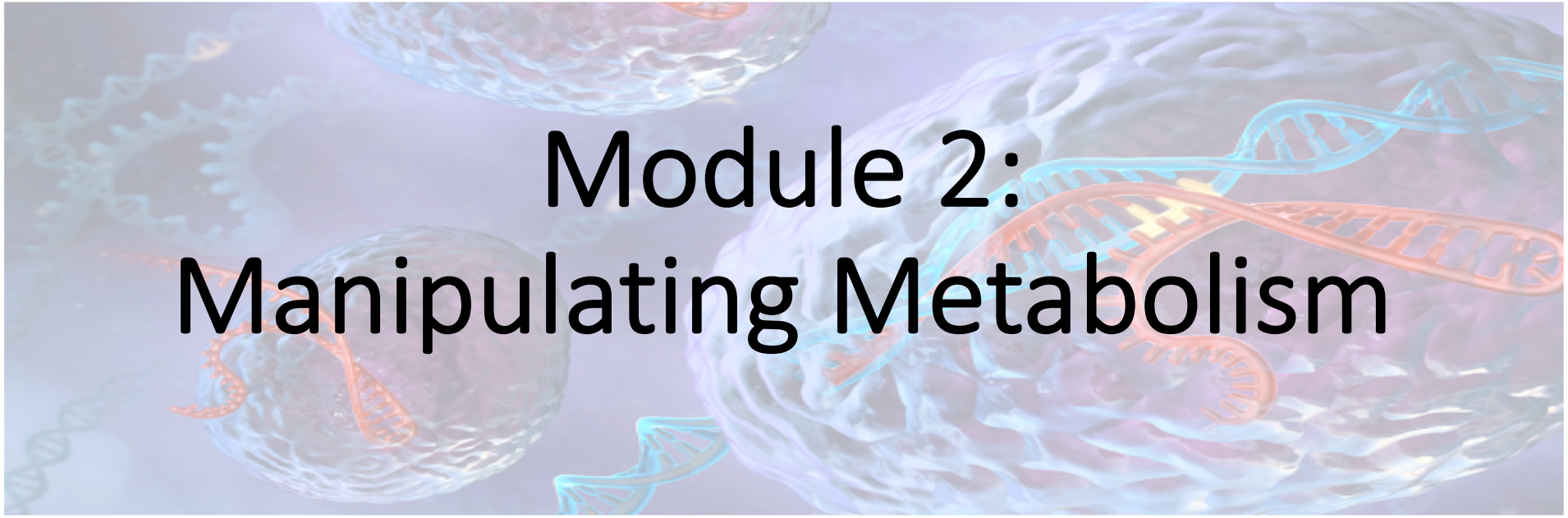
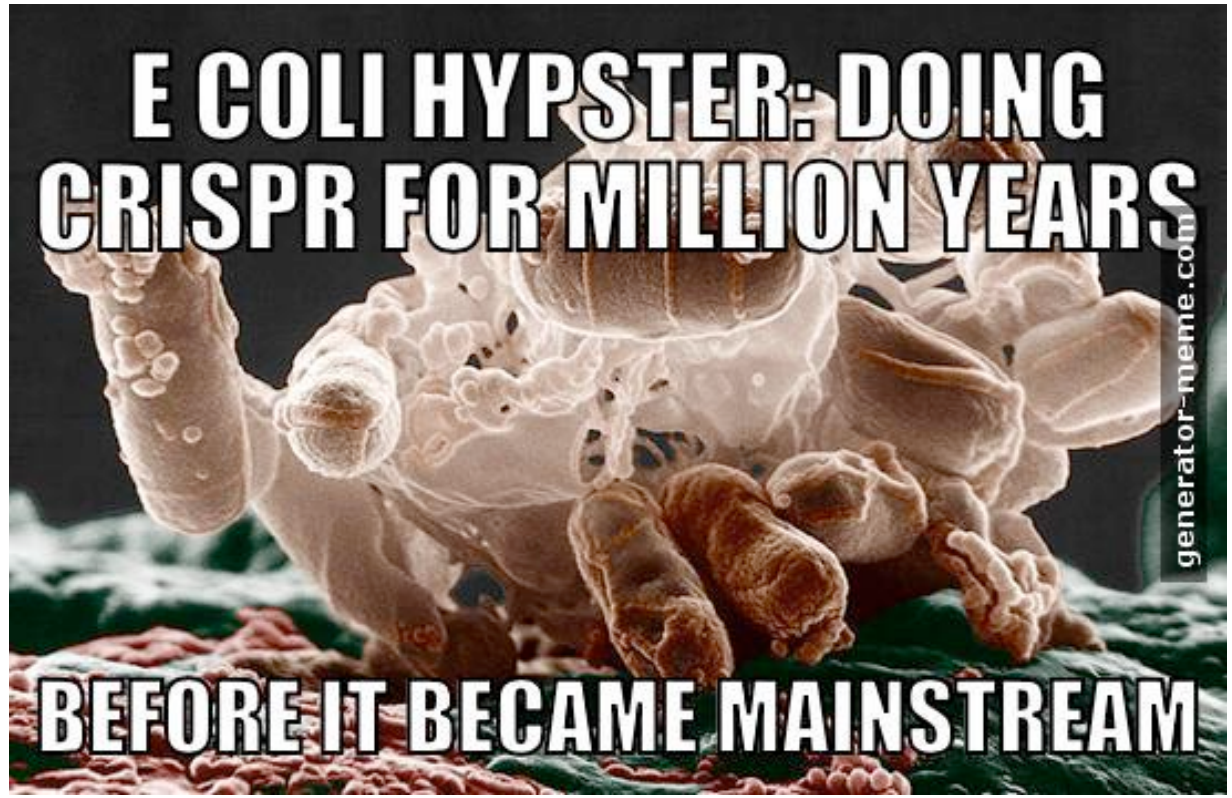


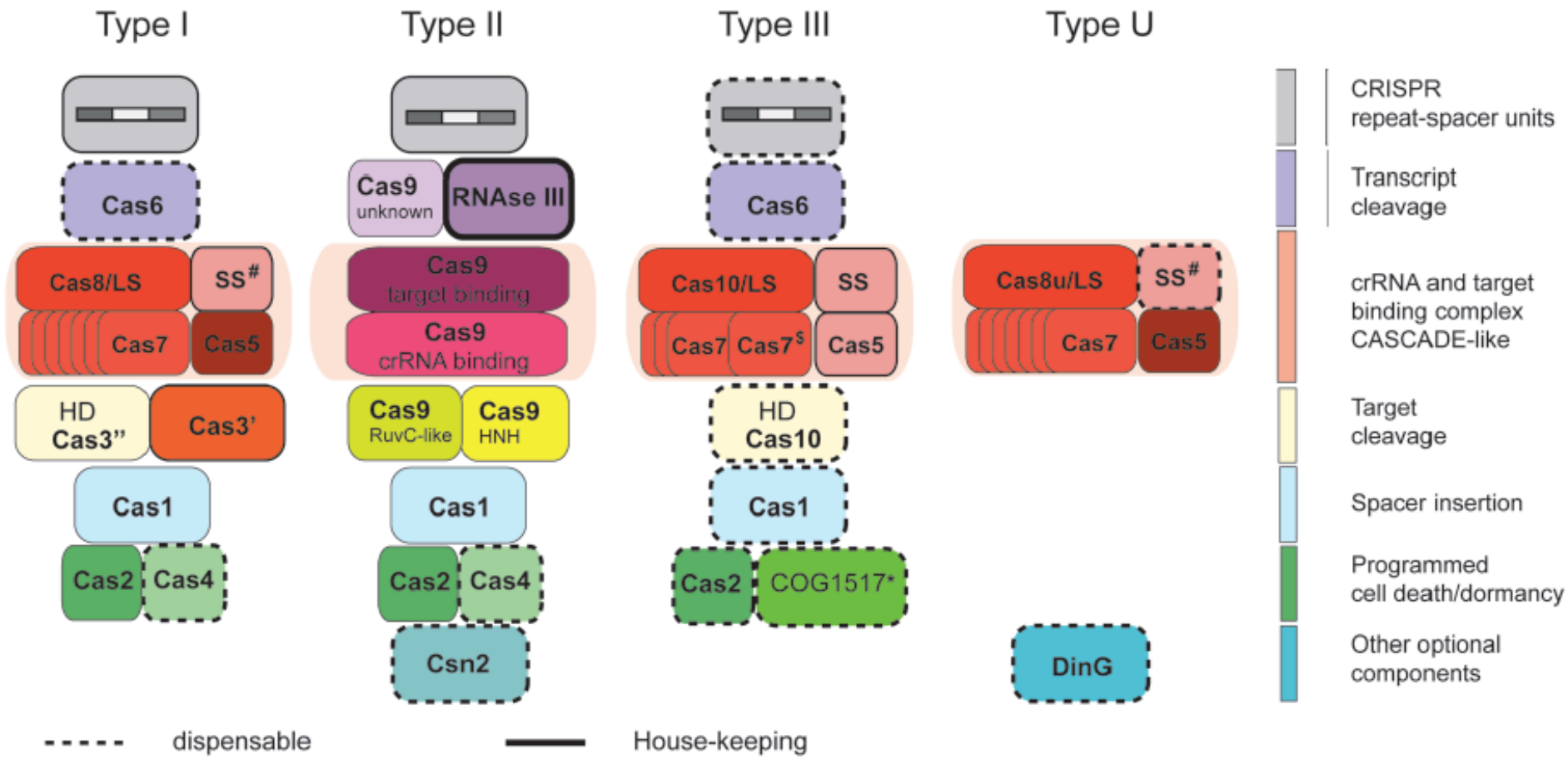
Module 2: Manipulating Metabolism

CRISPR: mechanism details

10/24/19


E COLI HYPSTER: DOING CRISPR FOR MILLION YEARS
BEFORE IT BECAME MAINSTREAM
generator-meme.com

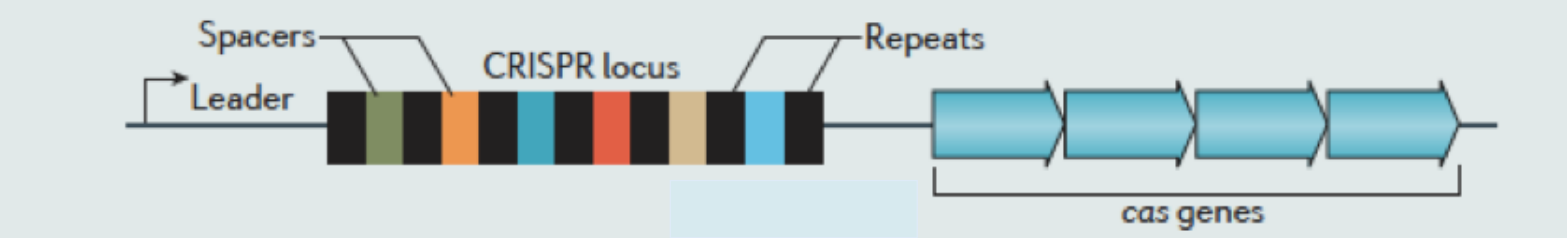
Several CRISPR systems have been identified



Koonin and Makarova. (2013) *RNA Biology*. 10:679-686.

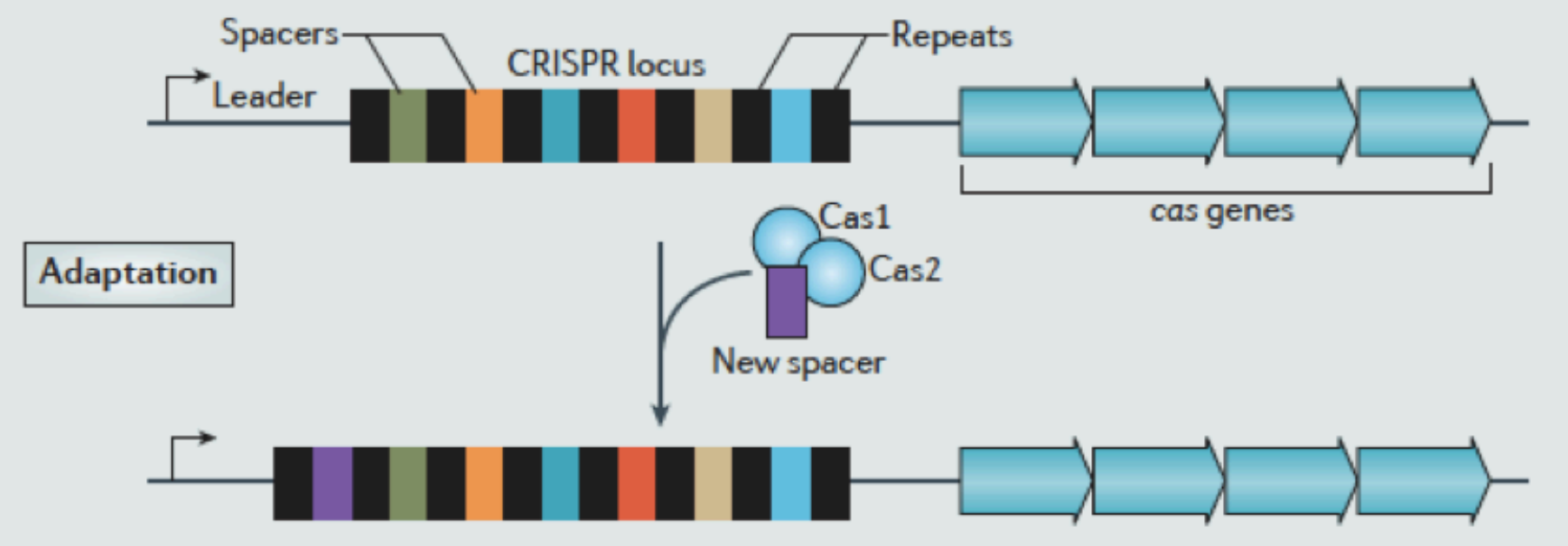
Native CRISPR system cleaves phage DNA

- Adaptive immune response encoded by CRISPR loci and Cas genes



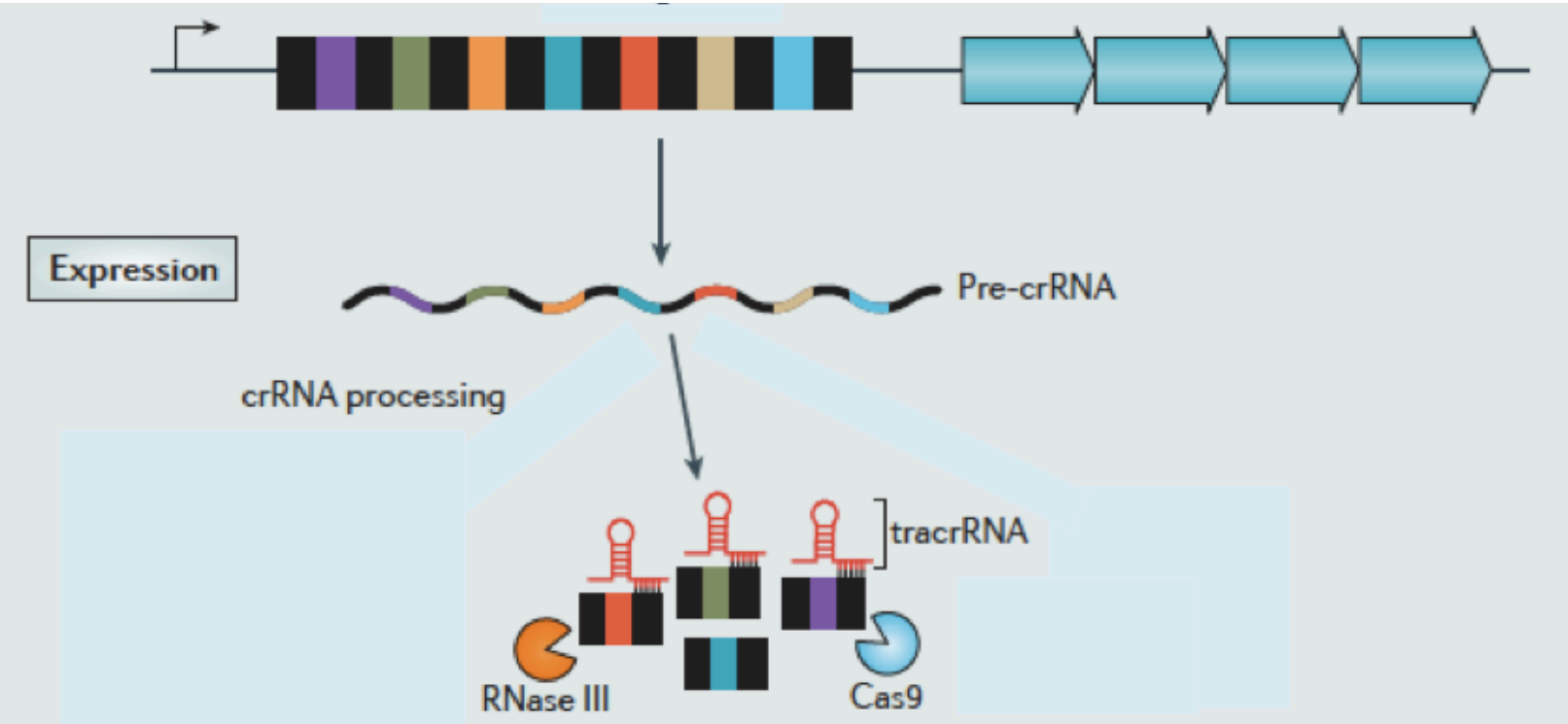
- Mechanism involves three stages:
  - Adaptation
  - Expression
  - Interference

CRISPR system: adaptation



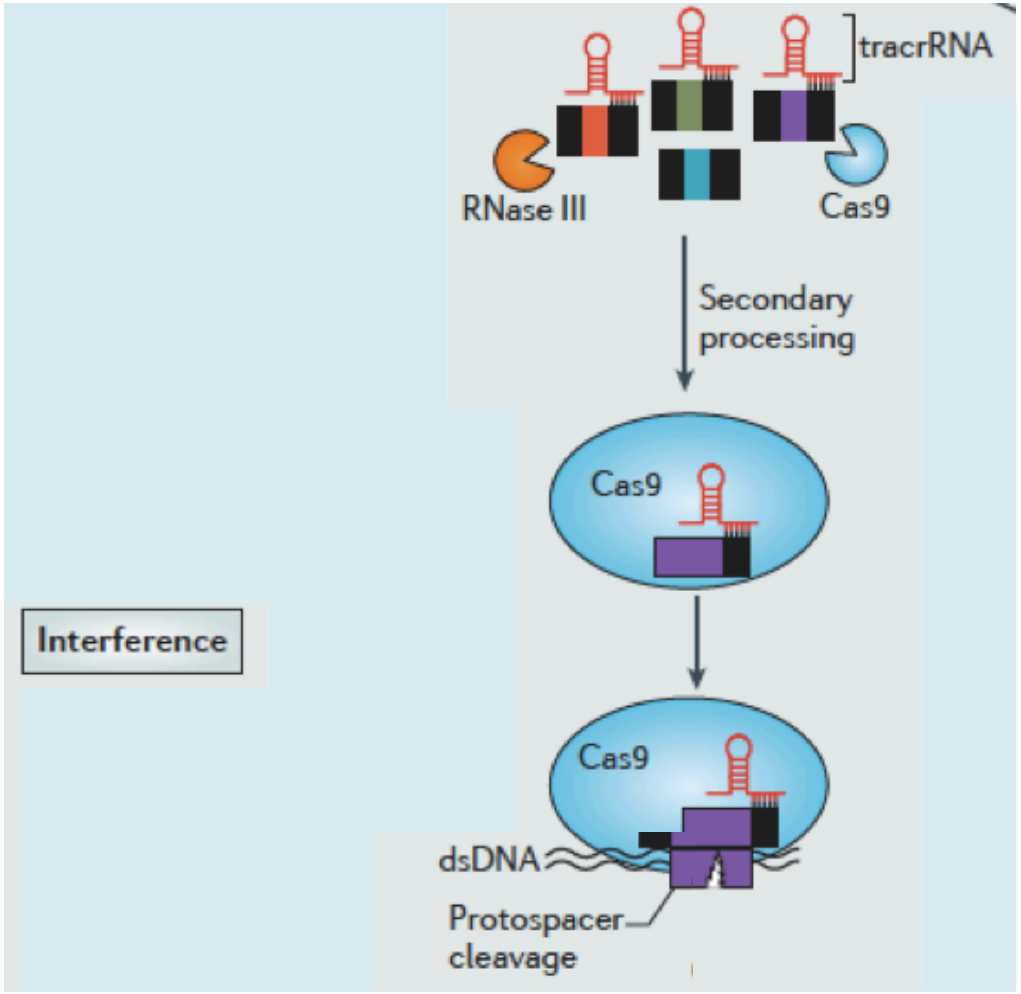
Phage DNA recognized and fragmented by restriction enzyme system, then ‘spacers’ incorporated into bacterial genome

CRISPR system: expression



Cas9 and Rnase III involved in processing pre-crRNA, then Cas9 forms complex with crRNA and tracrRNA

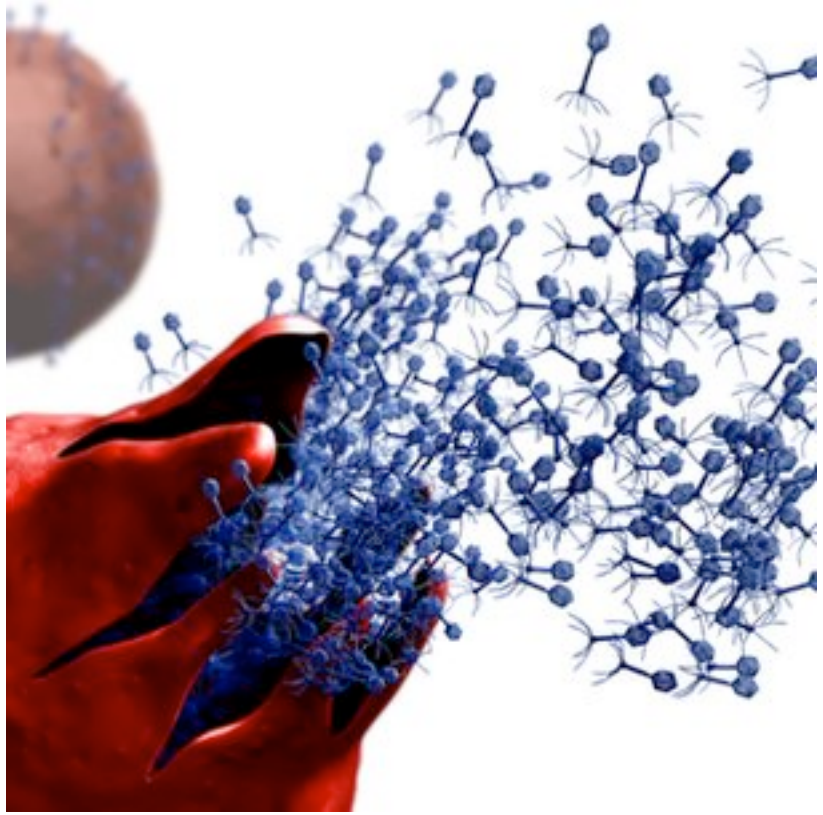
CRISPR system: interference



Cas9 / tracrRNA / crRNA bind invading phage DNA and cleave at target sequence that is complementary to ‘spacer’ sequence

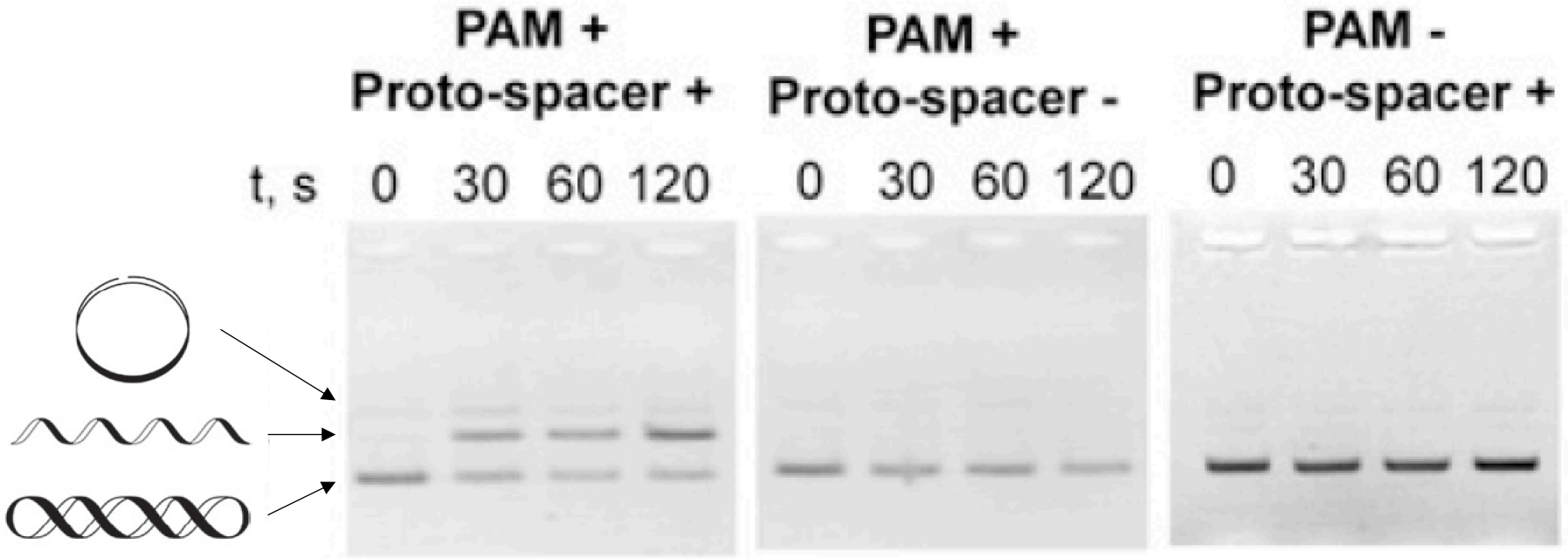
DNA vs RNA debate

Many in researchers in phage community convinced RNA interference by CRISPR too inefficient given explosive replication of phage during infection



What data support that Cas9 cleaves DNA?

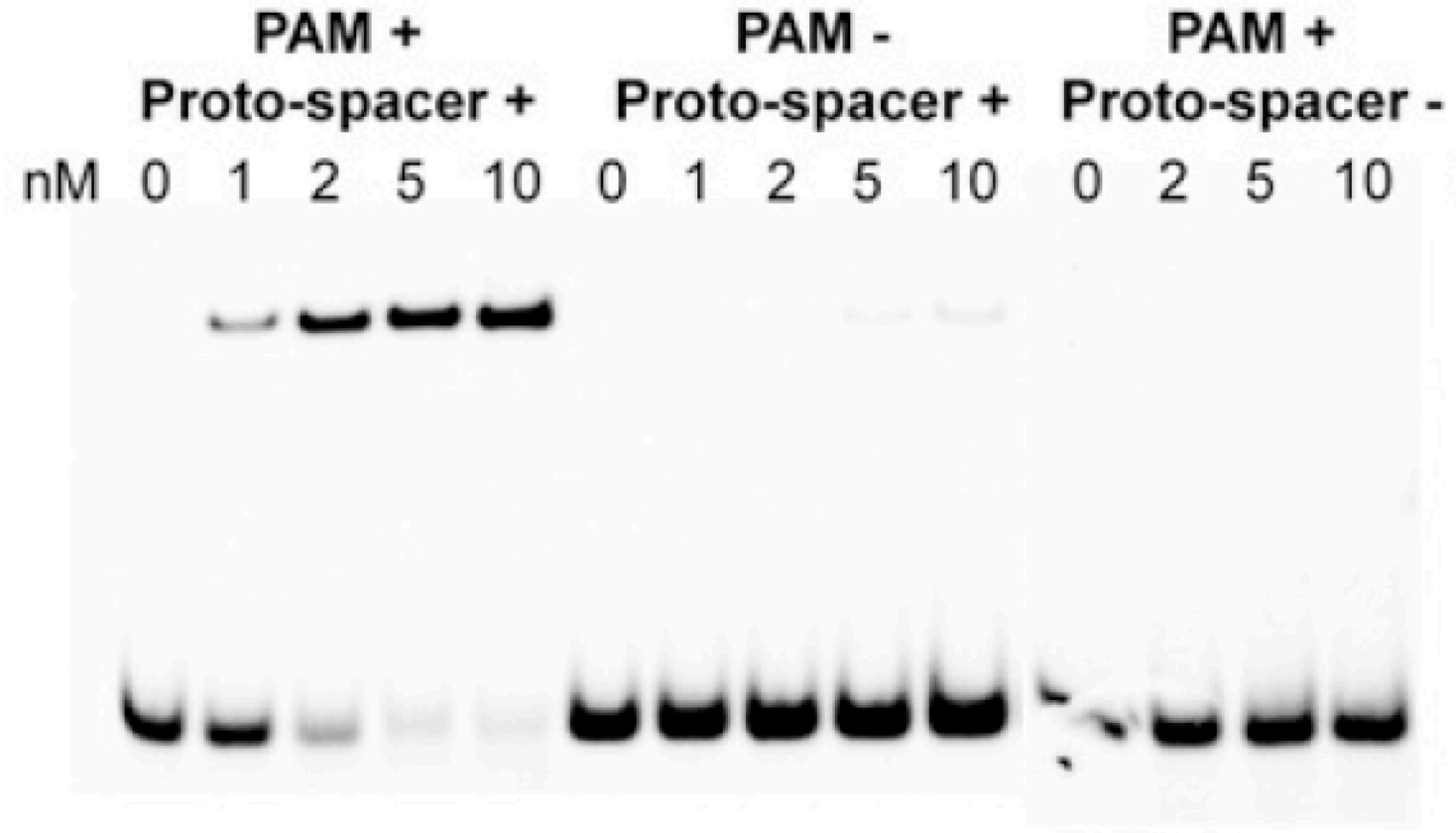
CRISPR / Cas9 recognizes ‘non-self’ DNA



Gasiunas et al. (2012) *Proc Nat Acad Sci*. 109:E2579-2586.

PAM required for DNA binding

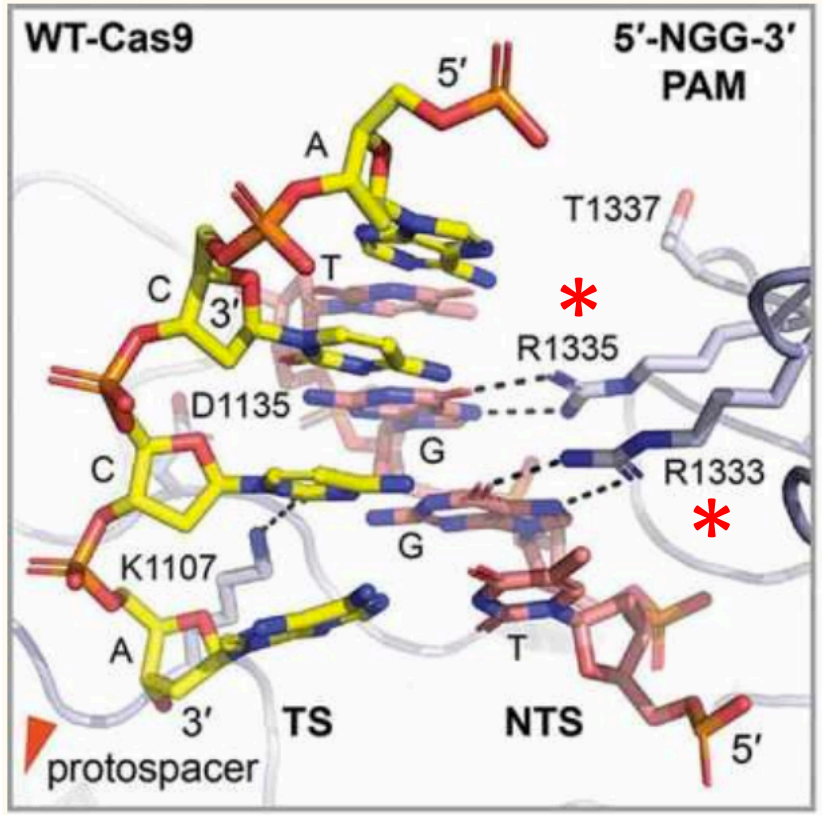
- dsDNA substrate was radio-labeled
- Cas9 protein added at increasing concentrations
- Binding observed via EMSA



Gasiunas et al. (2012) *Proc Nat Acad Sci*. 109:E2579-2586.

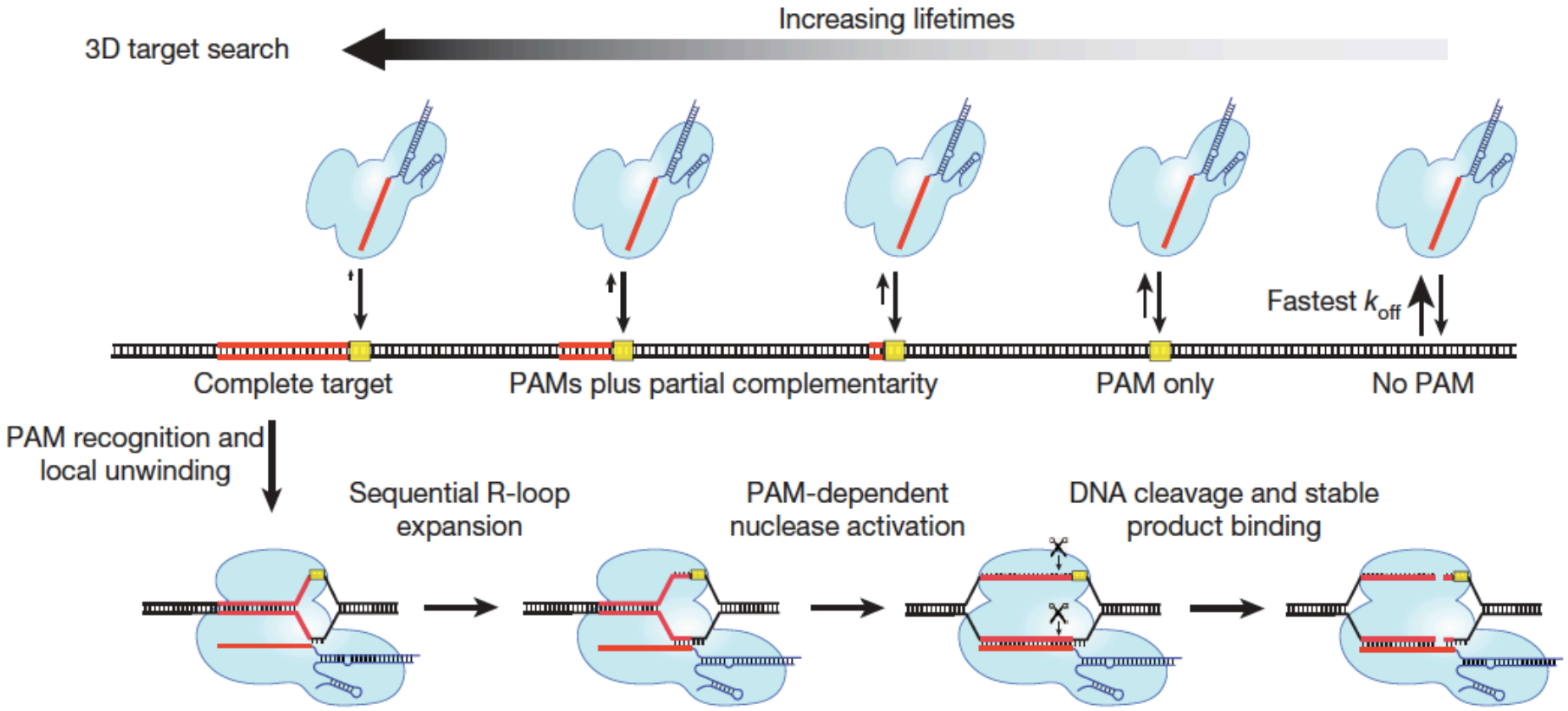
Cas9 contains PAM interacting (PI) domain

- Binding of tracrRNA-crRNA induces extensive conformational change in Cas9 structure
  - Results in formation of PI domain within the same lobe as the cleavage domain
- Two conserved arginine residues in PI domain interact with non-target strand GG bases
- Cleavage occurs 3 bp upstream of PAM sequence



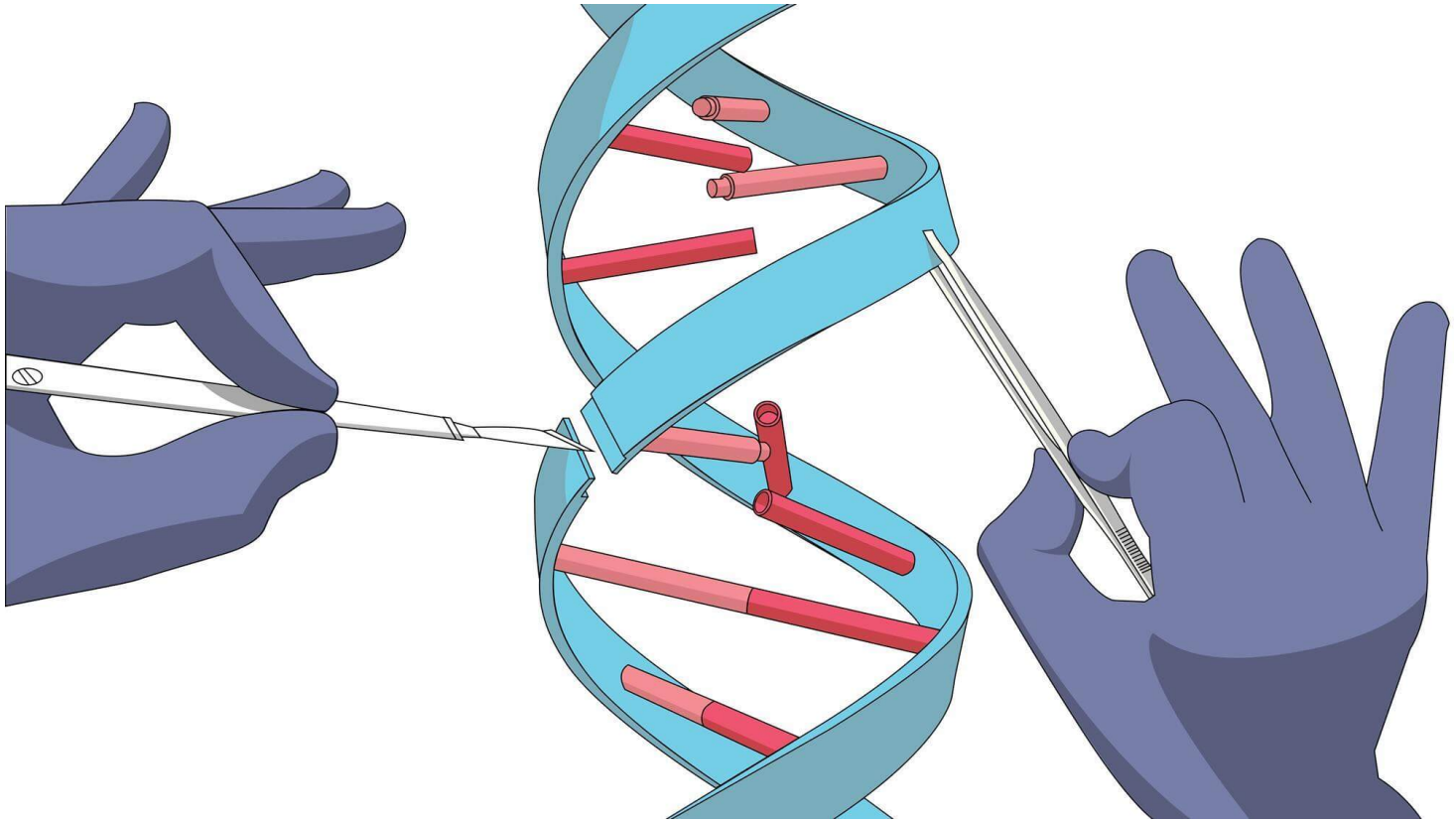
Gleditzsch et al. (2019) *RNA Biol*. 16:504-517.

Role of PAM in CRISPR system



Sternberg et al. (2014) *Nature*. 507:E62-67.

What features of CRISPR make it a useful tool in Biological Engineering?



Engineered CRISPRi system

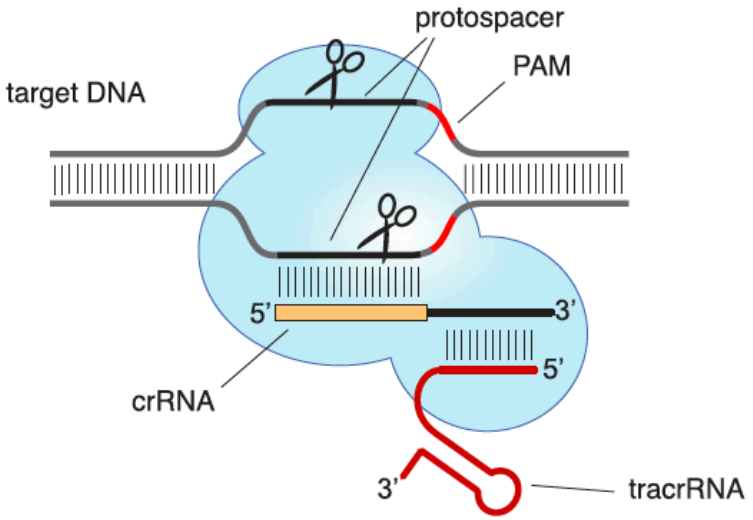
- Modifications to crRNA / tracrRNA complex?

- Modifications to Cas9?

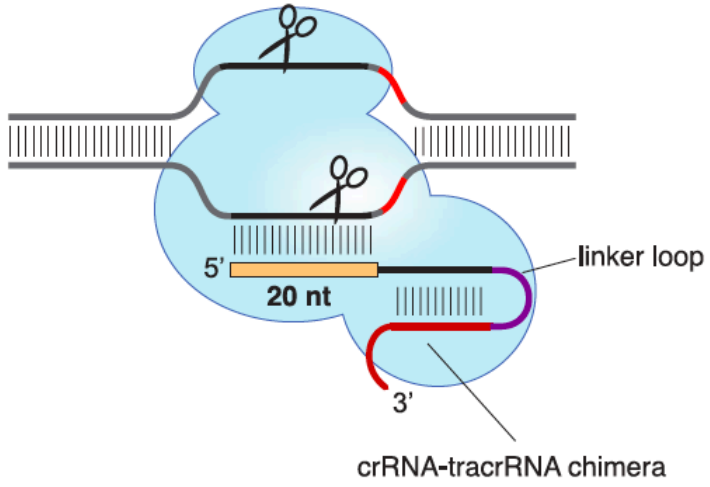
CRISPRi system: (s)gRNA

- (s)gRNA molecule is a target sequence and tracrRNA fused by a linker loop such that a single transcript used to direct Cas9 cleavage

crRNA / tracrRNA complex

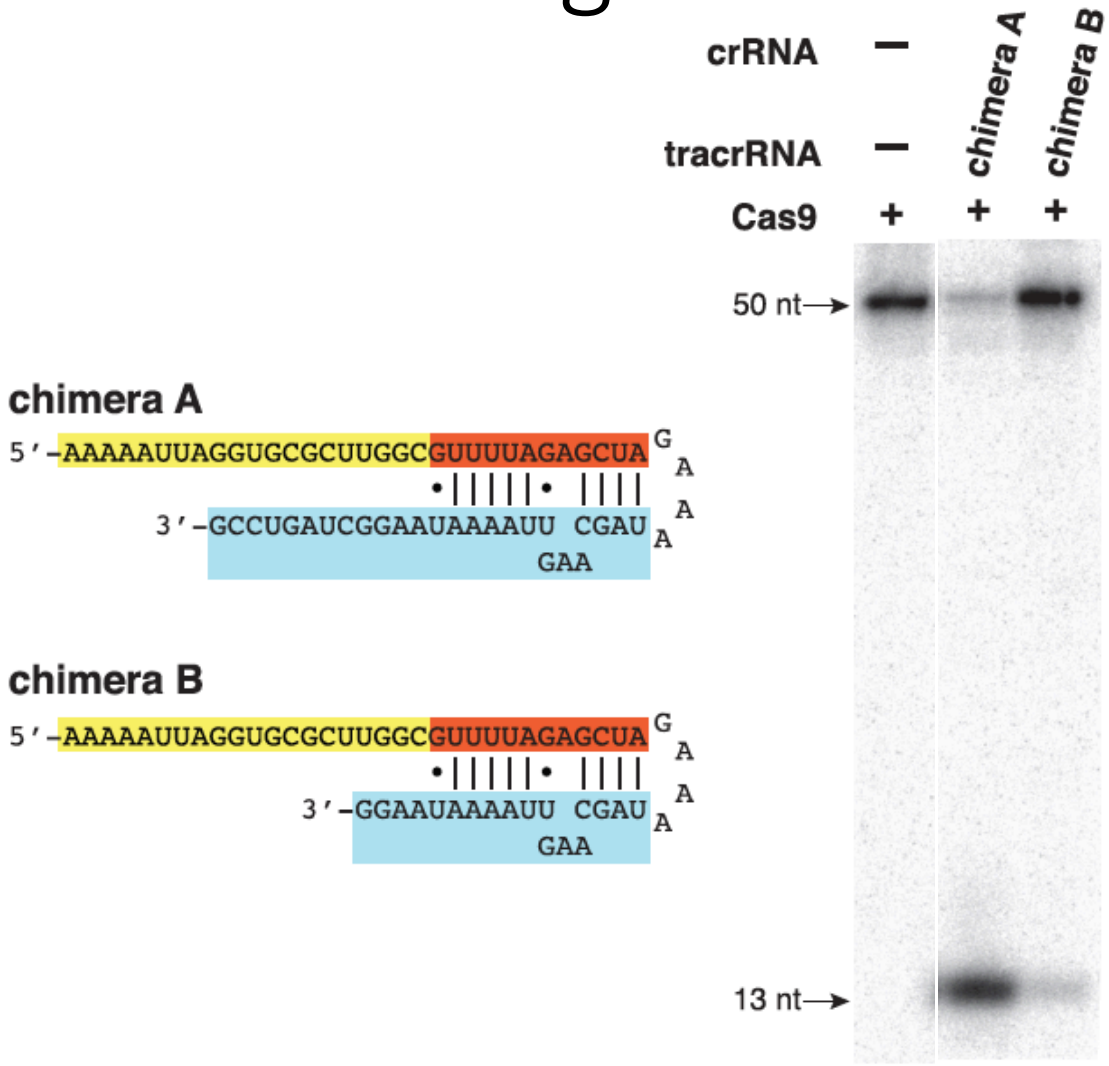


(s)gRNA



sgRNA able to target Cas9 cleavage

- 3’ end of crRNA fused to 5’ end of tracrRNA to generate chimera molecules (sgRNAs)
- dsDNA substrate 5’-labeled for cleavage assay

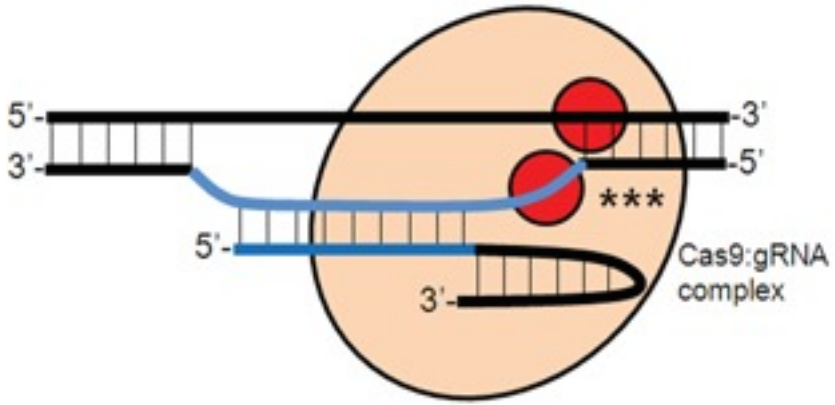


Jinek et al. (2012) *Science*. 337:816-820.

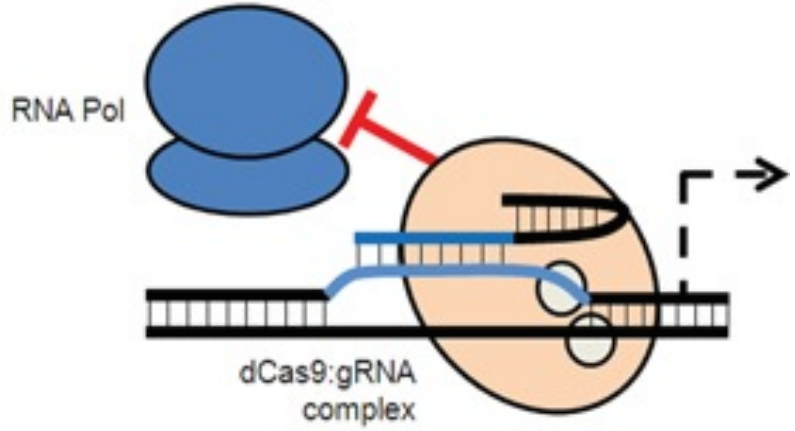
CRISPRi system: dCas9

- dCas9 protein contains mutated residues D10A and H840A that render it catalytically inactive and unable to cleave DNA, but still able to bind DNA

<u>Cas9</u>



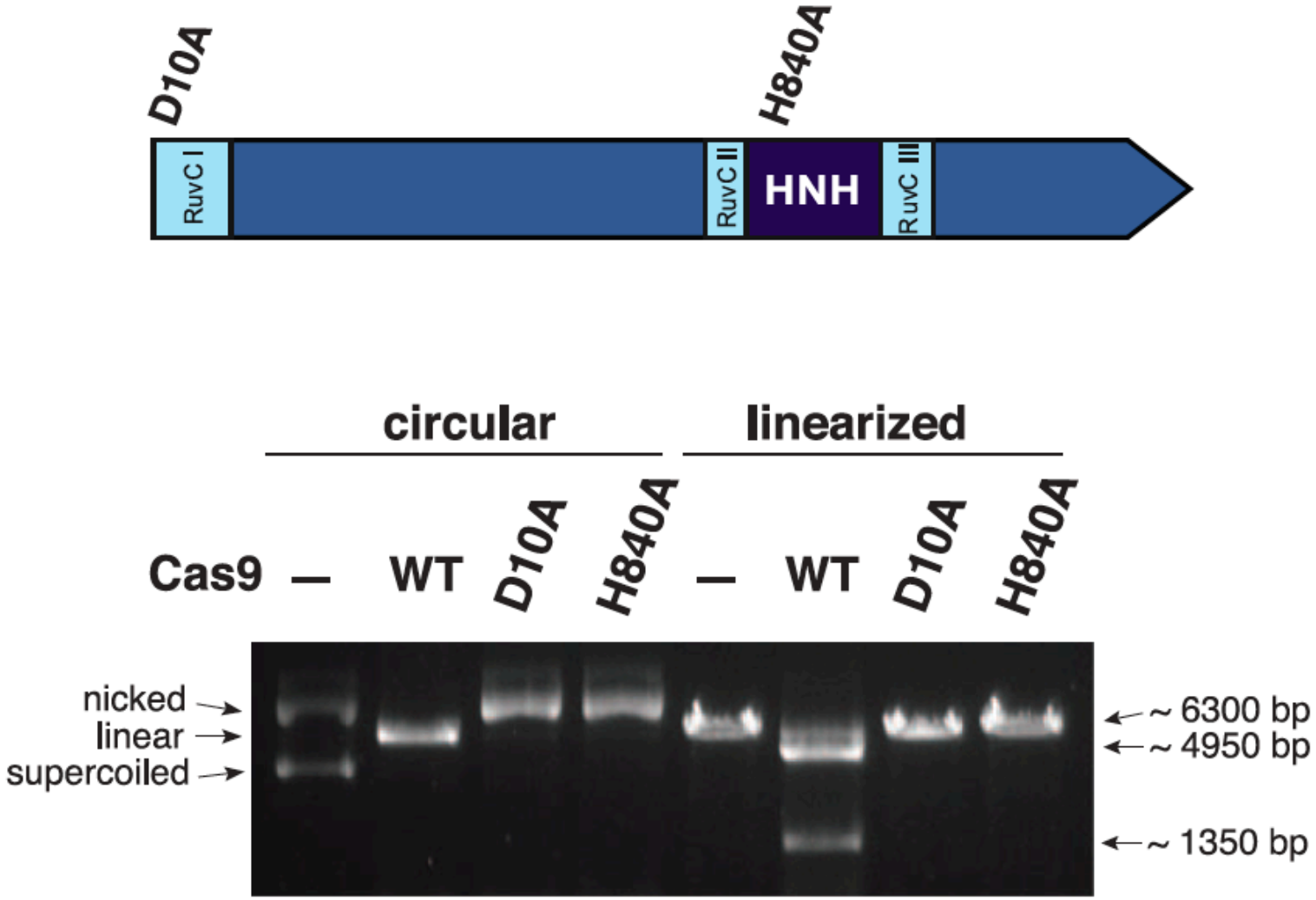
<u>dCas9</u>



HNH and RuvC endonuclease domains

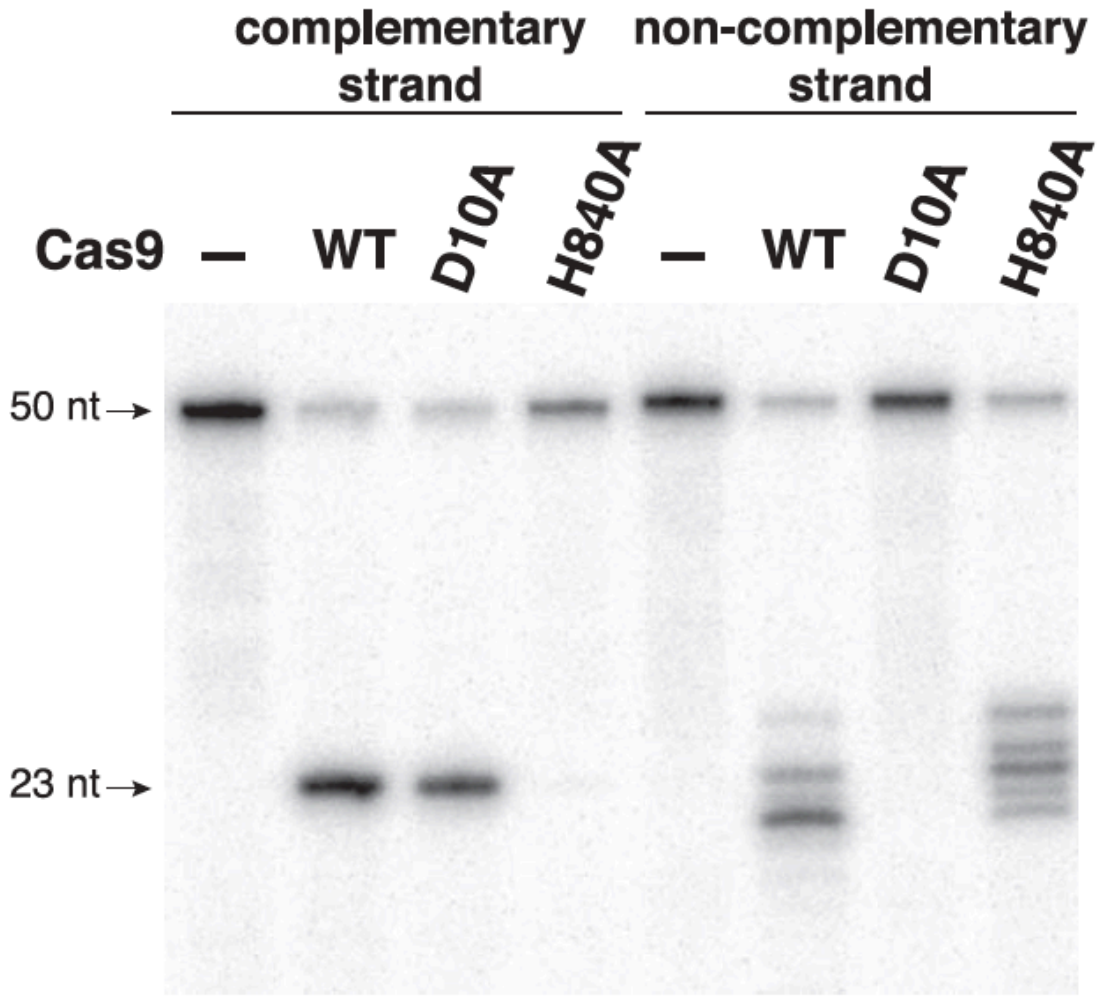
- RuvC
  - Endonuclease that resolves Holliday structure, intermediate structure in which dsDNA molecule is linked by single-stranded crossover
- HNH
  - Found in homing endonucleases, restriction endonucleases, transposases

Cleavage requires HNH and RuvC domains



Jinek et al. (2012) *Science*. 337:816-820.

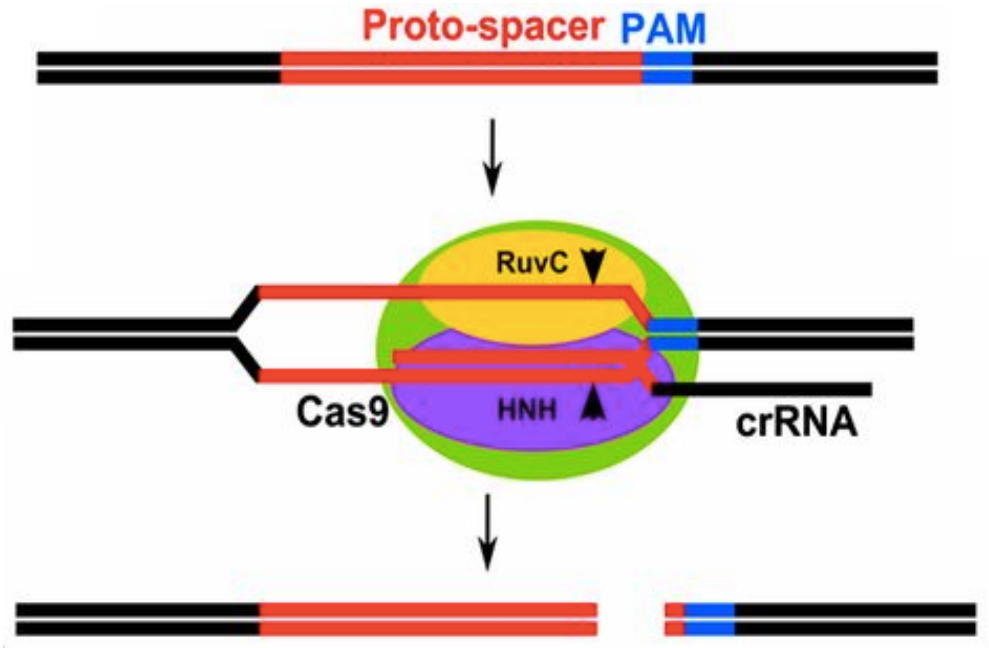
HNH and RuvC domains target specific DNA strands



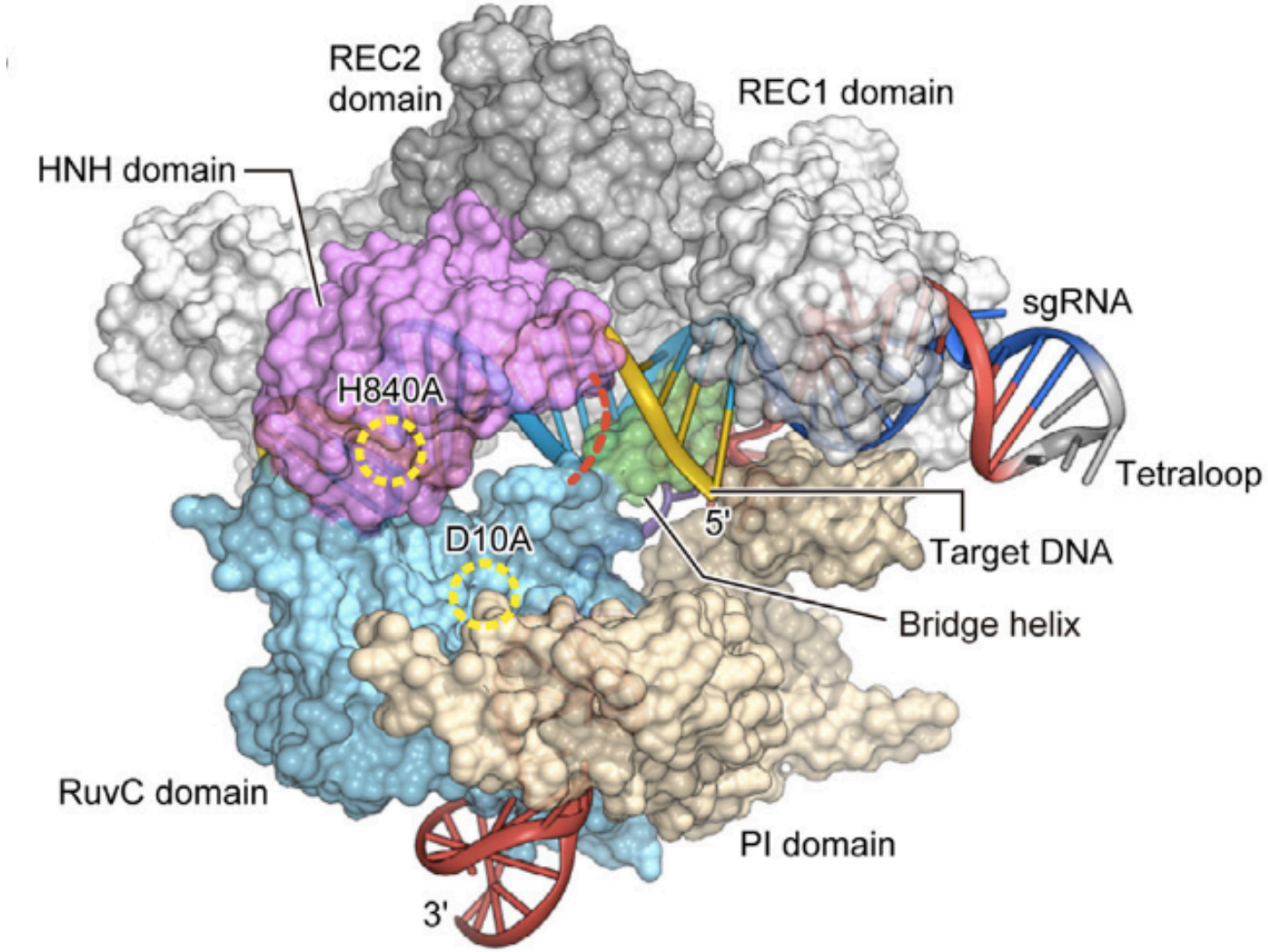
Jinek et al. (2012) *Science*. 337:816-820.

Schematic of Cas9 DNA cleavage

- RuvC domain (D10A) cleaves non-coding strand
- HNH domain (H840A) cleaves coding strand
- Results in blunt end cut 3 bp from PAM site



Crystal structure of Cas9 / sgRNA complex



In the laboratory…

- Mini-prep psgRNA_target clones
- Co-transform CRISPRi system components into competent *E. coli* MG1655 cells
  - pdCas9
  - psgRNA_target
- Send psgRNA_target clones for sequencing