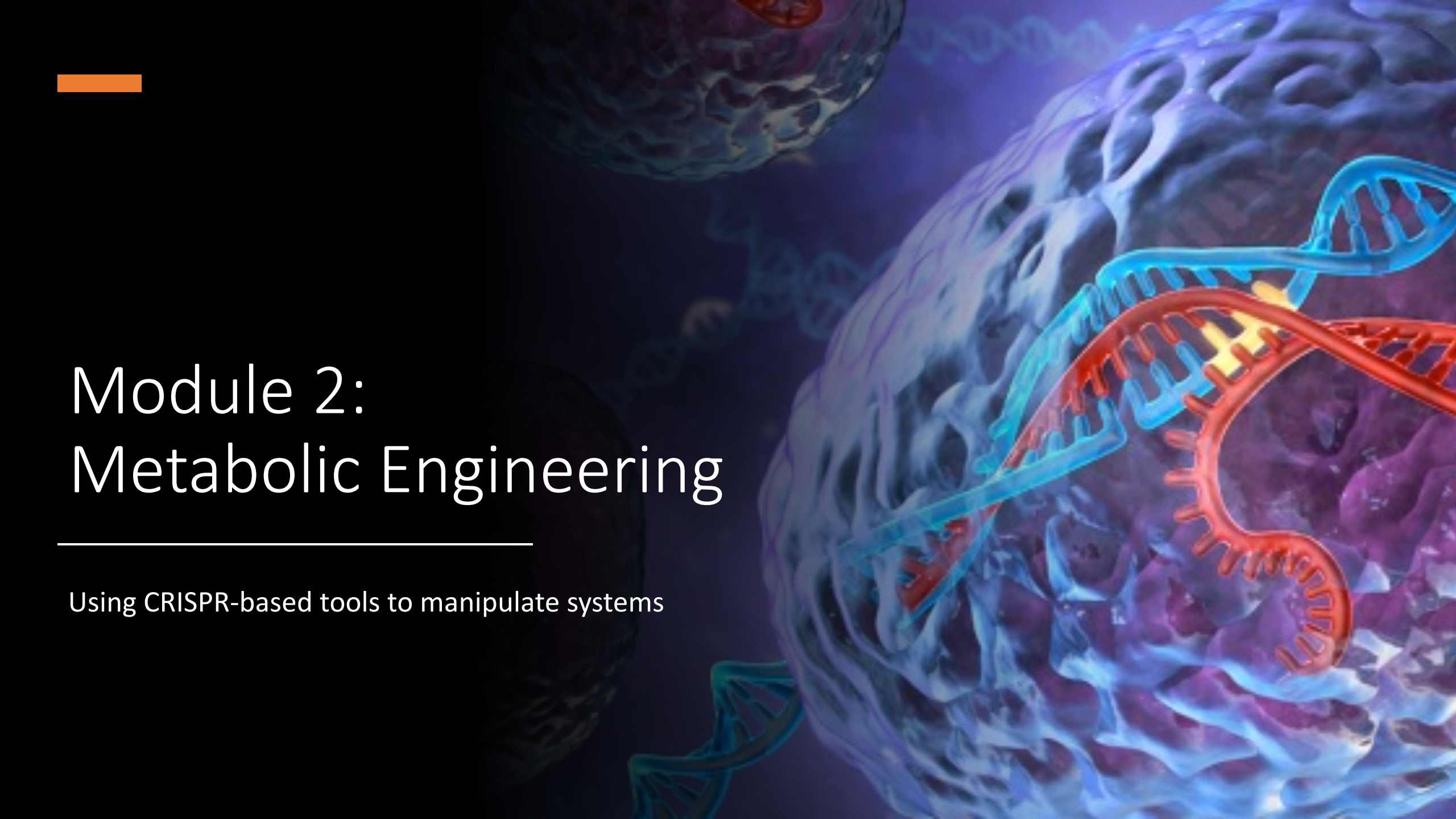
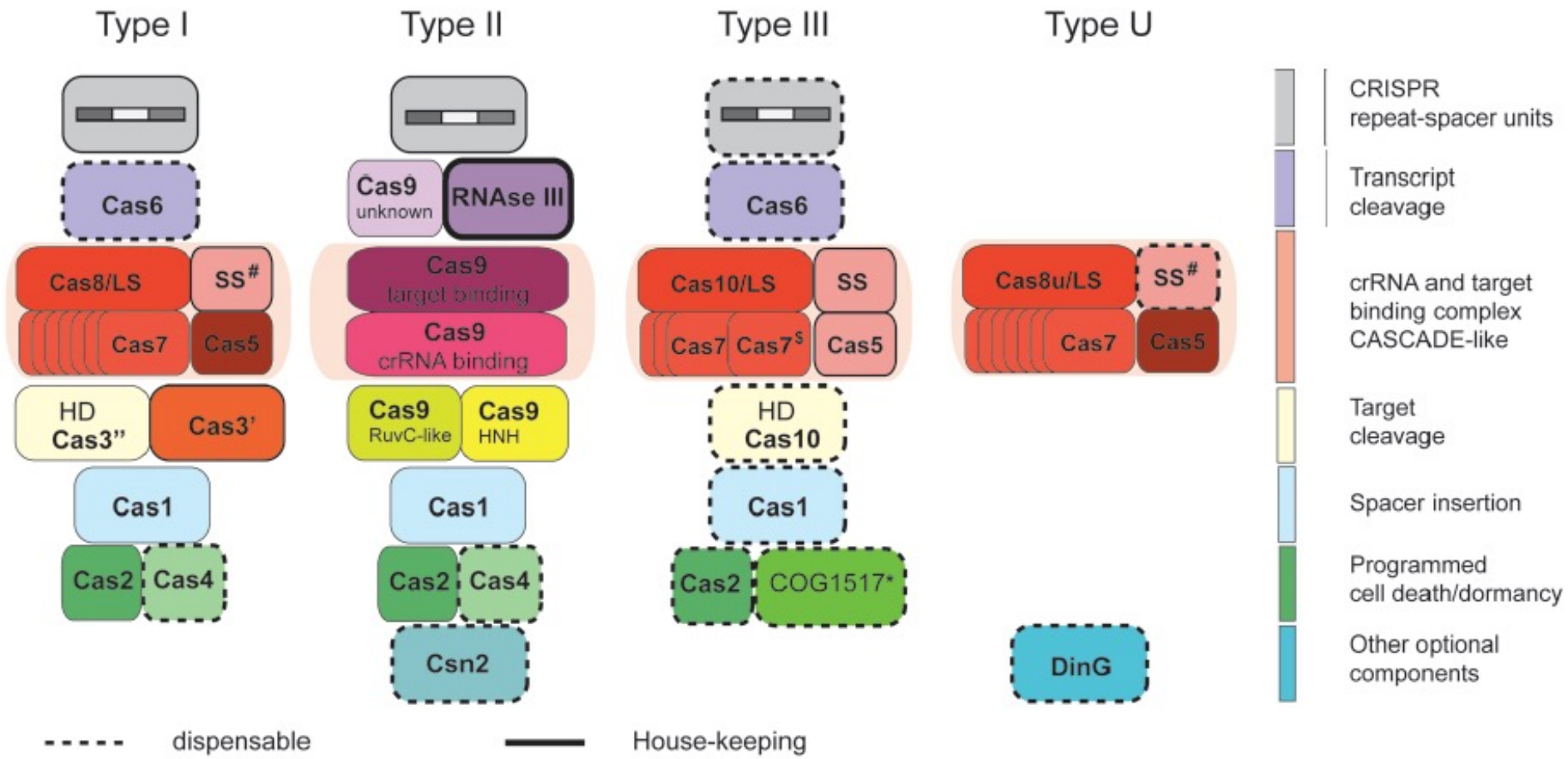


Module 2: Metabolic Engineering

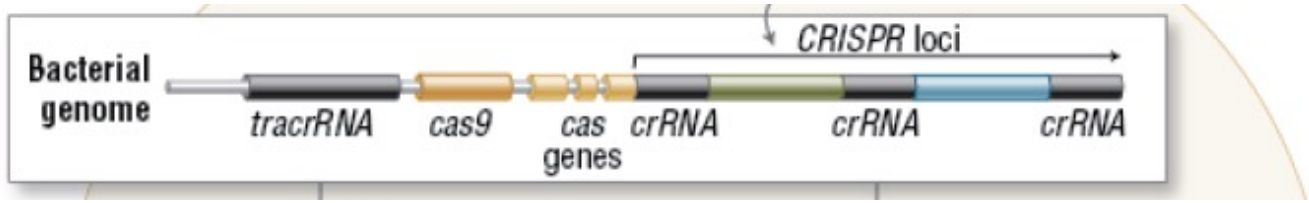
Using CRISPR-based tools to manipulate systems

Several CRISPR systems have been identified

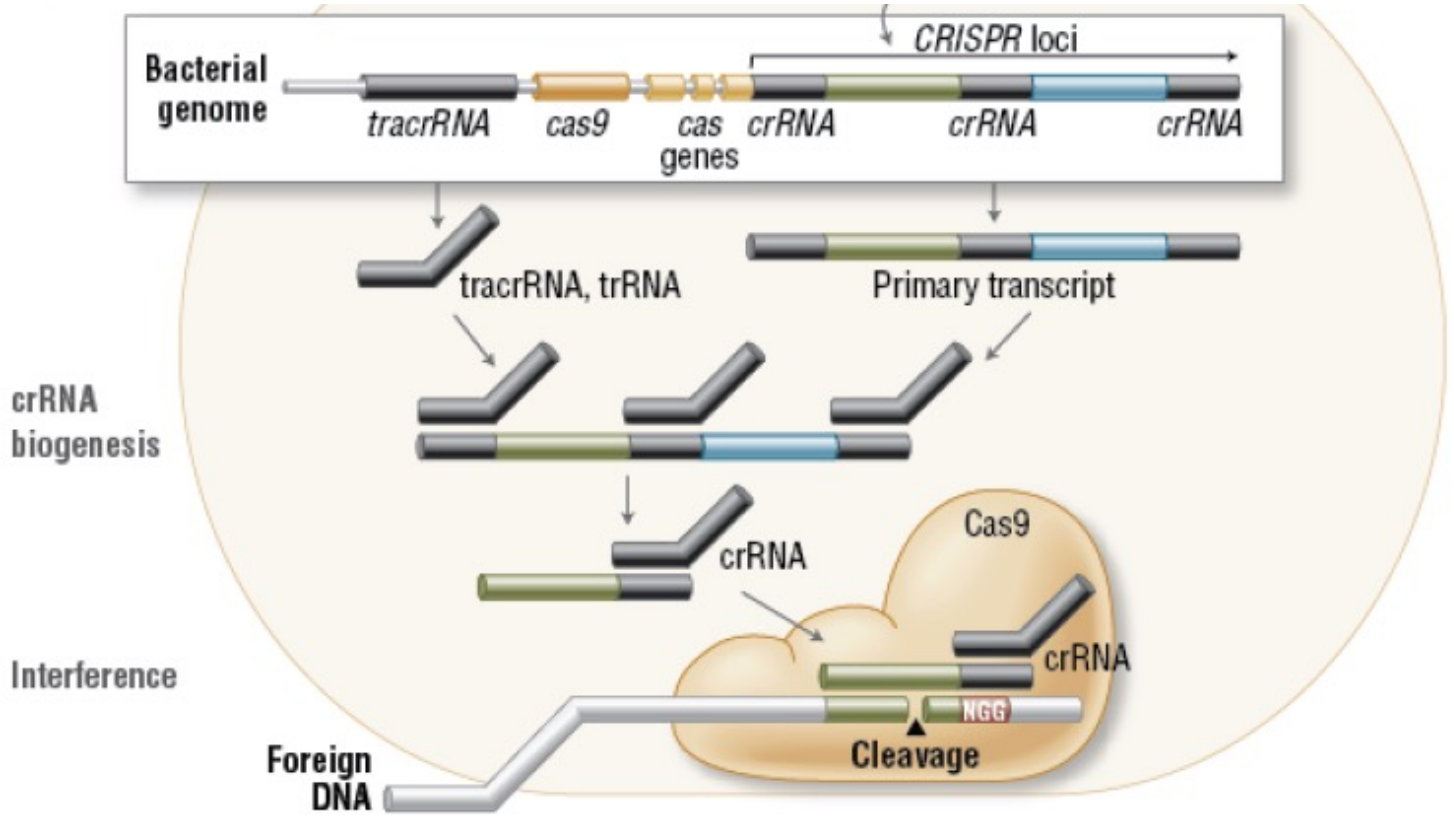


Koonin and Makarova. (2013) *RNA Biology*. 10:679-686.

Let’s review…


CRISPR loci
Bacterial genome
tracrRNA
cas9
cas genes
crRNA
crRNA
crRNA

Let’s review…


CRISPR loci
Bacterial genome
tracrRNA
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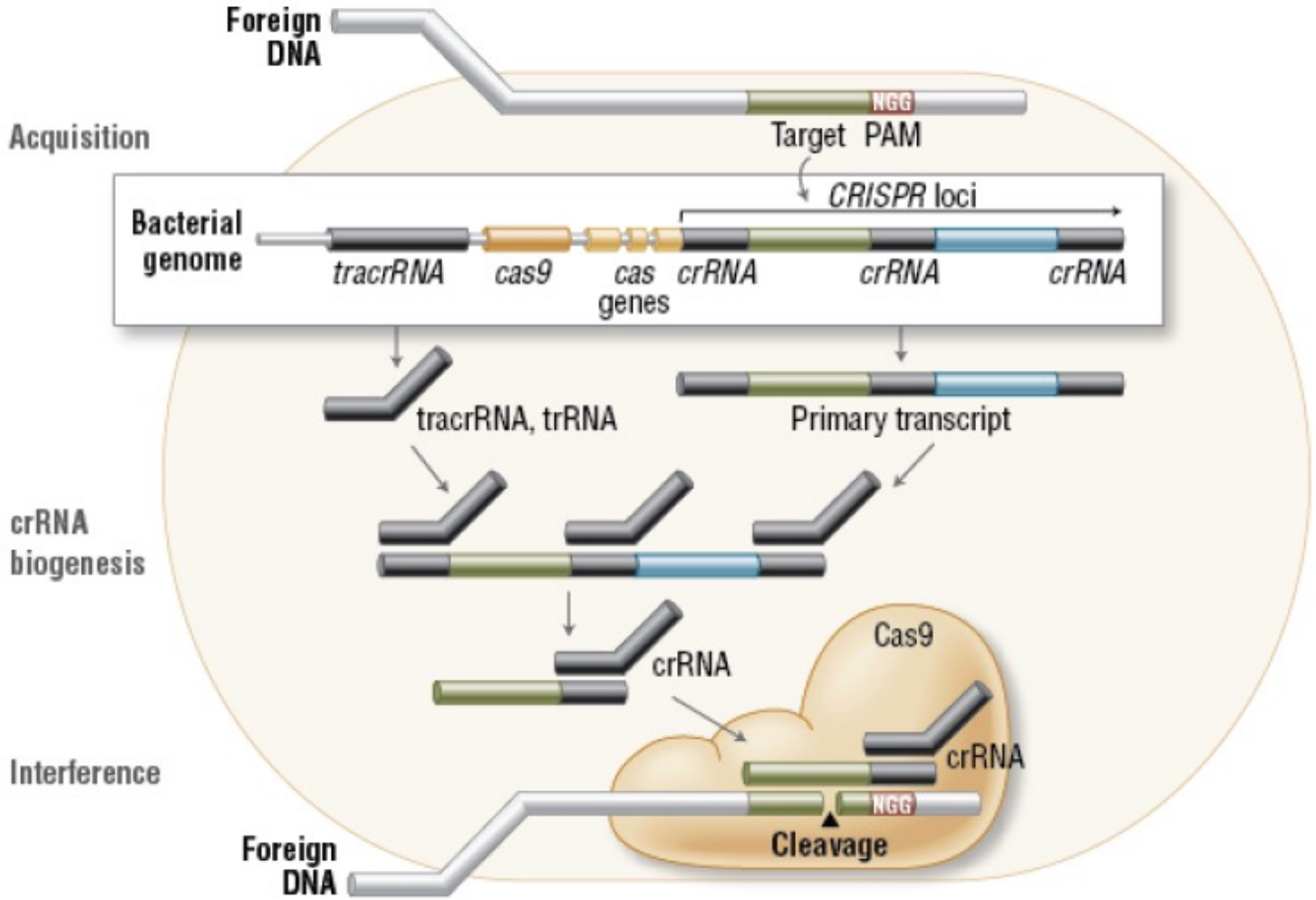
crRNA
crRNA
crRNA
tracrRNA, trRNA
Primary transcript
crRNA biogenesis
Cas9
crRNA
crRNA
Interference
NGG
Cleavage
Foreign DNA

Let’s review…


Foreign DNA
NGG
Target PAM
Acquisition
CRISPR loci
Bacterial genome
tracrRNA
cas9
cas genes
crRNA
crRNA
crRNA
tracrRNA, trRNA
Primary transcript
crRNA biogenesis
Cas9
crRNA
crRNA
Interference
NGG
Cleavage
Foreign DNA

How does the engineered CRISPRi system differ from native CRISPR?

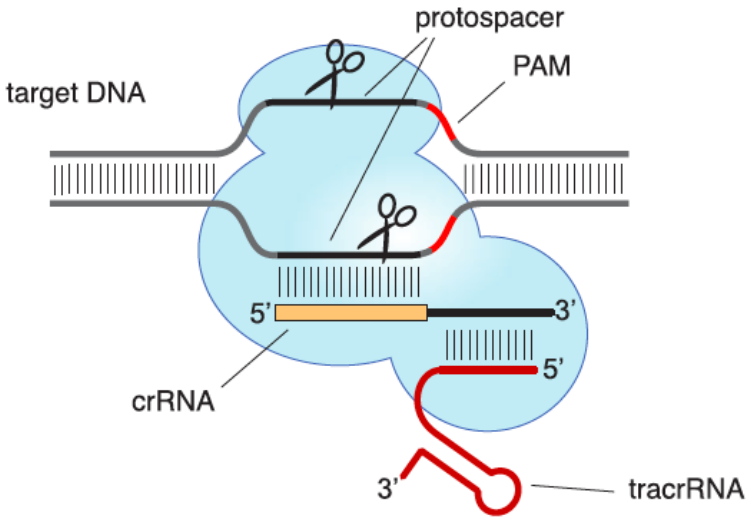
- Modifications to crRNA / tracrRNA complex

- Modifications to Cas9

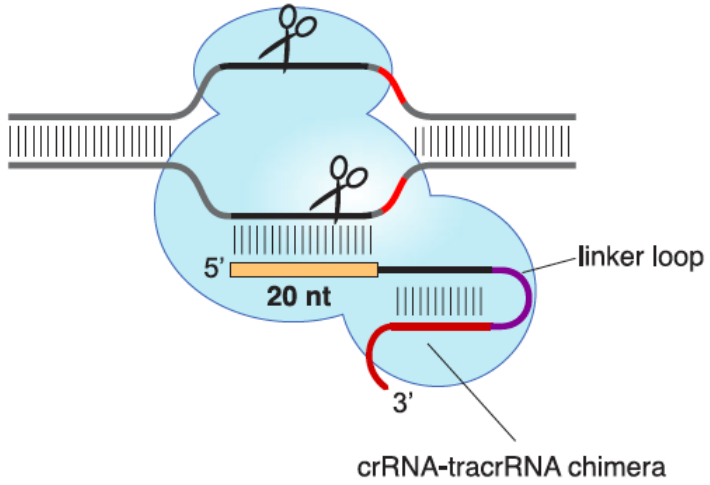
CRISPRi system: sgRNA

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

<u>crRNA / tracrRNA complex</u>

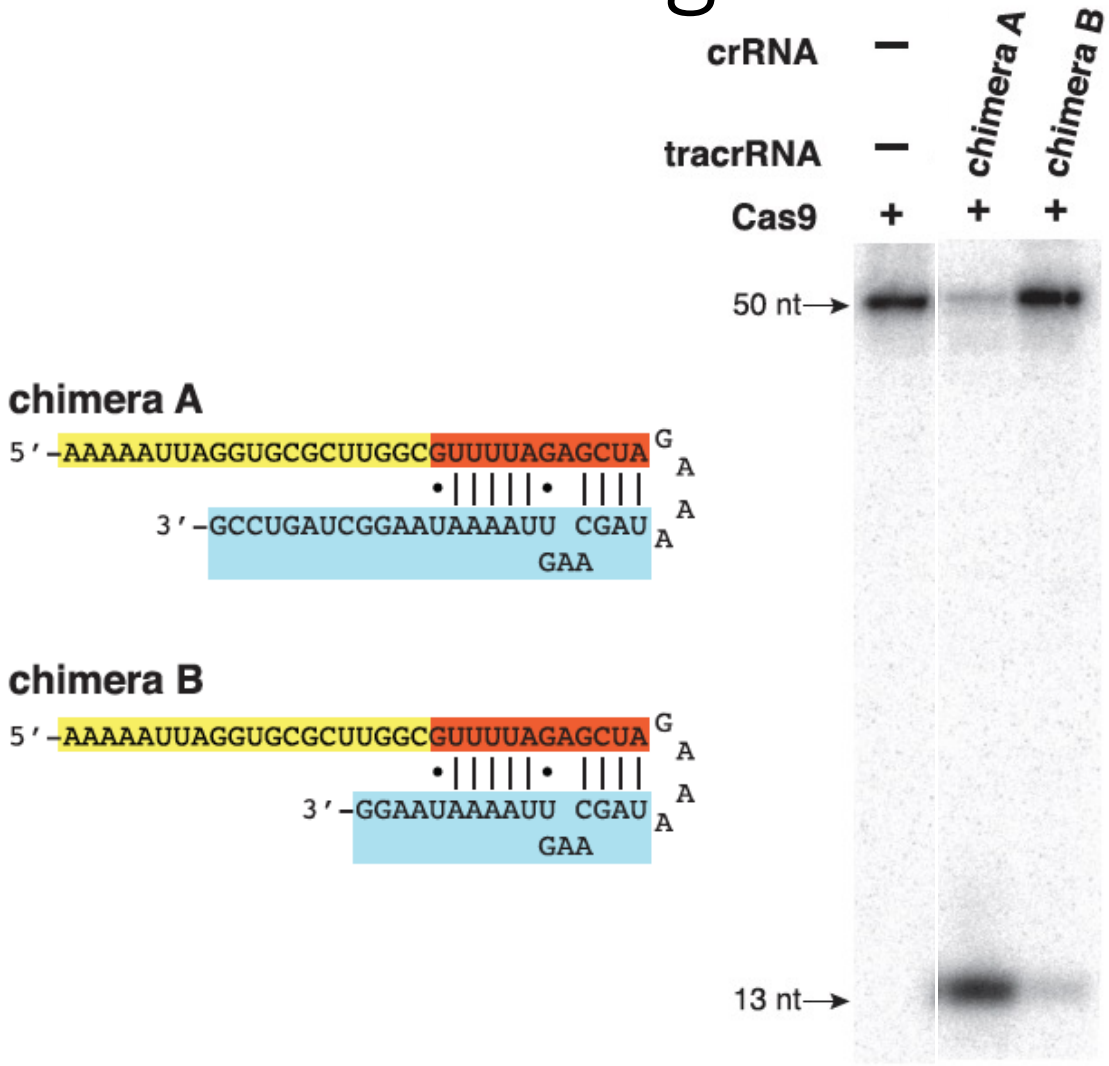


<u>(s)gRNA</u>



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

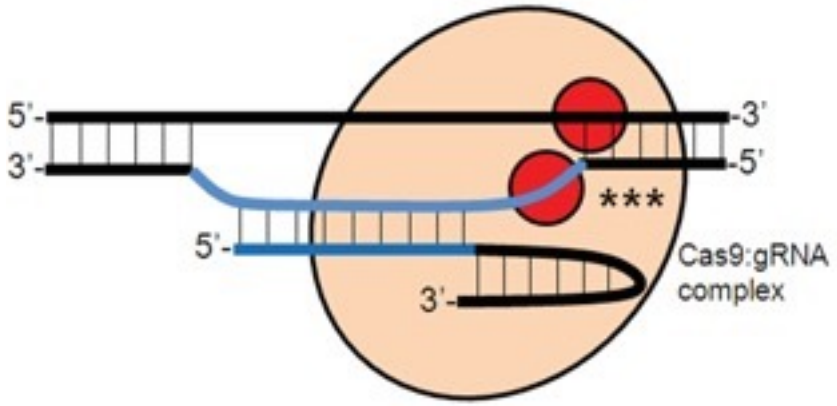


Jinek et al. (2012) *Science*. 337:816-820.

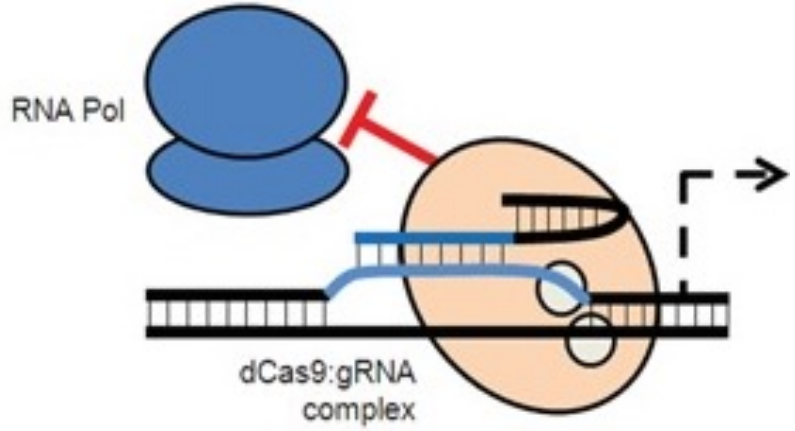
CRISPRi system: dCas9

- 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>



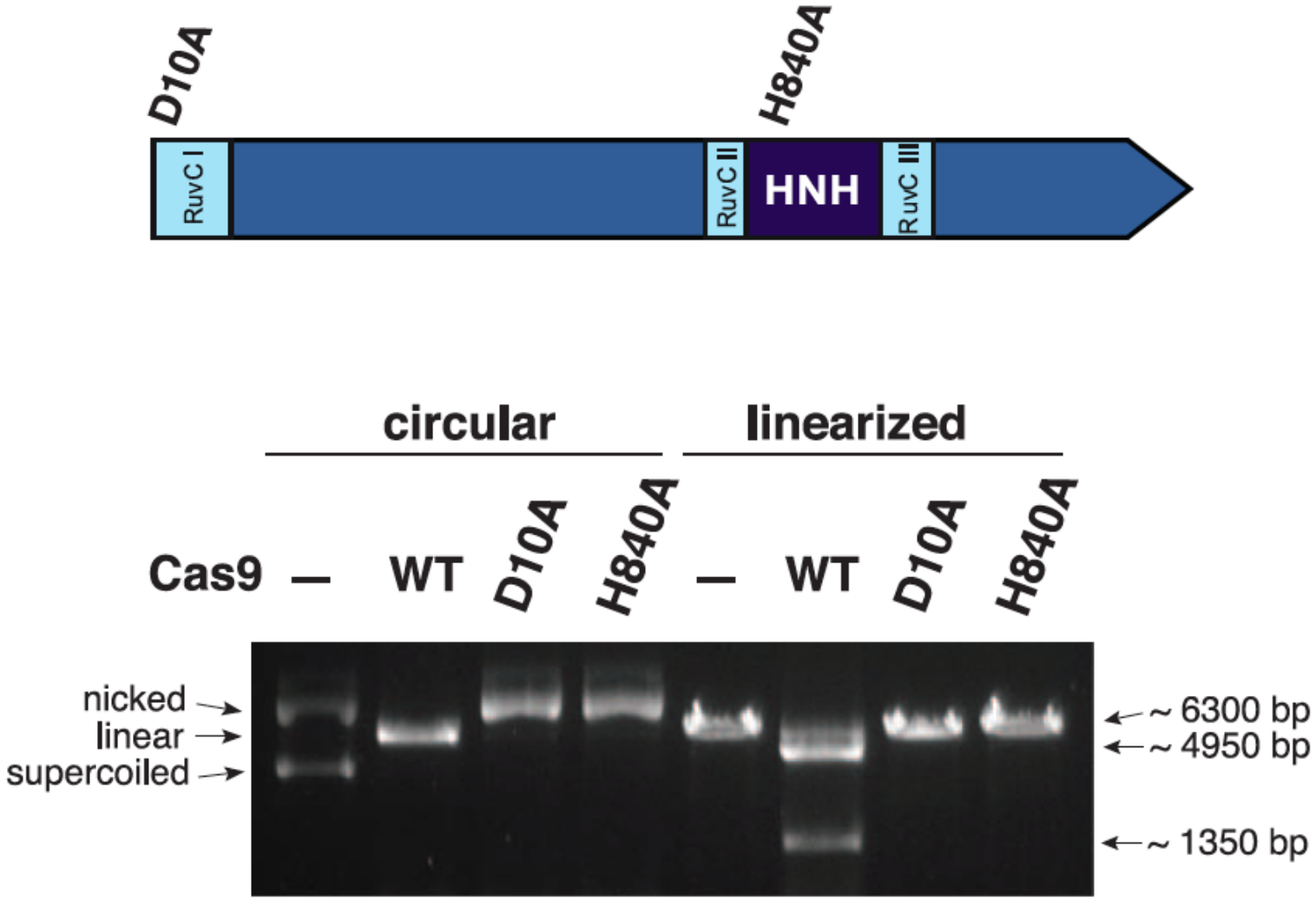
<u>dCas9</u>



HNH and RuvC endonuclease domains

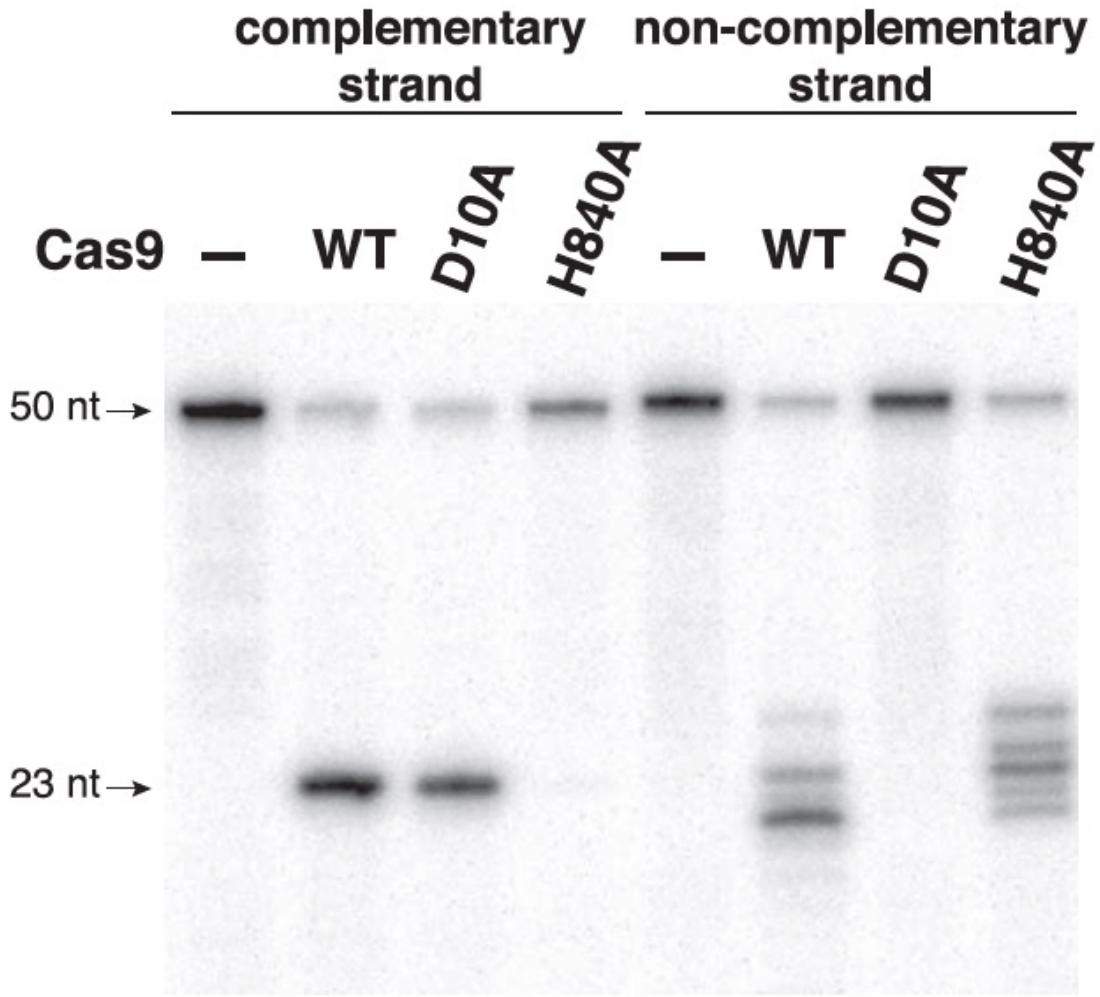
- RuvC
  - Endonuclease that resolves Holliday structure, intermediate structure in which dsDNA molecule is linked by single-stranded crossover
- HNH
  - Found in homing endonucleases, restriction endonucleases, transposases

Cleavage requires HNH and RuvC domains



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

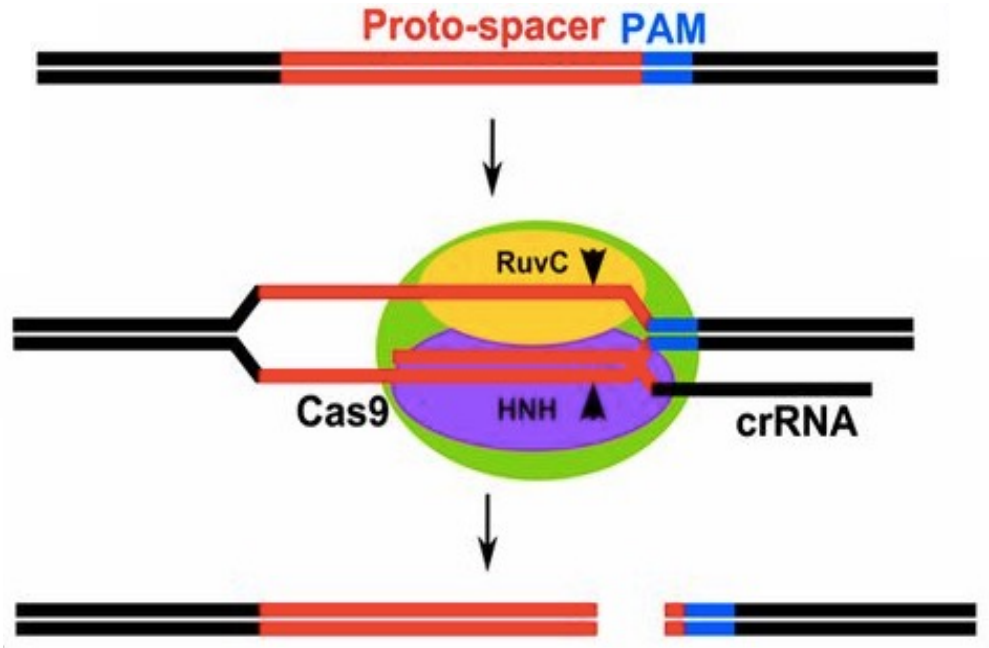
HNH and RuvC domains target specific DNA strands



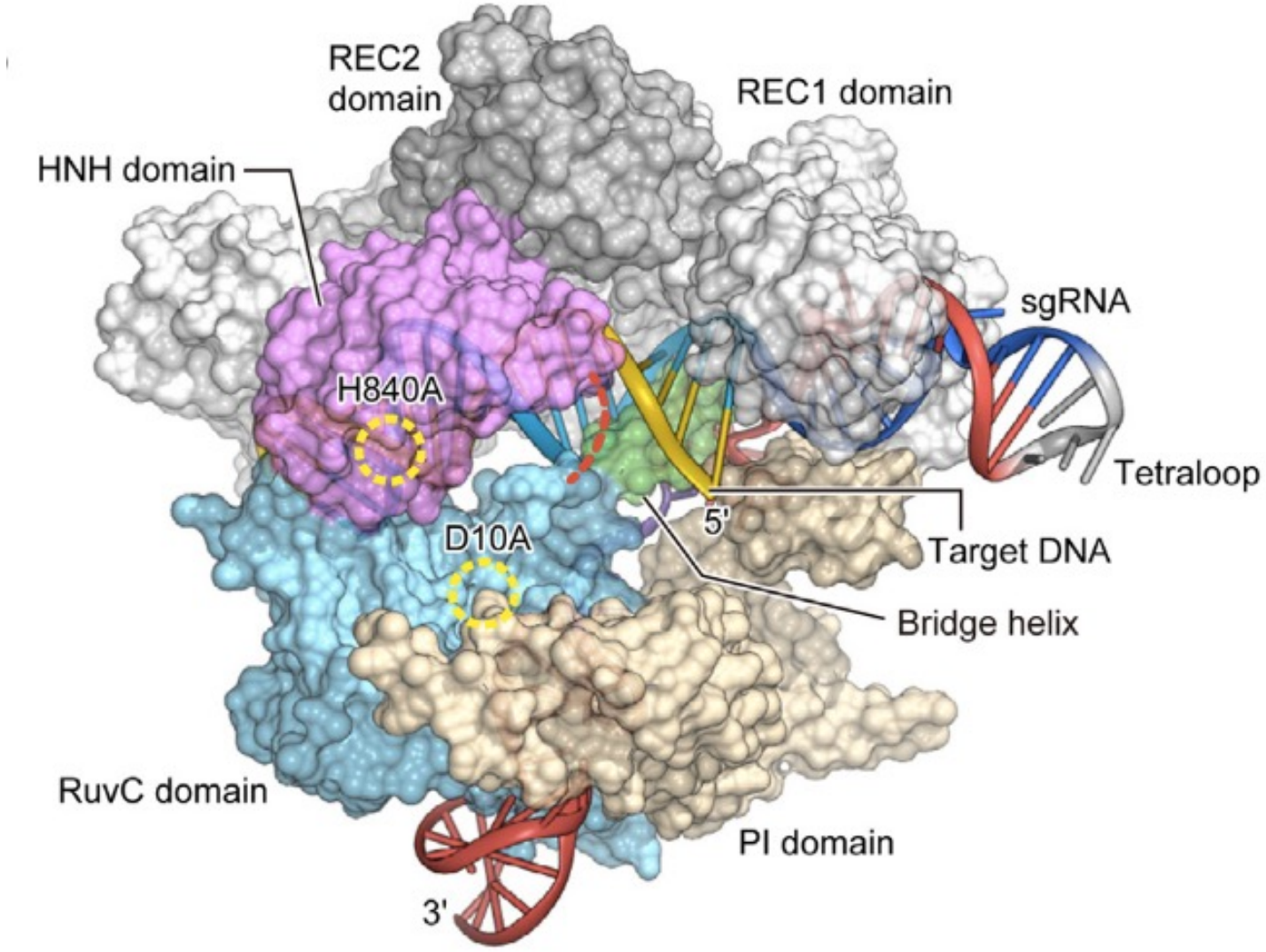
Jinek et al. (2012) *Science*. 337:816-820.

Schematic of Cas9 DNA cleavage

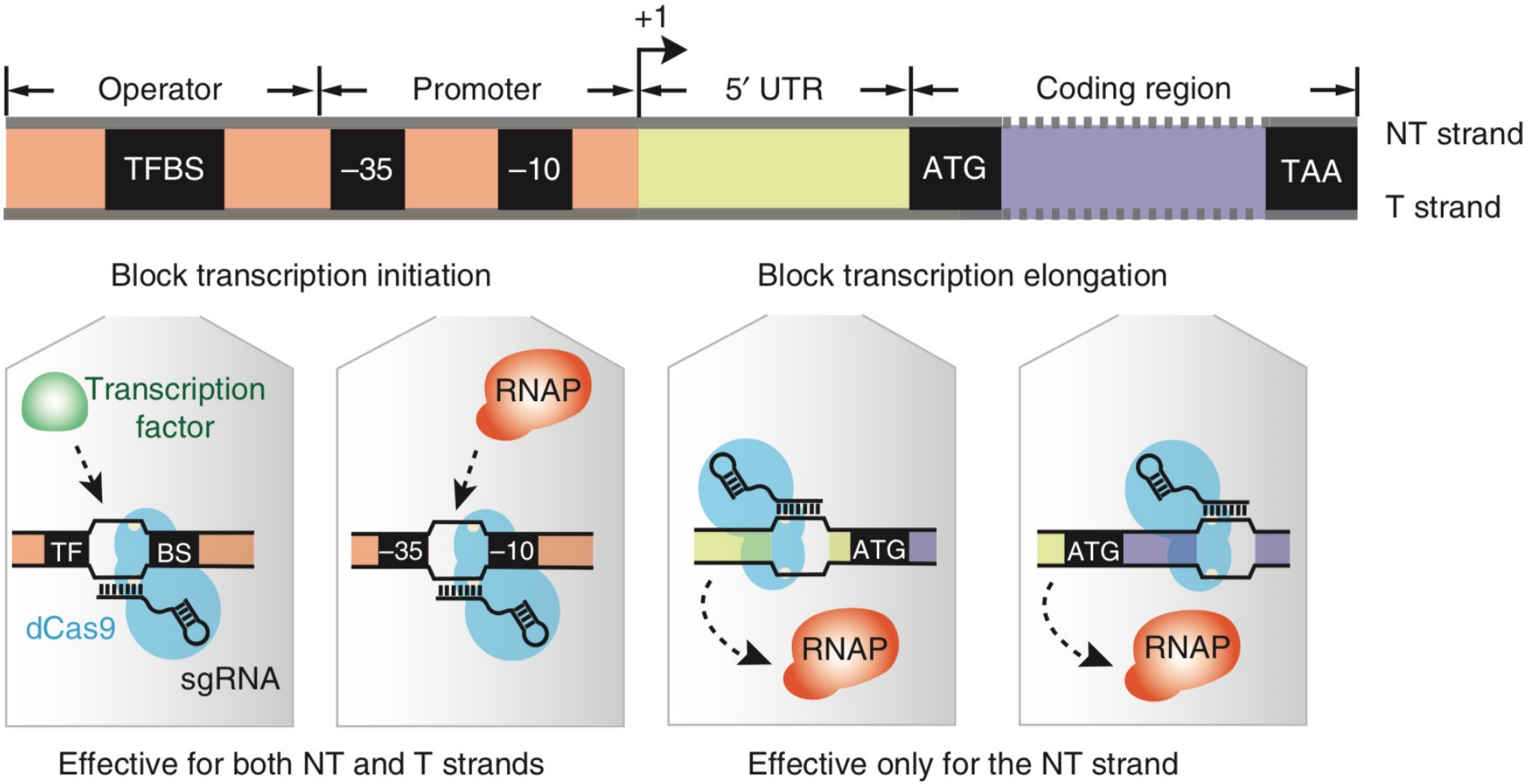
- RuvC domain (D10A) cleaves non-template / coding strand
- HNH domain (H840A) cleaves template / non-coding strand
- Results in blunt end cut 3 bp from PAM site



Crystal structure of Cas9 / sgRNA complex

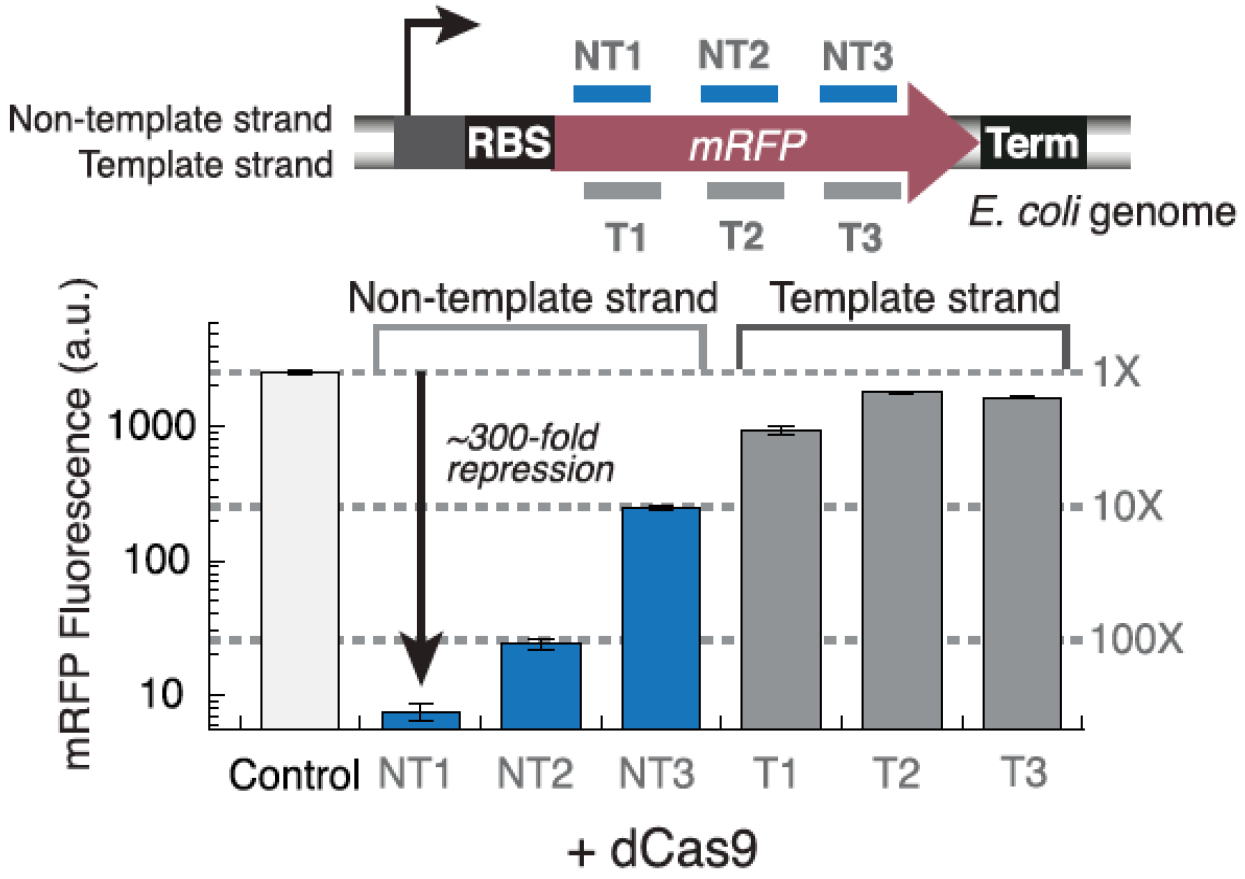


CRISPRi inhibition of gene expression



Targeting the coding region with CRISPRi system

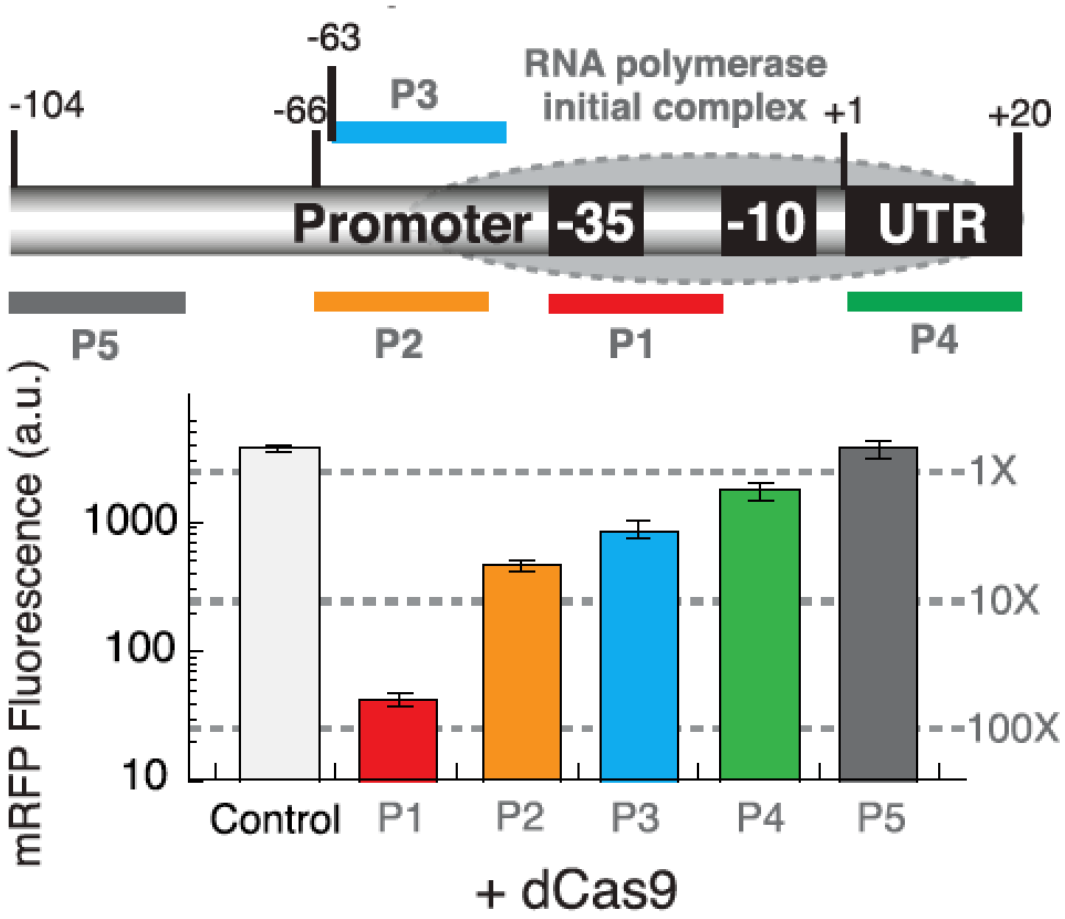
- sgRNA sequences designed to target coding region upstream within gene that encodes RFP
  - sgRNA sequences specific to non-template (NT1 – NT3) and template strand (T1 – T3)



Lei et al. (2013) *Cell*. 152:1173-1183.

Targeting the promoter with CRISPRi svstem

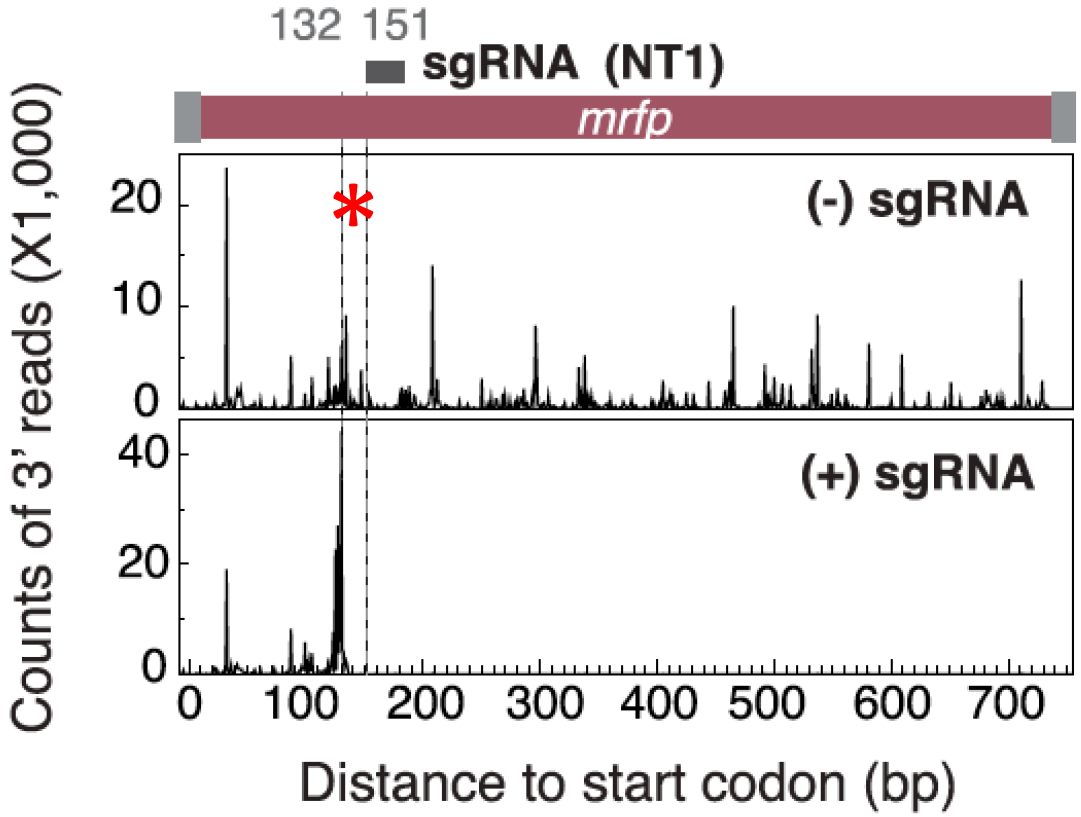
- sgRNA sequences designed to target promoter region upstream of gene that encodes RFP
  - sgRNA sequences = P1 – P5



Lei et al. (2013) *Cell*. 152:1173-1183.

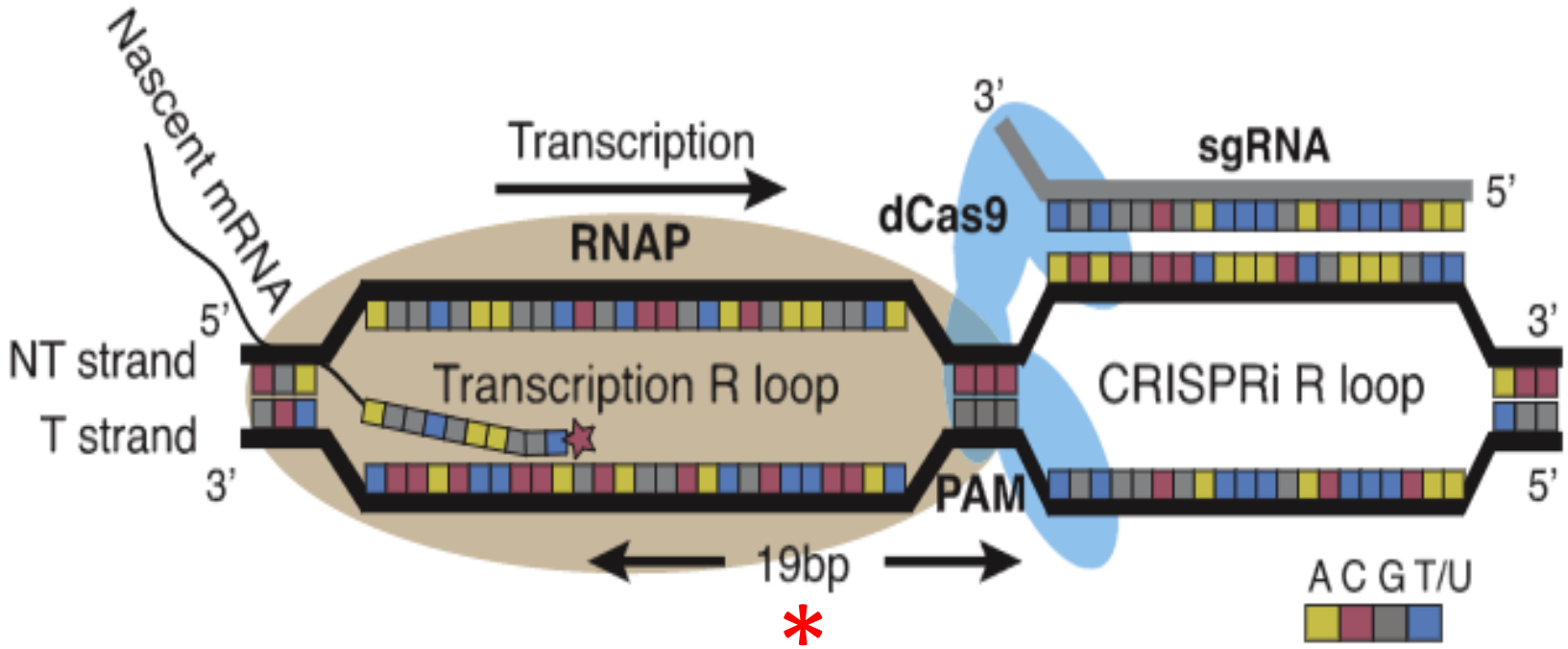
CRISPRi blocks transcription

- FLAG-tagged RNAP separated from cellular components
- Associated / bound mRNA were sequenced
- mRNA specific to RFP counted and graphed according to read length



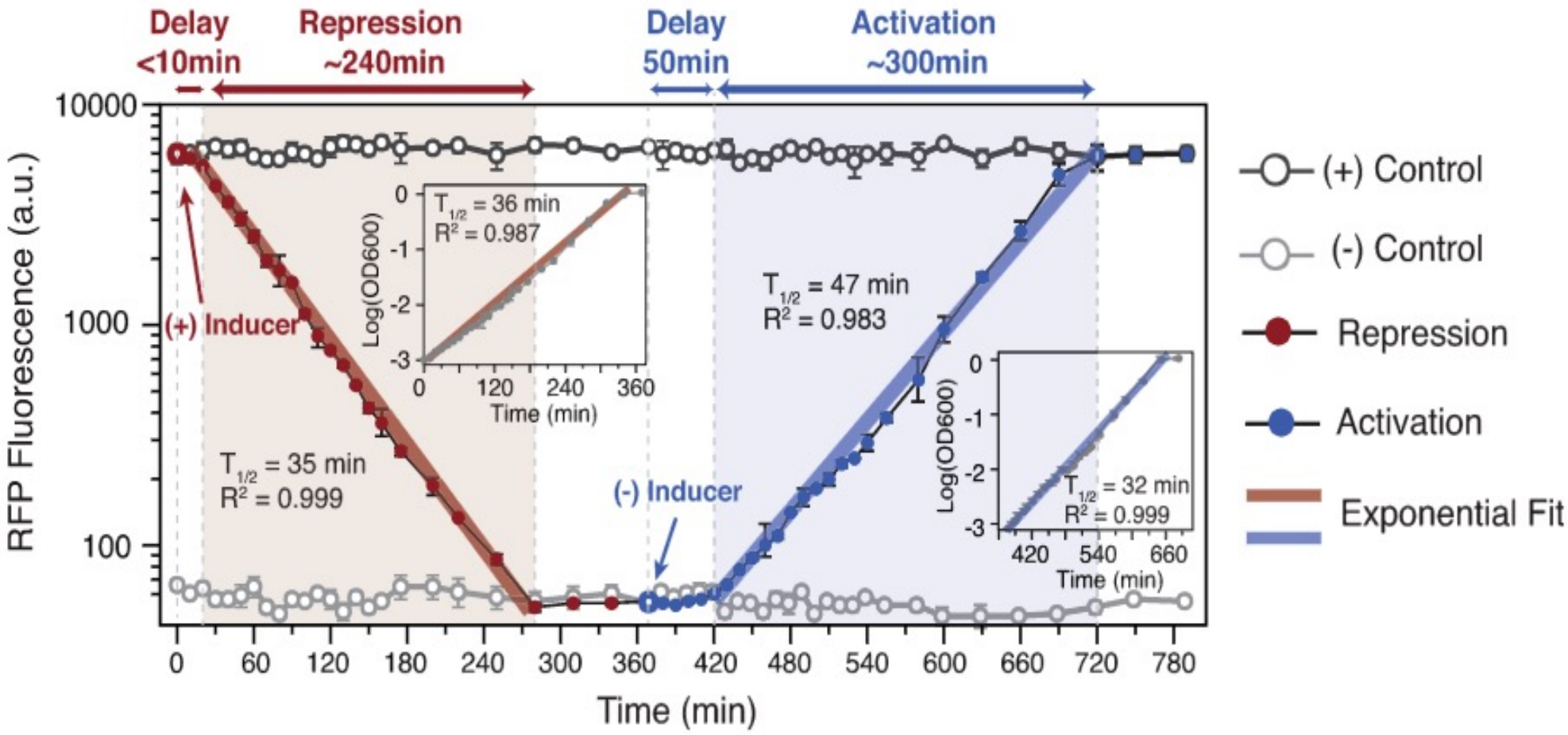
Lei et al. (2013) *Cell*. 152:1173-1183.

CRISPRi collision model



Lei et al. (2013) *Cell*. 152:1173-1183.

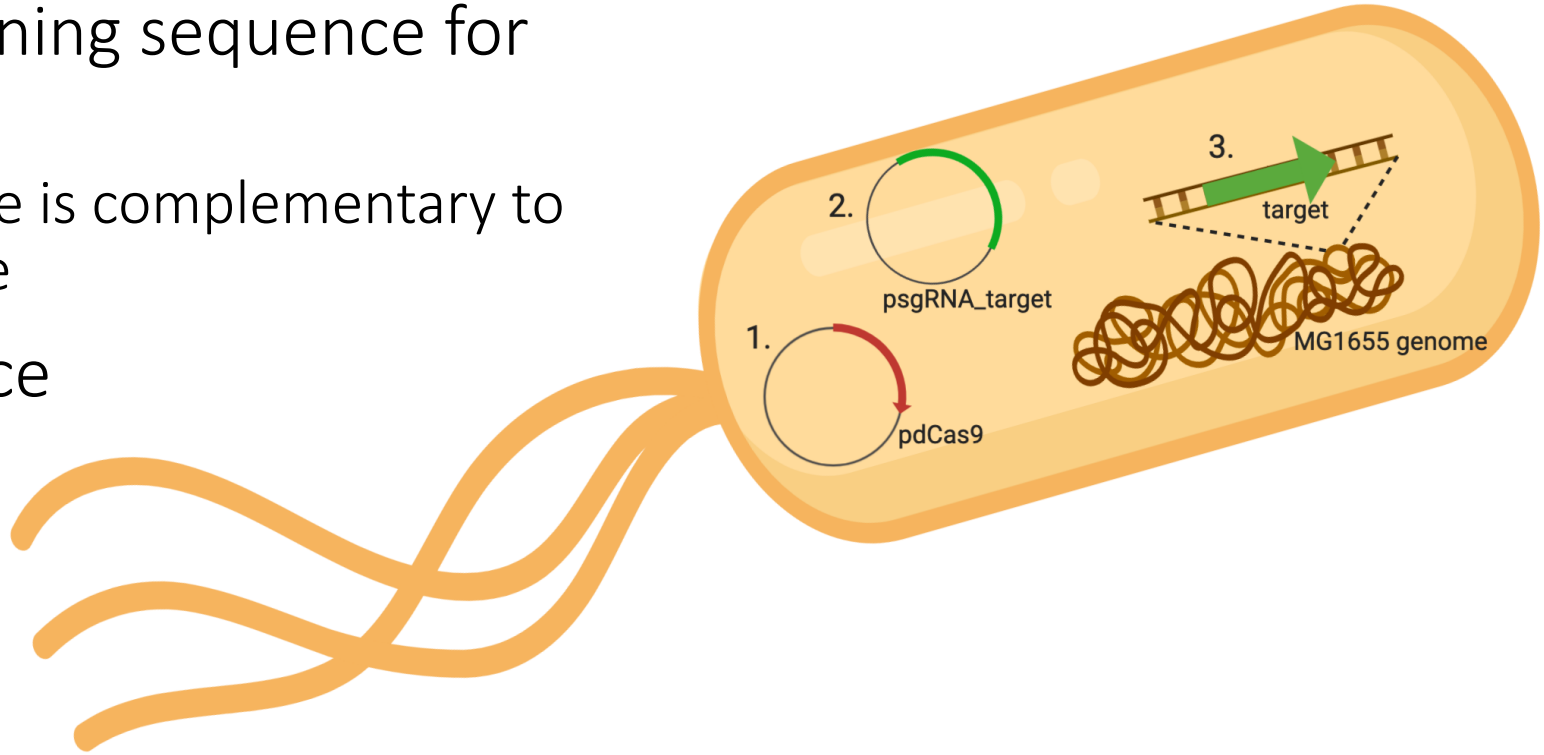
aTc-inducible promoter used to control CRISPRi inhibition of targeted gene



Lei et al. (2013) *Cell*. 152:1173-1183.

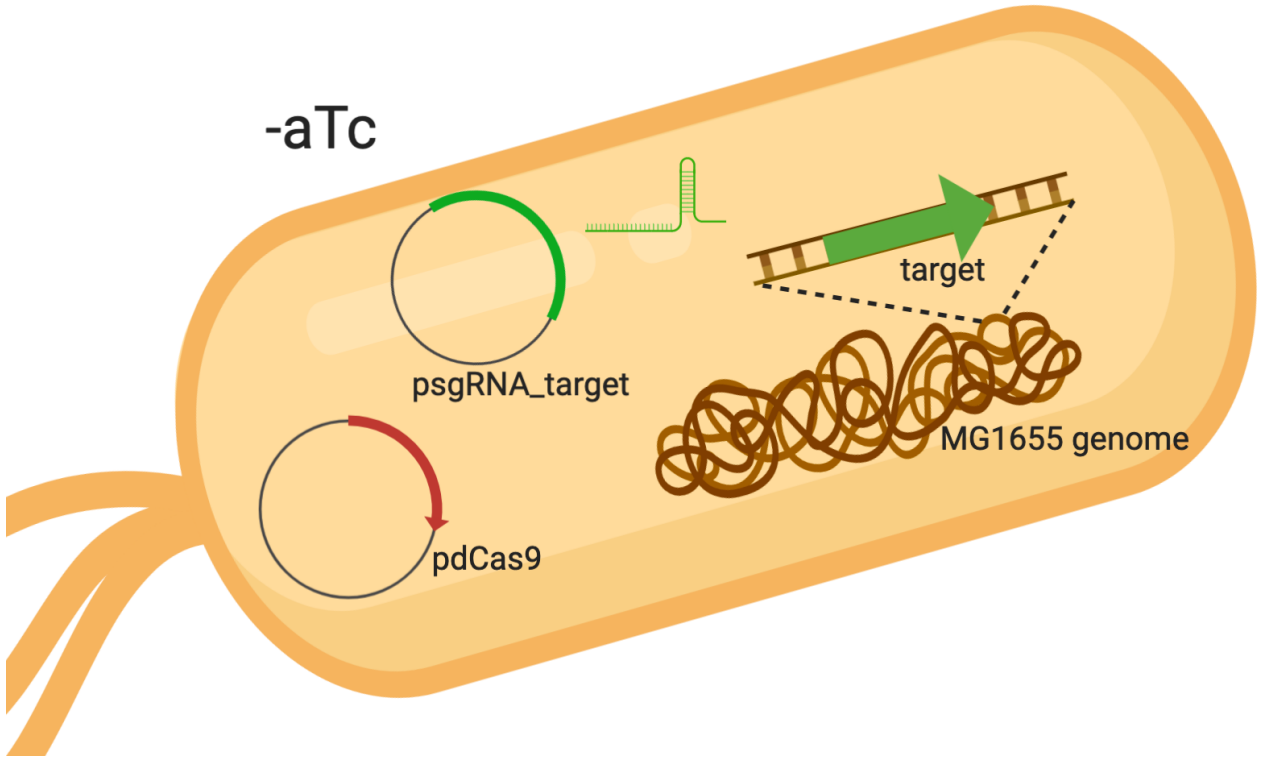
Components of CRISPRi system

1. Plasmid containing gene that encodes dCas9
2. Plasmid containing sequence for sgRNA
   - sgRNA sequence is complementary to target sequence
3. Target sequence



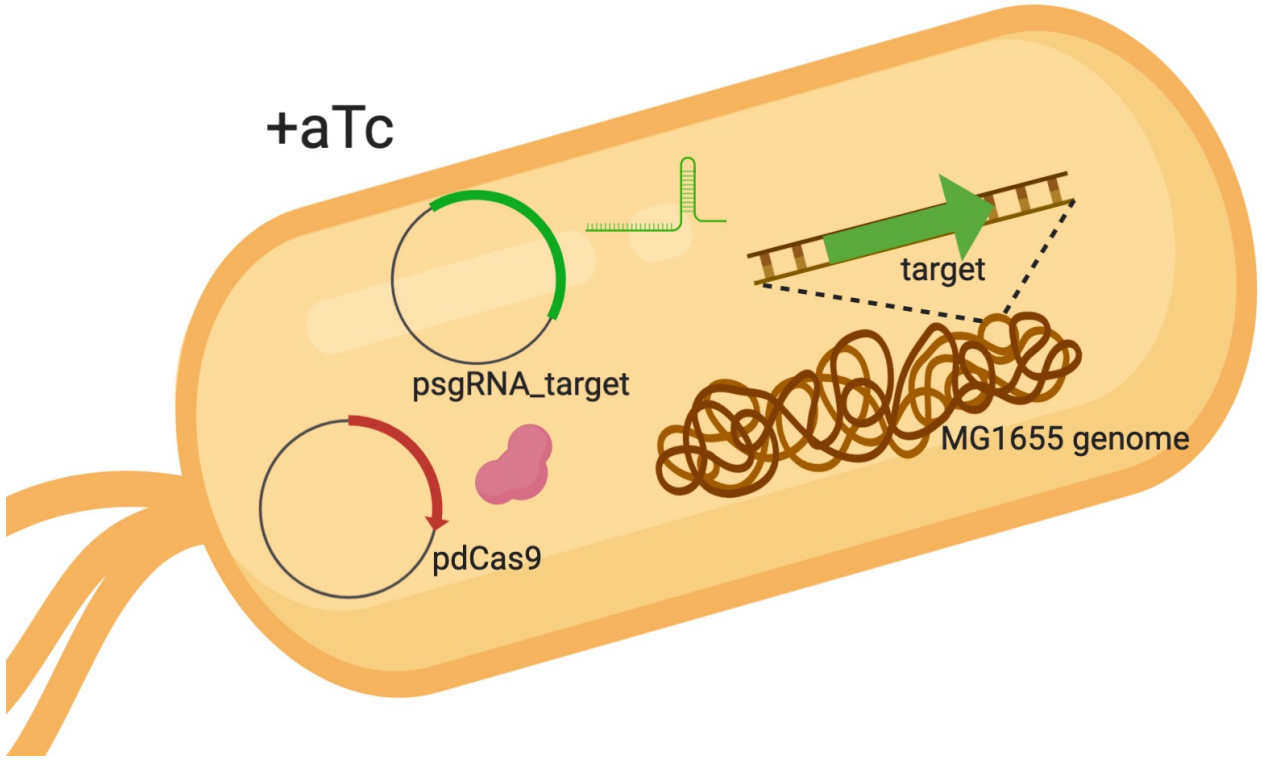
CRISPRi inactive in absence of inducer

- pgRNA_target expressed constitutively
  - Always transcribed and binding to target gene



CRISPRi inhibits gene expression in presence of inducer

- pdCas9 expressed when aTc added
  - When transcribed associates with pgRNA_target / target gene



What is the take-home message?

- CRISPRi is a tool for non-permanent genetic manipulation
- Know how CRISPRi differs from the native CRISPR system
- Know how the CRISPRi system inhibits expression of a targeted gene