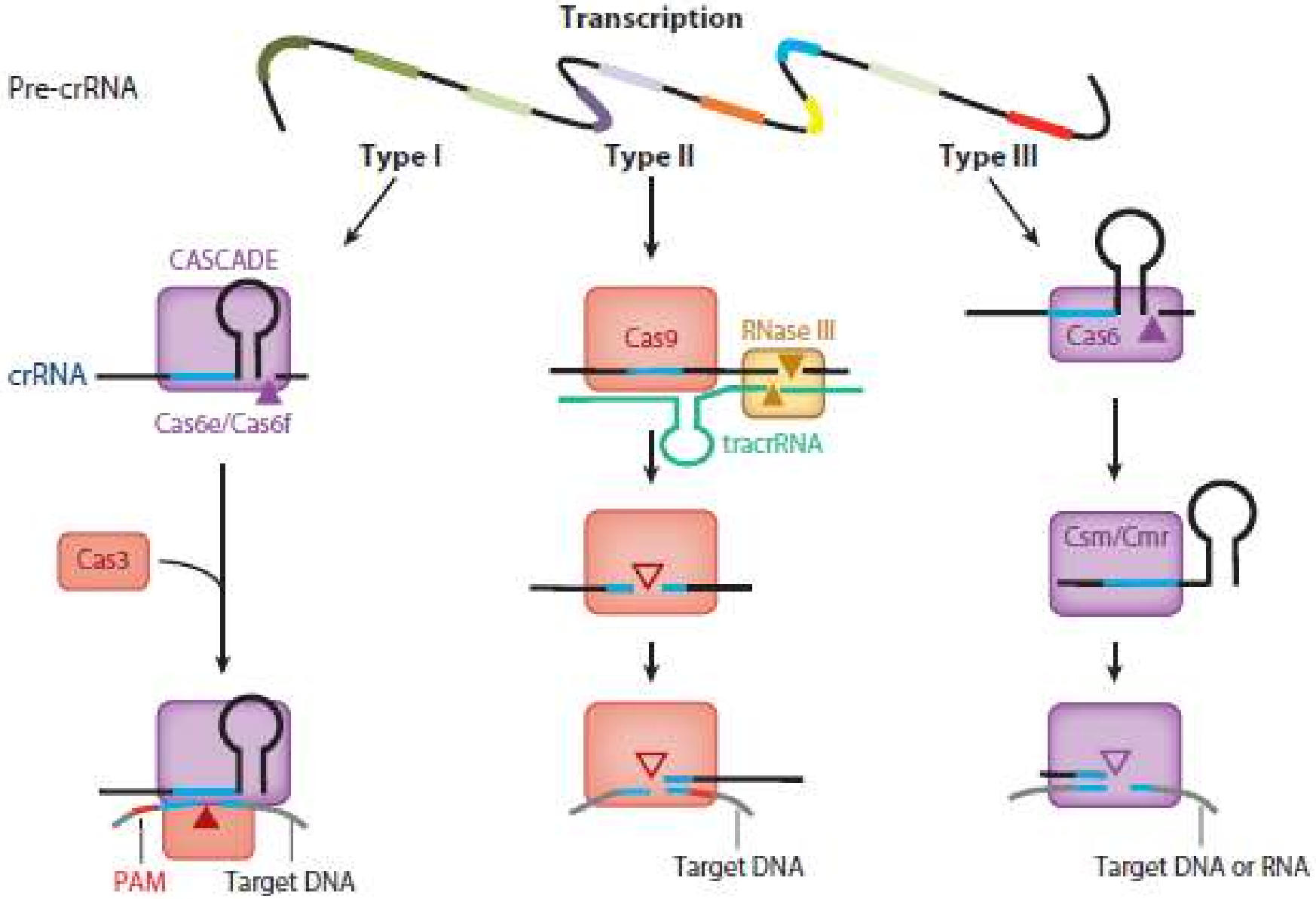
Bhaya et al., Annu Rev Genet, 2011

CRISPR vs RNAi



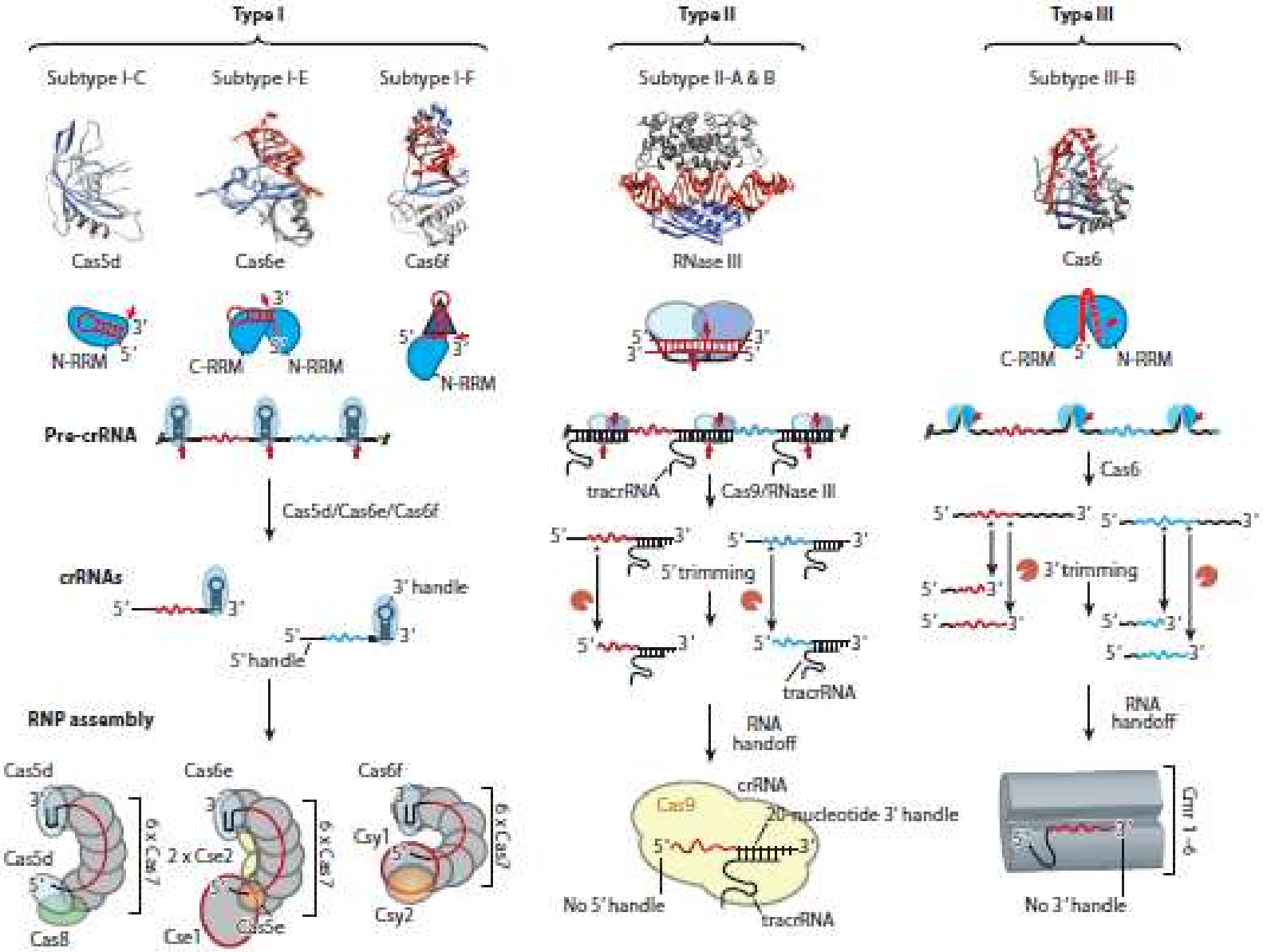
Wiedenheft et al. Nature, 2012

CRISPR/CAS system types



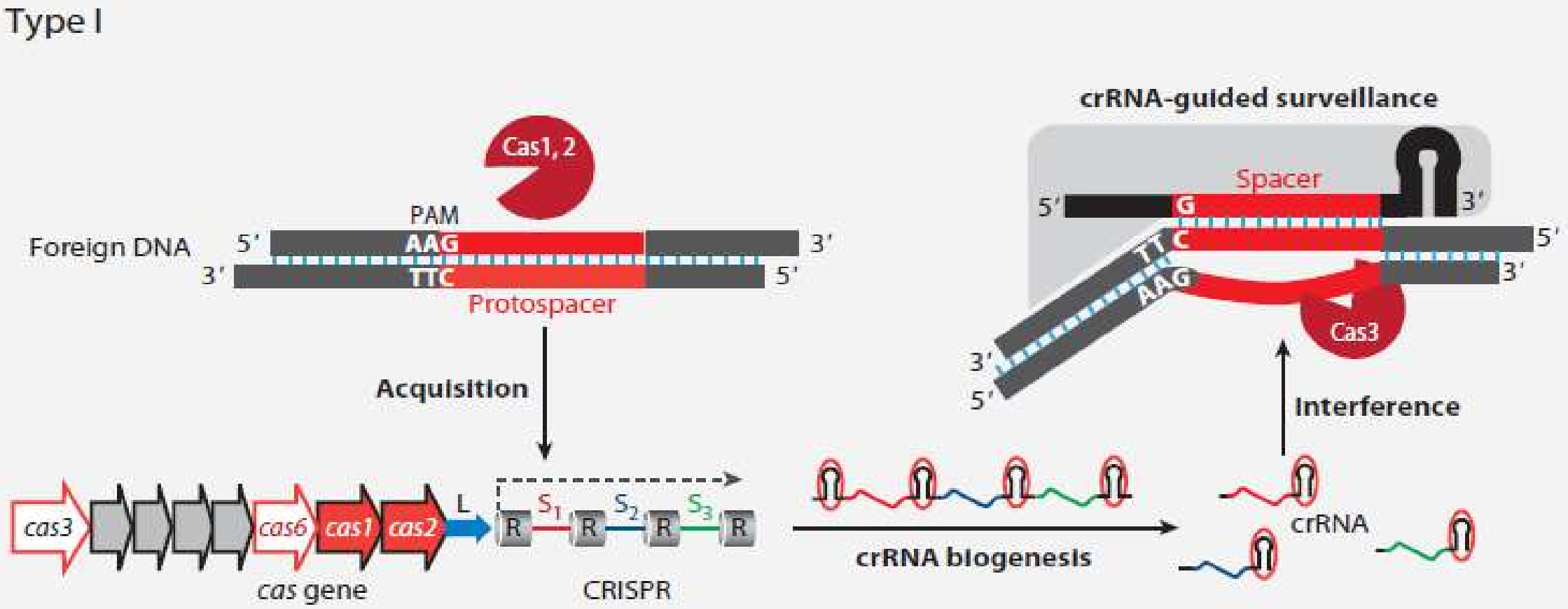
Bhaya et al., Annu Rev Genet, 2011

CRISPR/CAS system types



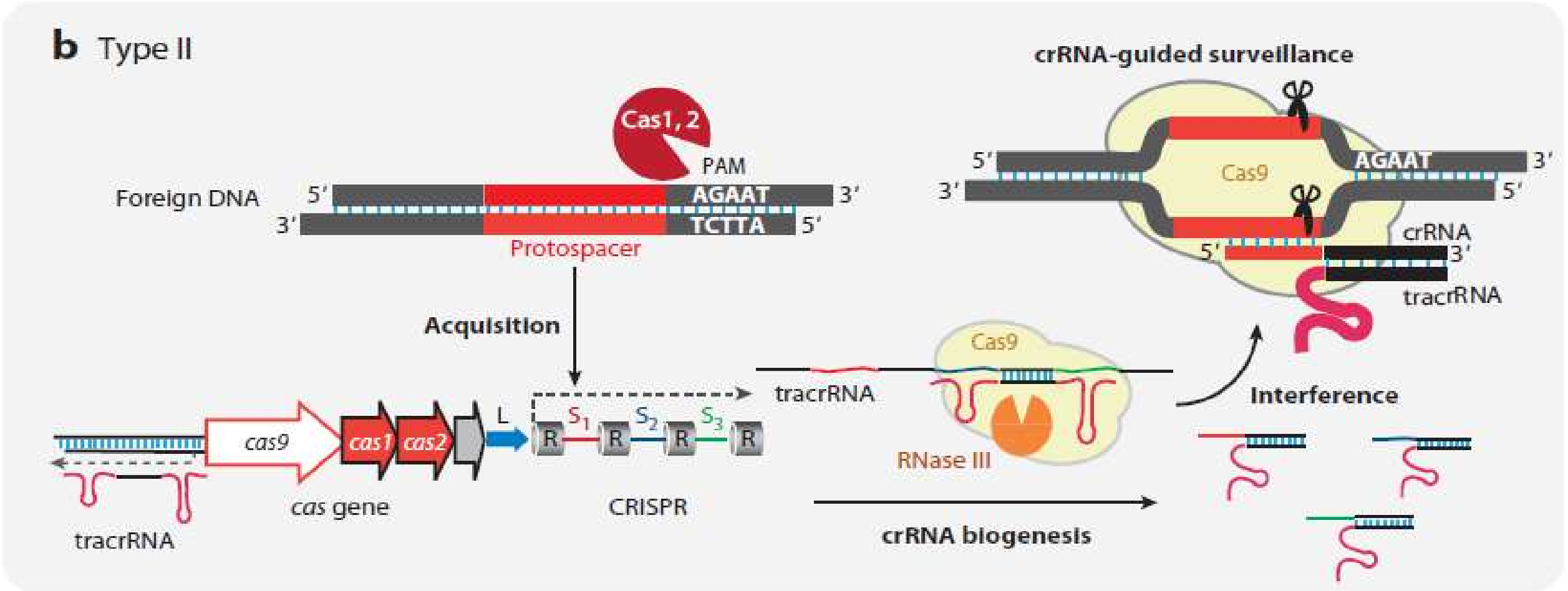
Sorek et al.,Annu. Rev. Biochem. 2013

CRISPR/CAS system type I



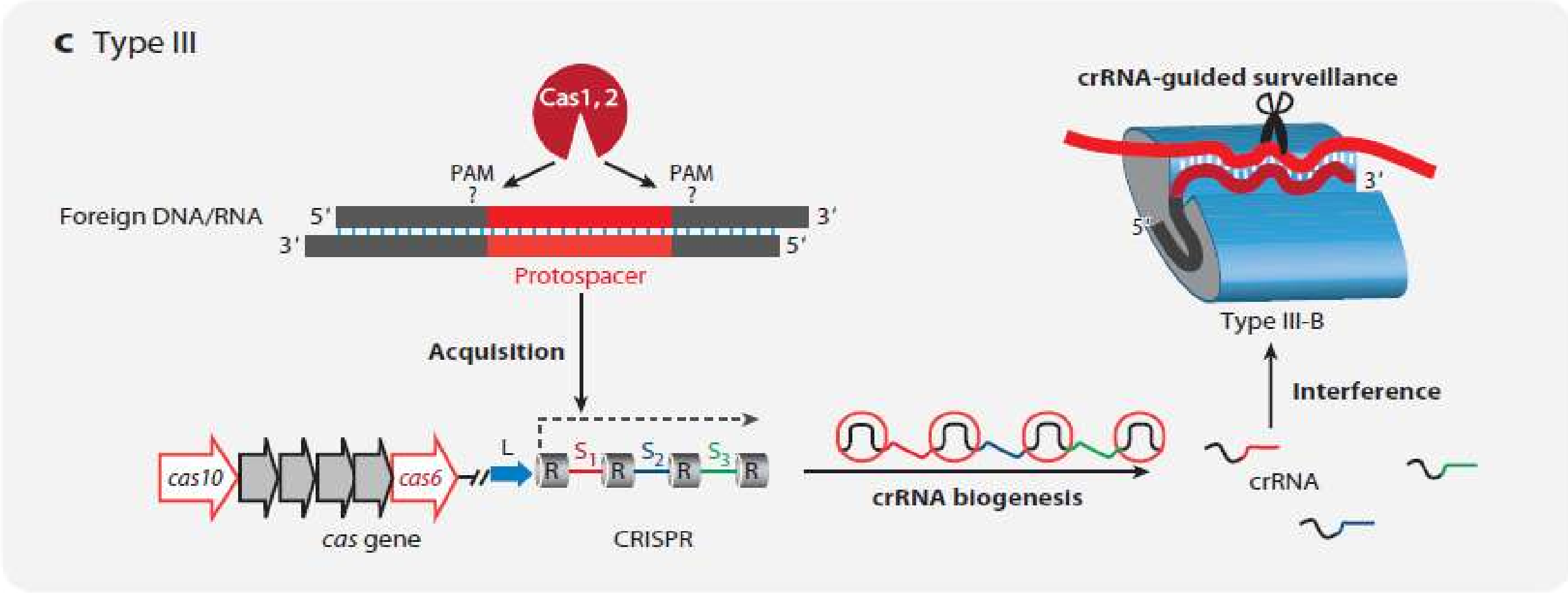
Sorek et al., Annu Rev Biochem, 2013

CRISPR/CAS system type II



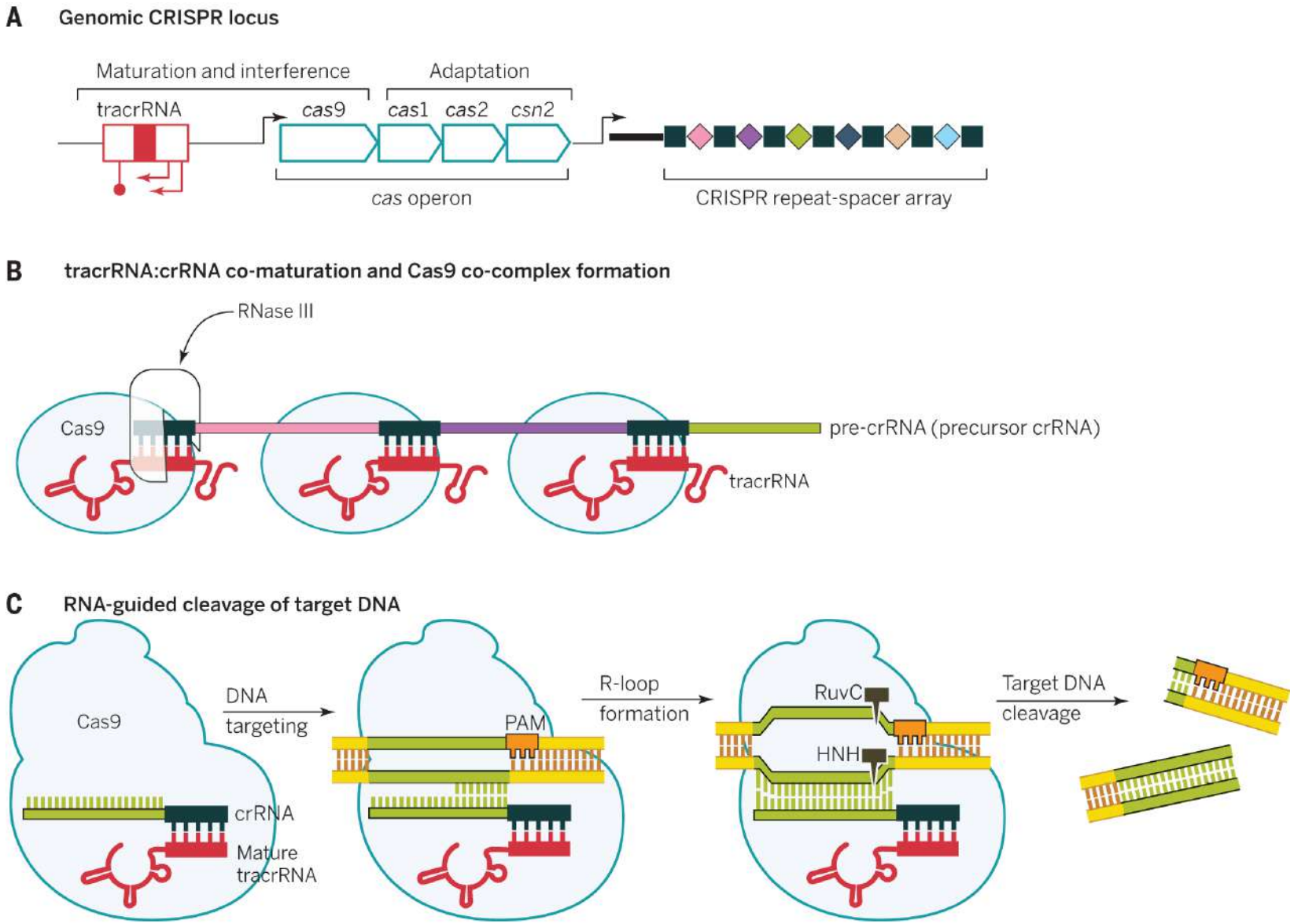
Sorek et al., Annu Rev Biochem, 2013

CRISPR/CAS system type III



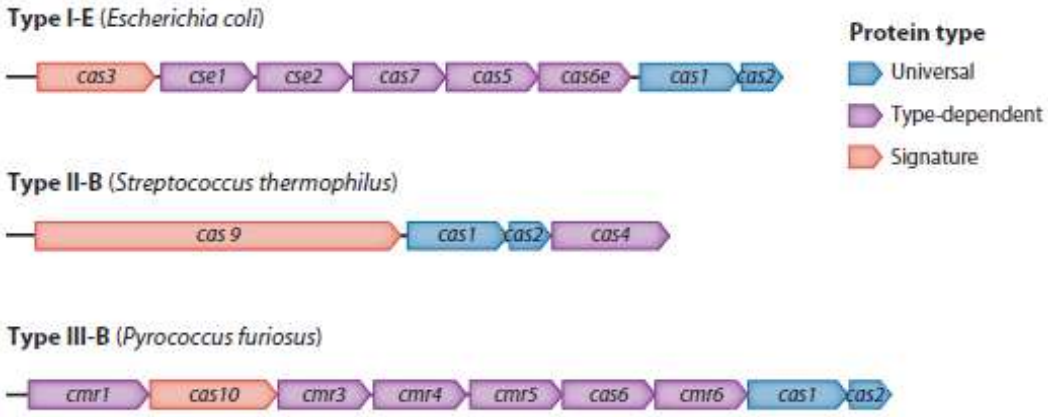
Sorek et al., Annu Rev Biochem, 2013

Type II of CRISPR / CAS system



Doudna et al. (2014) Science

CAS proteins



Protein	Distribution	Process	Function
Cas1	Universal	Spacer acquisition	DNAse, not sequence specfic, can bind RNA; present in all Types; structure available for several Cas 1 proteins
Cas2	Universal	Spacer acquisition	Small RNAse specific to U-rich regions; present in all Types; structure available from *Thermus thermophilus* and *Sulfolobus solfataricus* and others
Cas3	Type I signature	Target interference	DNA helicase; most proteins have a fusion to HD nuclease
Cas4	Type I, II	Spacer acquisition	RecB-like nuclease with exonuclease activity homologous to RecB
Cas5	Type I	crRNA expression	RAMP protein, endoribonuclease involved in crRNA biogenesis; part of CASCADE
Cas6	Type I, III	crRNA expression	RAMP protein, endoribonuclease involved in crRNA biogenesis; part of CASCADE; structure available from *P. furiosus*
Cas7	Type I	crRNA expression	RAMP protein, endoribonuclease involved in crRNA biogenesis; part of CASCADE
Cas8	Type I	crRNA expression	Large protein with McrA/HNH-nuclease domain and RuvC-like nuclease; part of CASCADE
Cas9	Type II signature	Target interference	Large multidomain protein with McrA-HNH nuclease domain and RuvC-like nuclease domain; necessary for interference and target cleavage
Cas10	Type III signature	crRNA expression and interference	HD nuclease domain, palm domain, Zn ribbon; some homologies with CASCADE elements

Bhaya et al., Annu Rev Genet, 2011

Different Cas proteins

Table 1 Naturally occurring major CRISPR-Cas enzymes

	Size	PAM sequence	Size of sgRNA guiding sequence	Cutting site	Reference
spCas9	1368	NGG	20 bp	~ 3 bp 5′ of PAM	Jinek et al.[42] Gasiunas et al.[43]
FnCas9	1629	NGG	20 bp	~ 3 pb 5′ of PAM	Hirano et al.[60]
SaCas9	1053	NNGR RT	21 bp	~ 3 pb 5′ of PAM	Mojica et al.[57]
NmCas9	1082	NNNNG ATT	24 bp	~ 3 bp 5′ of PAM	Hou et al.[53]
St1Cas9	1121	NNAGA AW	20 bp	~ 3 bp 5′ of PAM	Gasiunas et al.[43] Cong et al.[45]
St3Cas9	1409	NGGNG	20 bp	~ 3 bp 5′ of PAM	Gasiunas et al.[43] Cong et al.[45]
CjCas9	984	NNNNACAC	22 bp	~ 3 bp 5′ of PAM	Kim et al.[56]
AsCPf1	1307	TTTV	24 bp	19/24 bp 3′ of PAM	Yamano et al.[50] Kim et al. 2016
LbCpf1	1228	TTTV	24 bp	19/24 bp 3′ of PAM	Yamano et al.[50] Kim et al. 2016
Cas13	Multiple orthologs	RNA targeting	28 bp		Abudayyeh et al. 2017

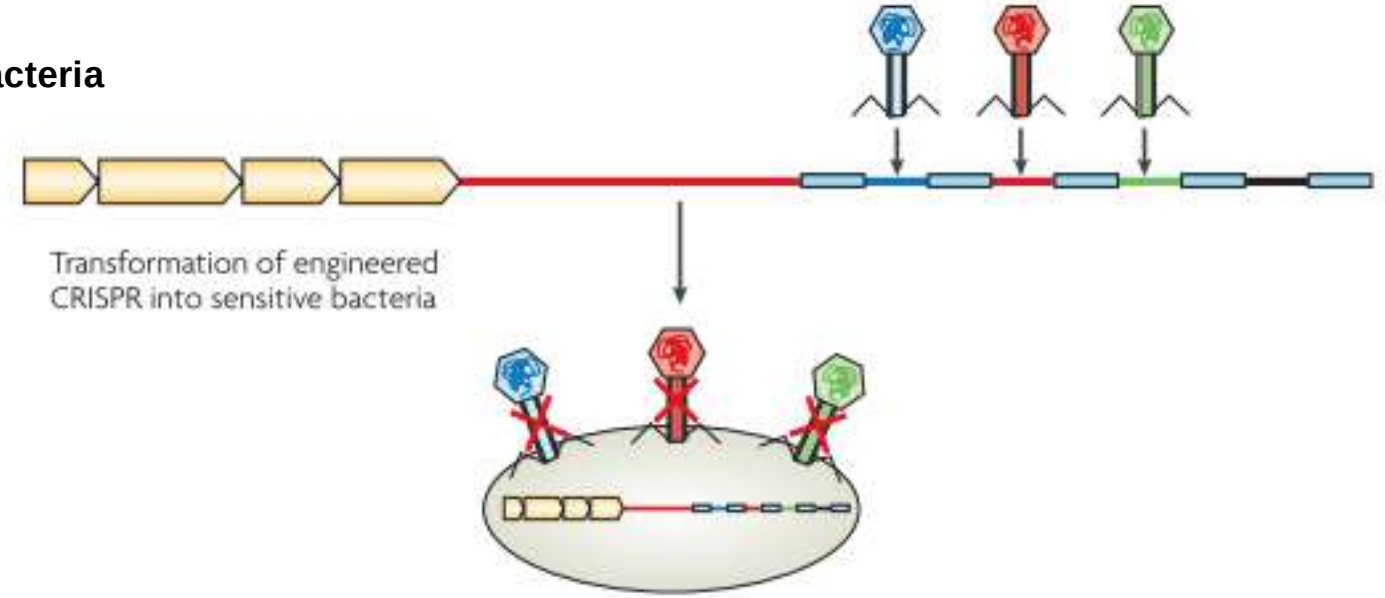
Web resources for CRISPR analysis

Resource and web page	Description
PILER-CR; http://www.drive5.com/pilercr/	A software tool for the detection of CRISPRs in microbial genomic sequences; based on local alignments in the genome that are represented by mathematical graphs*
CRISPR Recognition Tool; http://www.room220.com/crt/	A software tool for the detection of CRISPRs in microbial genomic sequences; based on the detection of exact k-mer matches that are separated by similar distances*
CRISPRFinder; http://crispr.u-psud.fr/crispr/	A software tool for the detection of CRISPRs in microbial genomic sequences; based on enhanced suffix arrays*
CRISPRdb; http://crispr.u-psud.fr/crispr/	Automatically updated database of CRISPR arrays in published microbial genomes; also contains CRISPR analysis tools that allow the alignment and comparison of repeats and spacers against the public databases
Pygram; http://www.irisa.fr/symbiose/projets/Modulome/article.php3?id_article=18	Visualization application that provides a graphical browser for studying repeats
TIGR Comprehensive Microbial Resource; http://rice.tigr.org/tigr-scripts/CMR2/genome_property.spl?subproperty=CRISPR%20region!&select_count=1	Provides a 'clickable' table that depicts, for each sequenced genome, the presence or absence of the 45 Cas protein families that are defined in Ref. 17

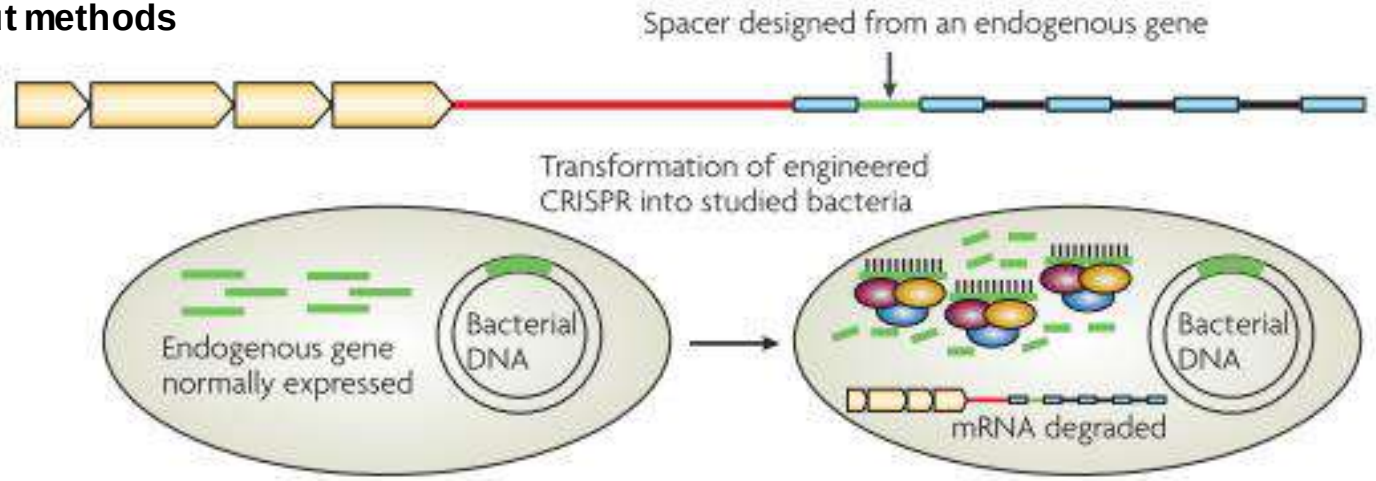
Sorek et al., Nat. Rev. Micro, 2008

Early applications of CRISPR

- **Engineering of phage resistance into sensitive industrial bacteria**



- **Silencing of endogenous genes as an alternative to knockout methods**



Sorek et al., Nat. Rev. Micro, 2008

CRISPR/CAS9 in genome engineering

Sciencexpress

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]†

Sciencexpress / http://www.sciencemag.org/content/early/recent / 03 January 2013 / Page 1/ 10.1126/science.1231143

Sciencexpress

RNA-Guided Human Genome Engineering via Cas9

Prashant Mali,[1,5] Luhan Yang,[1,3,5] Kevin M. Esvelt,[2] John Aach,[1] Marc Guell,[1] James E. DiCarlo,[4] Julie E. Norville,[1] George M. Church[1,2*]

Sciencexpress / http://www.sciencemag.org/content/early/recent / 03 January 2013; / Page 1/ 10.1126/science.1232033

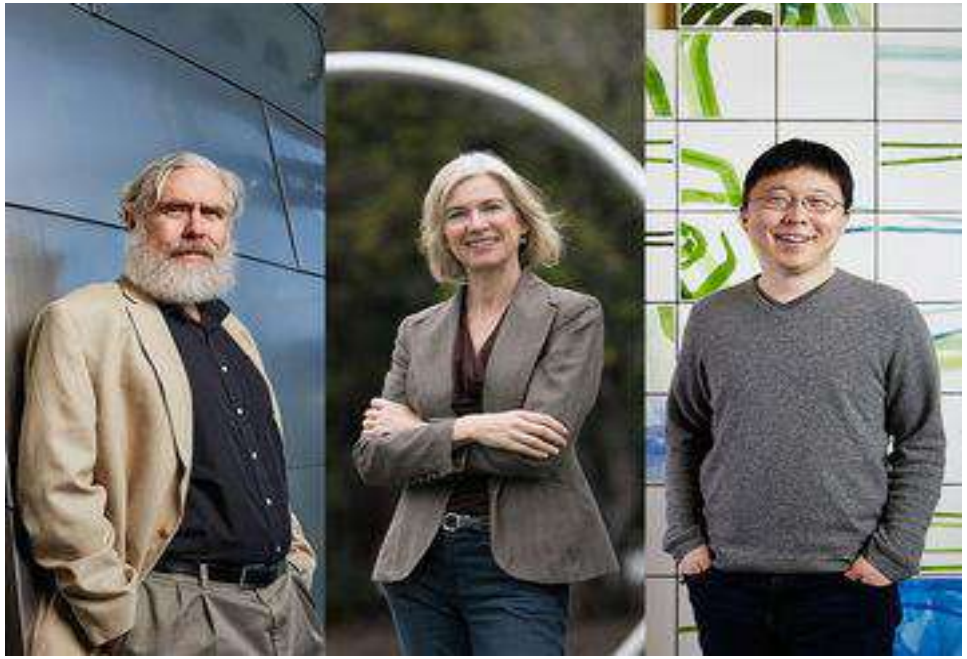
eLIFE
elife.elifesciences.org

RESEARCH ARTICLE

RNA-programmed genome editing in human cells

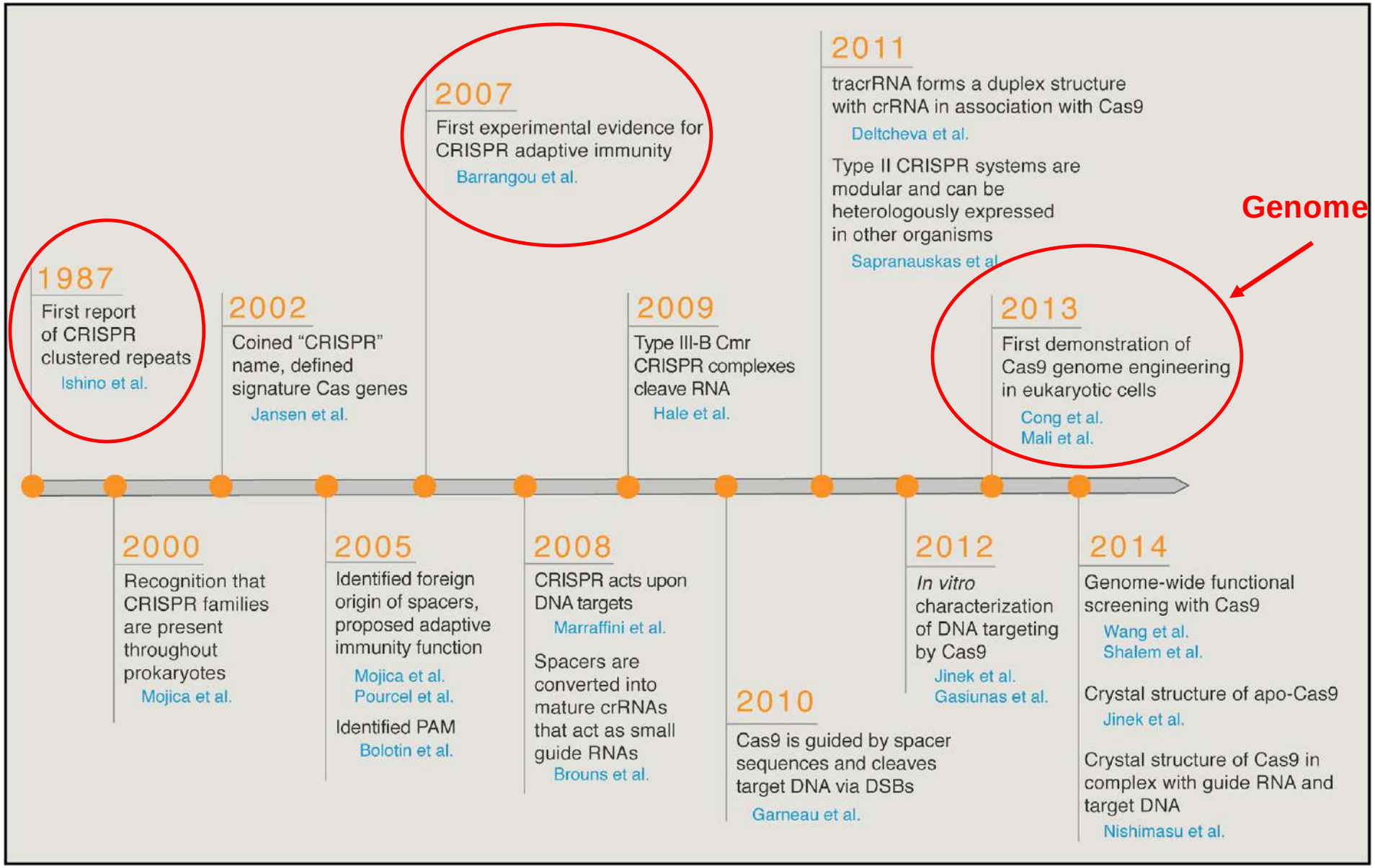
Martin Jinek[1,2], Alexandra East[2], Aaron Cheng[2], Steven Lin[1,2], Enbo Ma[2], Jennifer Doudna[1,2,3,4*]

Jinek *et al*. eLife 2013;2:e00471. DOI: 10.7554/eLife.00471



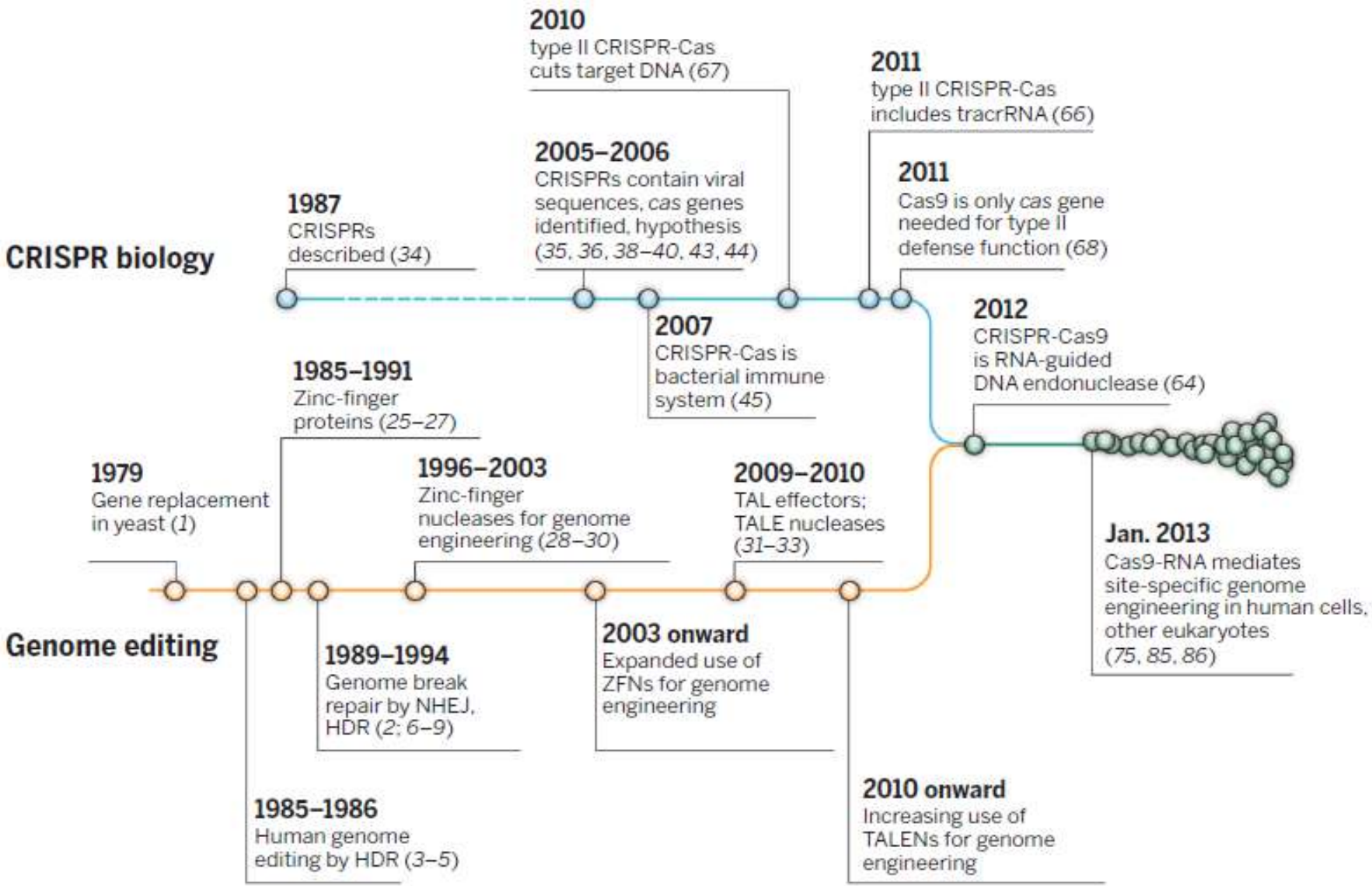
George Church Jennifer Doudna Feng Zhang

Discovery of CRISPR



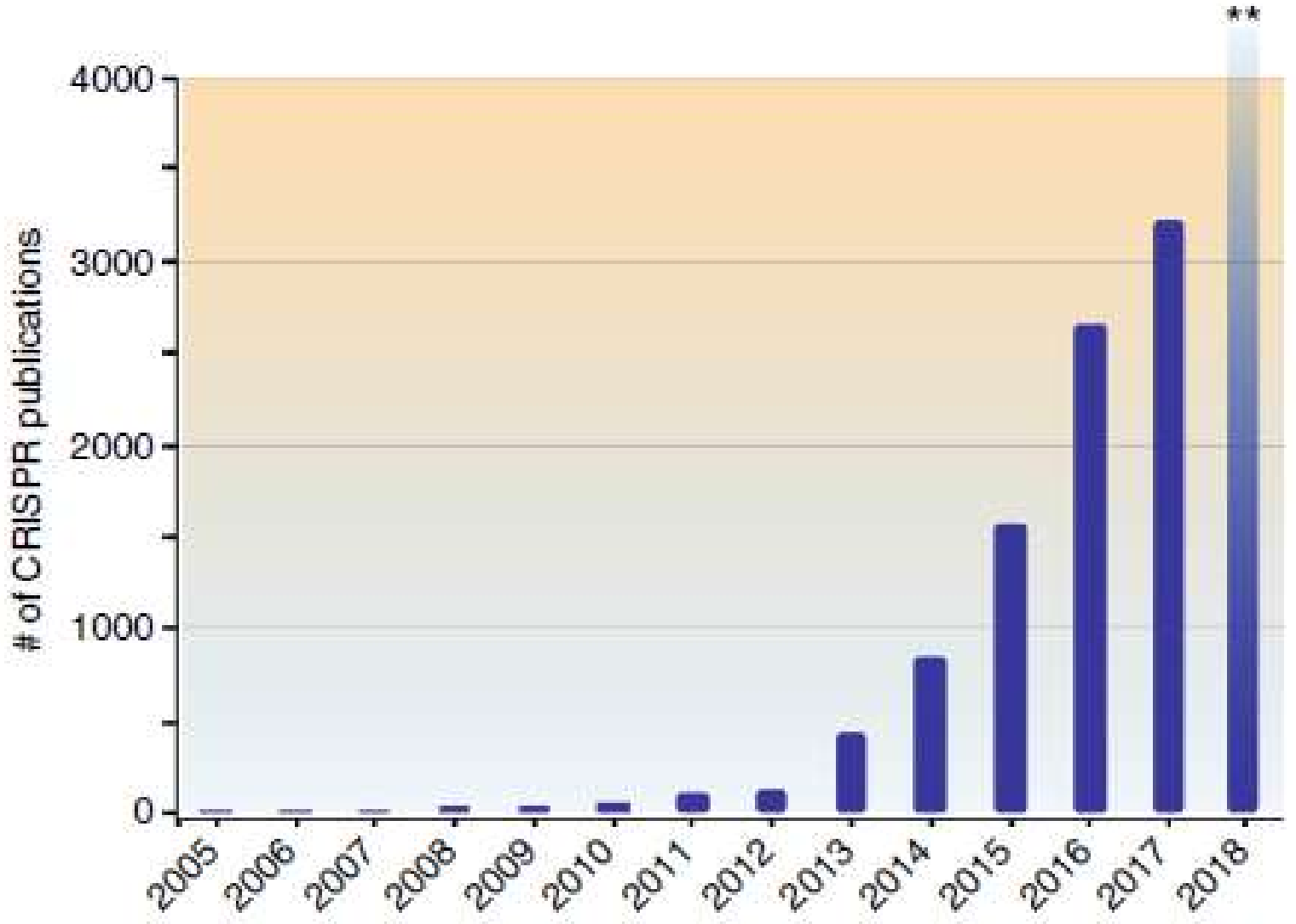
Hsu et al., Cell, 2014

Timeline of CRISPR research



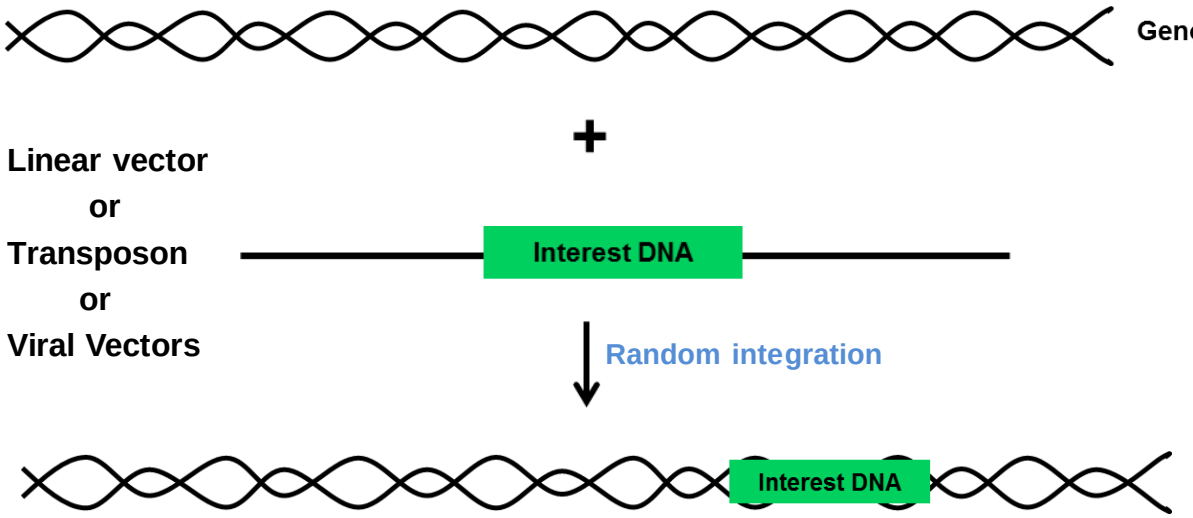
Doudna et al. (2014) Science

CRISPR publications

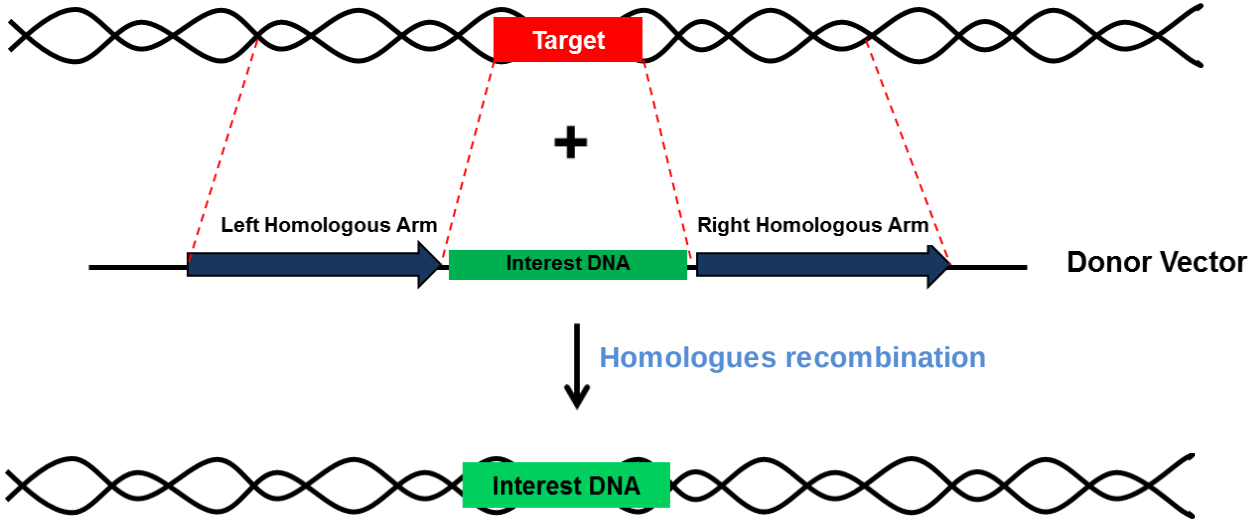


Genome engineering

Random



Site Specific

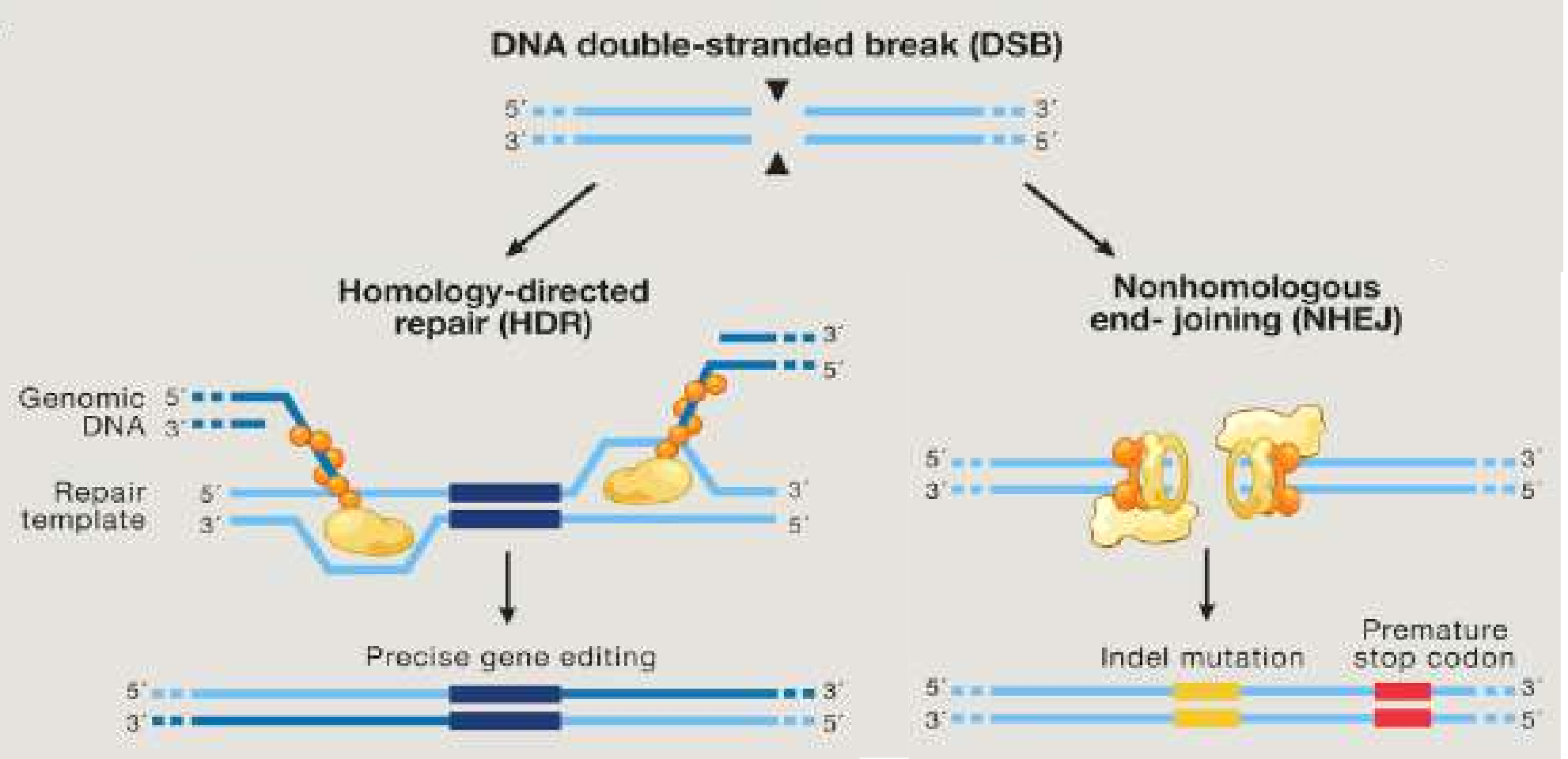


The Nobel Prize in Physiology or Medicine 2007

- A crucial first step for performing targeted genome editing is the creation of a DSB at the genomic locus to be modified.

Carroll et al., Genetics, 2011

Mechanism of site specific integration

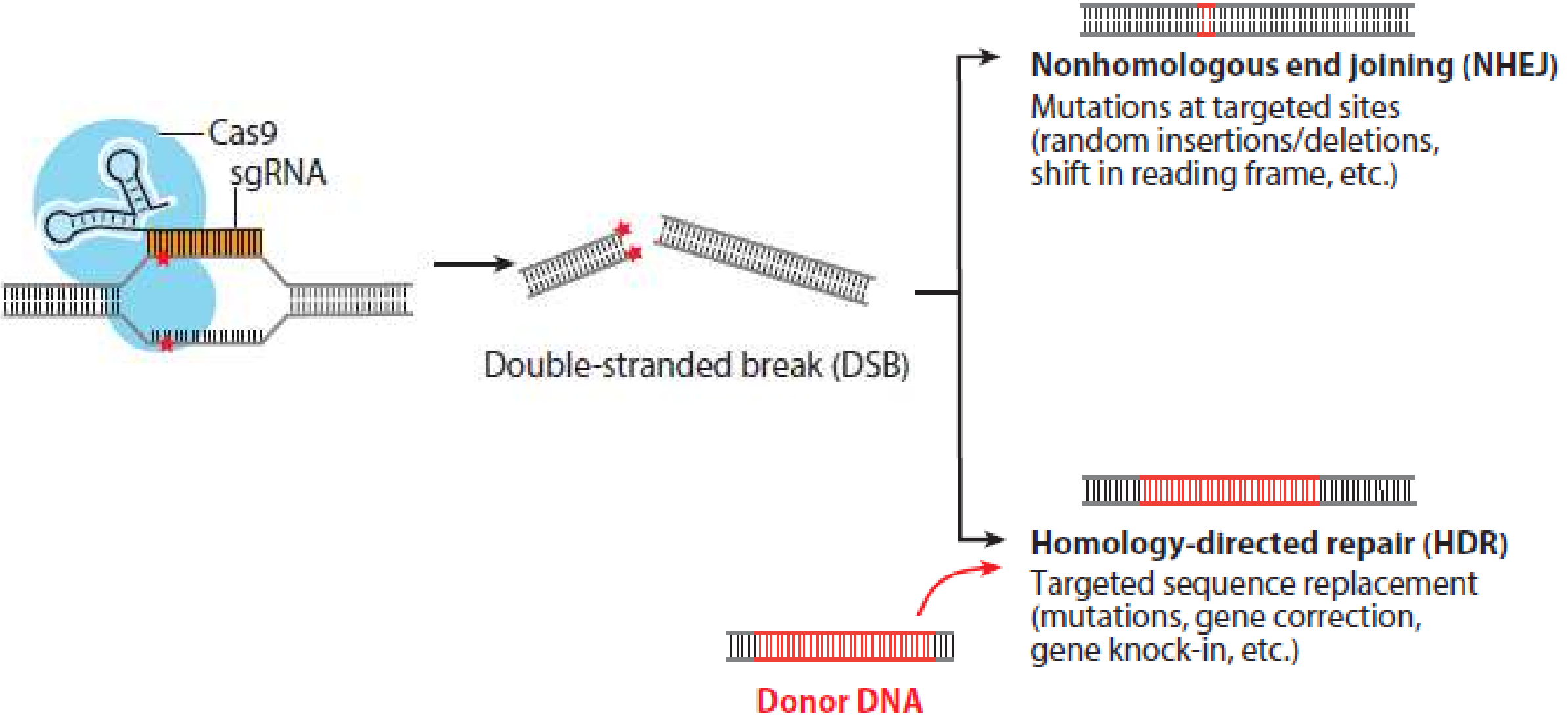


- DSBs can be repaired by one of at least two different pathways that operate in nearly all cell types and organisms: homology directed repair (HDR) and non-homologous end-joining (NHEJ)

Hsu et al., Cell, 2014

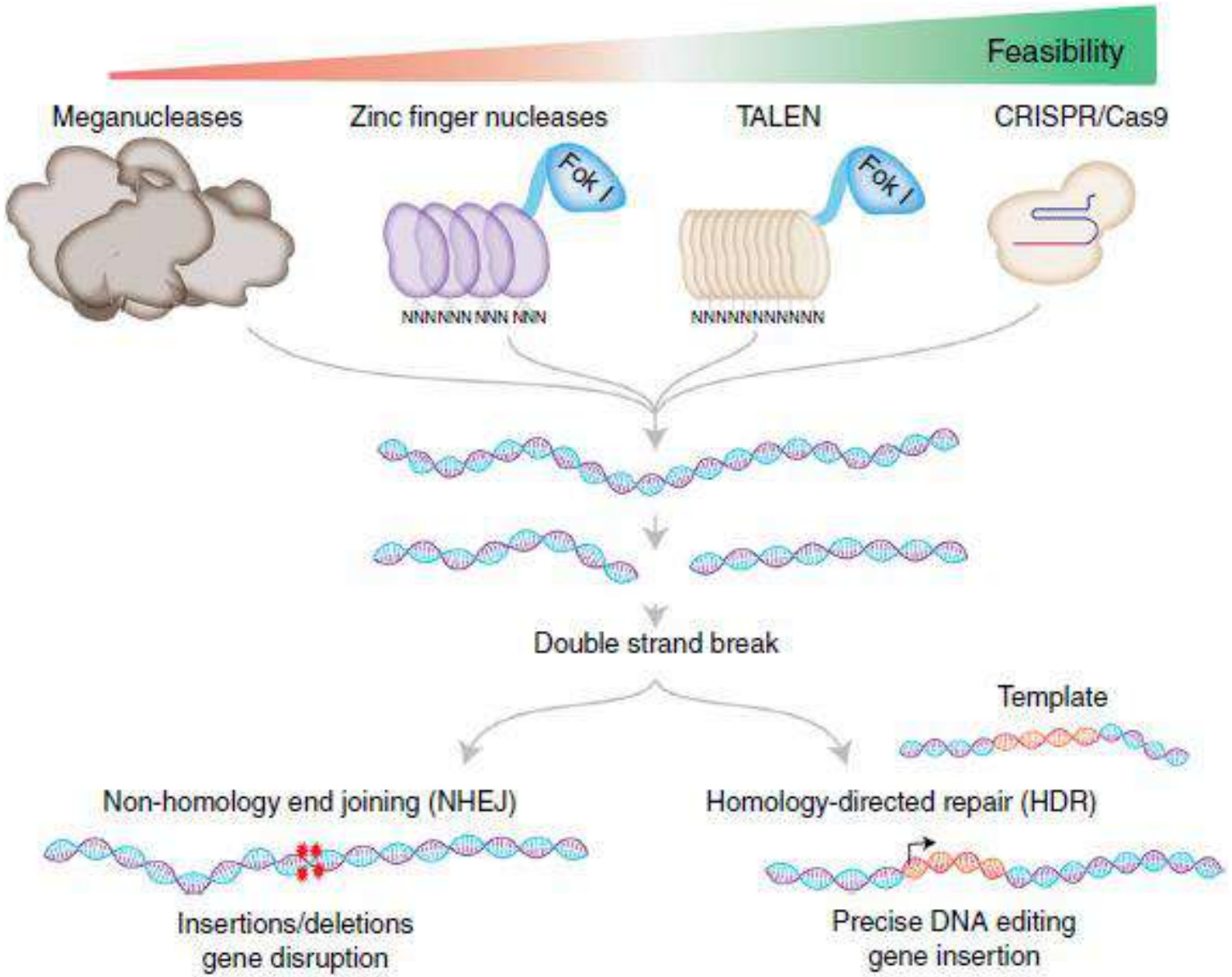
Sander & Joung, Nature Biotechnology, 2014

Using CRISPR/Cas for DSB creation

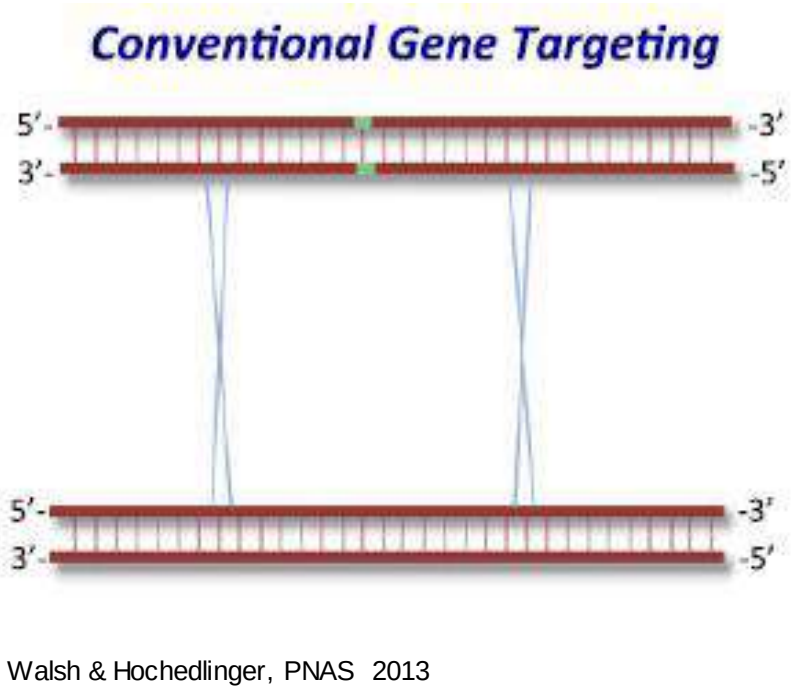


Wang et al., Annual review of biochemistry 2016

Other strategies for DSB creation

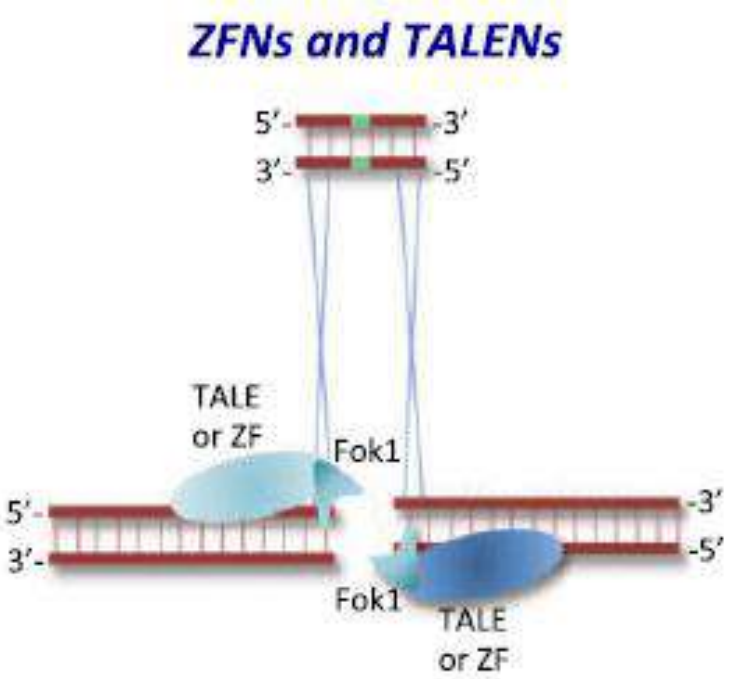


New strategies for site specific genome engineering

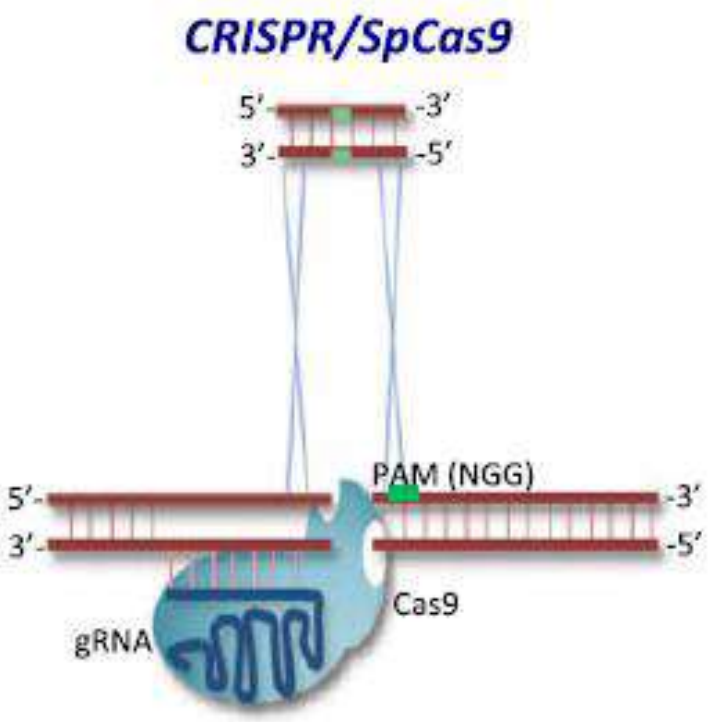


Walsh & Hochedlinger, PNAS 2013

- Complex
- Time-consuming
- Low efficiency



- High efficiency (1- 50 %)
- Complex
- Time-consuming design

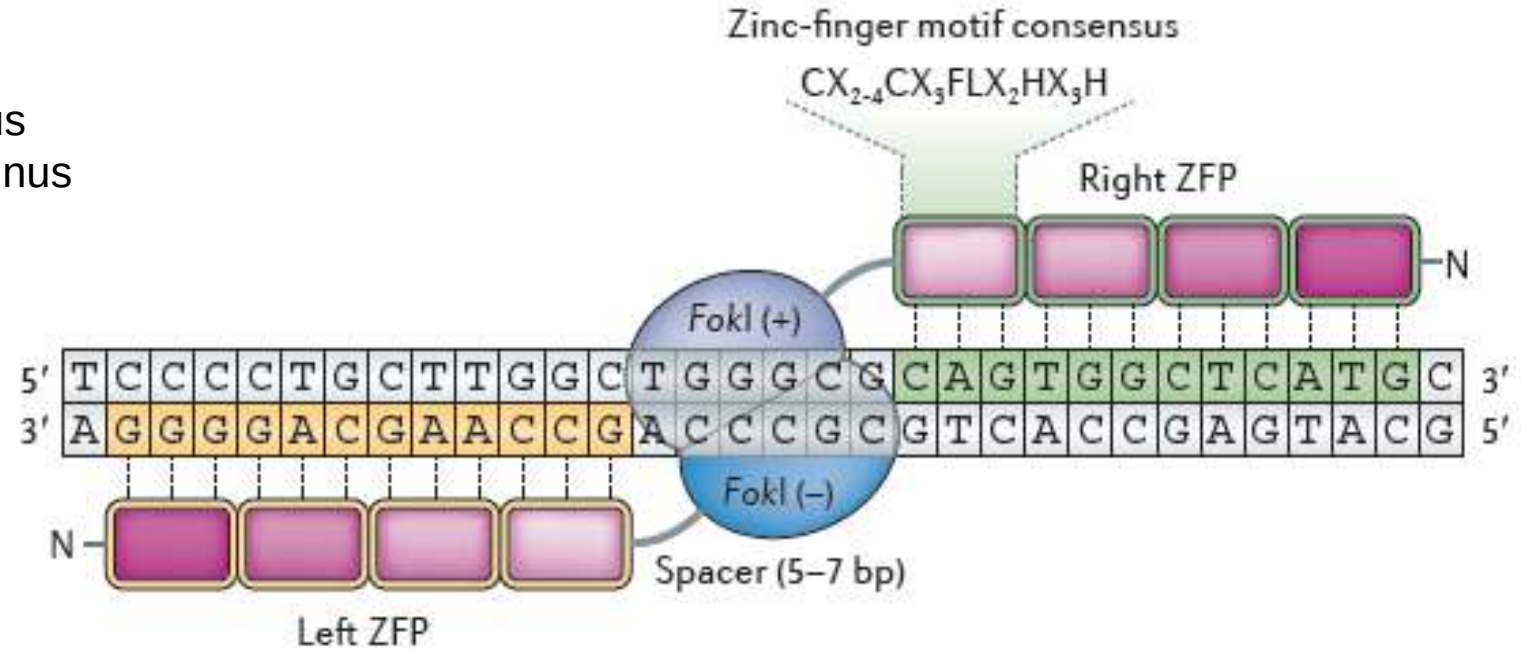


- Facile design
- High efficiency

Capecchi , Nat. Rev. Genet, 2005
Gaj et al. Trends in Biotechnology, 2013

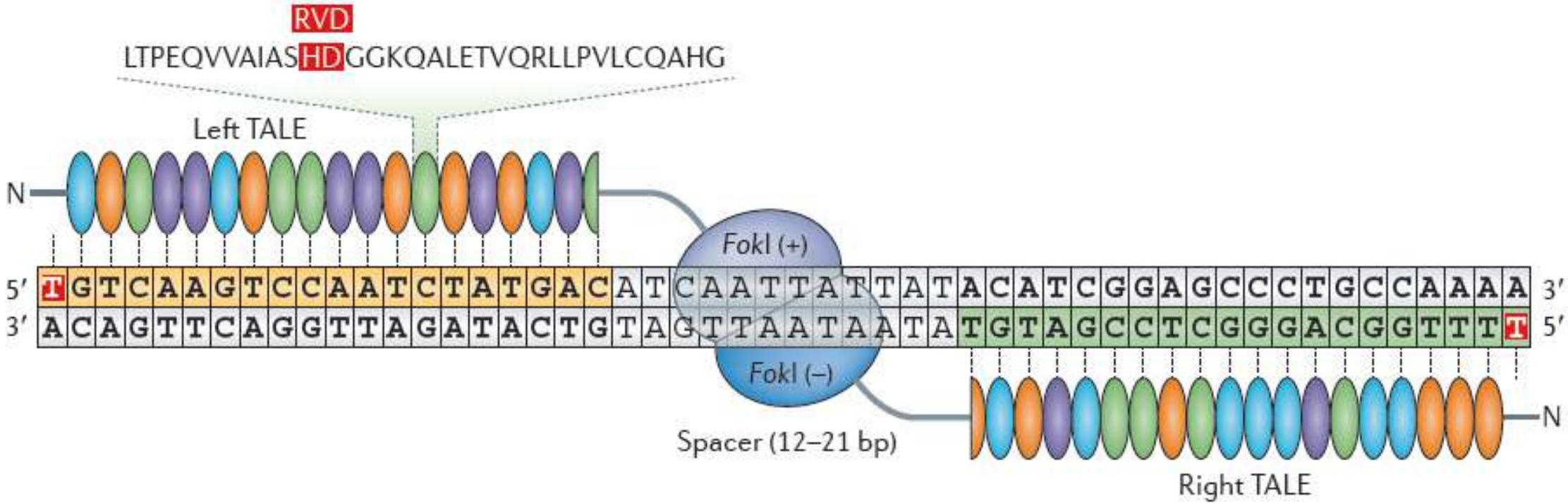
Zinc finger nuclease (ZFN)

- **Each ZFN is composed of:**
  A zinc-finger protein (ZFP) at the amino terminus
  The FokI nuclease domain at the carboxyl terminus



Kim $ Kim, Nat. Rev. Genet, 2014

Transcription activator-like effector nuclease (TALEN)



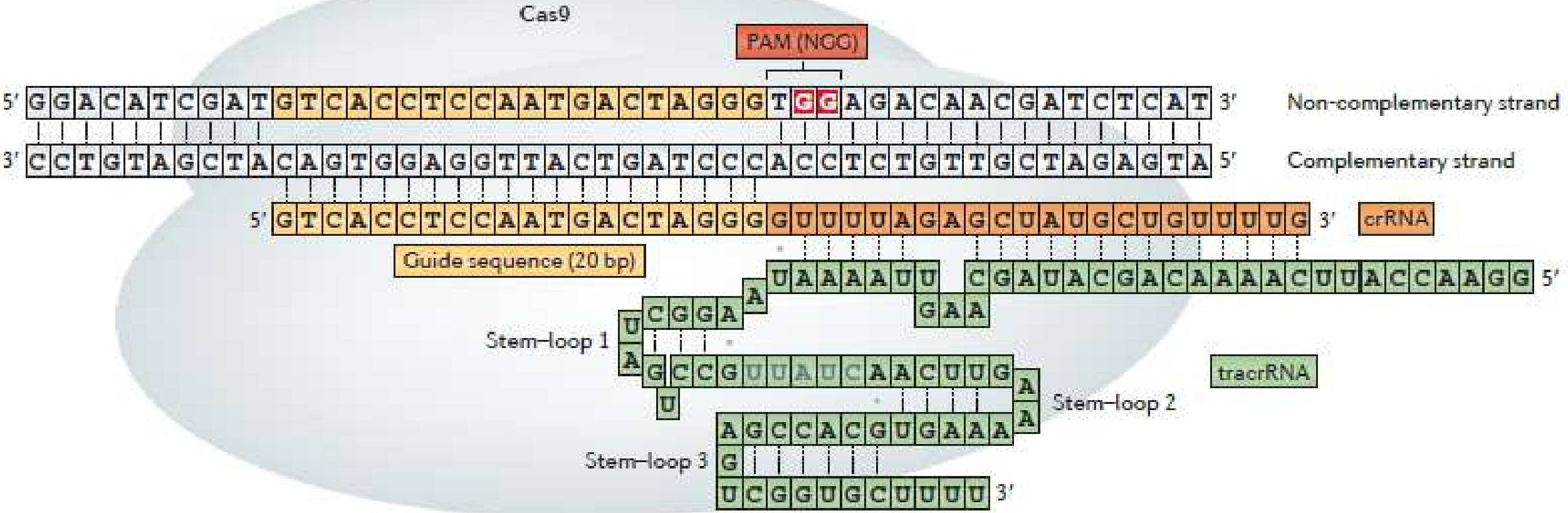
- **Each TALEN is composed of:**
  Transcription activator-like effectors (TALEs) at the amino terminus
  The *Fok*I nuclease domain at the carboxyl terminus

- Each TALE repeat is comprised of 33–35 amino acids and recognizes a single base pair through the amino acids at positions 12 and 13, which is called the repeat variable diresidue (RVD).

Kim $ Kim, Nat. Rev. Genet, 2014

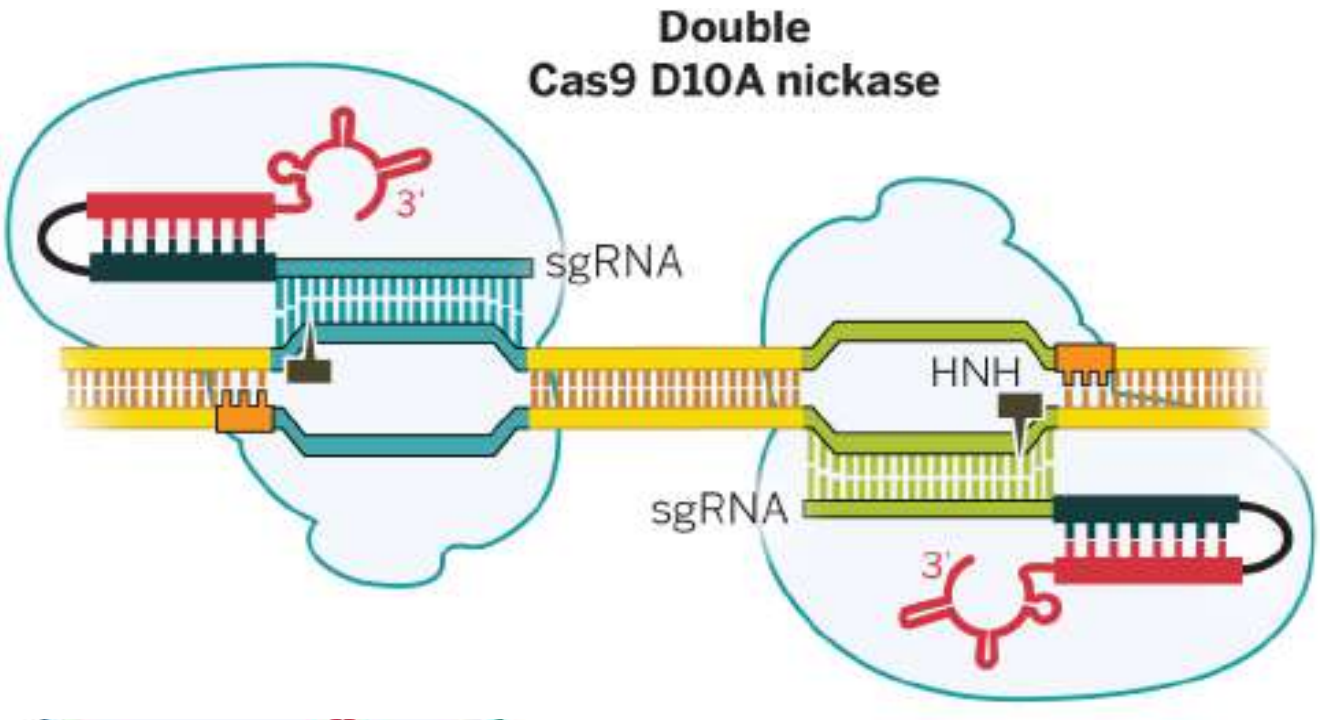
Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) / CRISPR Associated Protein (CAS)

- **RNA Guided Nuclease (RGN)**



Kim $ Kim, Nat. Rev. Genet, 2014

Cas9 modifications



Depression of off-targets
Enhancement of specificity

Doudna et al. Science, 2014

Cpf1 as an alternative for Cas9

Cell

Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System

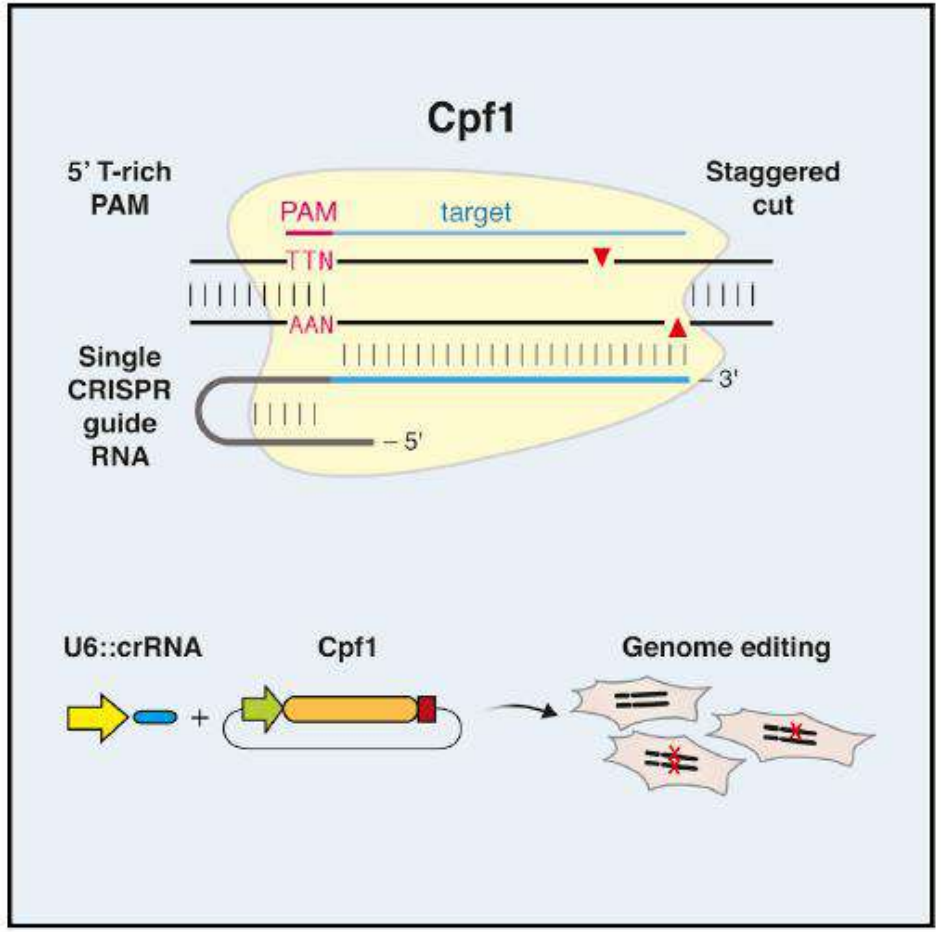
Bernd Zetsche,[1,2,3,4,5,10] Jonathan S. Gootenberg,[1,2,3,4,6,10] Omar O. Abudayyeh,[1,2,3,4] Ian M. Slaymaker,[1,2,3,4] Kira S. Makarova,[7] Patrick Essletzbichler,[1,2,3,4] Sara E. Volz,[1,2,3,4] Julia Joung,[1,2,3,4] John van der Oost,[8] Aviv Regev,[1,9] Eugene V. Koonin,[7] and Feng Zhang[1,2,3,4,*]

Cell *163*, 1–13, October 22, 2015 ©2015 Elsevier Inc.

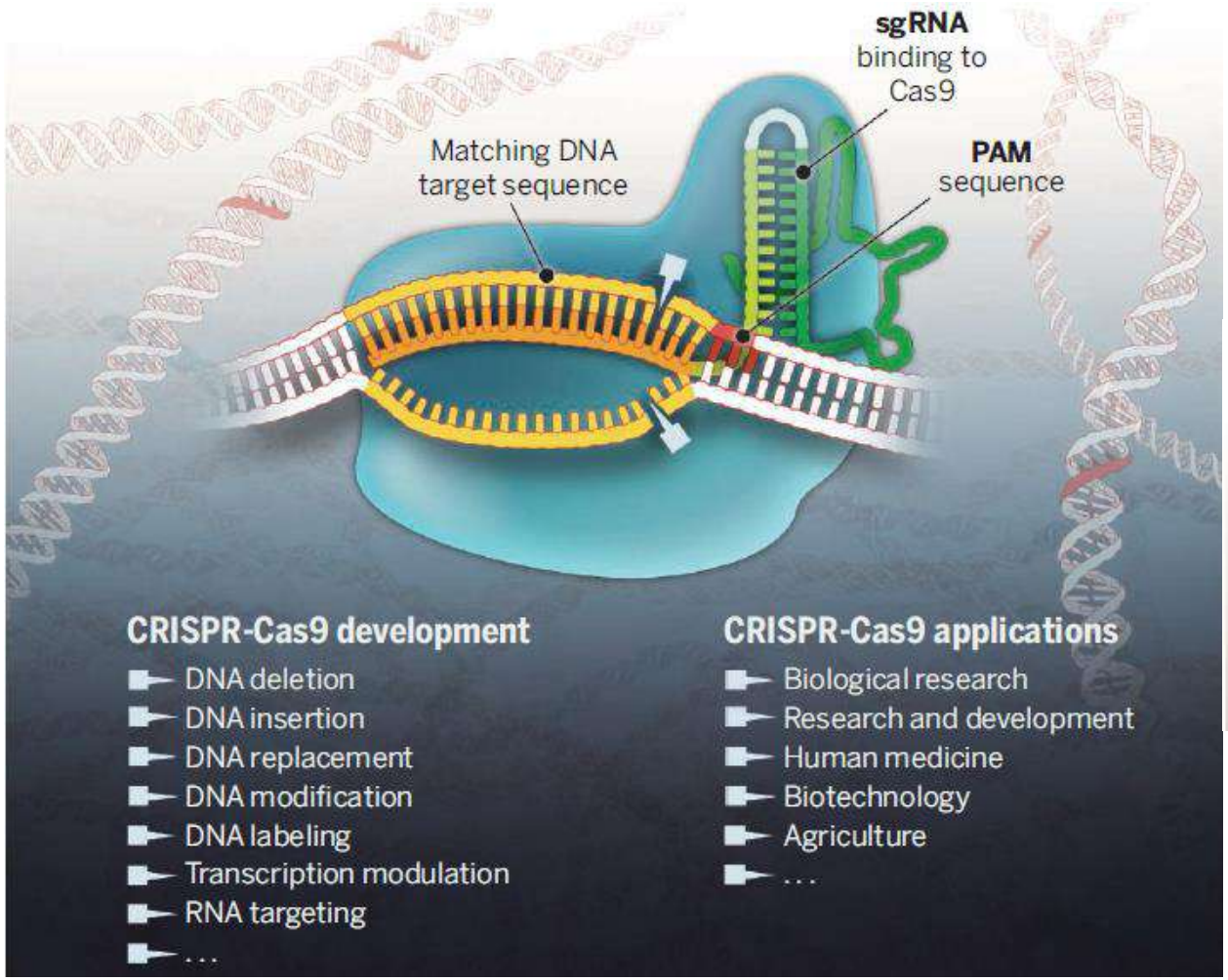
- This feature could simplify the design and delivery of genome-editing tools. For example, the shorter (42 nt) crRNA employed by Cpf1 has practical advantages over the long (100 nt) guide RNA in Cas9-based systems because shorter RNA oligos are significantly easier and cheaper to synthesize.

- Cpf1 generates a staggered cut with a 50 overhang, in contrast to the blunt ends generated by Cas9. This structure of the cleavage product could be particularly advantageous for facilitating nonhomologous end joining (NHEJ)-based gene insertion into the mammalian genome. Being able to program the exact sequence of a sticky end would allow researchers to design the DNA insert so that it integrates into the genome in the proper orientation. Specifically, in non-dividing cells, in which genome editing via homology-directed repair (HDR) mechanisms is especially challenging ,Cpf1 could provide an effective way to precisely introduce DNA into the genome via non-HDR mechanisms.

Acidominococcus and Lachnospiraceae



CRISPR/CAS9 Applications



Biology

Cell lines
HEK293
U2OS
K562

Model organisms
Mice
Rats
Fruit flies
Nematodes
Arabidopsis
Salamanders
Frogs
Monkeys

Biotechnology

Crop plants
Rice
Wheat
Sorghum
Tobacco

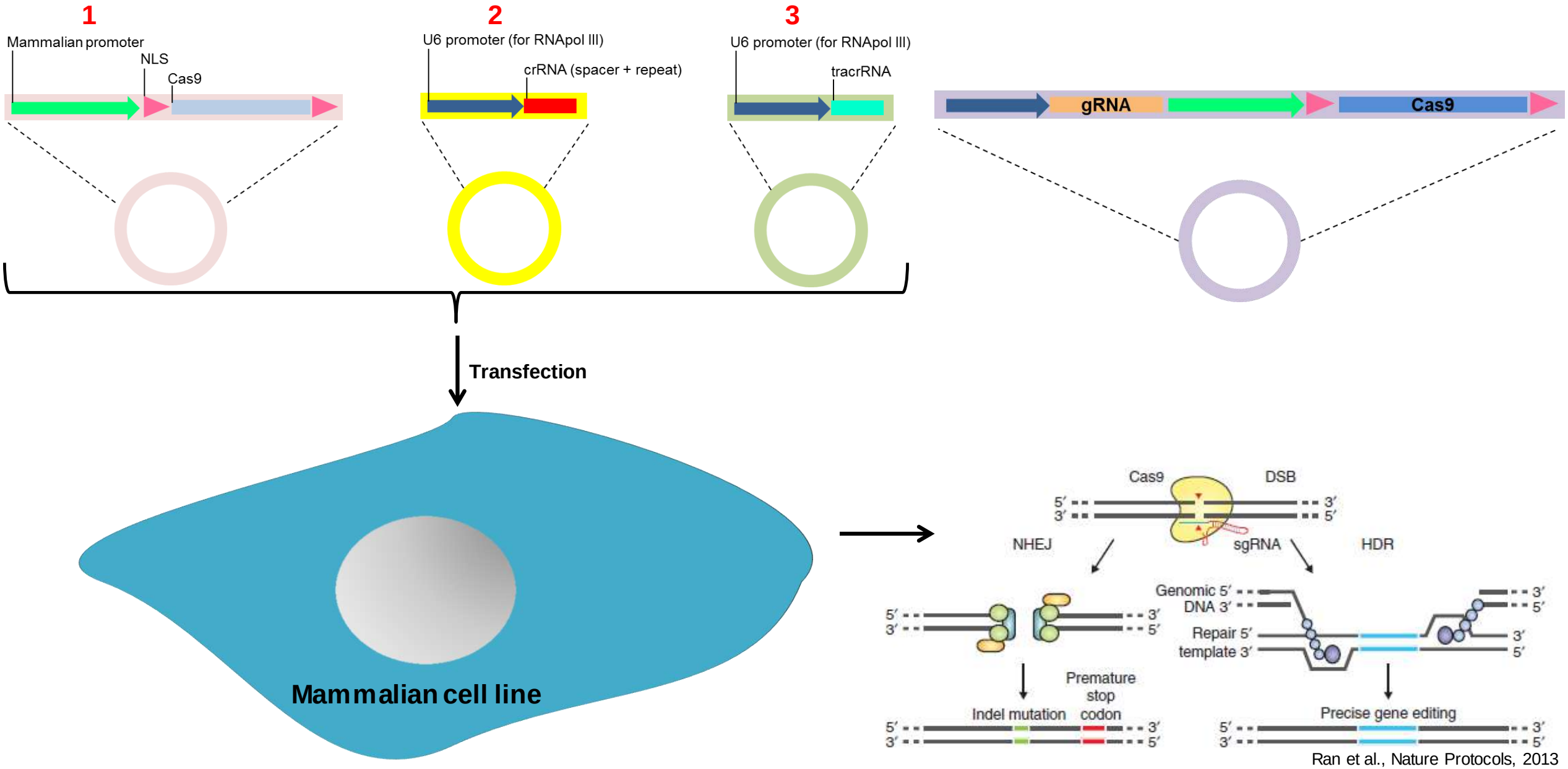
Fungi
Kluyveromyces
Chlamydomonas

Biomedicine

Organoids
hESCs
iPSCs

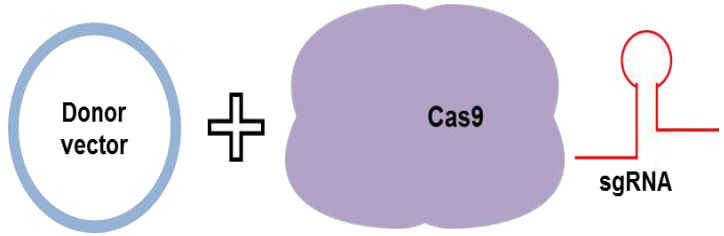
Doudna et al., Science 2014

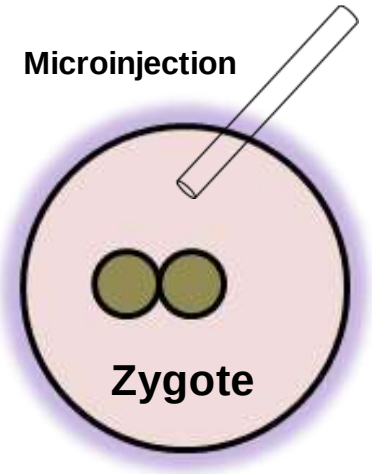
Genome engineering of cell lines via CRISPR/CAS9

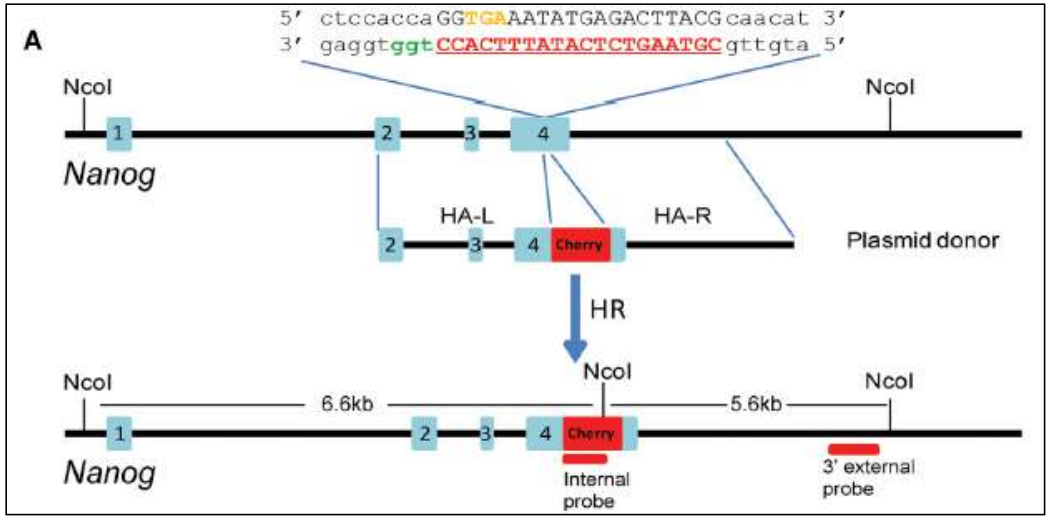


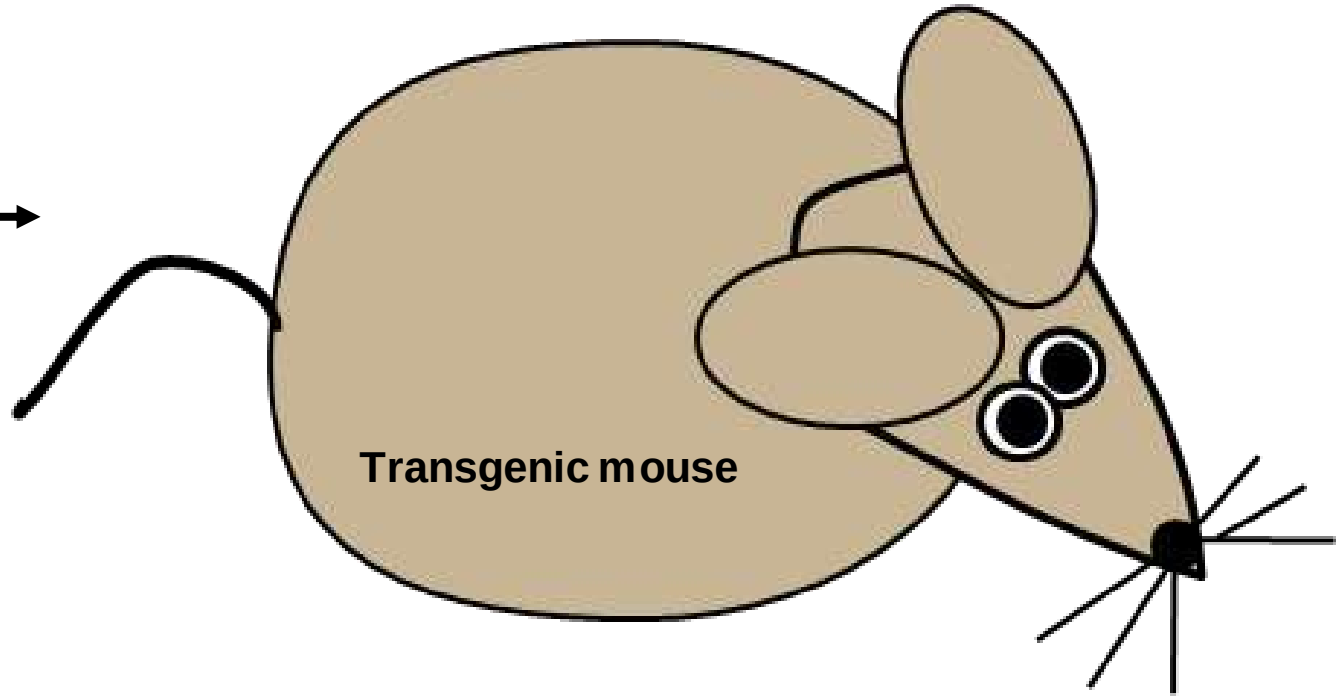
Ran et al., Nature Protocols, 2013

Genome engineering of mice via CRISPR









Yang et al. Cell, 2013

Transgenic monkey

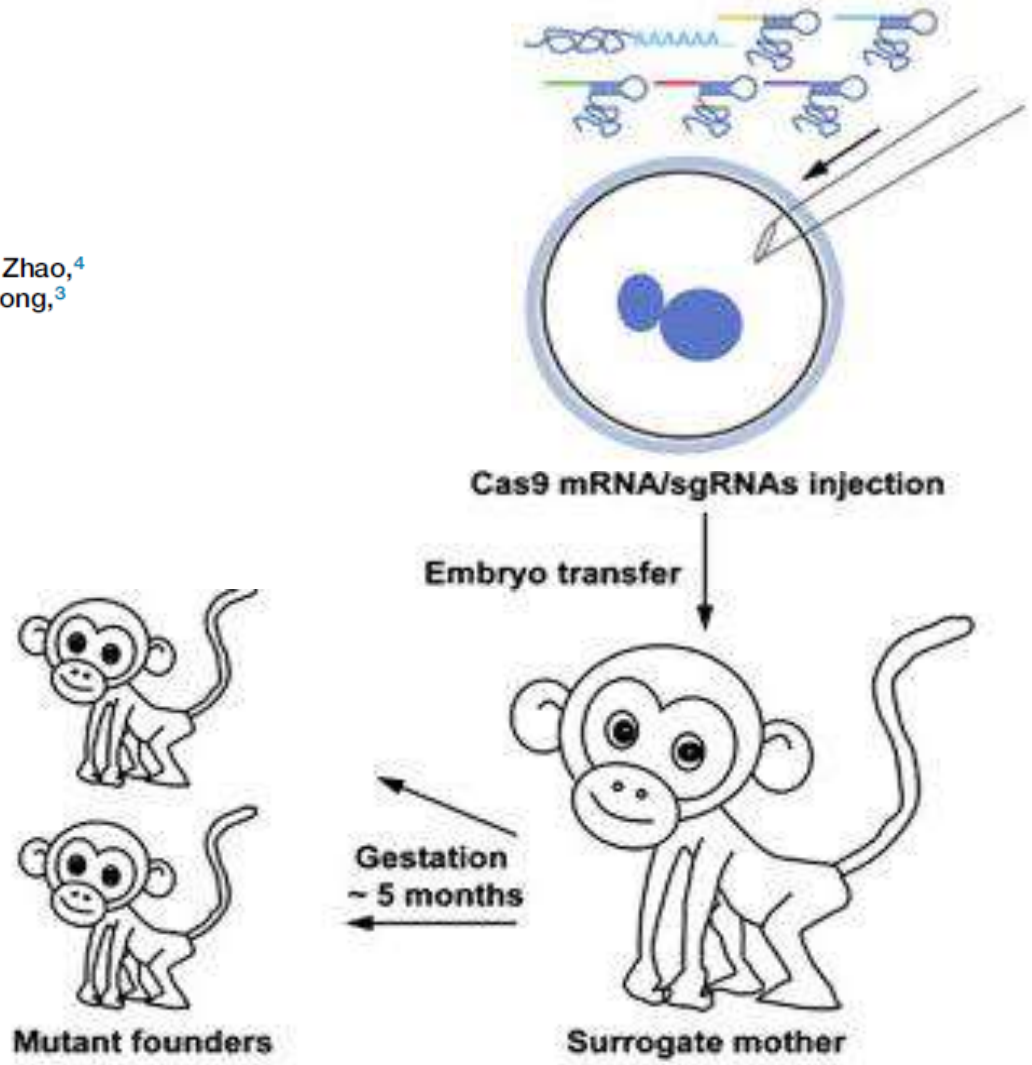
Cell

Generation of Gene-Modified Cynomolgus Monkey via Cas9/RNA-Mediated Gene Targeting in One-Cell Embryos

Yuyu Niu,[1,5,7] Bin Shen,[2,7] Yiqiang Cui,[3,7] Yongchang Chen,[1,5,7] Jianying Wang,[2] Lei Wang,[3] Yu Kang,[1,5] Xiaoyang Zhao,[4] Wei Si,[1,5] Wei Li,[4] Andy Peng Xiang,[6] Jiankui Zhou,[2] Xuejiang Guo,[3] Ye Bi,[3] Chenyang Si,[1,5] Bian Hu,[2] Guoying Dong,[3] Hong Wang,[1,5] Zuomin Zhou,[3] Tianqing Li,[1,5] Tao Tan,[1,5] Xiuqiong Pu,[1,5] Fang Wang,[1,5] Shaohui Ji,[1,5] Qi Zhou,[4] Xingxu Huang,[2,*] Weizhi Ji,[1,5,*] and Jiahao Sha[3,*]

Cell *156*, 836–843, February 13, 2014 ©2014 Elsevier Inc.

Ppar-g and Rag1



Genome engineering in human

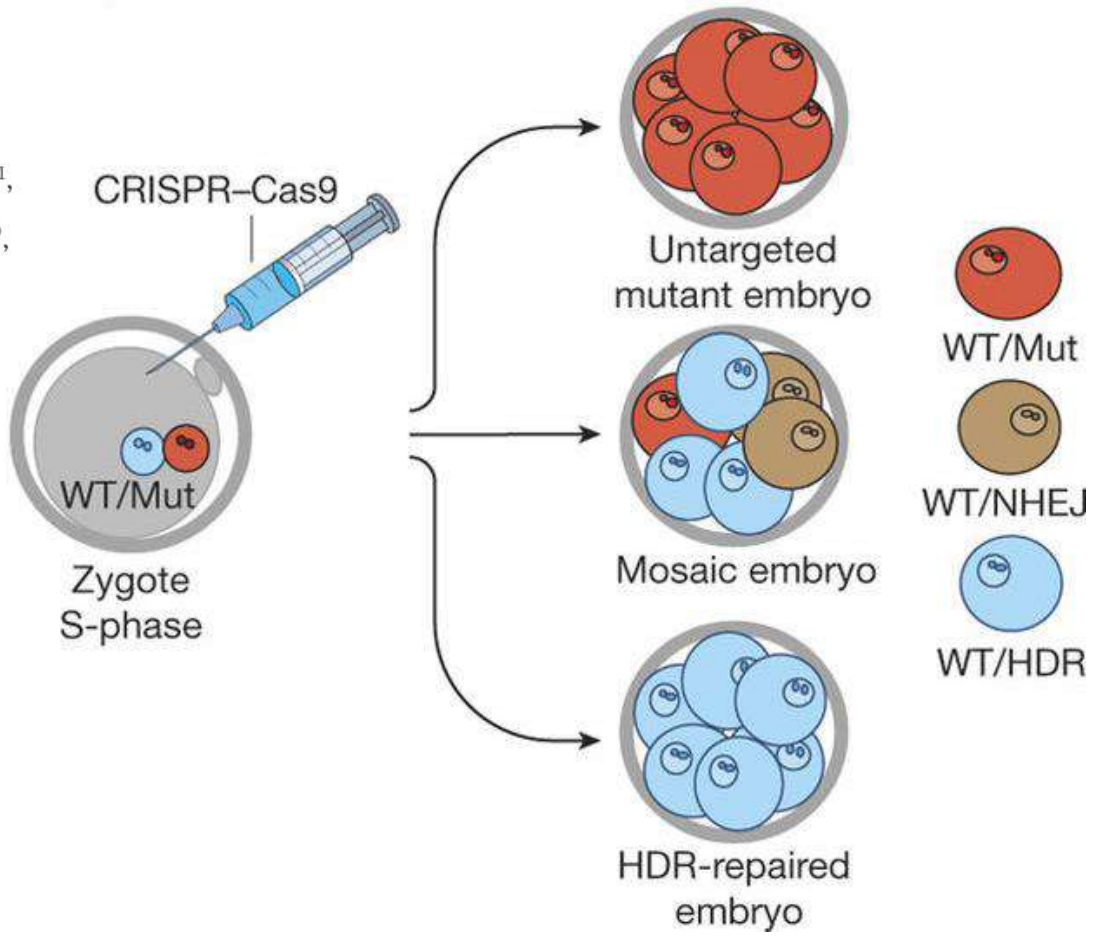
ARTICLE

doi:10.1038/nature23305

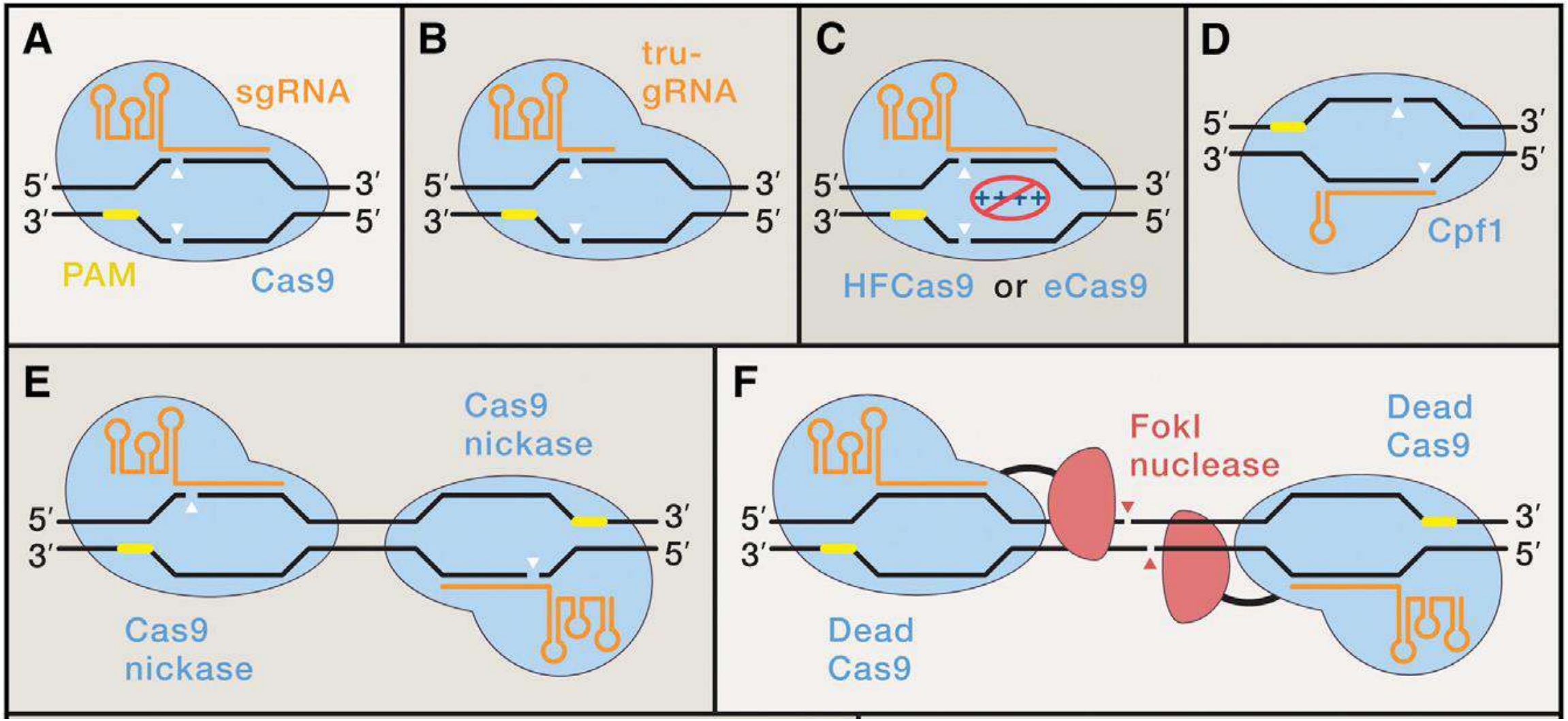
Correction of a pathogenic gene mutation in human embryos

Hong Ma[1]*, Nuria Marti-Gutierrez[1]*, Sang-Wook Park[2]*, Jun Wu[3]*, Yeonmi Lee[1], Keiichiro Suzuki[3], Amy Koski[1], Dongmei Ji[1], Tomonari Hayama[1], Riffat Ahmed[1], Hayley Darby[1], Crystal Van Dyken[1], Ying Li[1], Eunju Kang[1], A.-Reum Park[2], Daesik Kim[4], Sang-Tae Kim[2], Jianhui Gong[5,6,7,8], Ying Gu[5,6,7], Xun Xu[5,6,7], David Battaglia[1,9], Sacha A. Krieg[9], David M. Lee[9], Diana H. Wu[9], Don P. Wolf[1], Stephen B. Heitner[10], Juan Carlos Izpisua Belmonte[3]§, Paula Amato[1,9]§, Jin-Soo Kim[2,4]§, Sanjiv Kaul[10]§ & Shoukhrat Mitalipov[1,10]§

24 AUGUST 2017 | VOL 548 | NATURE | 413

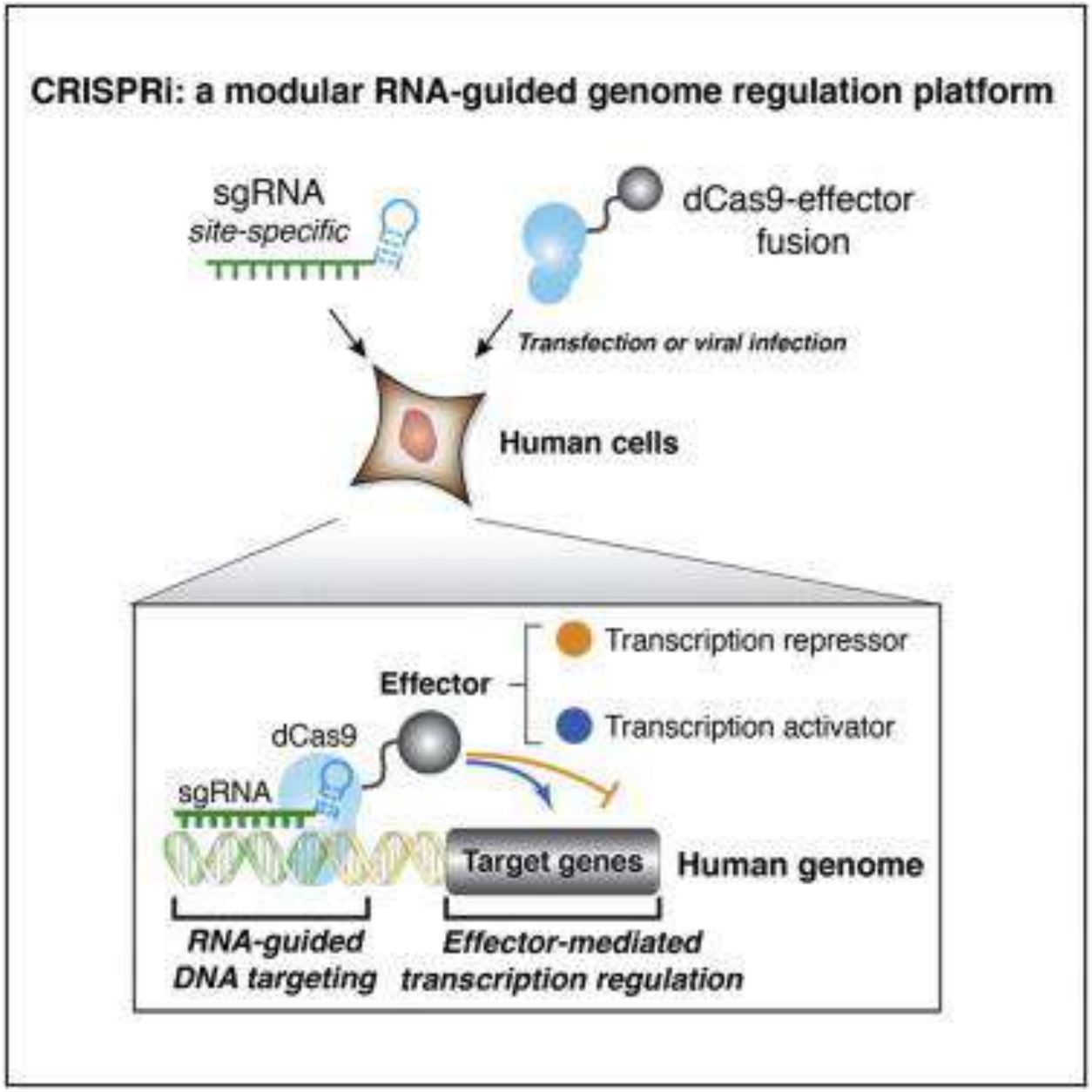


Strategies for improving specificity of CRISPR/Cas



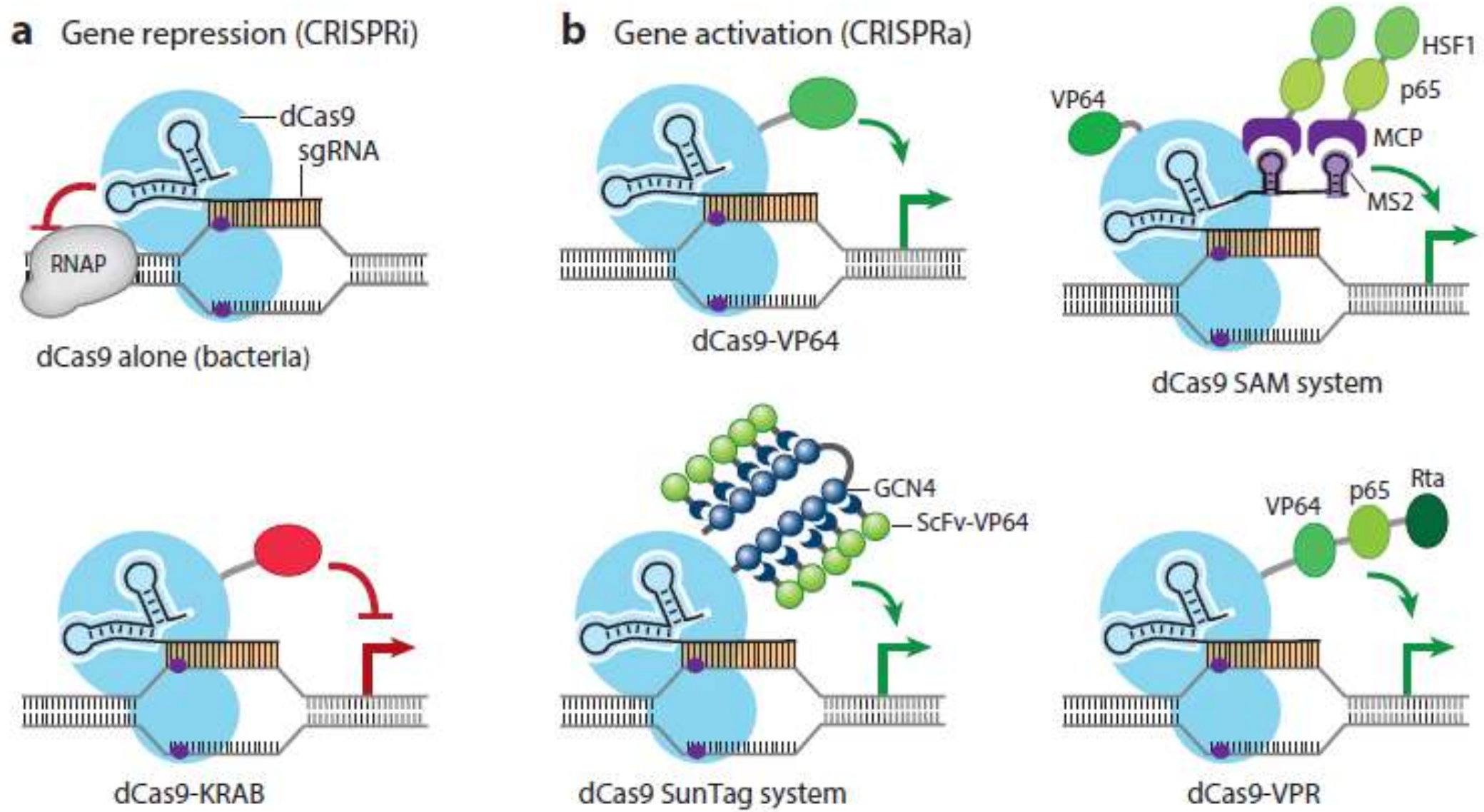
Komor et al., Cell, 2016

Genome regulation by CRISPR/dCAS9



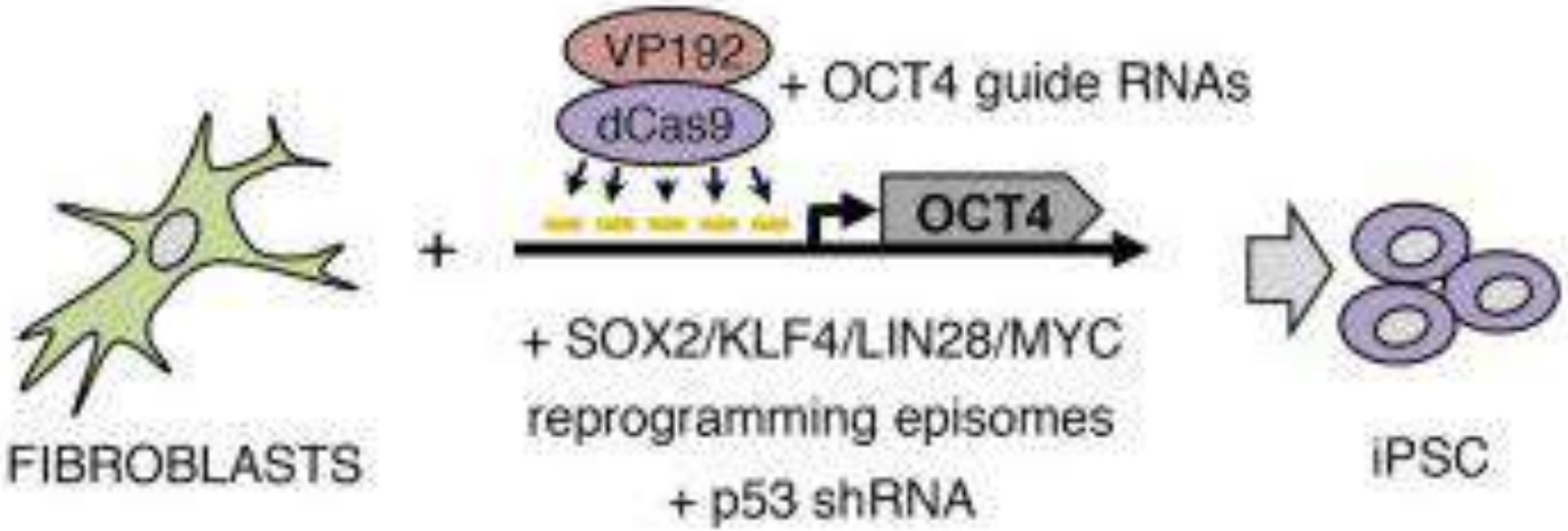
Gilbert et al., Cell, 2013
Lei et al., Cell, 2013

Genome regulation by CRISPR/dCAS9



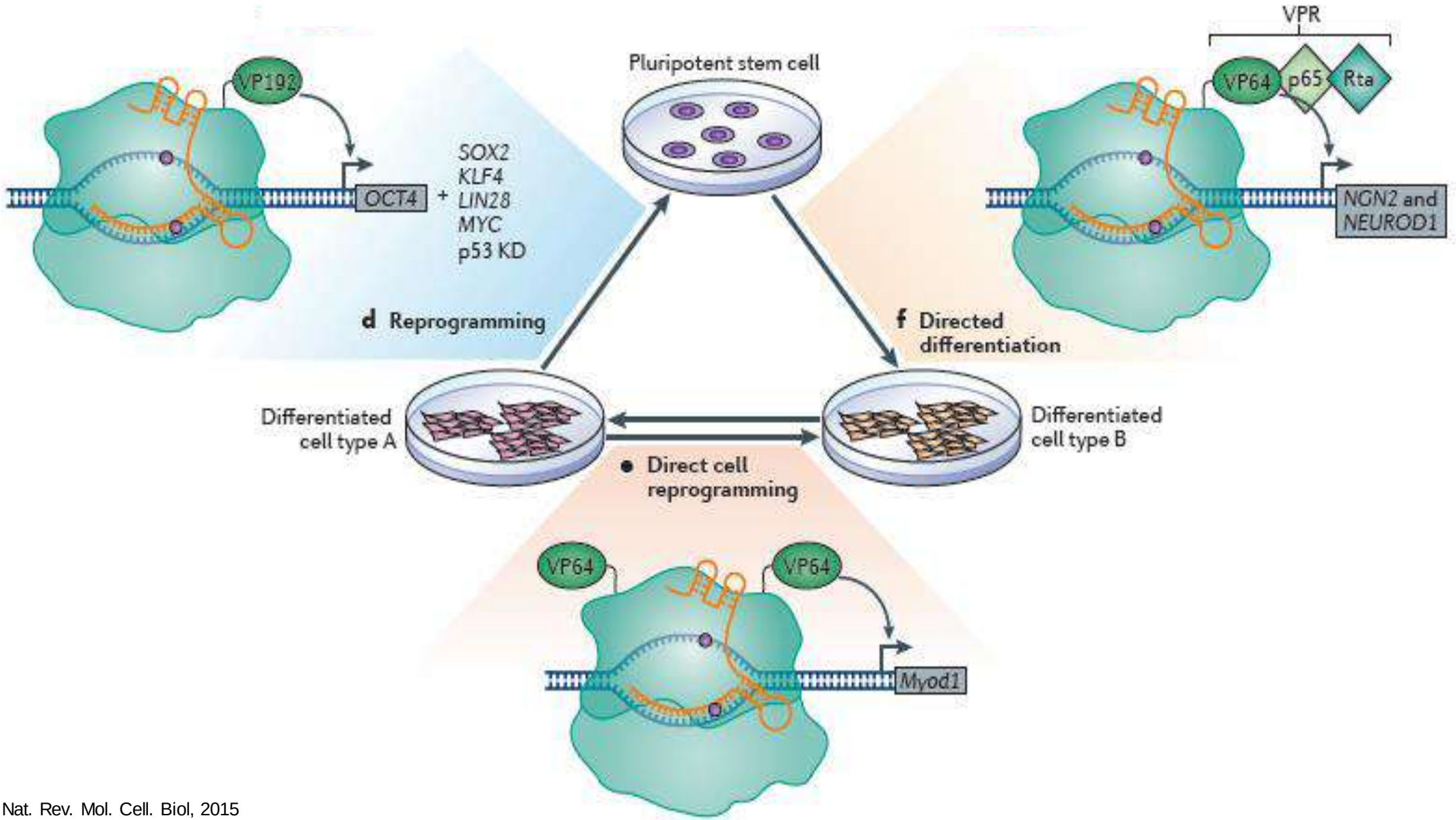
Wang et al., Annual review of biochemistry 2016

iPSC generation by CRISPR/dCAS9



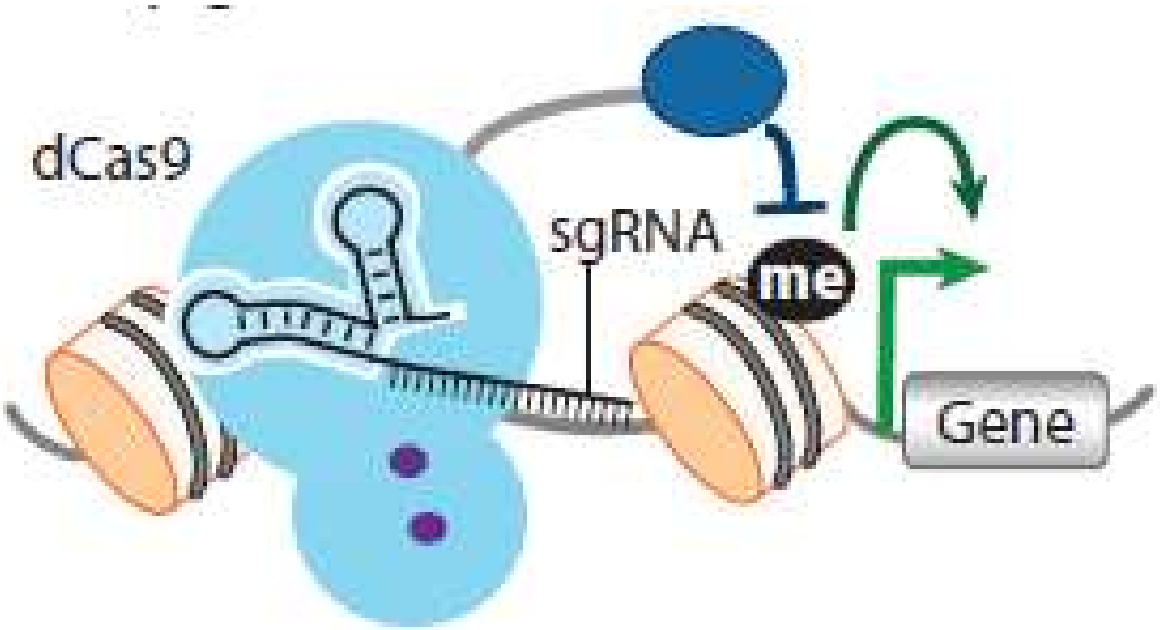
Balboa et al., Stem Cell Reports, 2015

Reprogramming, directed differentiation & trans-differentiation

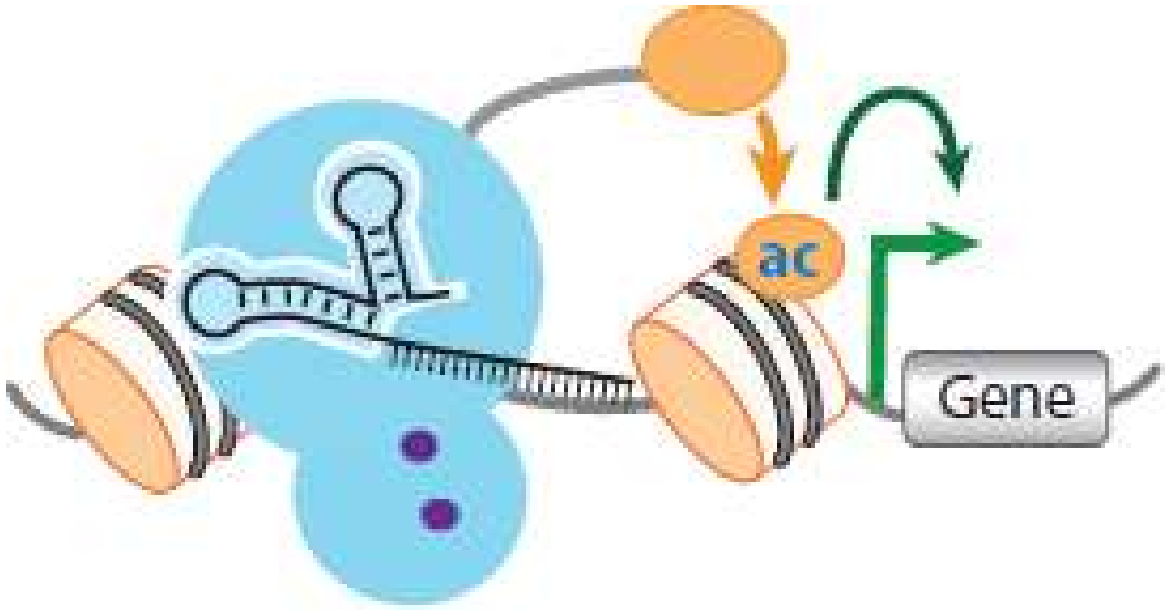


Dominguez et al., Nat. Rev. Mol. Cell. Biol, 2015

Epigenetic modifications by CRISPR/dCAS9



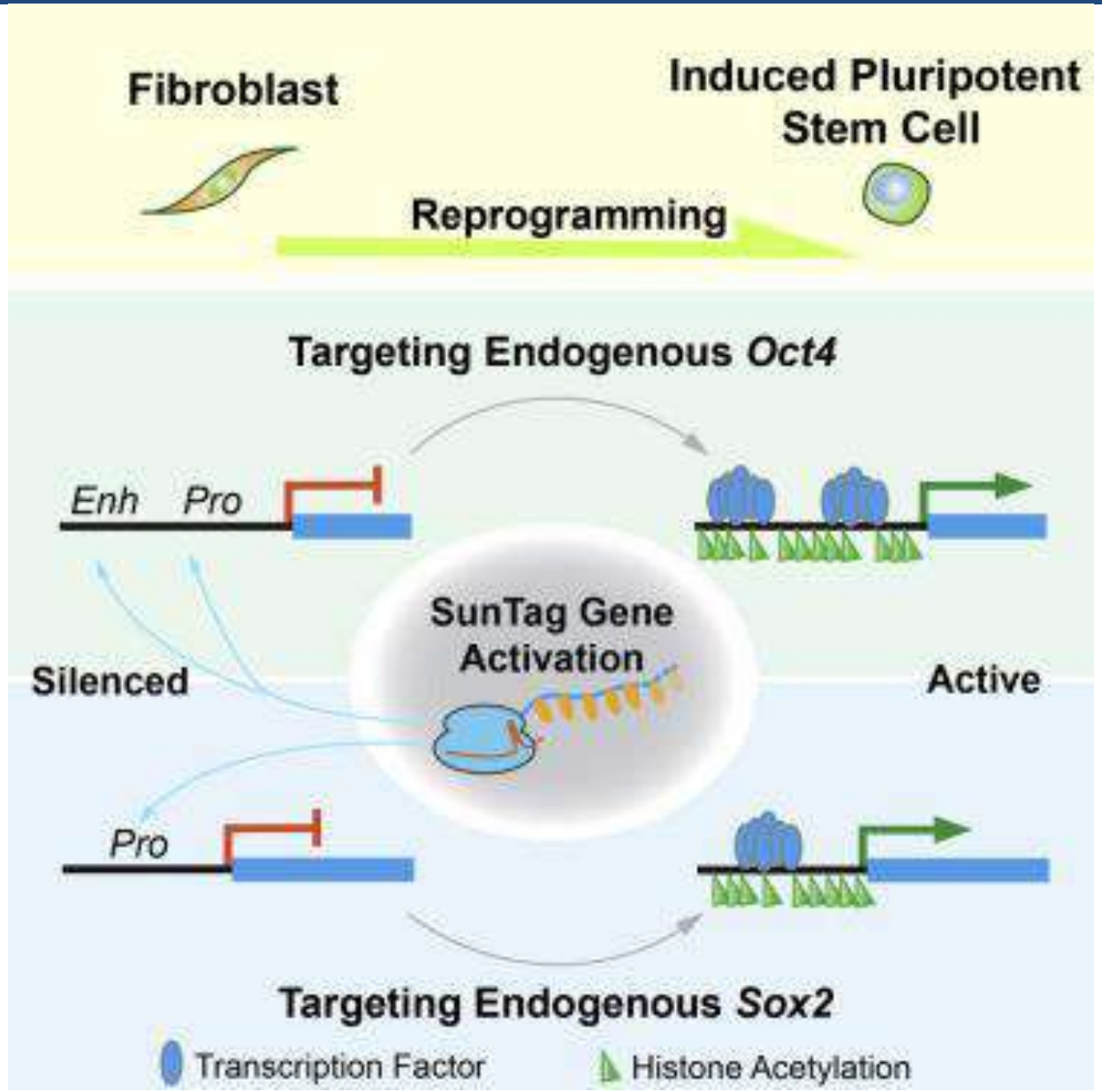
dCas9-LSD1



dCas9-p300 core

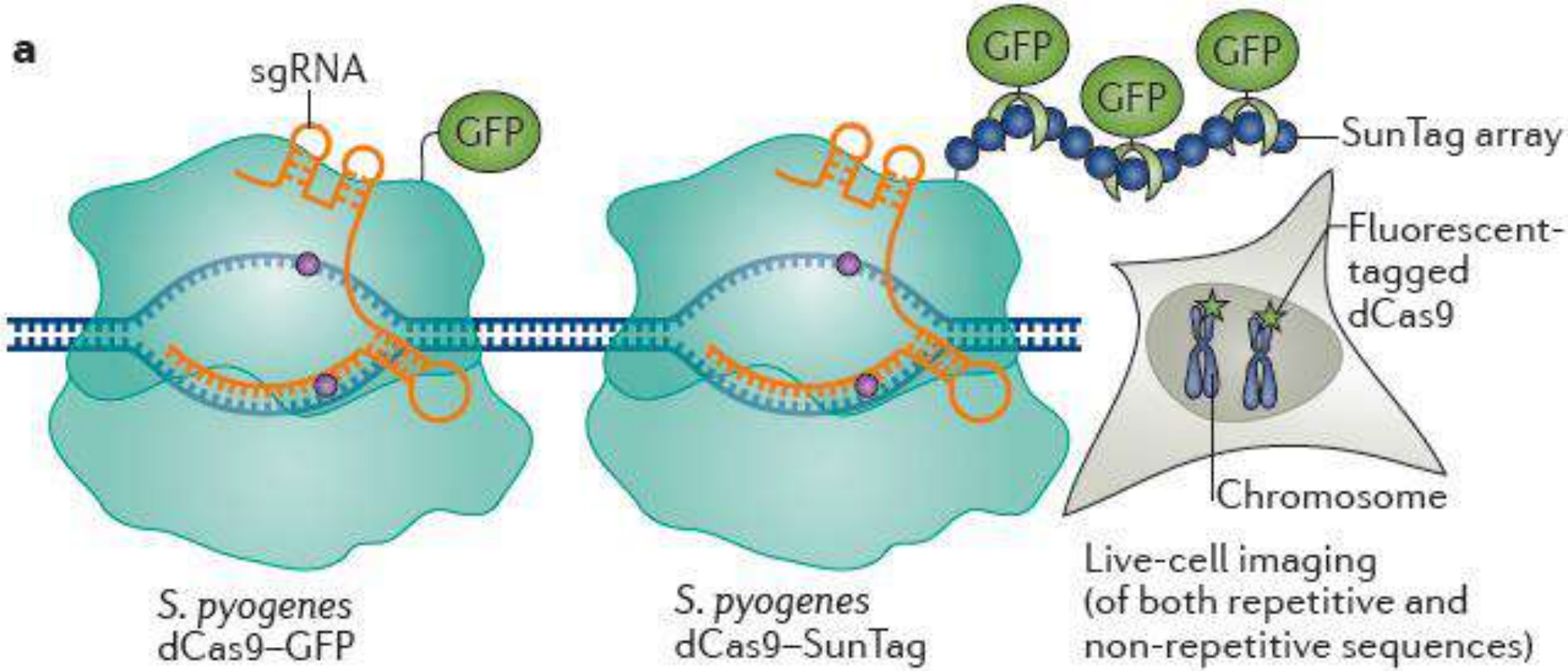
Wang et al., Annual review of biochemistry 2016

iPSC generation by CRISPR/dCAS9



Liu et al., Cell Stem Cells, 2018

Live cell imaging by CRISPR/dCAS9



Dominguez et al., Nat. Rev. Mol. Cell. Biol, 2015

Disease Modeling (Cas9 knock in mouse)

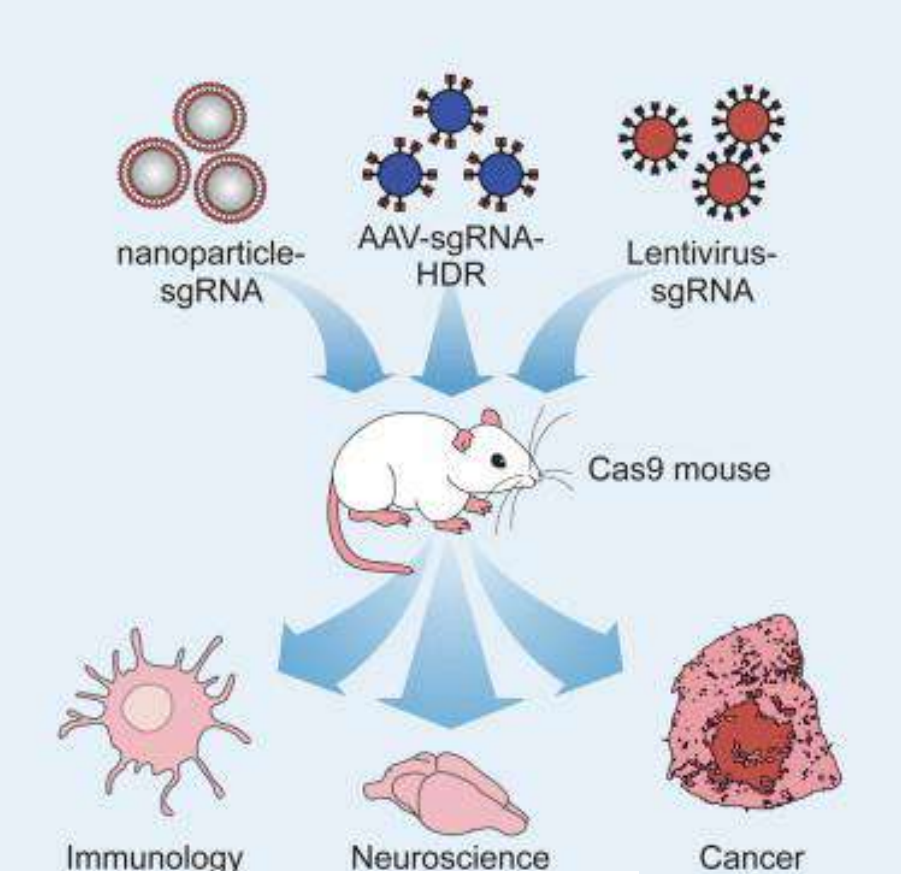
Cell

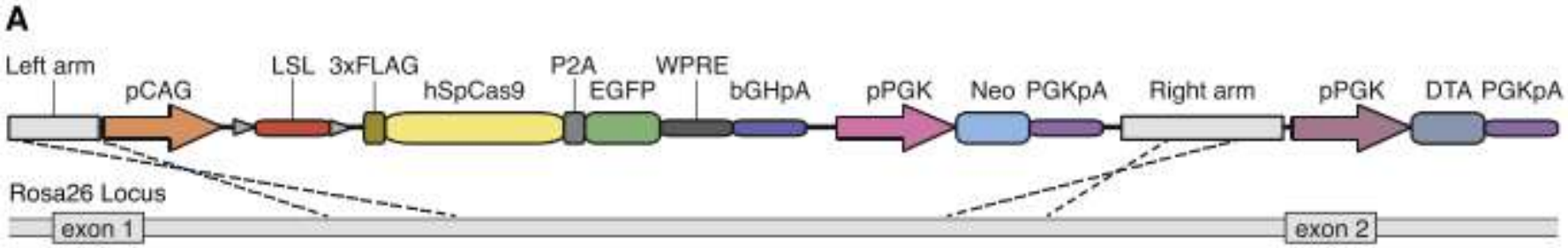
Resource

CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling

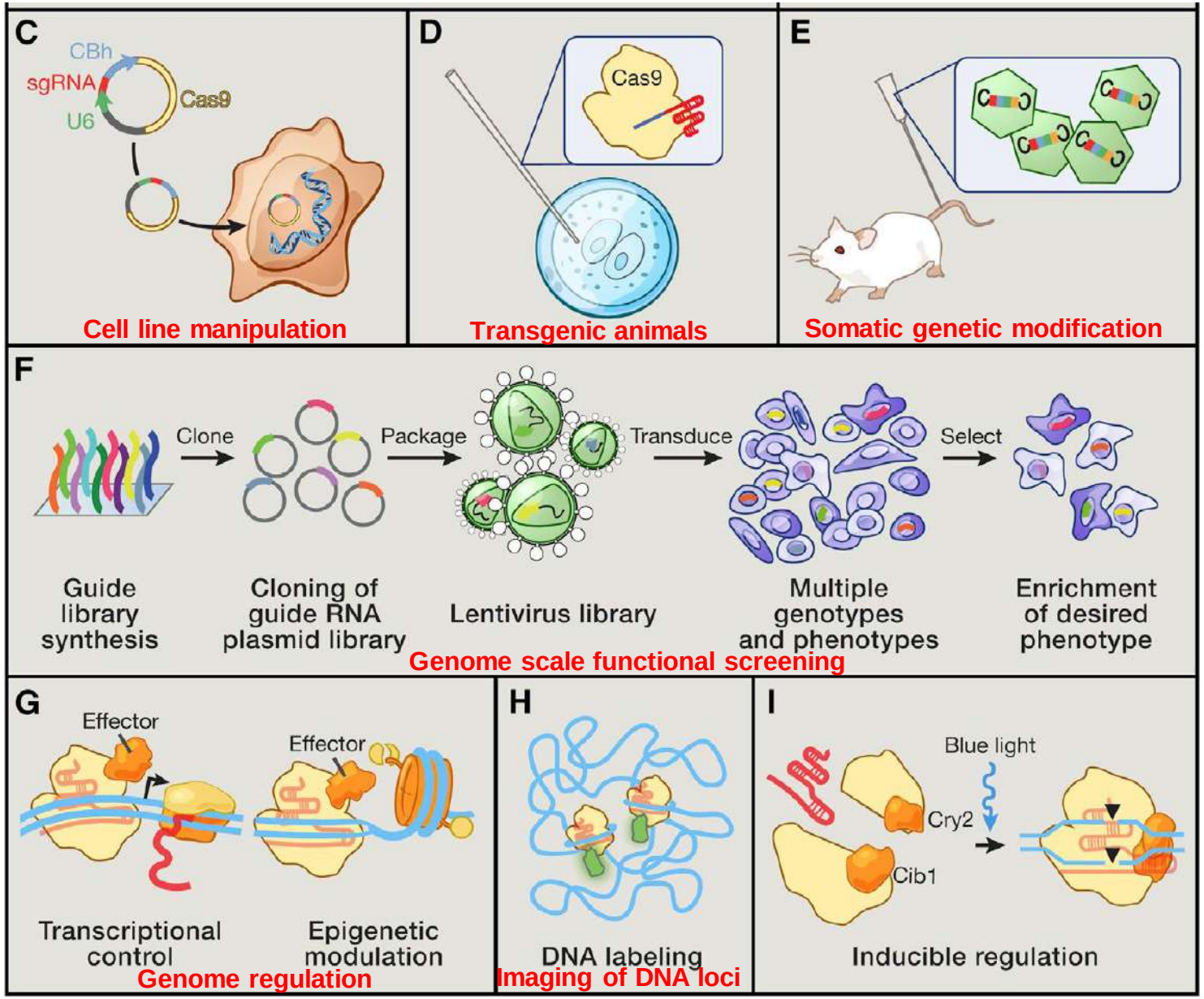
Randall J. Platt,[1,2,3,4,14] Sidi Chen,[5,6,14] Yang Zhou,[2,3] Michael J. Yim,[1,2,3,4] Lukasz Swiech,[1,2,3,4] Hannah R. Kempton,[1,2,4] James E. Dahlman,[5,7,8] Oren Parnas,[1] Thomas M. Eisenhaure,[1,11] Marko Jovanovic,[1] Daniel B. Graham,[1] Siddharth Jhunjhunwala,[5] Matthias Heidenreich,[1,2,3,4] Ramnik J. Xavier,[1] Robert Langer,[5,7,8,9] Daniel G. Anderson,[5,7,8,9] Nir Hacohen,[1,10,11] Aviv Regev,[1,6,12] Guoping Feng,[1,2,3,13] Phillip A. Sharp,[5,6,*] and Feng Zhang[1,2,3,4,13,*]

[1]Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA
[2]McGovern Institute for Brain Research
[3]Department of Brain and Cognitive Sciences
[4]Department of Biological Engineering
[5]David H. Koch Institute for Integrative Cancer Research
[6]Department of Biology
[7]Harvard-MIT Division of Health Sciences and Technology
[8]Institute for Medical Engineering and Science
[9]Department of Chemical Engineering
Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[10]Harvard Medical School, Boston, MA 02115, USA
[11]Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Charlestown, MA 02129, USA
[12]Howard Hughes Medical Institute, Chevy Chase, MD 20815, USA
[13]Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA
[14]Co-first authors
*Correspondence: sharppa@mit.edu (P.A.S.), zhang@broadinstitute.org (F.Z.)
http://dx.doi.org/10.1016/j.cell.2014.09.014



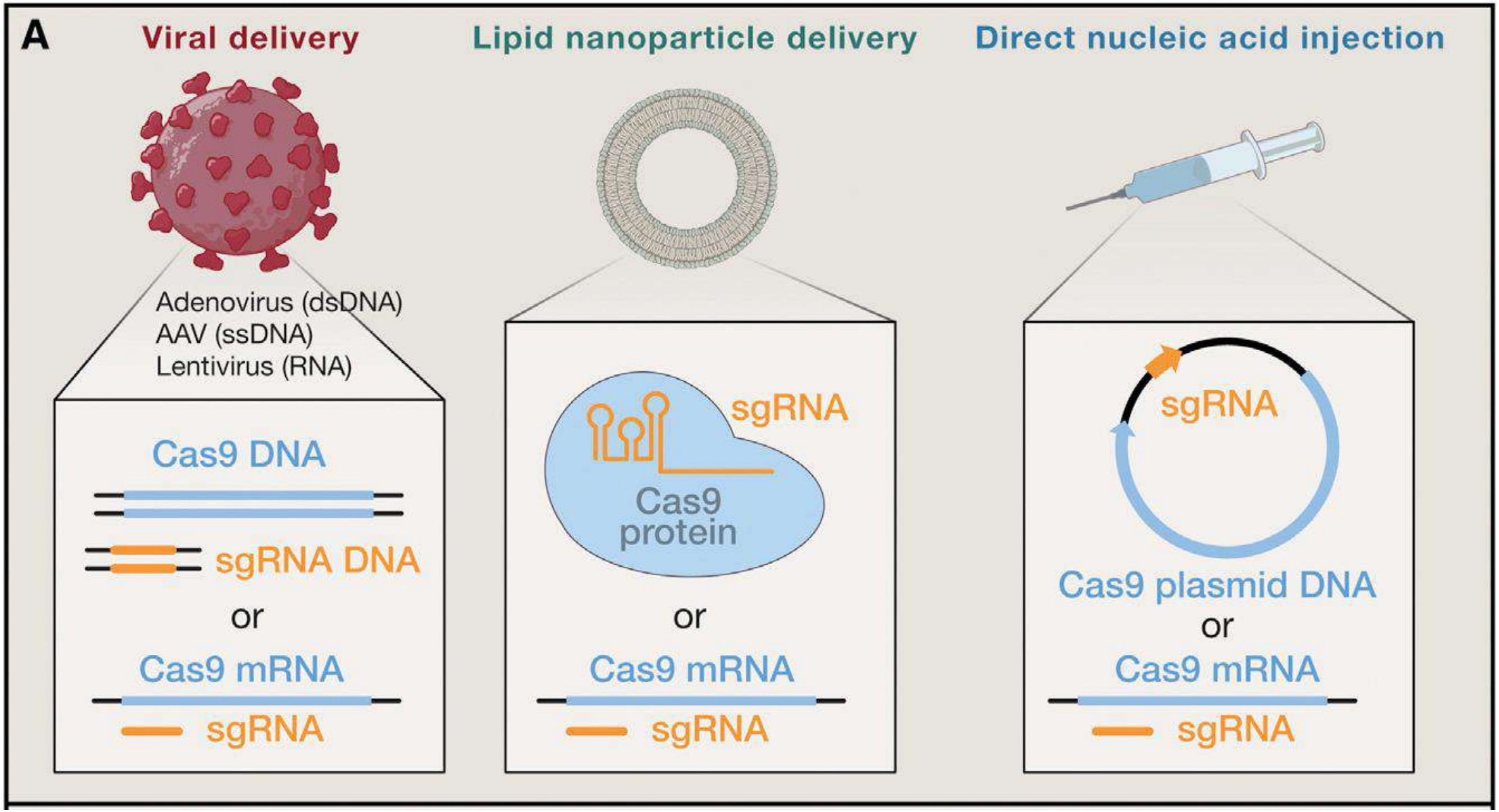


Applications of CRISPR/CAS



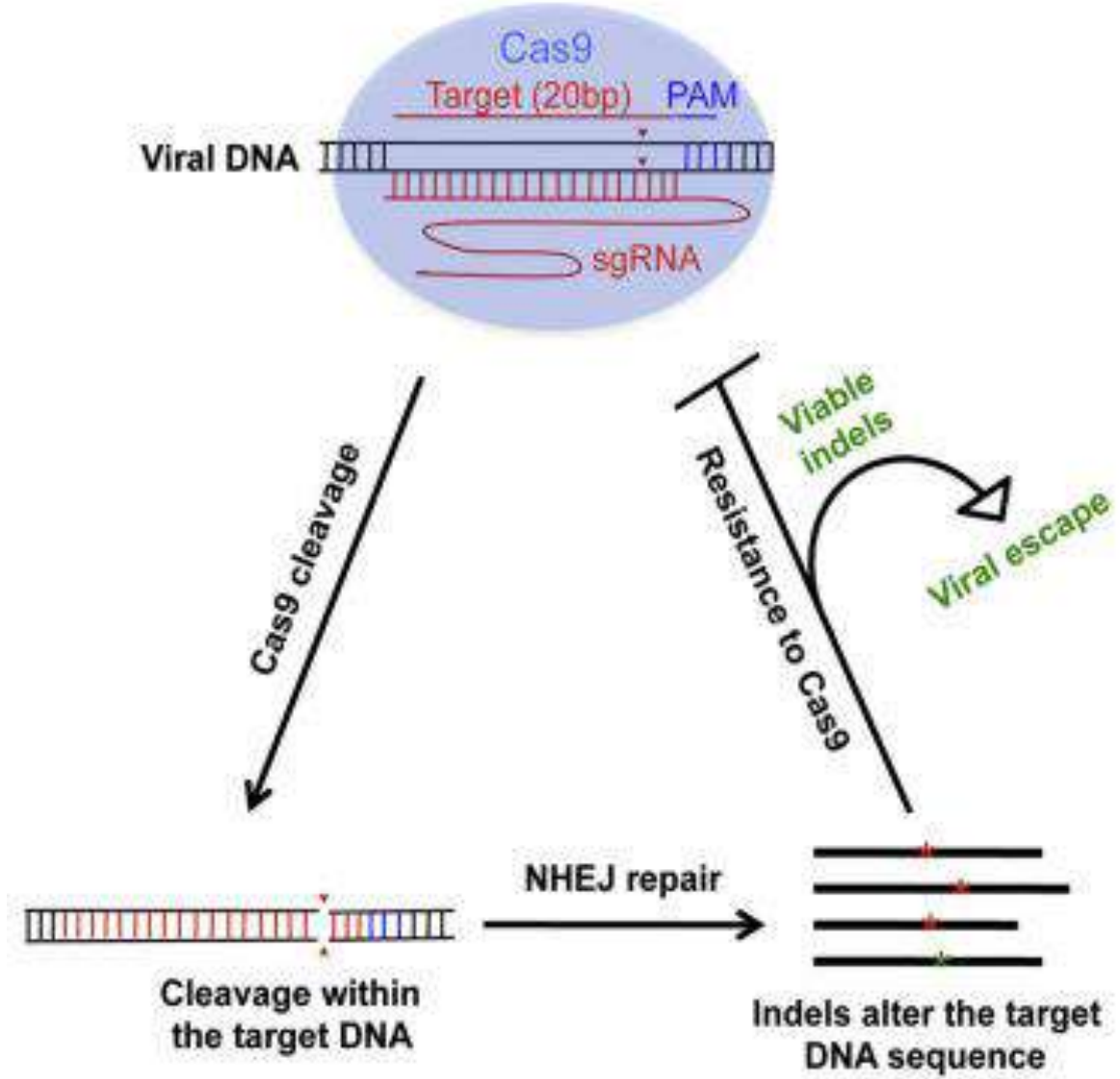
Hsu et al., Cell, 2014

Strategies for in vivo delivery of CRISPR/Cas



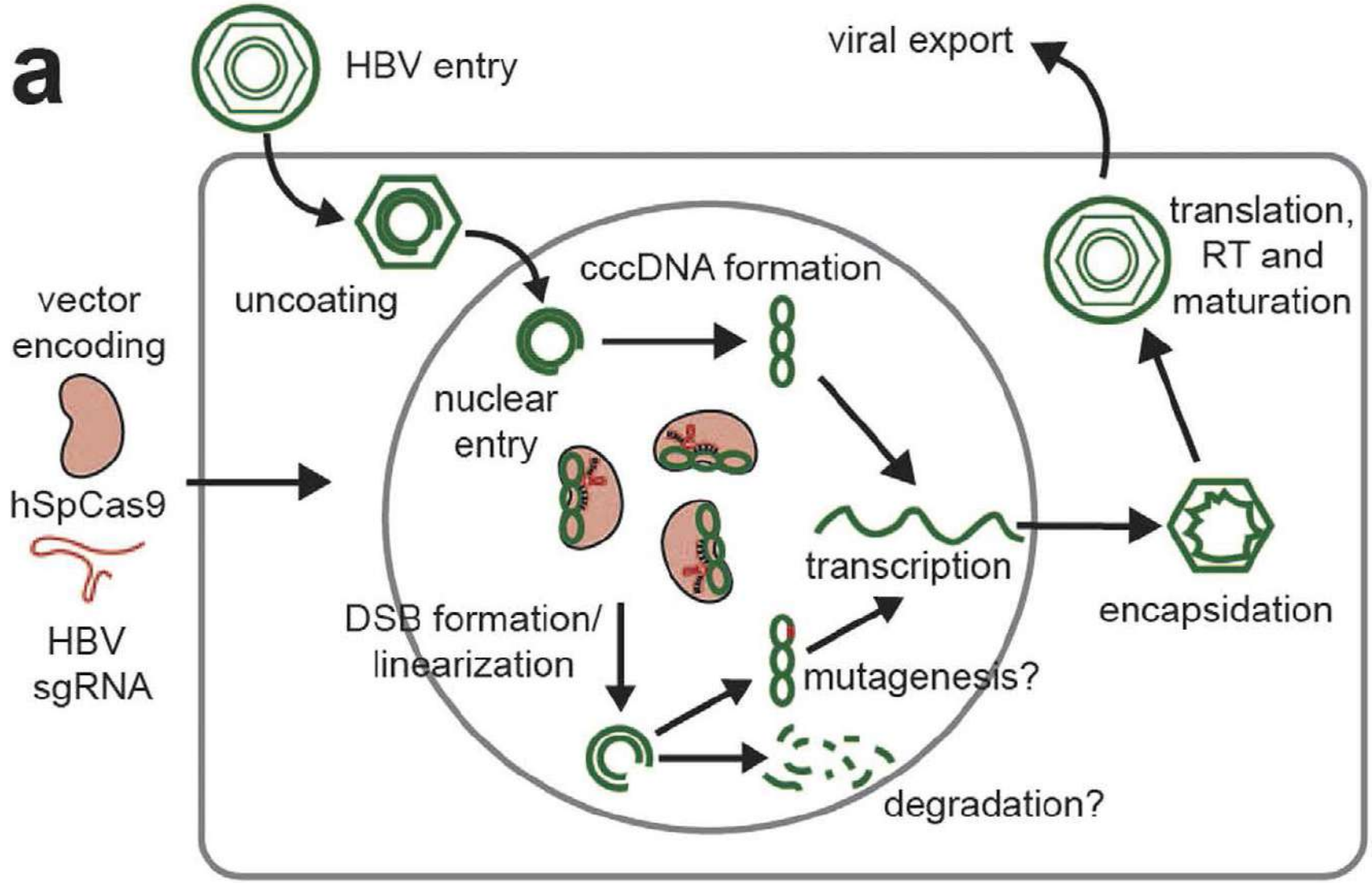
Komor et al., Cell, 2016

CRISPR/CAS9 for targeting HIV



Wang et al., Cell reports, 2016

CRISPR/CAS9 for targeting HBV cccDNA



Ramanan et al., Scientific Reports, 2015

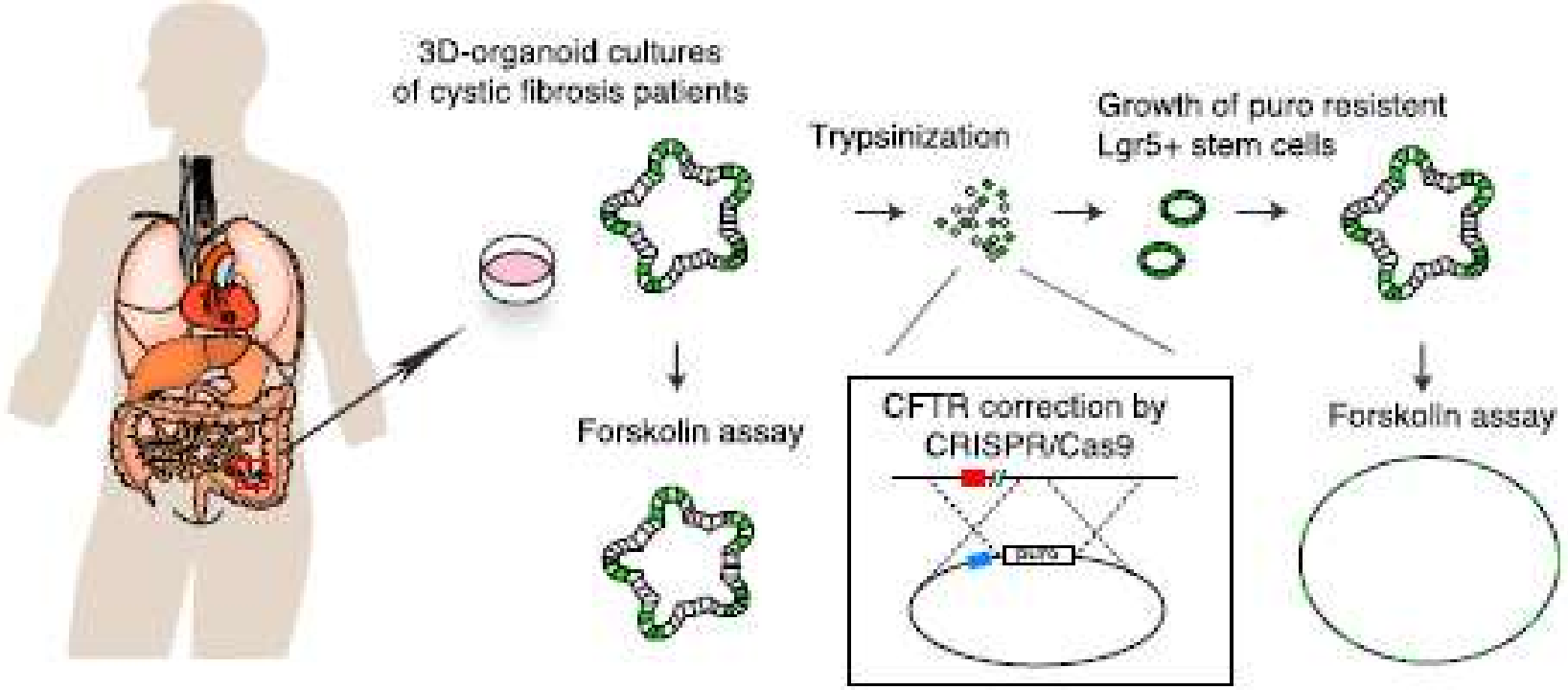
Genetic manipulation of intestinal organoids

Cell Stem Cell
Brief Report

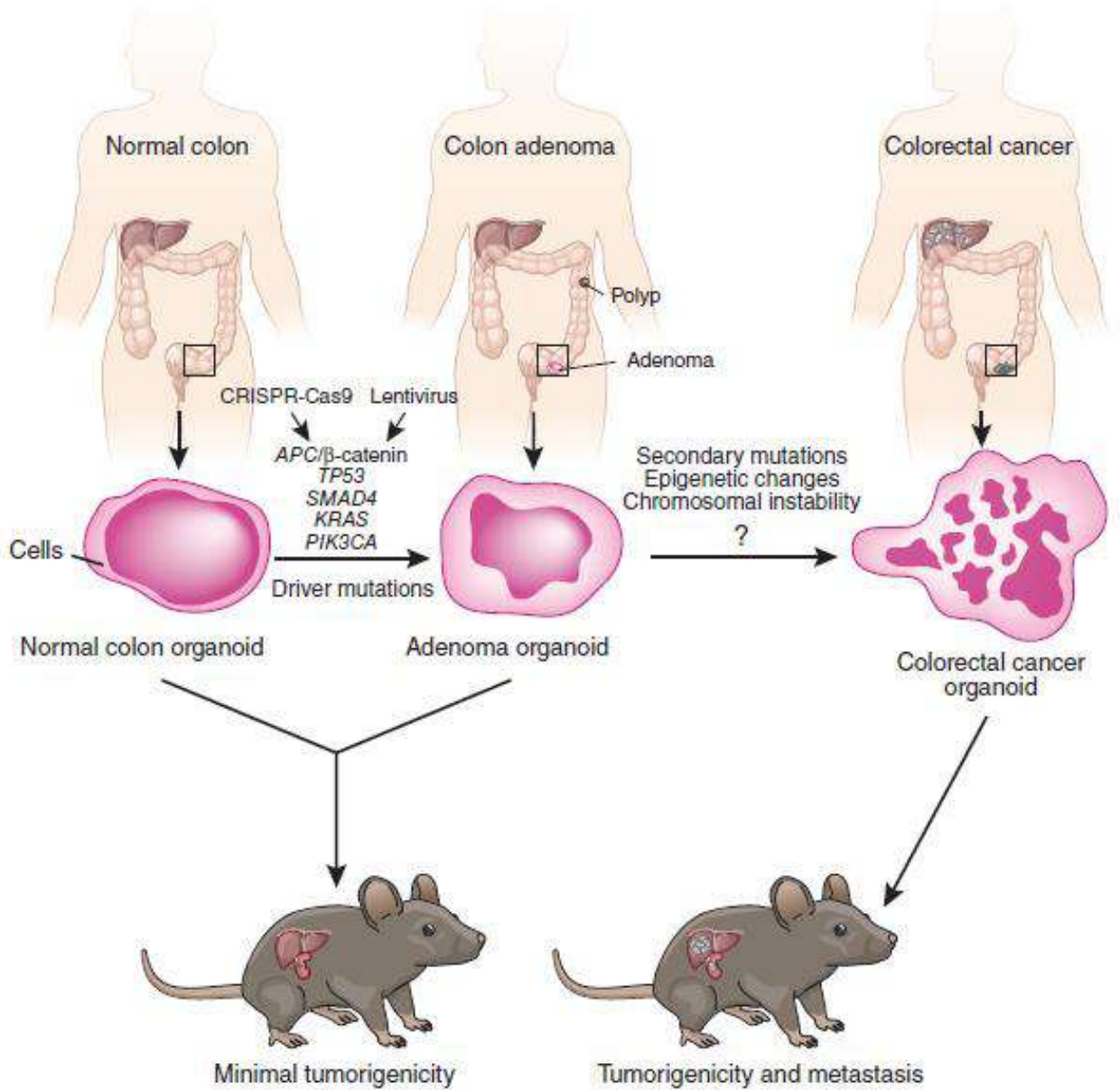
Cell Stem Cell *13*, 653–658, December 5, 2013 ©2013 Elsevier Inc.

Functional Repair of CFTR by CRISPR/Cas9 in Intestinal Stem Cell Organoids of Cystic Fibrosis Patients

Gerald Schwank,[1,2,7] Bon-Kyoung Koo,[1,2,7,8] Valentina Sasselli,[1,2] Johanna F. Dekkers,[3,4] Inha Heo,[1,2] Turan Demircan,[1] Nobuo Sasaki,[1,2] Sander Boymans,[1] Edwin Cuppen,[1,6] Cornelis K. van der Ent,[3] Edward E.S. Nieuwenhuis,[5] Jeffrey M. Beekman,[5,6] and Hans Clevers[1,2,*]
[1]Hubrecht Institute/KNAW



Colorectal cancer modeling



Salahudeen & Kuo., Nature Medicine, 2015

Applications of CRISPR/Cas diagnostic

1 Prepare sample, release and protect nucleic acids

Method: HUDSON

2 Amplify DNA and RNA

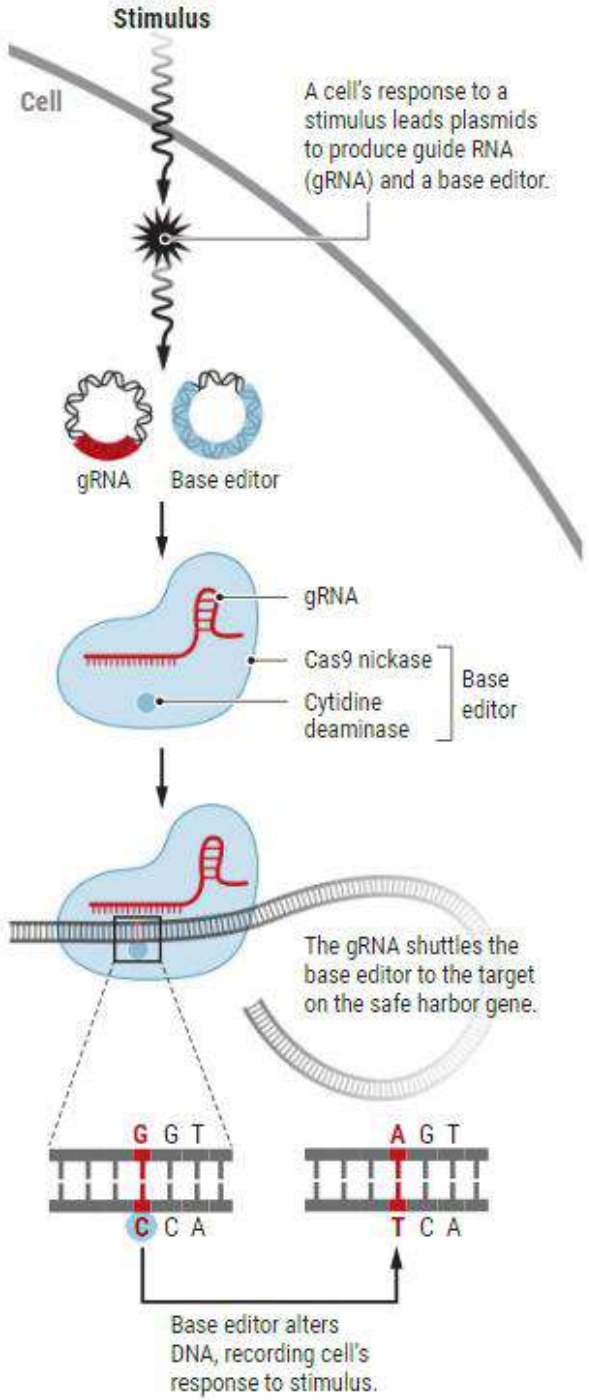
Method: RPA

3 Accurately detect target and amplify signal

Method: SHERLOCK, SHERLOCKv2, and DETECTR

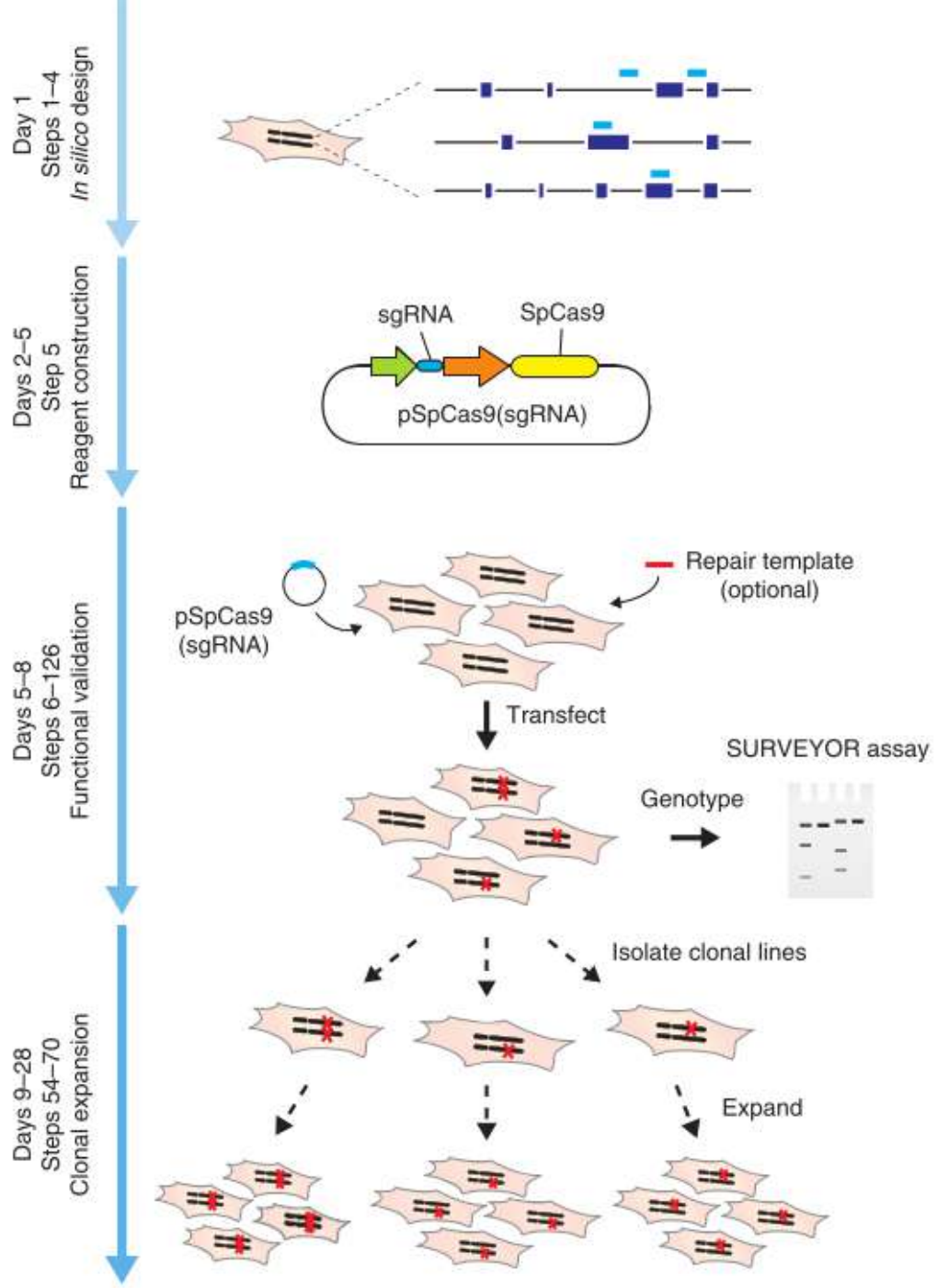
Molecular recorders (CAMERA)

- This tool can record exposure to light, antibiotics, and viral infection or document internal molecular events



Tang et al. Science 2018

Experimental Process



Ran et al., (2013) Nature Protocols

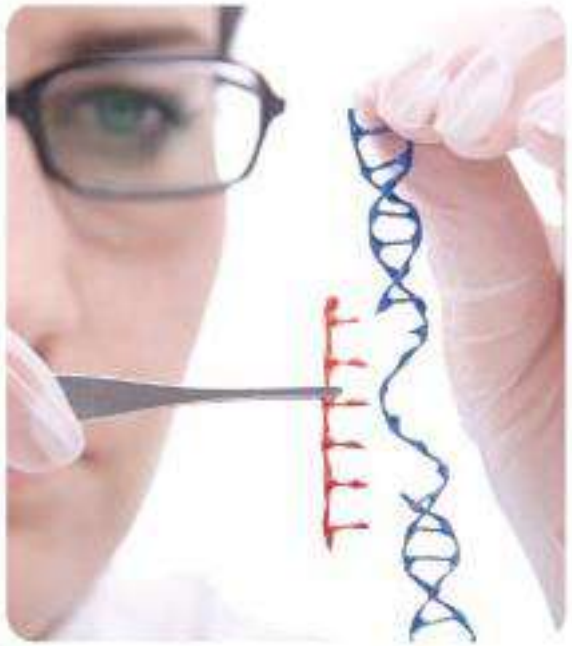
CRISPR tools

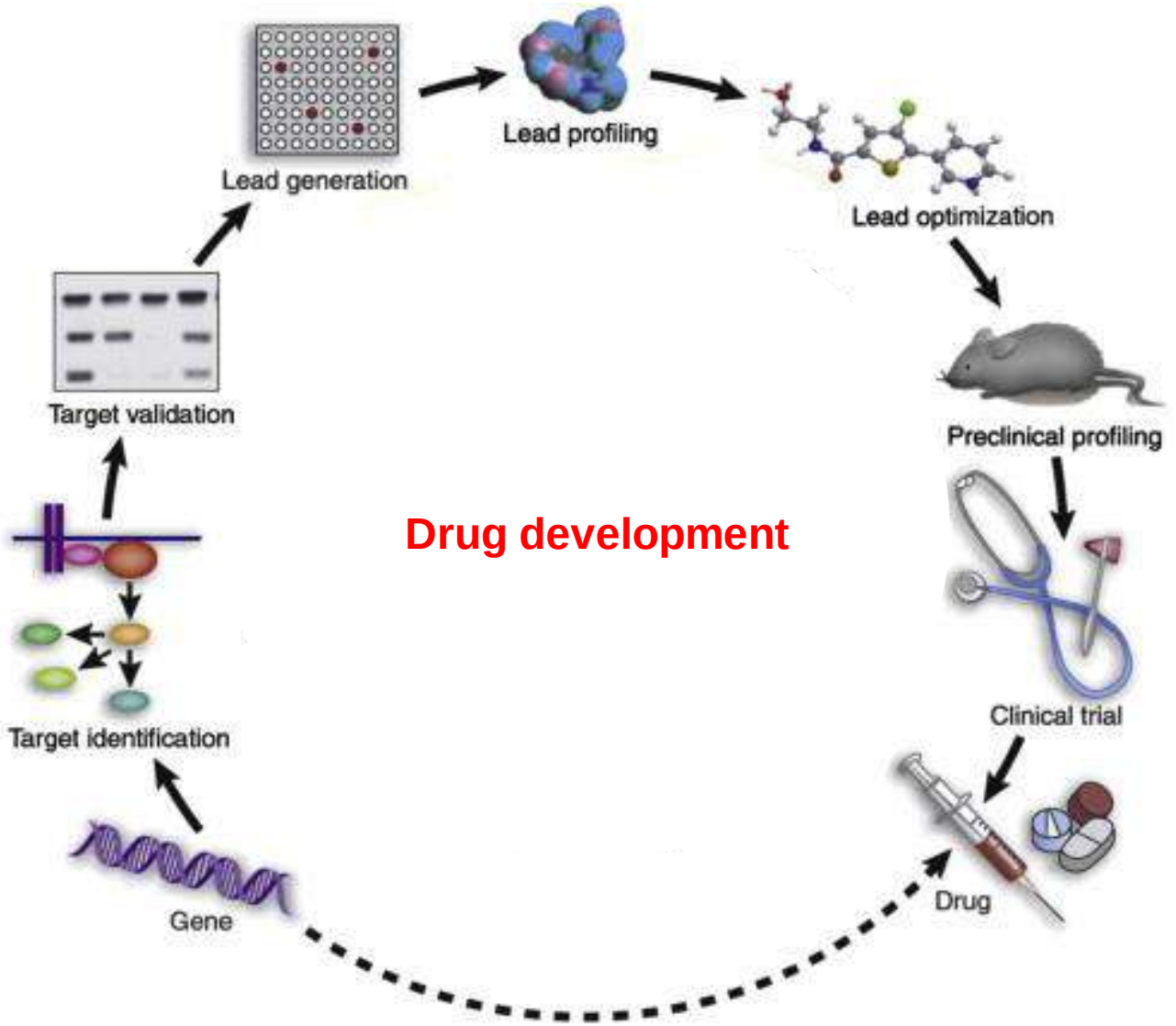
Tool Name	Provider	Searches whole genome for targets	Returns all targets of genome	Seed span and location can be defined	Maximum number of mismatches supported	Predicts gRNA activity	Available Protospacer adjacent motif (PAM) sequences	Annotation is reported	gRNA suggestion or scoring	External Link	References
Benchling CRISPR gRNA Design	Benchling	Yes	Yes	Yes	4	Yes	User customizable	Yes	Yes	Webserver	-
Breaking-Cas	Spanish National Center for Biotechnology	Yes (over 1000 genomes)	Yes	Yes (by weights)	4	No	User customizable	Yes	Yes	Webserver	[3]
Cas-OFFinder	Seoul National University	Yes	Yes	No	0-10	No	NGG, NRG, NNAGAAW, NNNNGMTT	No	Yes	Webserver Source code	[4]
CASTING	Caagle	Yes	Yes	No	3	No	NGG and NAG	No	Yes	Webserver	[5]
CCTop	University of Heidelberg	Yes	Yes	Partial	5 (0-5)	No	NGG, NRG, NNGRRT, NNNNGATT, NNAGAAW, NAAAAC	Yes	Yes	Webserver	[6]
CHOPCHOP	Harvard University	Yes	Yes	Partial	0, 2	No	NGG, NNAGAA, NNNNGANN	No	Yes	Webserver	[7]
CHOPCHOP v2	University of Bergen	Yes	Yes	Yes	3 (0-3)	Yes	User customizable	Yes	Yes	Webserver Source code	[8]
COD	Dayong Guo	No	No	No	0, 3, 5, 8	No	NGG and NAG	No	Yes	Webserver	-
CRISPOR	University of California, Santa Cruz TEFOR	Yes (over 200 genomes)	Yes	No	4	Yes	NGG, NGA, NGCG, NNAGAA, NGGNG, NNGRRT, NNNRRT, NNNNGMTT, NNNNACA, TTTN	Yes	Yes	Webserver Source code	[9]
CRISPR Design Tool	Horizon Discovery	Yes (over 30 species)	Yes	Yes	8 (gaps or mismatches)	Internally	NGG and NAG	mRNA exons, Links to UCSC genome browser annotations	No	CRISPR Design Tool CRISPR Specificity Tool	-
CRISPR Design	Zhang Lab, MIT	Yes	No	No	4	No	NGG and NAG	mRNA exons	Yes	Webserver	[10]
CRISPRdirect	Database Center for Life Science (DBCLS)	Yes (over 200 species)	Yes	No	Any number	No	NNN	Yes	Yes	Webserver	[11]
CRISPR gRNA Design Tool	DNA2.0	Yes	Yes	No	0-10	No	NGG, NAG	Genbank annotations: Gene, misc_RNA, ncRNA, CDS, exon	Yes	Webserver	-
CRISPR LifePipe	Life and Soft	Yes	Yes	Yes	0-5	yes	NGG, NGA, NGCG, TTTN, NNGRRT	Yes	Yes	Webserver	-
CRISPRseek	Bioconductor	Yes	Yes	No	Any number	No	User customizable	mRNA exons	Yes	Source code	[12]
DESKGEN	Desktop Genetics	Yes	Yes	Yes	Any number	Yes	Fully user customizable	Yes	Yes	Webserver	[13]

Activate Windows
Go to PC settings to activate Windows.

https://en.wikipedia.org/wiki/CRISPR/Cas_Tools

Genome engineering applications





Basic research

Drug development

Disease modeling

Gene therapy

DELTA BIOTECHNOLOGY

Many Thanks

Majid Lotfinia

PhD in Pharmaceutical Biotechnology

Majid.lotfinia@gmail.com