CRISPR/Cas9: From Fundamental Research to Billion Dollar Technology

Casey Roos
Knowles Lab
Group Meeting
May 4, 2019

Off topic slide: The capybara

-Size 3-4 ft long, ~2 ft tall, 80-146 lbs,
-Herbivores, autocoprophagous.
-Teeth grow continuously.
-The live 8-10 years, but less than 4 in the wild
"favourite food of jaguar, puma, ocelot, eagle, and caiman.The capybara is also the preferred prey of the anaconda."

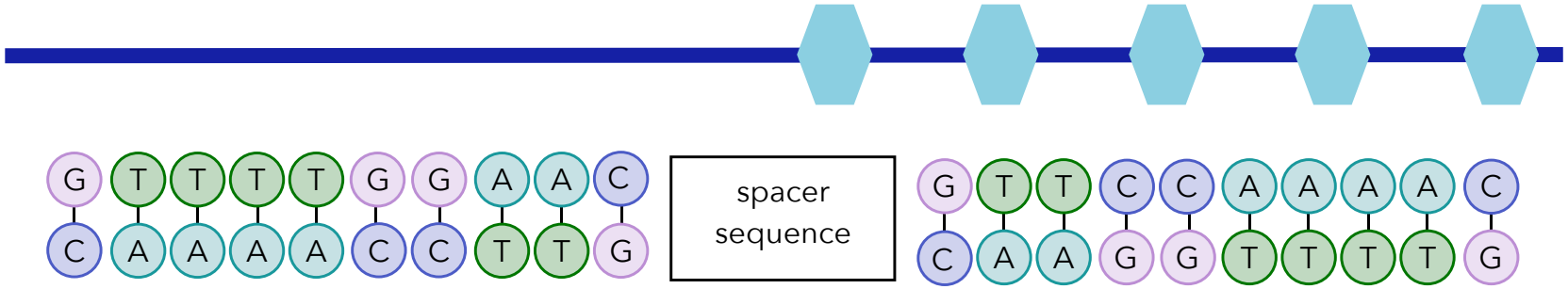
Class:	Mammalia
Order:	Rodentia
Family:	Caviidae
Genus:	*Hydrochoerus*
Species:	***H. hydrochaeris***

https://www.youtube.com/watch?v=XHYKTgzs9_0

Timeline of Crispr/Cas9 Discovery

1987
CRISPRs described

1987: CRISPRs described with the genome sequencing of *E. coli* genome



"clustered regularly interspaced short palindromic repeats"

J. Bacteriol. **1987,** *169,* 5429.
Nucleic Acids Res. **2002**, *30*, 482.
Microbiology, **2005**, *151*, 2551
J. Mol. Evol. **2005**, *60*, 174.
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Biol. Direct **2006**, *1*, 7.
Science **2007**, *135*, 1709.

Timeline of Crispr/Cas9 Discovery

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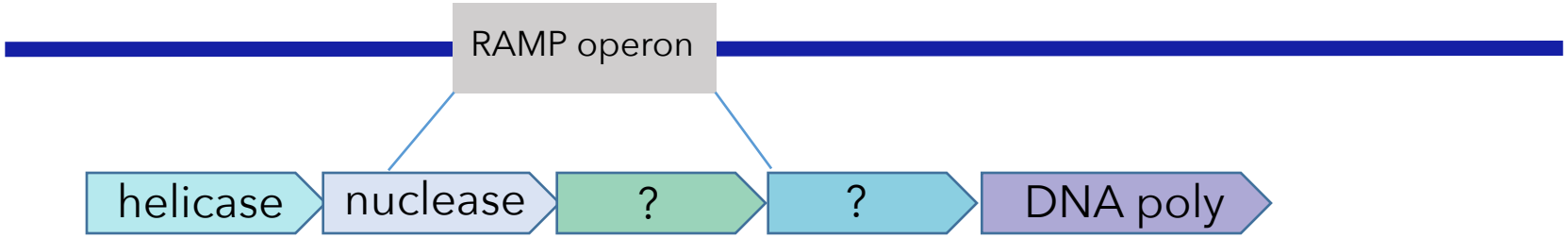
2002
Repair associated
mysterious proteins
(RAMPs)

2002: Koonin discovers clusters of 5 genes that are highly conserved in some Archaea and Bacteria.



Predicted functions of the proteins by sequence similarity to known proteins involved in DNA repair.

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2005: Nelsen realizes that these RAMPs are always near CRISPR loci

Suggests role of RAMPs in somehow modifying or maintaining CRISPR spacer sequences?

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2004-2005
Spacer sequences
are from viruses

2004-2005: Multiple groups realize spacer sequences derive from exogenous/viral DNA.

How and why is the cell incorporating viral
DNA into it's chromosome?

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RAMPs actually CRISPR associated

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Spacer sequences are from viruses

2006
Bacterial immunity hypothesis

2006: Koonin revises hypothesis about DNA repair. Actually a bacterial immune response.

Bacterial Immune Response?

J. Bacteriol. **1987,** *169,* 5429.
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Microbiology, **2005**, *151*, 2551
J. Mol. Evol. **2005**, *60*, 174.
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RAMPs actually CRISPR associated

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Spacer sequences are from viruses

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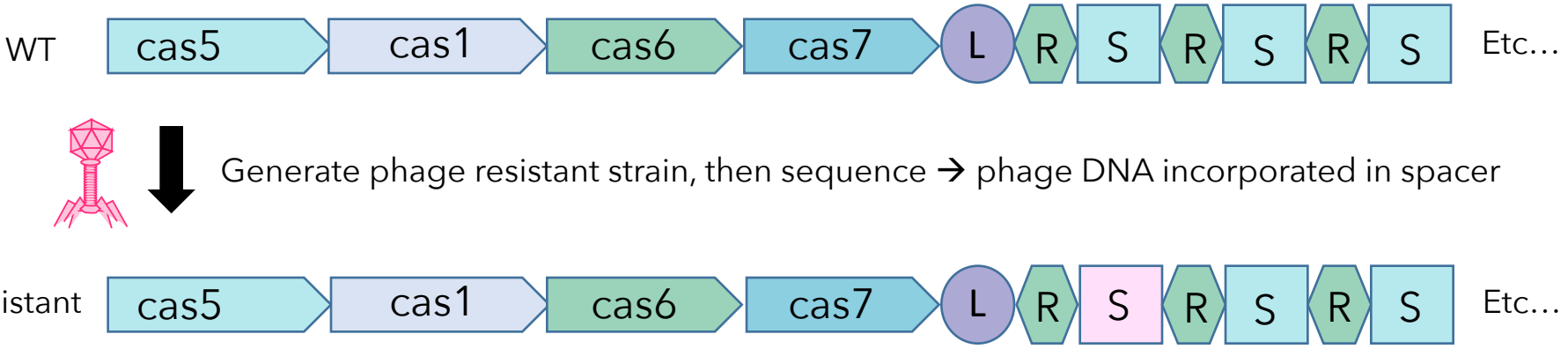
Bacterial immunity hypothesis

2007
Hypothesis verified

2007: Horvath confirms acquisition of foreign DNA correlates with phage resistance.



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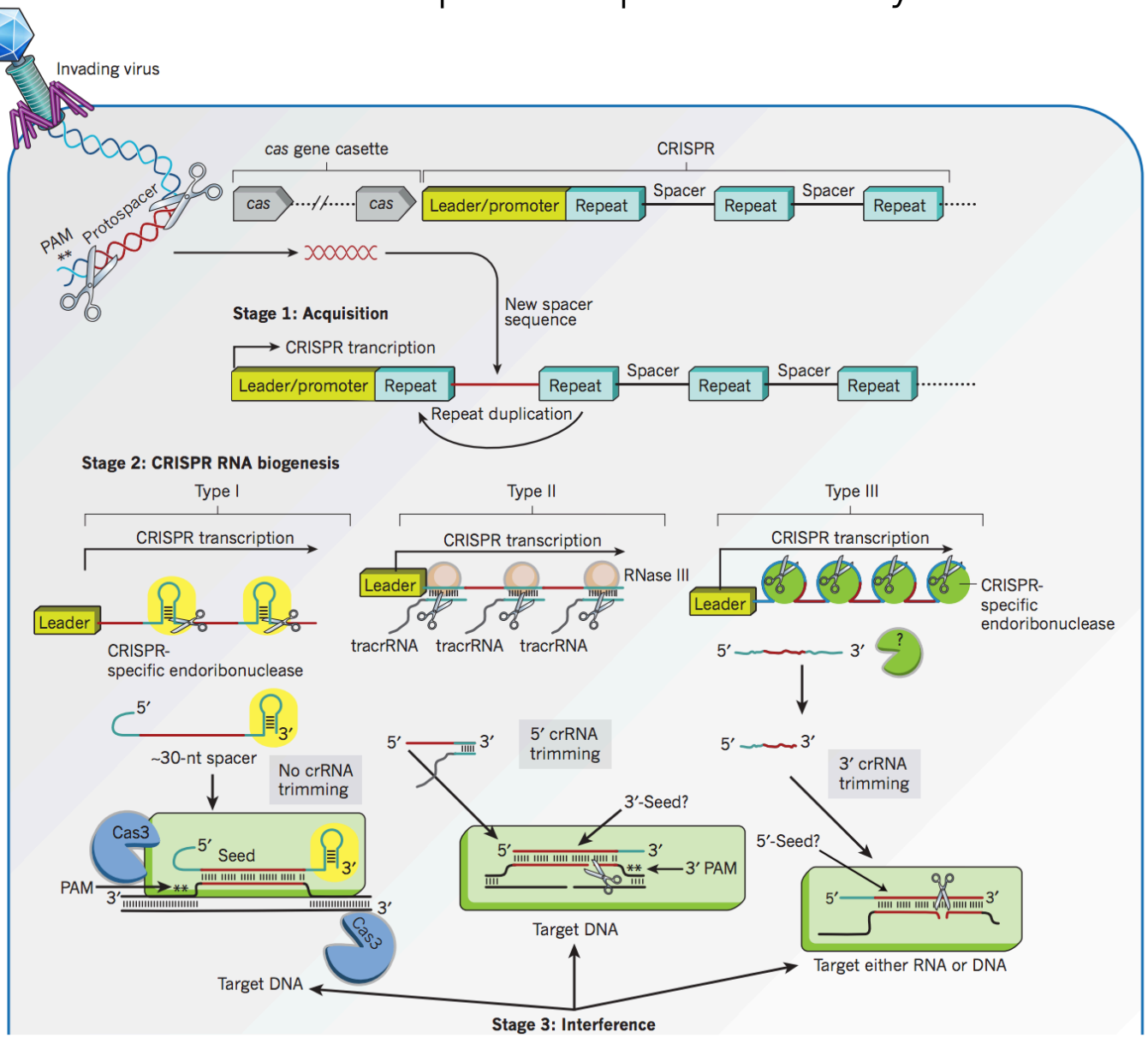
Steps for Acquired Immunity in Bacteria



1. Acquisition of foreign DNA

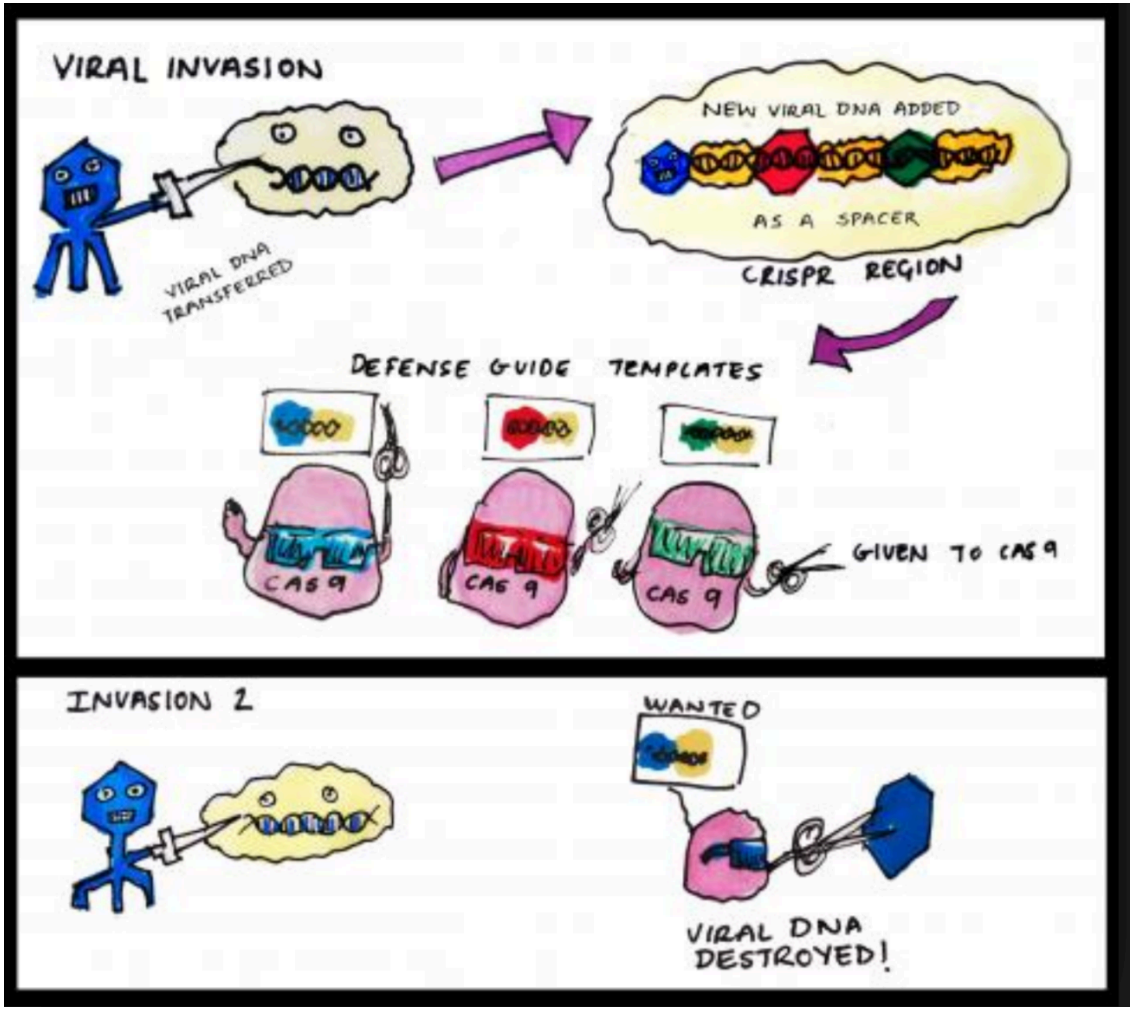
2. Transcribing and processing CRISPR information

3. Destroying DNA from future viral attacks

Steps 2,3 can proceed through 3 different mechanisms depending on the type of CRISPR system

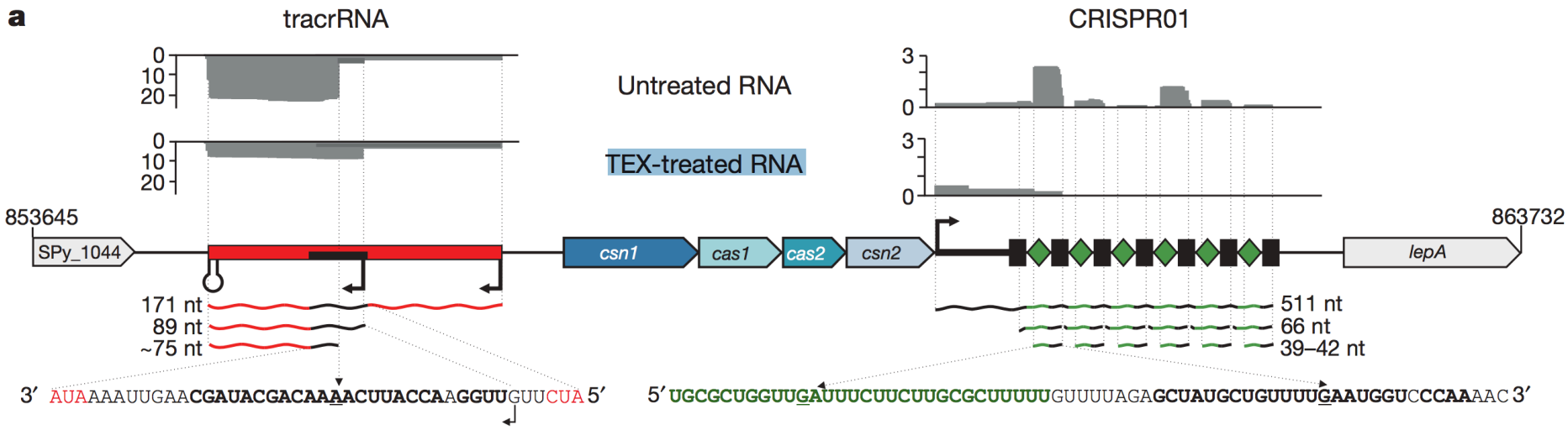
Cas9 technologies are based on the Type II system

Steps for Acquired Immunity in Bacteria



https://www.eurostemcell.org/crispr-changing-gene-editing-landscape

Steps for Acquired Immunity in Bacteria



Key observations:

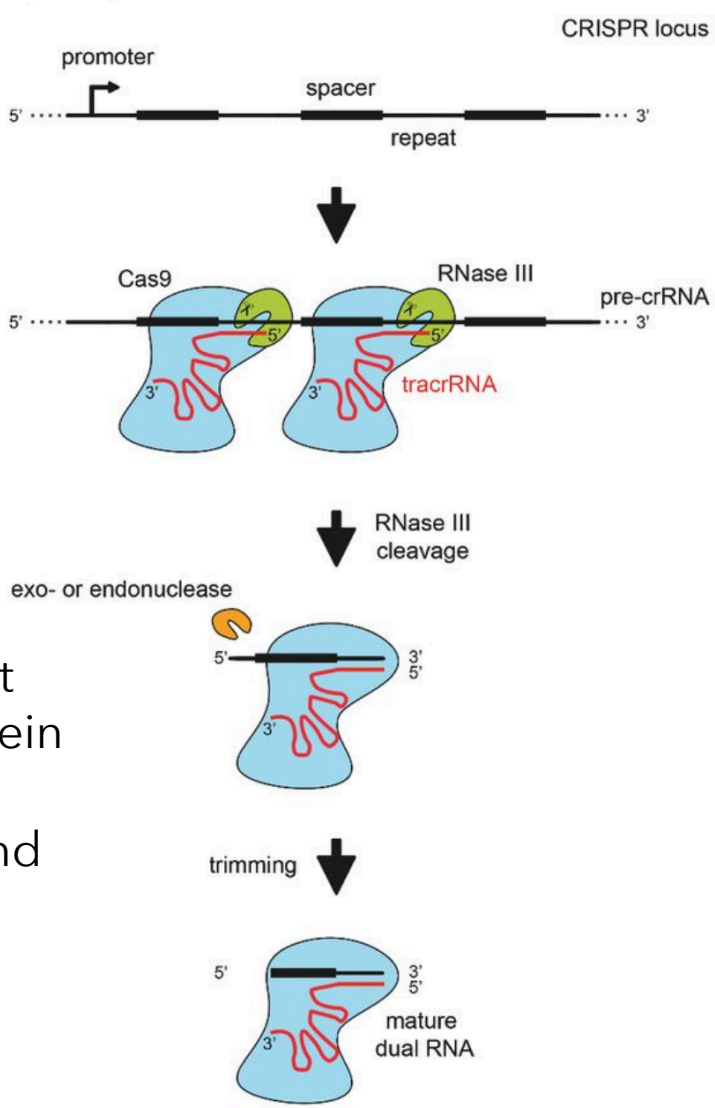
1. Notice a significant amount of transcription upstream, portions complementary to CRISPR repeat– "trans activating CRISPR RNA" or "tracrRNA"

2. In ΔtracrRNA mutants, they don't observe processed crRNA

3. Cleavage sites for crRNA look like RNase III

4. Various deletion controls, determined that only csn1 and RNase III required for crRNA maturation.

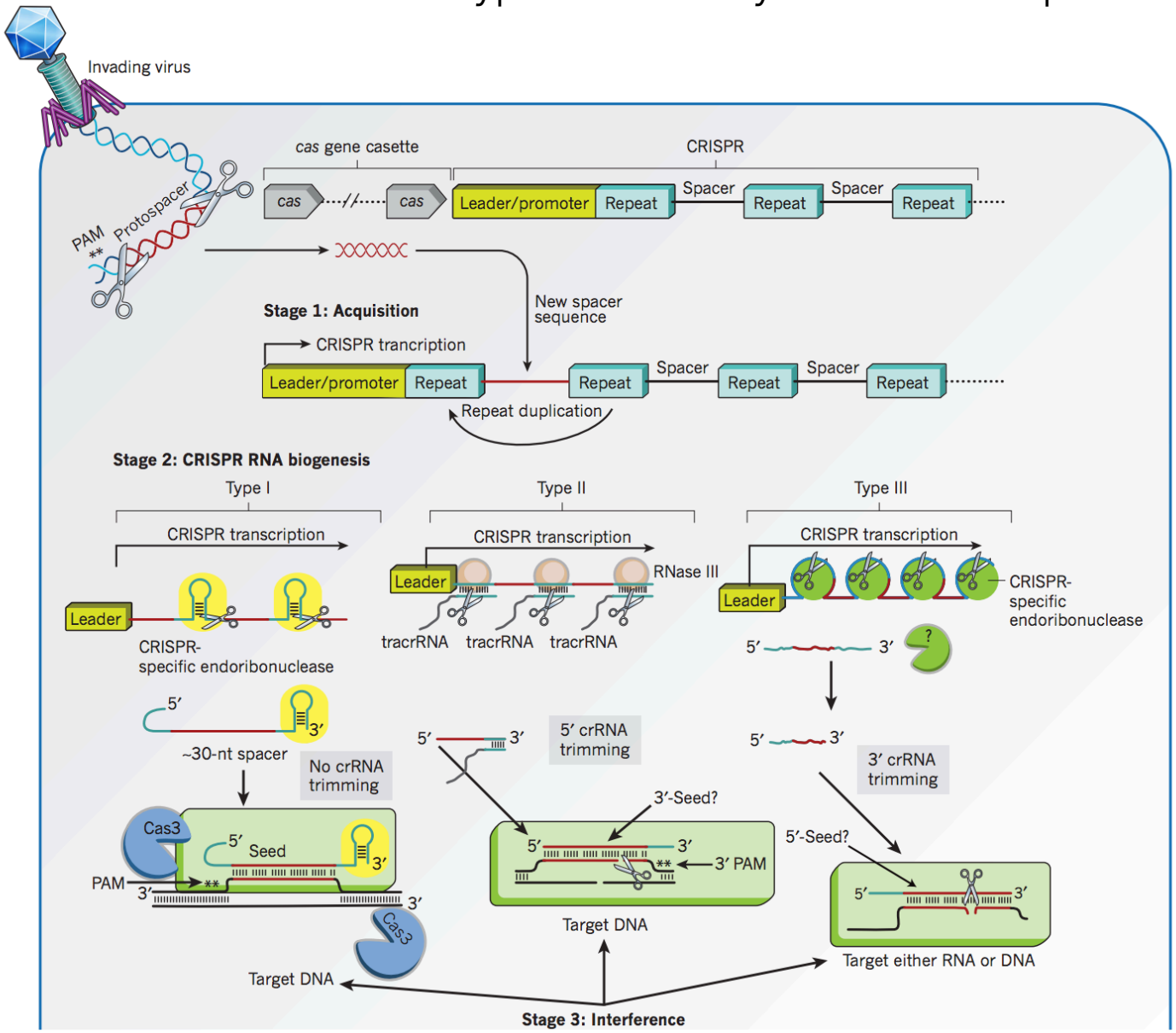
Maturation of *Cas9*/RNA Complex: Summary



Also determined that *Cas9* is the only protein necessary for DNA target recognition and cleavage.

FEMS Microbiology Reviews, 2015, 39, 428.

Type II CRISPR Systems are Unique



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2. Transcribing and processing CRISPR information

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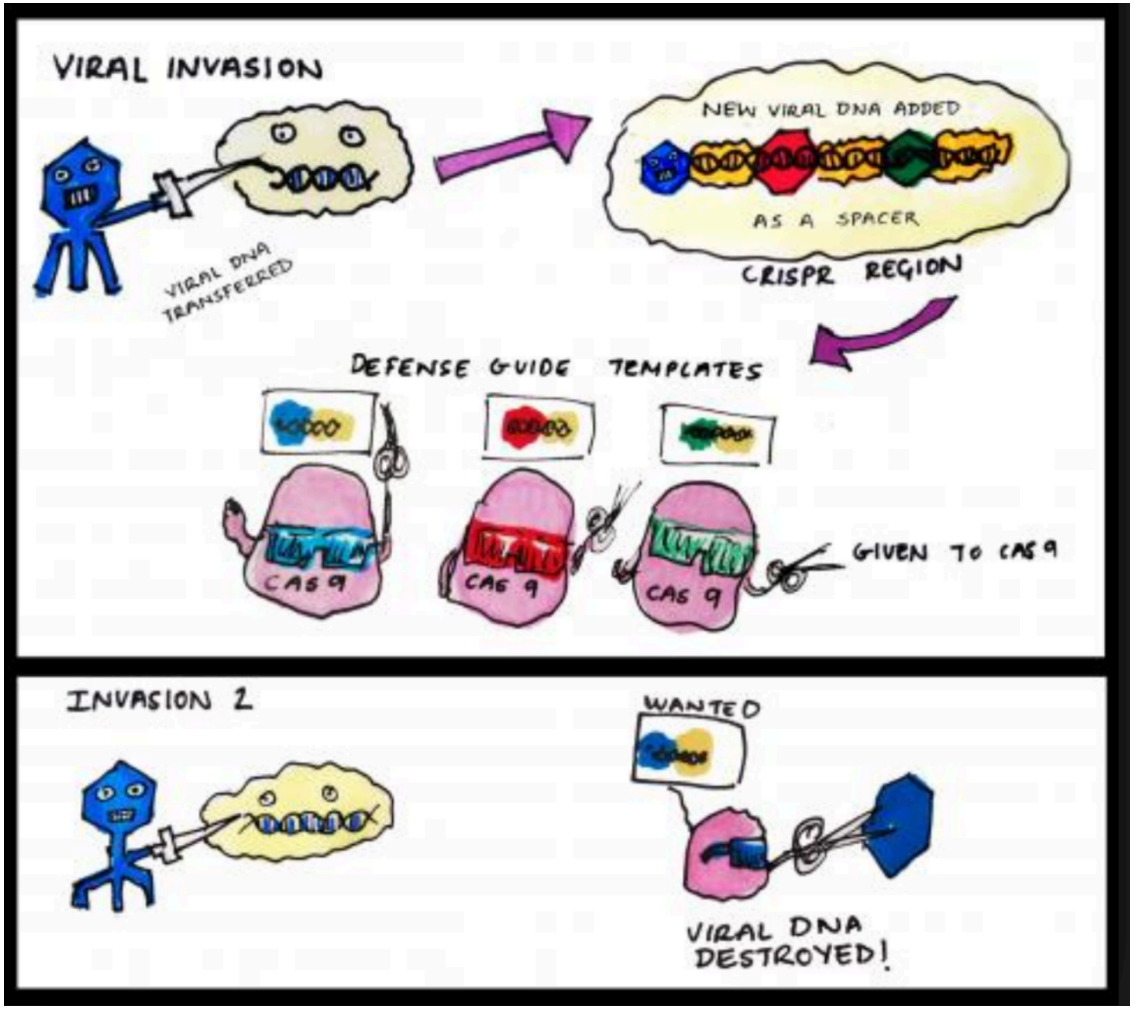
Steps for Acquired Immunity in Bacteria



1. Acquisition of foreign DNA

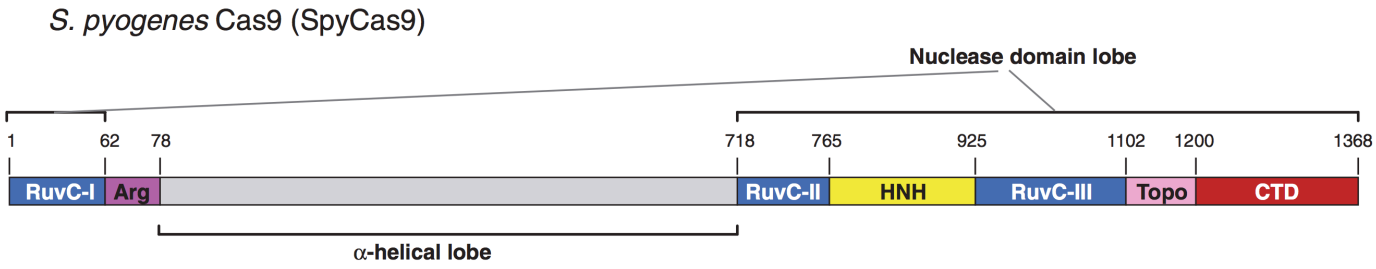
2. Transcribe CRISPR sequences-- Make some kind of RNA complex that can recognize foreign DNA

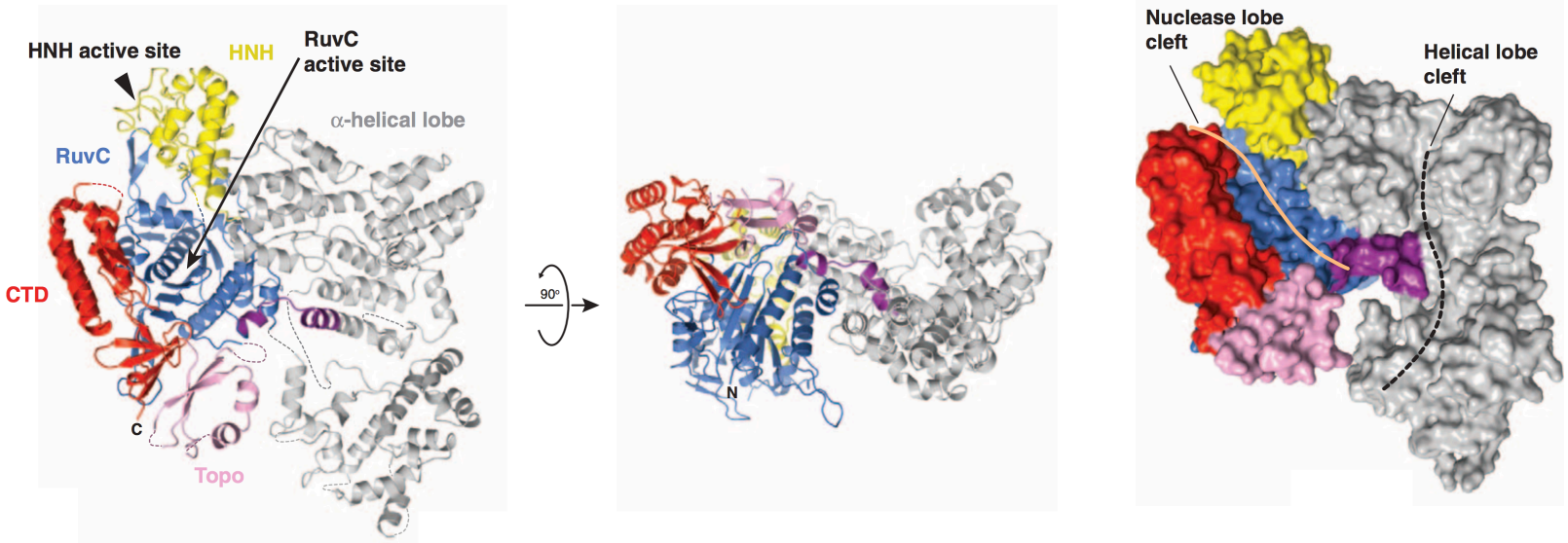
3. Destroy foreign DNA from future attacks.

https://www.eurostemcell.org/crispr-changing-gene-editing-landscape

Apo Protein Structure of *Cas9*

Primary gene structure:



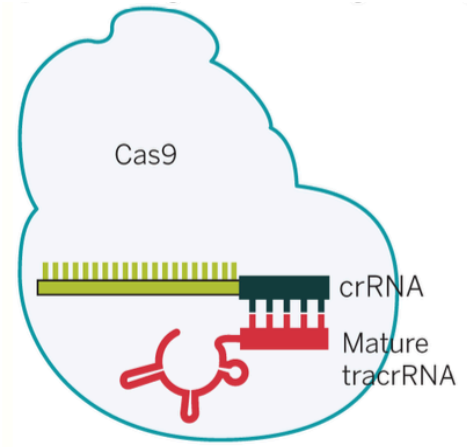


Two lobes REC and NUC, connected by an argenine rich helix and disordered region.
REC made up of 3 helical domains, not similar to known proteins.
NUC region has domains that share similarity to RuvC and HNH, nucleases

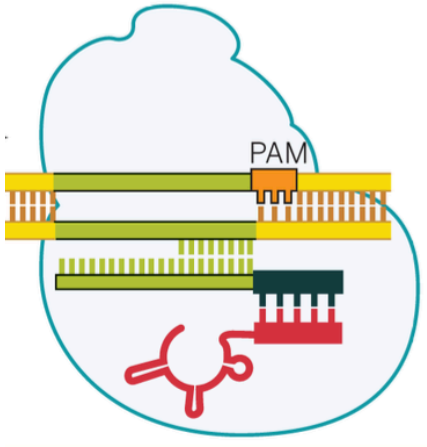
Two clefts for DNA binding.

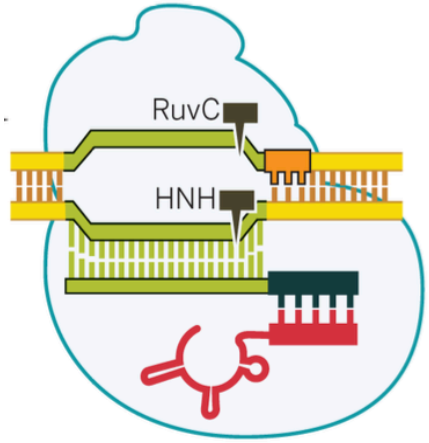
Science, **2014**, *343*, 1215.

Cas9 Induced DNA Cleavage: Summary

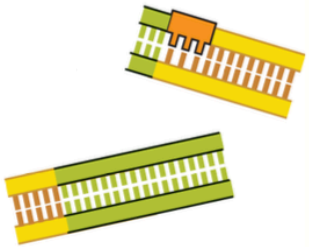


Target DNA binds to the complementary strand of exposed crRNA





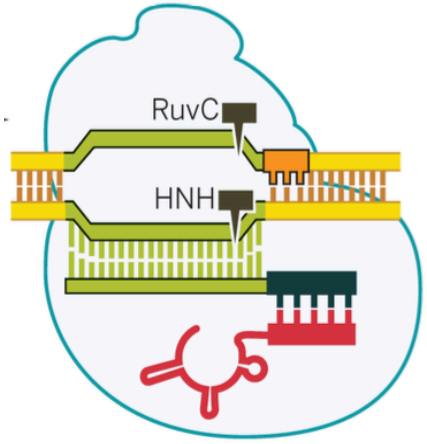
Endonucleases cleave at specific points relative to PAM

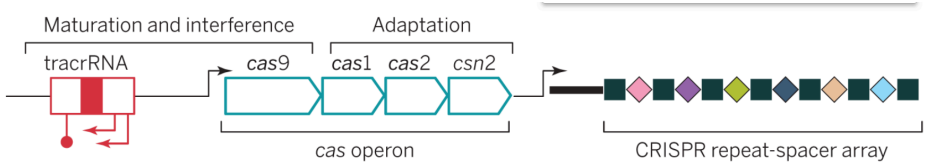


Science **2014**, *346*, 1077.

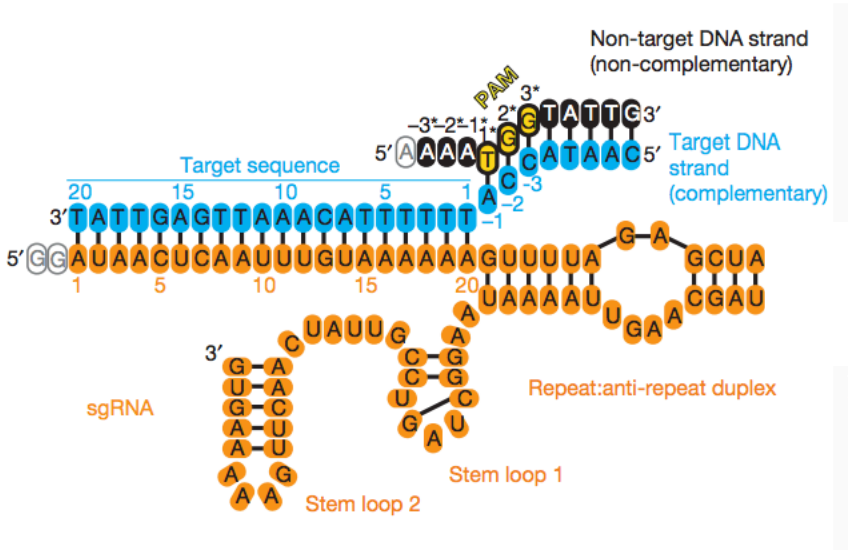
Importance of Protospacer Adjacent Motif (PAM)

How does the bacteria distinguish between infecting viral DNA and it's own CRISPR spacer sequences?





Cas9 recognizes NGG sequence in viral DNA, but in the bacterial chromosome (spacer)GTT.

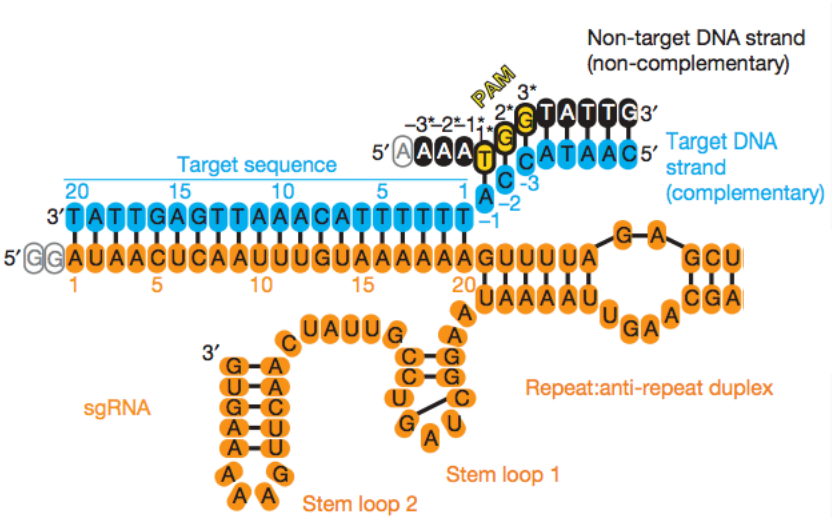


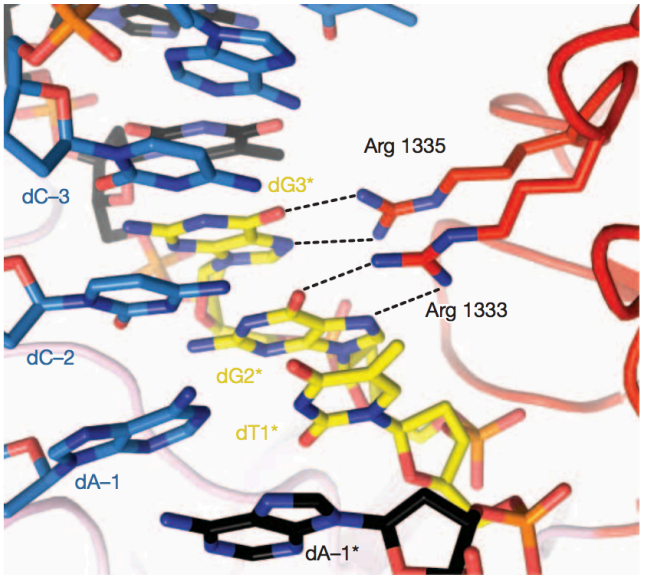
Independent of the crRNA sequence, as it is on the non-complementary strand

What facilitates this specific recognition?

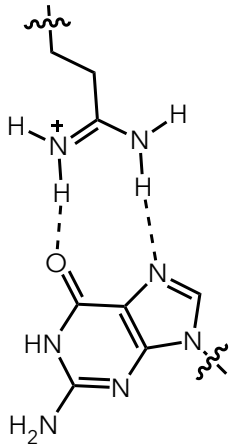
Nature **2014**, *513*, 569.

Hydrogen Bonding Enables PAM Recognition

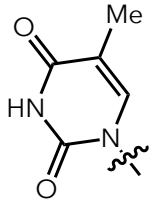




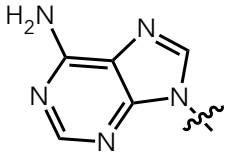
Sequence specific hydrogen bonding interactions between NGG sequence and arginine residues in the CTD inform the bacteria whether the probed DNA is its own or viral.



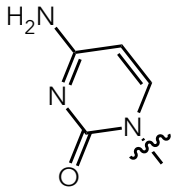
Guanine



Thymine



Adenosine



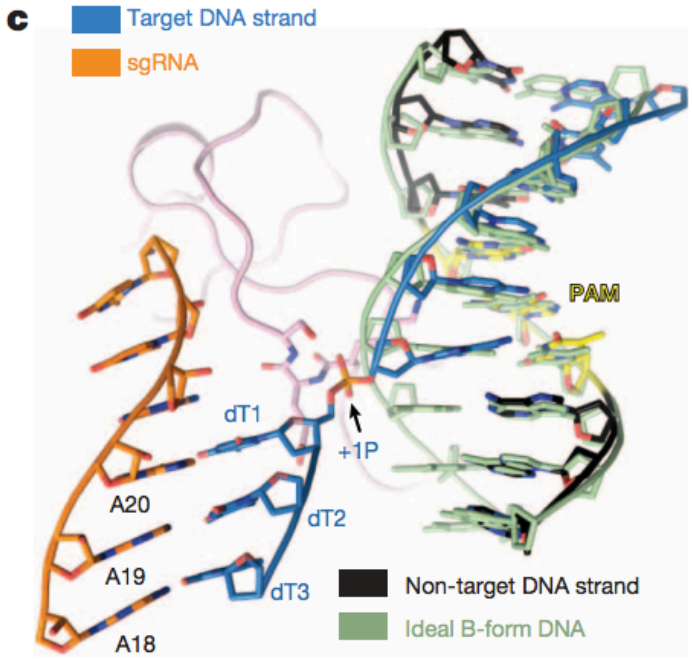
Cytosine

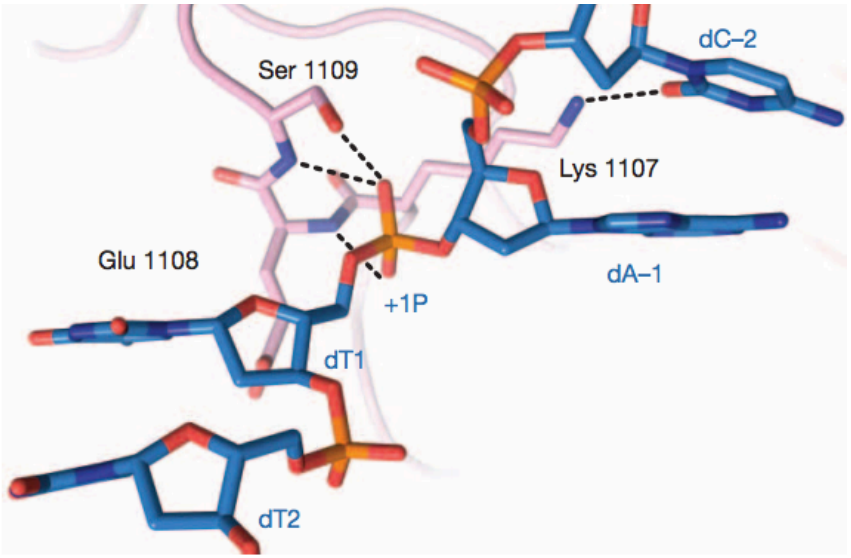
These ineractions are specific to the DD–AA pattern between arginine and guanine

Nature **2014**, *513*, 569.

Target Search and Recognition

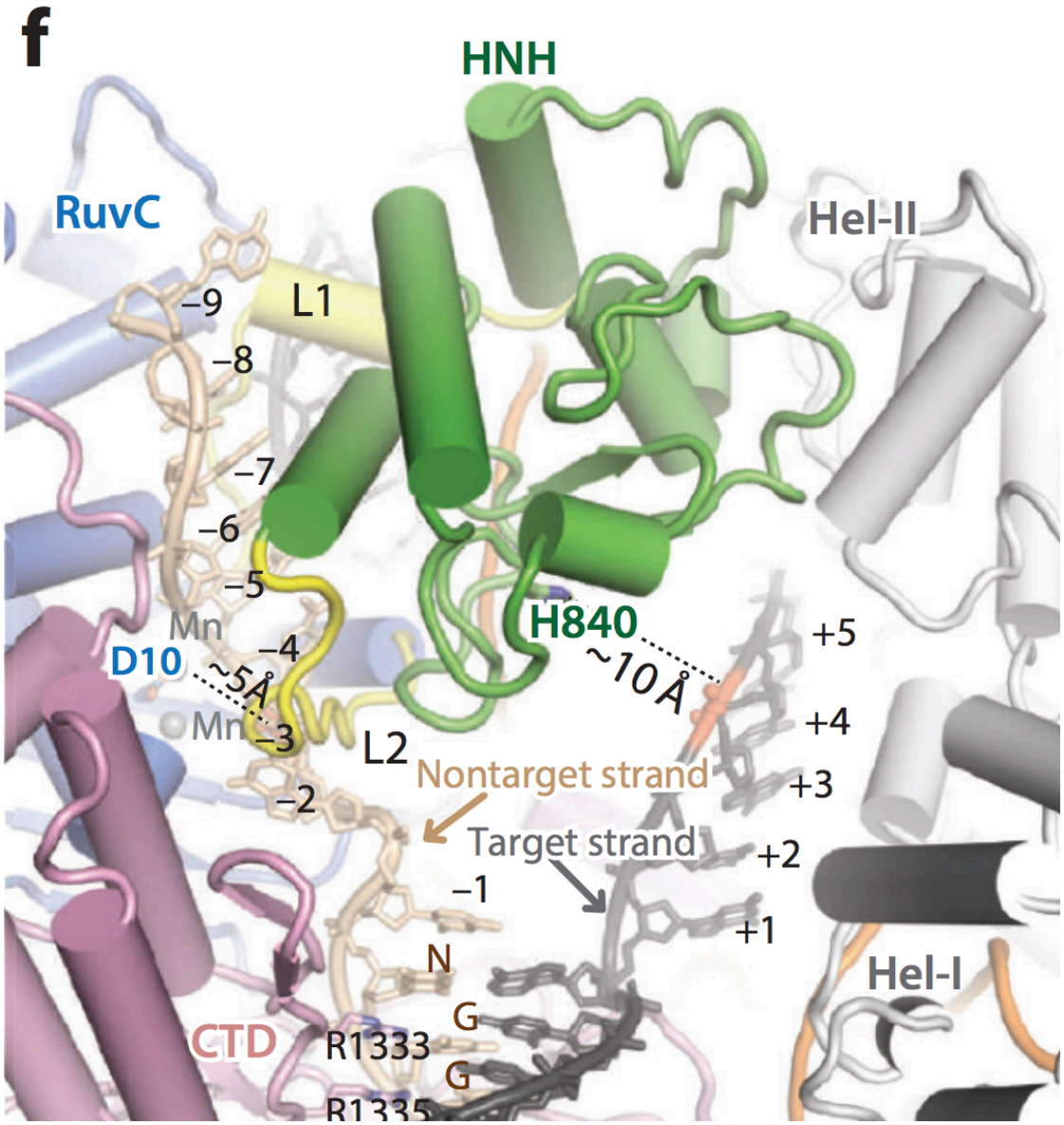
If PAM sequence detected, DNA is unwound to probe for sequence complementarity.





Phosphate on complementary strand (+1P) is kinked out of conformation, stabilized by hydrogen bonding lock loop.

Target Search and Recognition

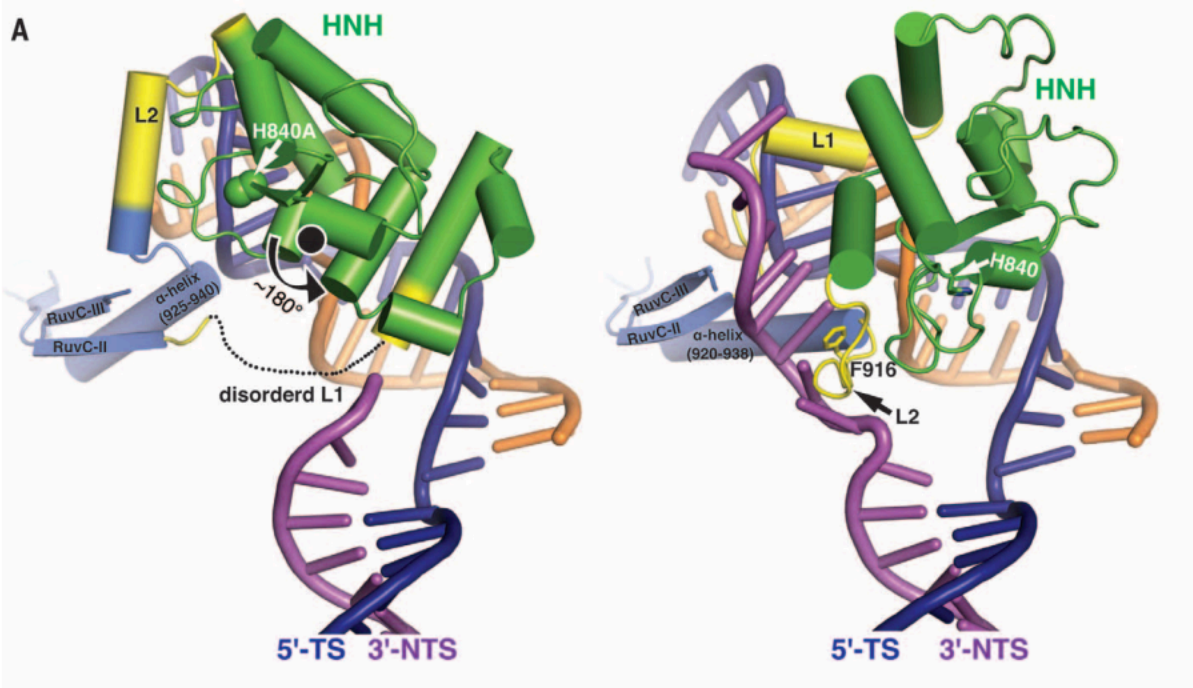


Non-target strand is kinked at -1P position (relative to PAM)

The conformation is stabilized by Van der Waals and hydrophobic interactions and helps to compensate for DNA melting.

Science **2016**, *351*, 869.

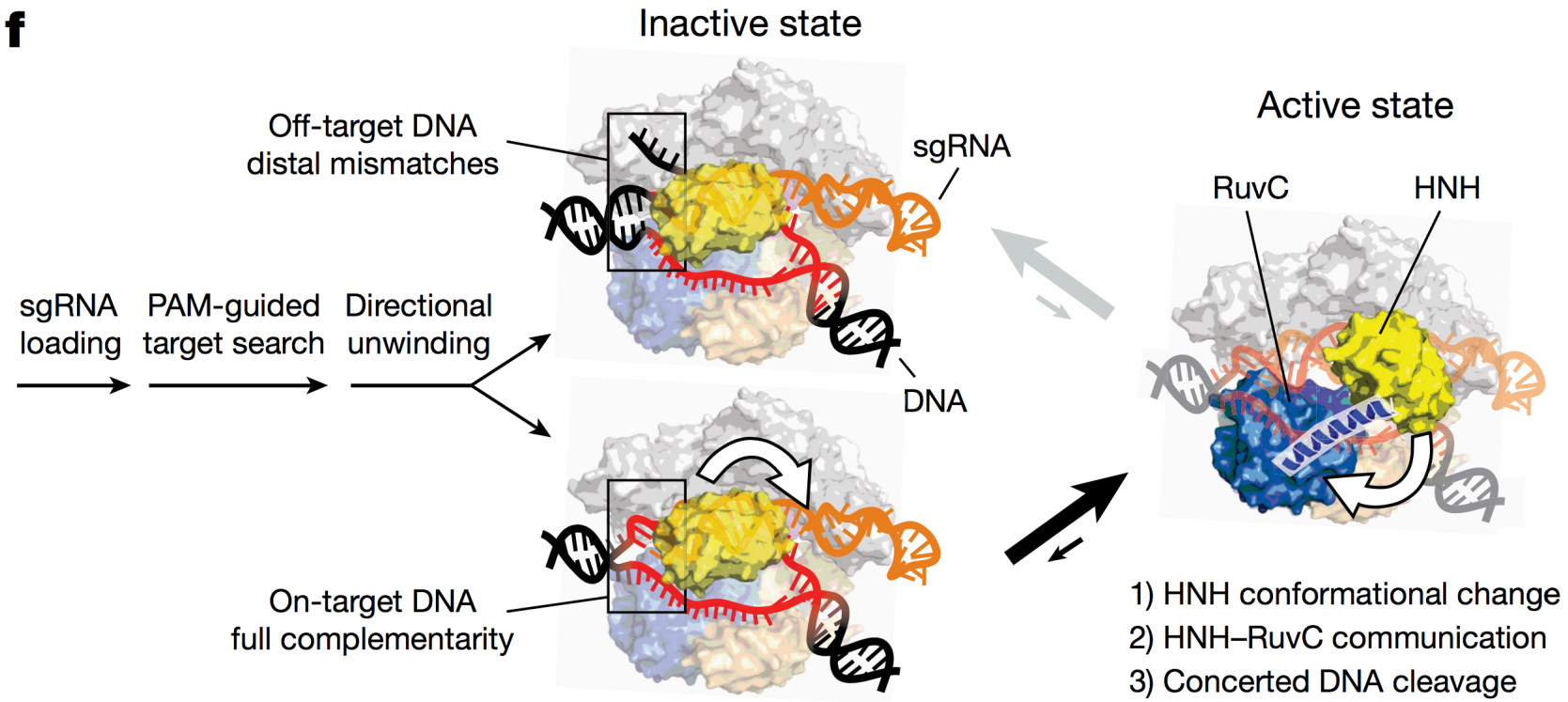
Conformational Change in Protein with Full Complementarity



Feeding the non-complementary strand through the DNA binding cleft induces ordering in L1 linker and causes major conformational change in protein structure.

Science **2016**, *351*, 869.

Allostery Places Cleavage Sites in Proximity to Respective Nucleases

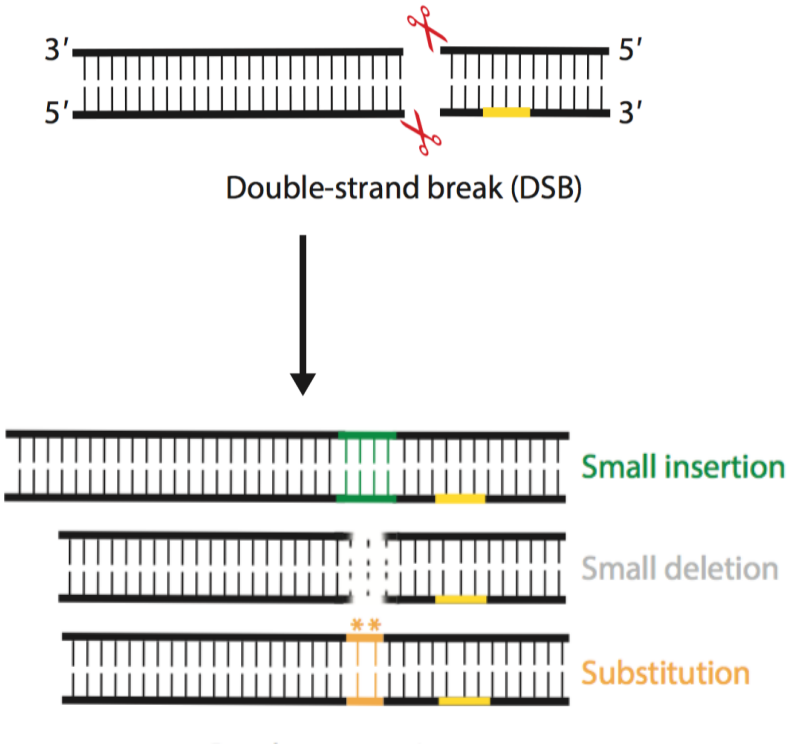


Science **2016**, *351*, 869.

DNA Repair Mechanisms

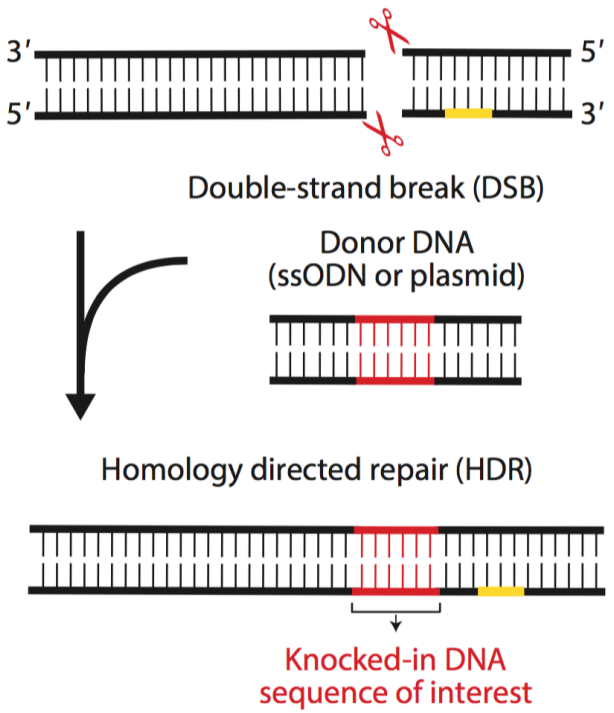
If there is too much DNA damage in a cell dormancy, cancer, or apoptosis can occur.
Cells are constantly identifying and repairing damaged DNA.

Non-Homologous End Joining



Error prone, can result in gene disruption through small base pair deletions, insertions, or substitutions

Homology Directed Repair



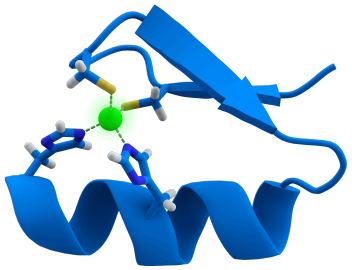
Homologous sequences in donor DNA (black) can guide recombination, resulting in the addition of a gene (red)

Annu. Rev. Biophys. **2017**, *46*, 505

Zinc Finger Based Technologies

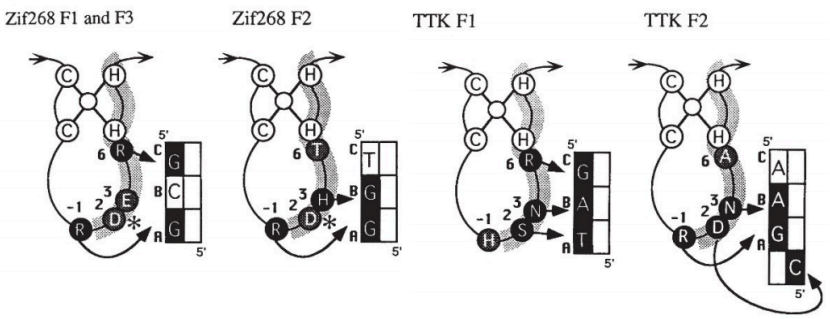
Category of protein fold stabilized by Zinc ion coordination, typically binds DNA, RNA, or peptides.

Best studied category is His_2Cys_2

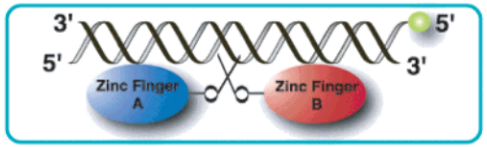


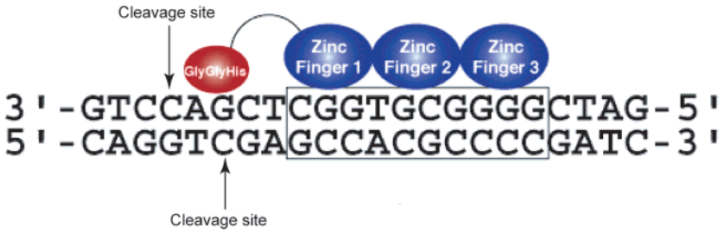
[Thomas Splettstoesser](), via scistyle

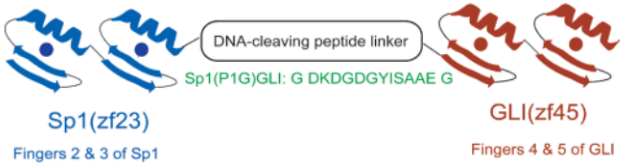
-binds specific DNA sequences based on composition of protein alpha helix



Artificial nuclease engineering:





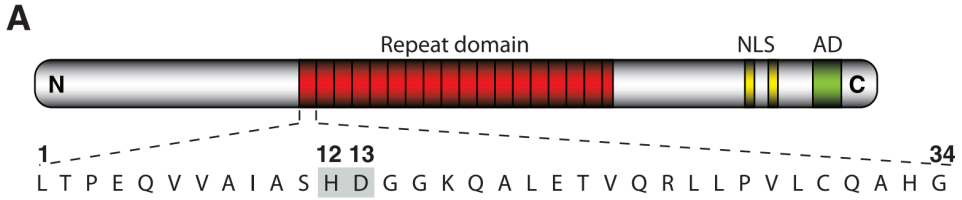


-Need to design a completely new protein for each targeted DNA sequence

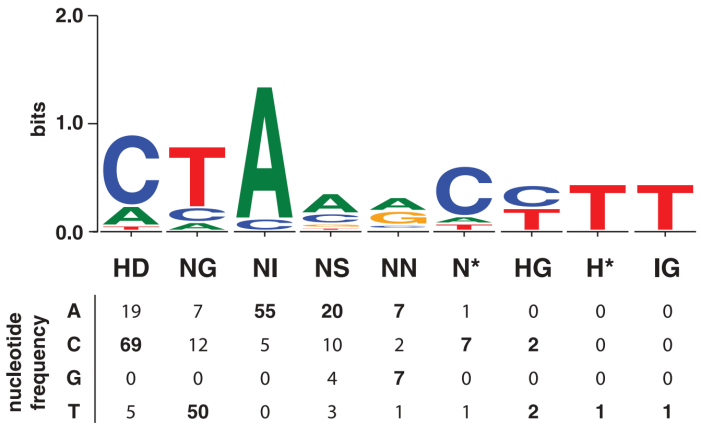
Nature **1993**, *366*, 483
Acc. Chem. Res. **2006**, *39*, 45-52

Transcription Activator Like Effector Nuclease (TALEN)

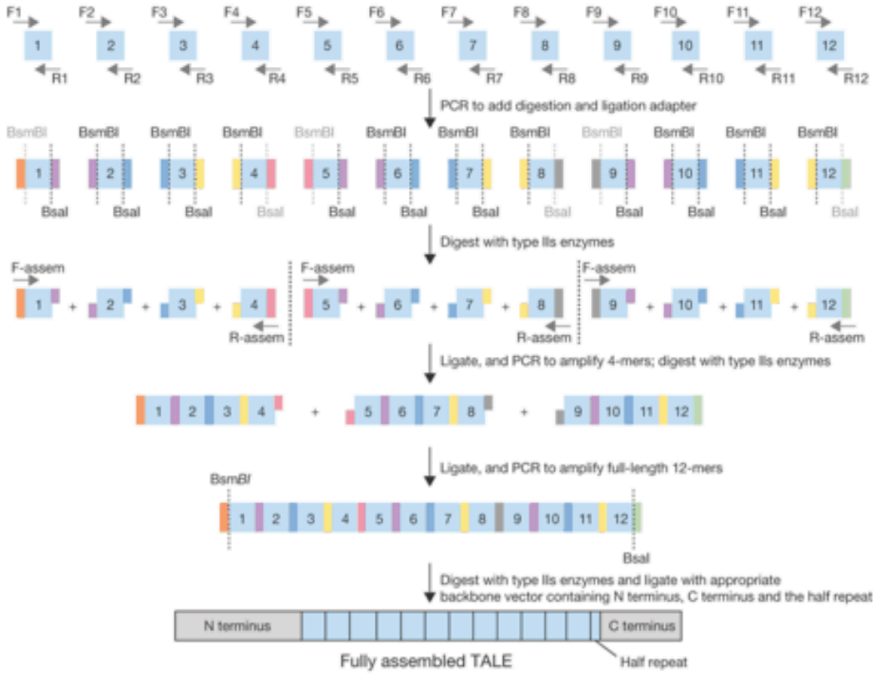
Pathogen *Xanthomonas* proteins secreted into plant cells to bind specific host DNA and disrupt cellular activity.



Repeat domain with high variability at the 12,13 positions.



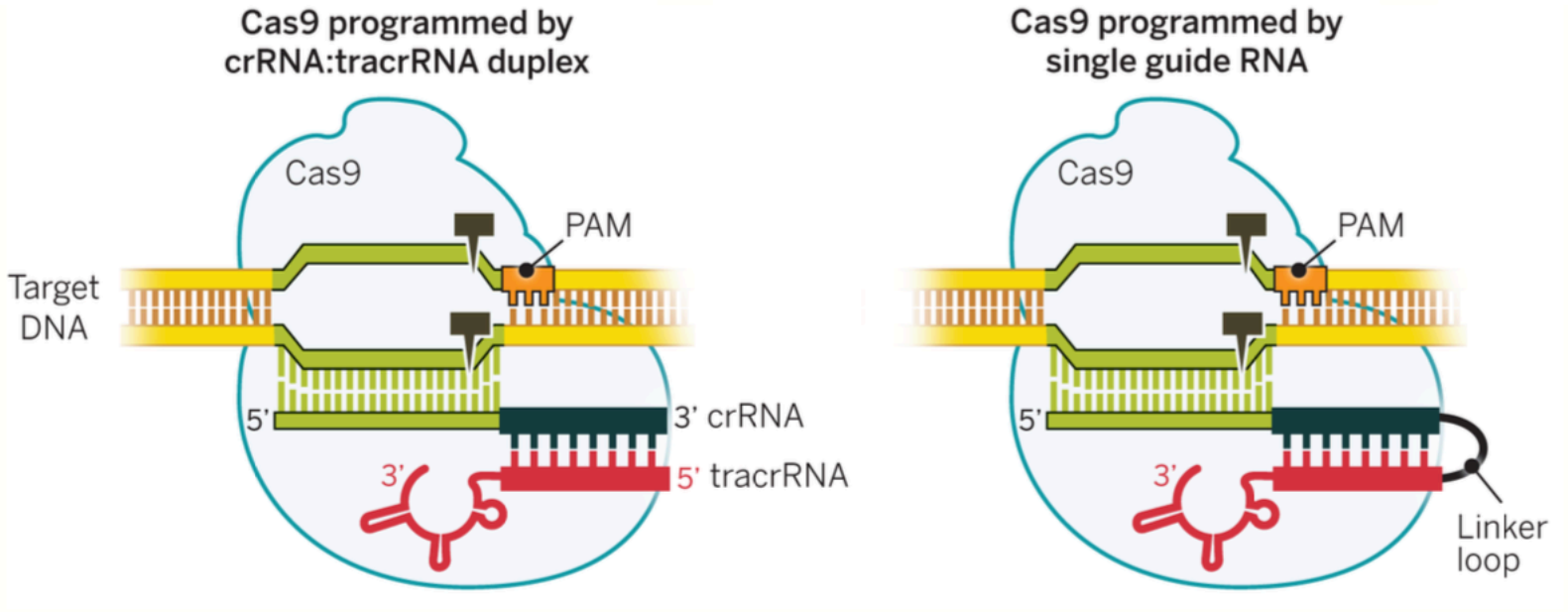
Diresidue corresponds to base pair binding frequency



Science **2009**, *326*, 1501

https://www.addgene.org/talen/guide/

Can You Simplify Nature's Approach?

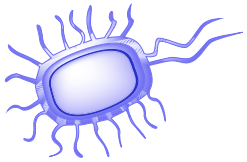


"Zinc-finger nucleases and transcription-activator–like effector nucleases have attracted considerable interest as artificial enzymes engineered to manipulate genomes (35–38). We propose an alternative methodology based on RNA-programmed Cas9 that could offer considerable potential for gene-targeting and genome-editing applications."

Science **2014**, *346*, 1077.

Rapid Adoption of Experimental Technique

prokaryotes



Doudna, Charpentier, Siksnys

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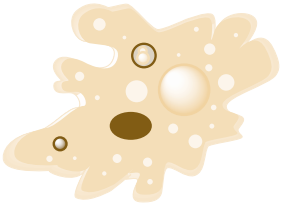
Human and mouse cells



Zhang and Church, Doudna

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Mouse embryo

Zhang, Jaenisch
Multiple mutations, Study F0

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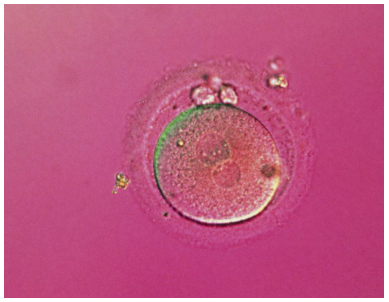

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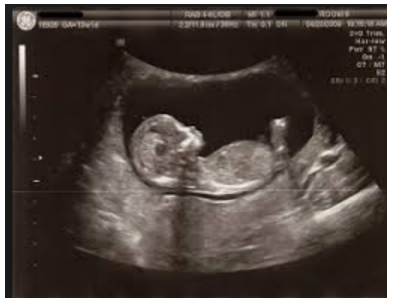
First clinical trial

Lu You, 2016

First CRISPR edited babies

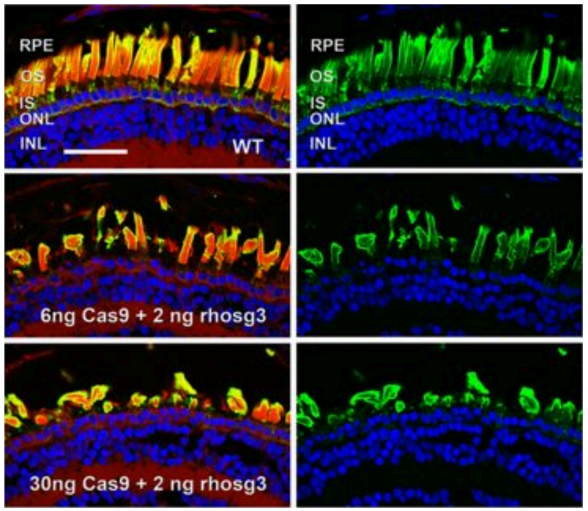


2018–Jiankui He edits genes in human embryo

Some Applications of CRISPR/*Cas9*

Disease models

Model for Retinitis Pigmentosa:
Editing with Cas9 causes loss of rod opsin levels and retinal degredation in *Xenopus laevis*

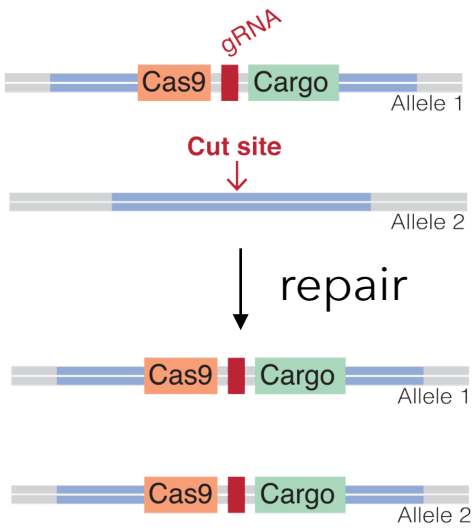


Accelerates the rate of research, by allowing studies on the F0 generation

Scientific Reports **2017**, *7*, 6920.

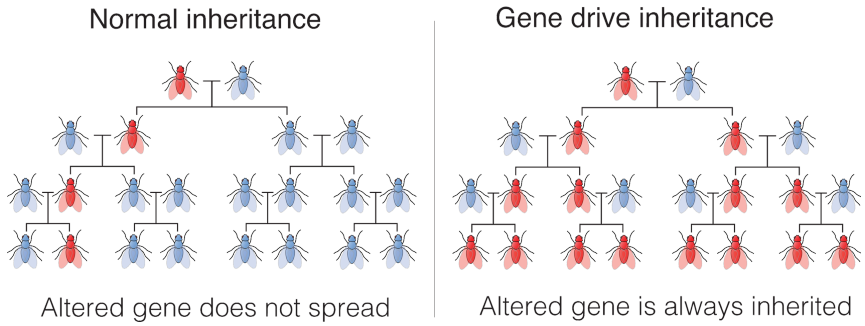
Gene drive

Used to eradicate populations of invasive, or disease carrying species. Highly ethically questionable.



heterozygote

homozygote

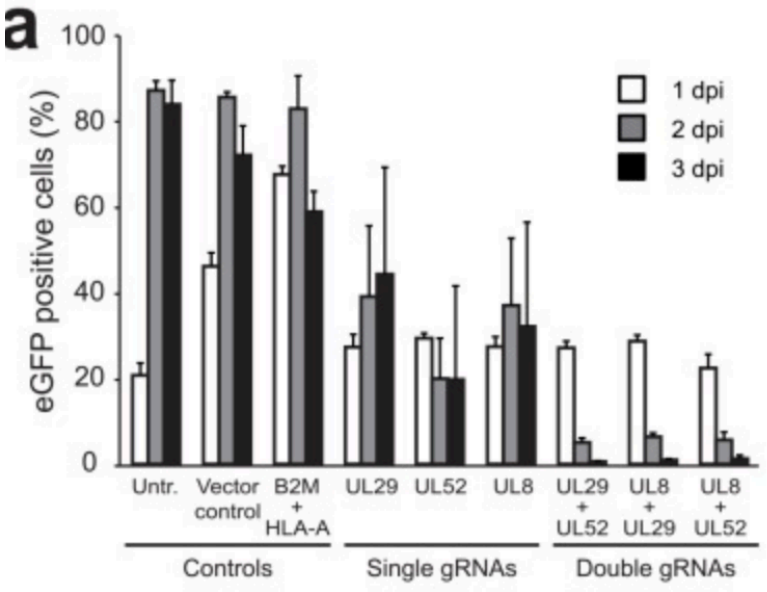


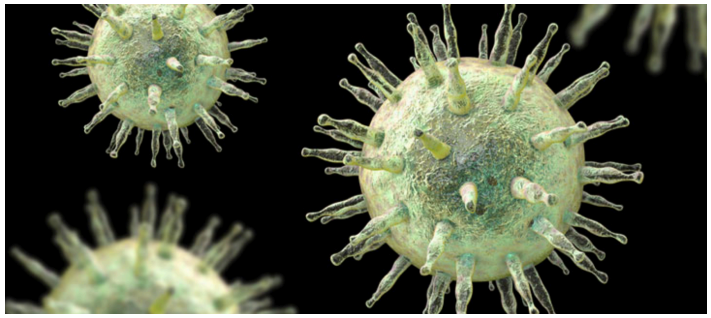
Nature **2016**, *34*, 78.

Some Applications of CRISPR/*Cas9*

Infectious diseases

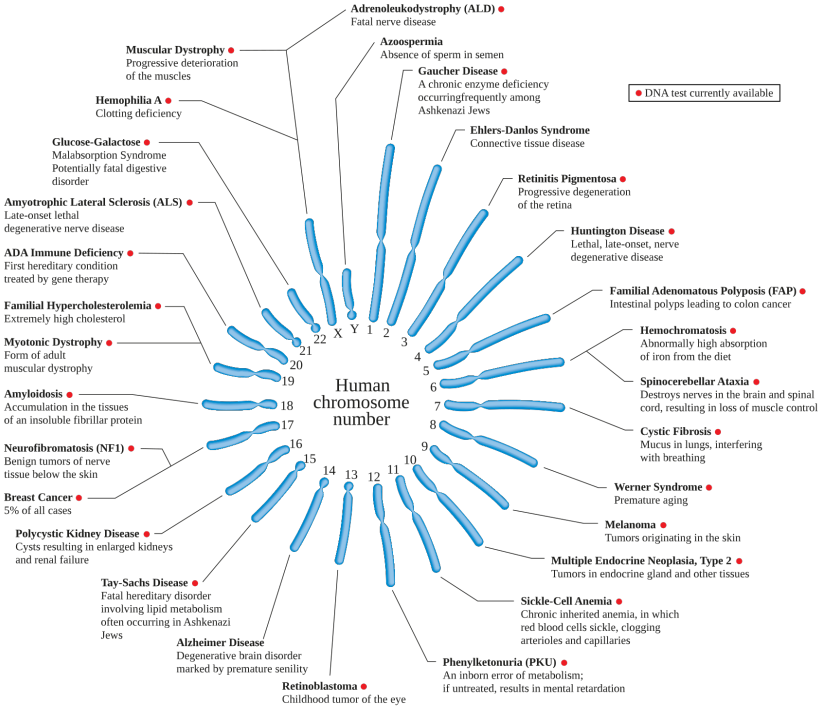
Treatment of cells infected with Epstein-Barr virus with Cas9 and viral crRNA reduces viral load.





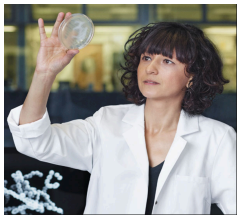
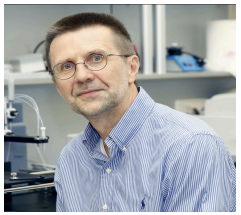
PLoS Pathog. **2016**, *12*, 1005701.

Modification of Pathological Genes



https://en.wikipedia.org/wiki/Genetic_disorder

Patent Dispute


Berkeley UNIVERSITY OF CALIFORNIA

UNIVERSITY OF VIENNA

Vilnius University

BROAD INSTITUTE MIT

March 2012
Virginijus Siksnys + others submit first patent request

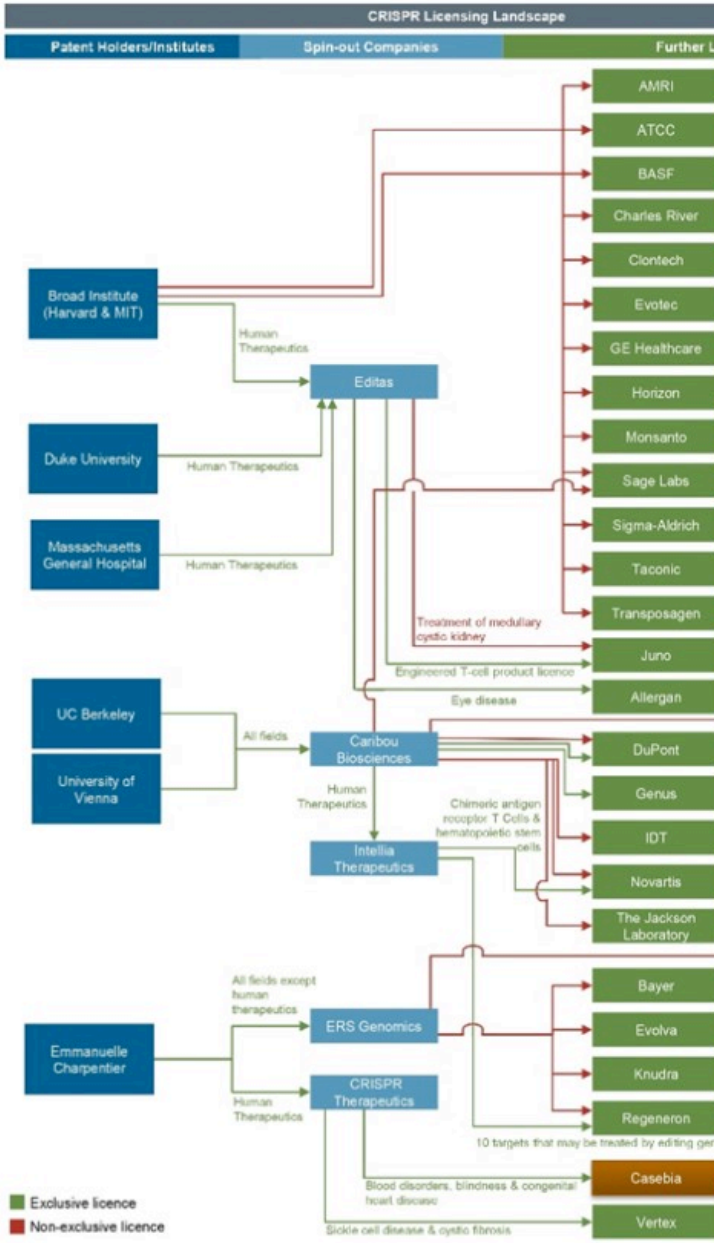
May 2012
UC Berkeley submits patent request

December 2012
Broad Institute submits patent with accelerated examination

April 2014
Broad receives patent

2015
UC Berkeley declares interference: “first to invent” provision

Unfortunately for UC Berkeley, the PTO rules the extension of the technology’s use to eukaryotes (Broad) from prokaryotes (UCB) is ruled to be non-obvious. Trials do not procede to invention hearings.



https://labiotech.eu/features/crispr-patent-dispute-licensing/
https://www.broadinstitute.org/crispr/journalists-statement-and-background-crispr-patent-process

Rapid Adoption of Experimental Technique

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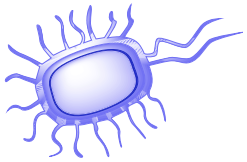

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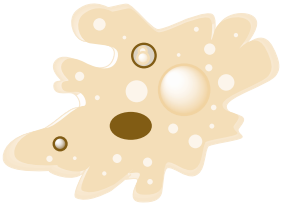

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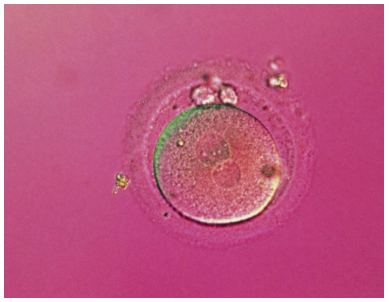

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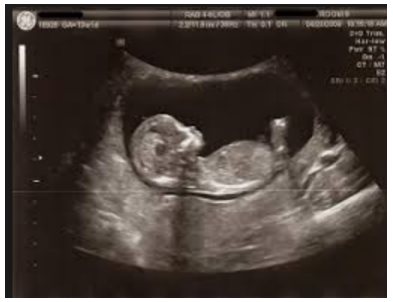


2018–Jiankui He edits genes in human embryo

“CRISPR Babies”

Jiankui recruits researchers for a study to birth the first Cas9 modified babies.
-Germline editing, pass on traits to offspring
-trial conducted prior to adequate testing in animals

Recruited couples with HIV positive men, goal is to inactivate CCR5 gene to protect offspring from HIV.

- There are other ways to prevent HIV from passing to the child.
- CCR5 inactivation only protects from one strain of HIV
- HIV positive individuals experience great discrimination, banned from IVF treatment in China
- Sent in substitutes for blood tests, Faked/retroactively registered ethics approval document
- Unclear informed consent for both patients and other researchers/doctors involved

Twin girls Lulu and Nana born.

- Reported mutations don’t match the desired Δ32 deletion, instead random insertion/deletion–mosaic genotypes
- One study suggests that mutations in CCR5 can cause cognative enhancement in mice (unlikely in humans)
- Furthermore, CCR5 mutations can cause more health problems

- https://twitter.com/tictoc/status/1067762866370023426

American scientists knew about Jiankui’s intentions but remained silent.

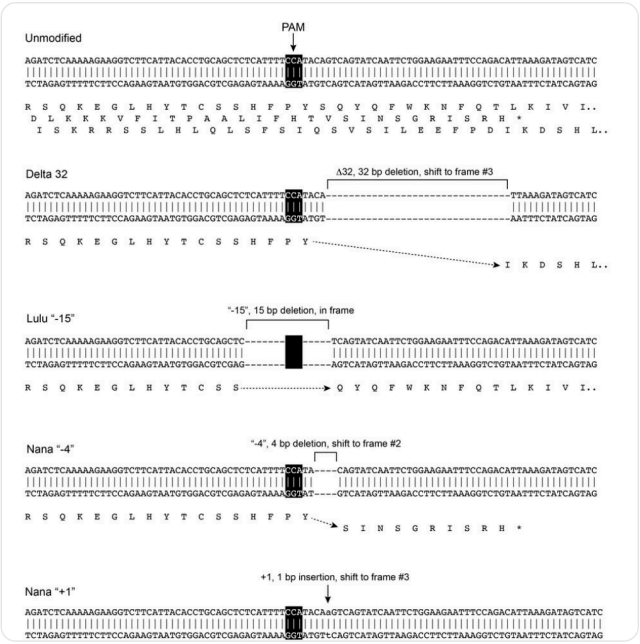
- Absence of global governing body
- Thought he had been dissuaded.
- Contrast between dominant values of science and role of science in society

He Jiankui

Pinned Tweet

Sean Ryder @RyderLab · 29 Nov 2018

I made a new figure to try to help explain the nature of the "reported" mutations in #CRISPRbabies. The point is that none of the three match the well studied delta 32 mutation, and as far as I can tell, none have been studied in animal models. Unconscionable. #GeneEditSummit



46 648 1.0K

Summary and Outlook

In less than 10 years CRISPR/Cas9 has gone from bacterial immune system to clinical trials for cancer therapy.

RNA guided system allows for sequence specific recognition and cleavage of DNA.

Powerful technology with a wide variety of applications… and ethical concerns...

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Patent:
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Human Germline Editing
https://techcrunch.com/2018/11/26/hospital-denies-gene-edited-babies-china/
http://www.chictr.org.cn/showprojen.aspx?proj=32758
http://www.globaltimes.cn/content/1132670.shtml
https://www.nature.com/articles/d41586-018-07713-2
https://www.nature.com/articles/d41586-019-00662-4
https://www.statnews.com/2019/01/31/crispr-babies-michael-deem-rice-he-jiankui/
https://www.nature.com/articles/d41586-019-00246-2
https://www.technologyreview.com/s/612458/exclusive-chinese-scientists-are-creating-crispr-babies/
https://www.nature.com/articles/d41586-018-07713-2
https://www.statnews.com/2018/12/14/china-aids-history-crispr-babies/
https://www.nature.com/articles/nbt.3227
https://www.npr.org/sections/health-shots/2018/02/21/585336506/doctors-in-china-lead-race-to-treat-cancer-by-editing-genes

Clinical trials
http://www.bu.edu/khc/files/2018/10/CRISPR-Ethics-reading.pdf
http://fortune.com/2016/11/15/first-crispr-trial-humans-china/
https://www.nature.com/news/crispr-gene-editing-tested-in-a-person-for-the-first-time-1.20988?WT.mc_id=TWT_NatureNews