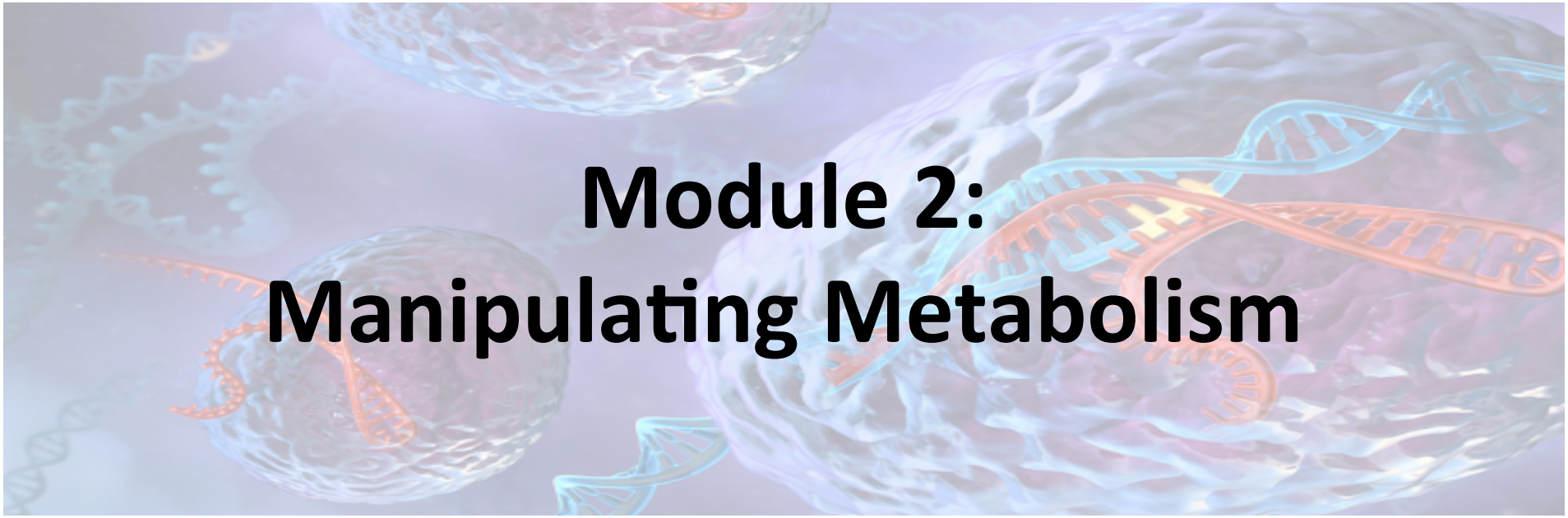


Module 2: Manipulating Metabolism

CRISPR and genetic engineering

10/31/17


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

Identifying the cleavage target of Cas9

- Adaptive immune response that confers phage resistance
- Requires crRNA, tracrRNA, and Cas9

What is the target of the native system?

Incoming viral DNA or host-transcribed viral mRNA?

DNA vs RNA debate

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

Data support that Cas9 cleaves DNA

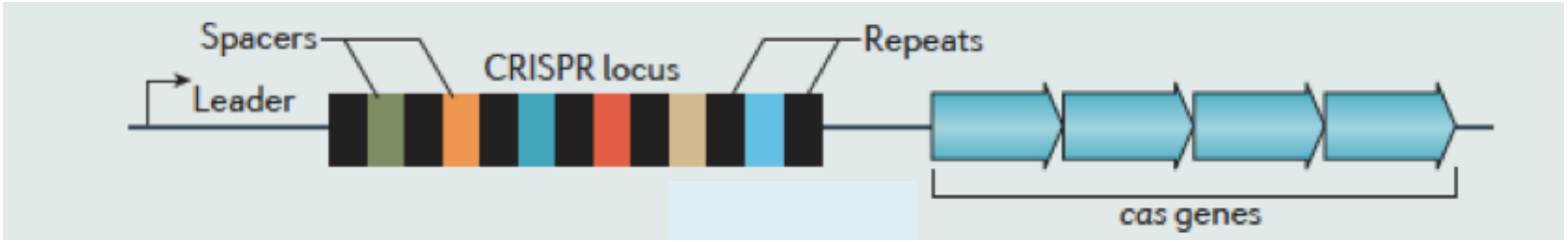
1. Targets in direction of ‘sense’ DNA more efficient than those of ‘anti-sense’

2. Transformation of plasmid DNA blocked

3. Presence of self-splicing RNA sequence in DNA target abolished CRISPR activity

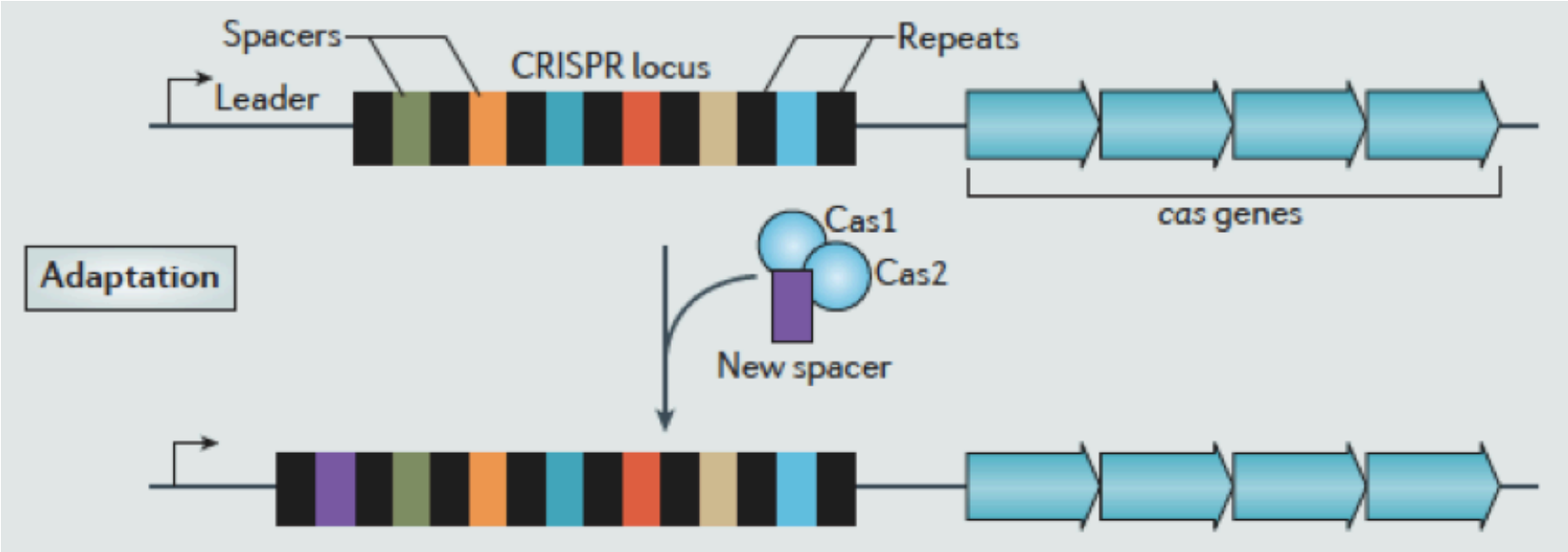
Native CRISPR system cleaves phage DNA

- Adaptive immune response encoded by CRISPR loci and Cas genes



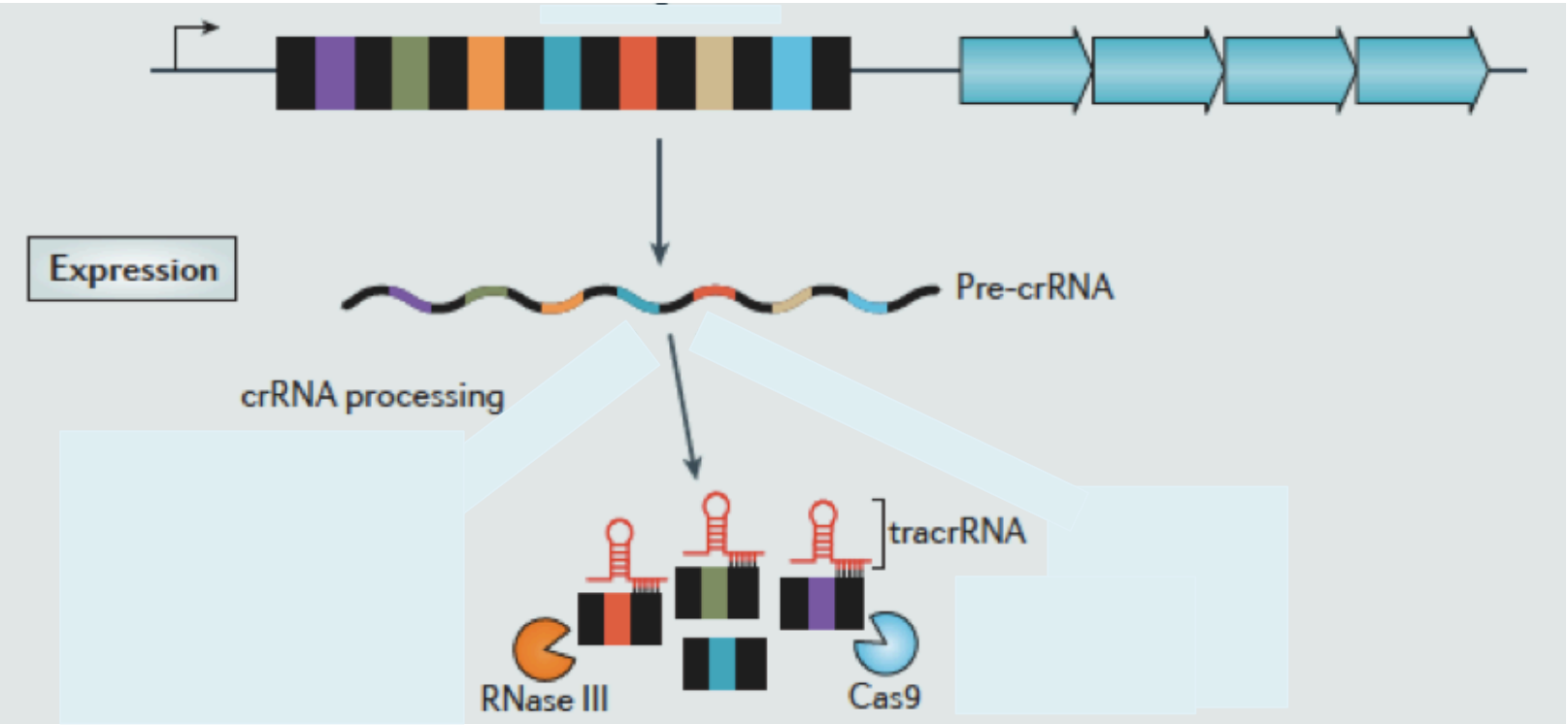
- Mechanism involves three stages:
  - Adaptation
  - Expression
  - Interference

CRISPR system: adaptation



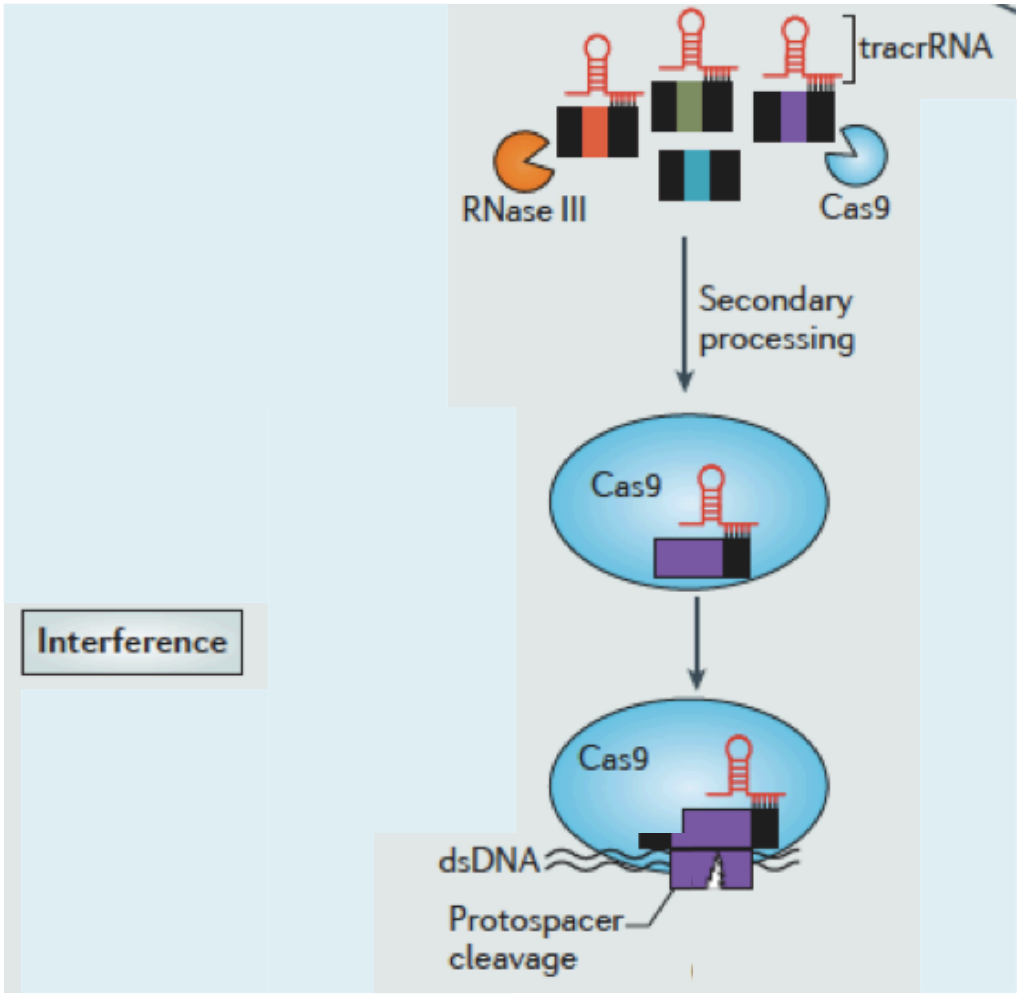
Phage DNA recognized and fragmented by restriction enzyme system, then ‘spacers’ incorporated into bacterial genome

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

Engineered CRISPRi system

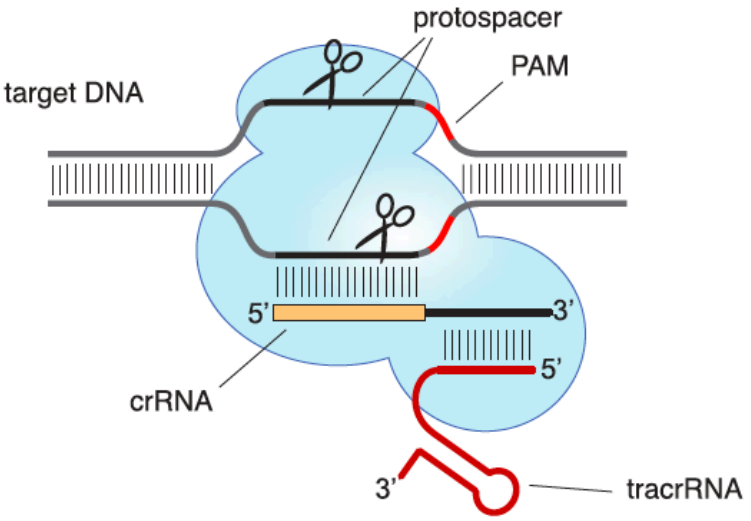
- Modifications to crRNA / tracrRNA complex?

- Modifications to Cas9?

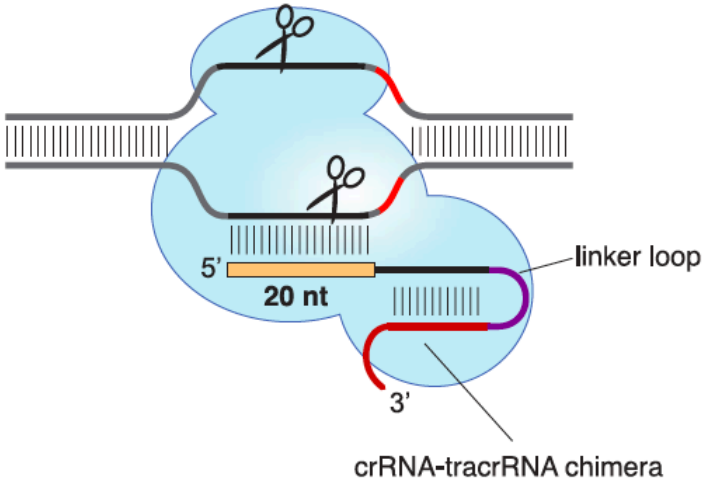
CRISPRi system: (s)gRNA

- (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



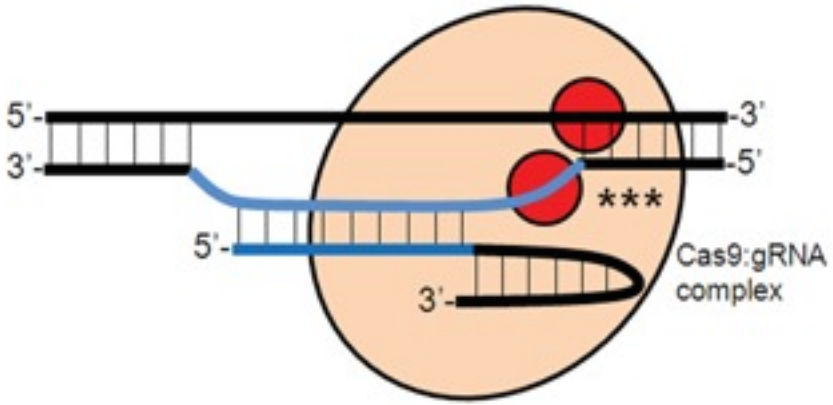
(s)gRNA



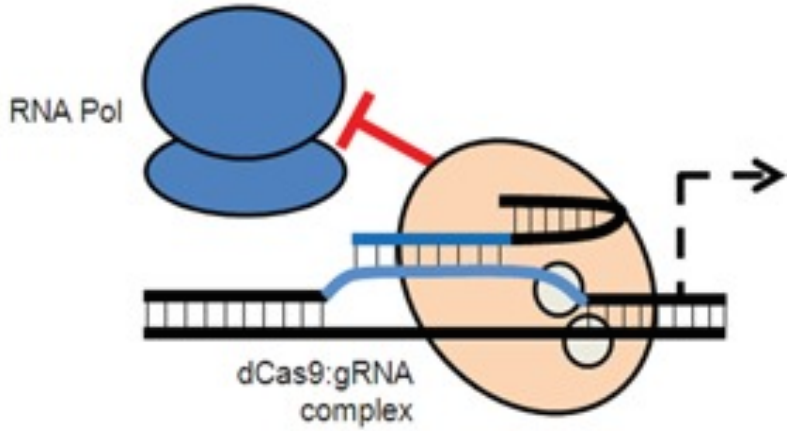
CRISPRi system: dCas9

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

Cas9



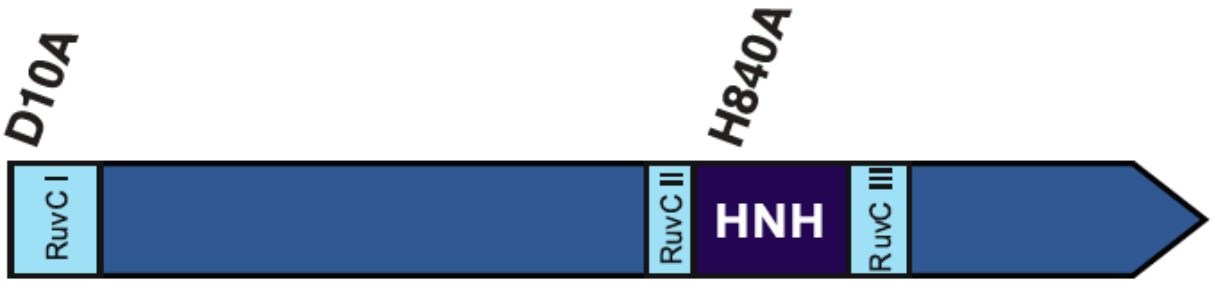
dCas9

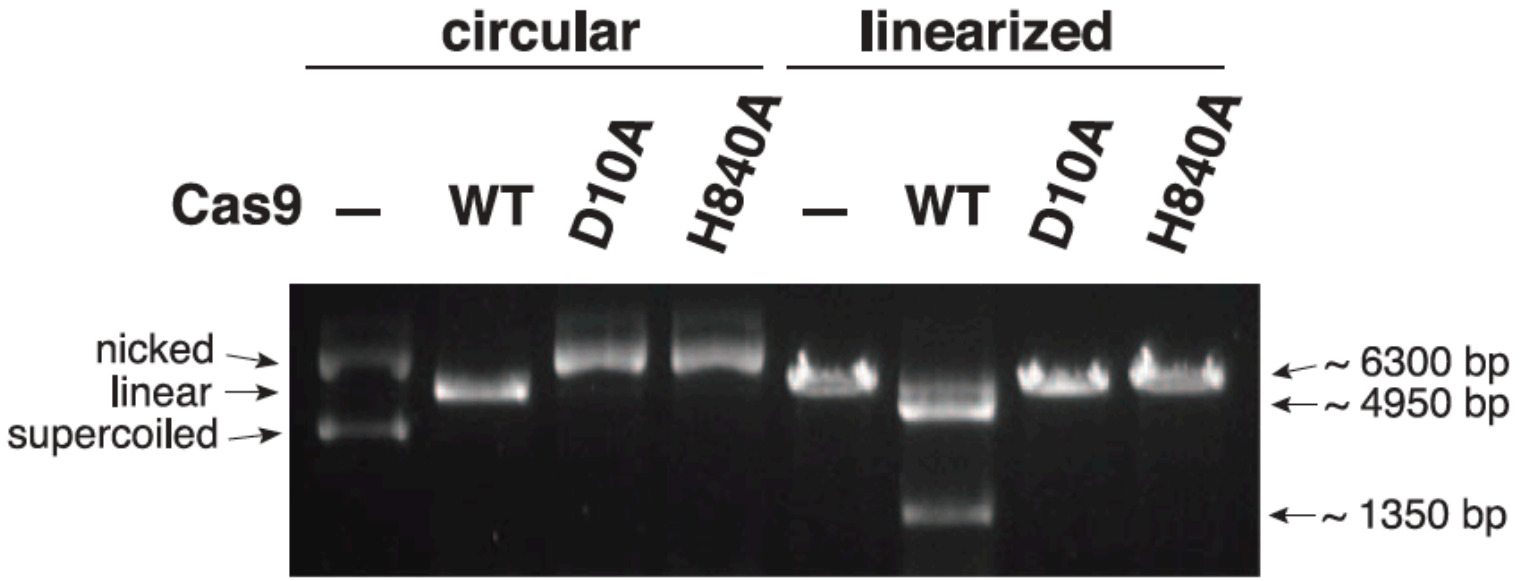


HNH and RuvC endonuclease domains

- 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

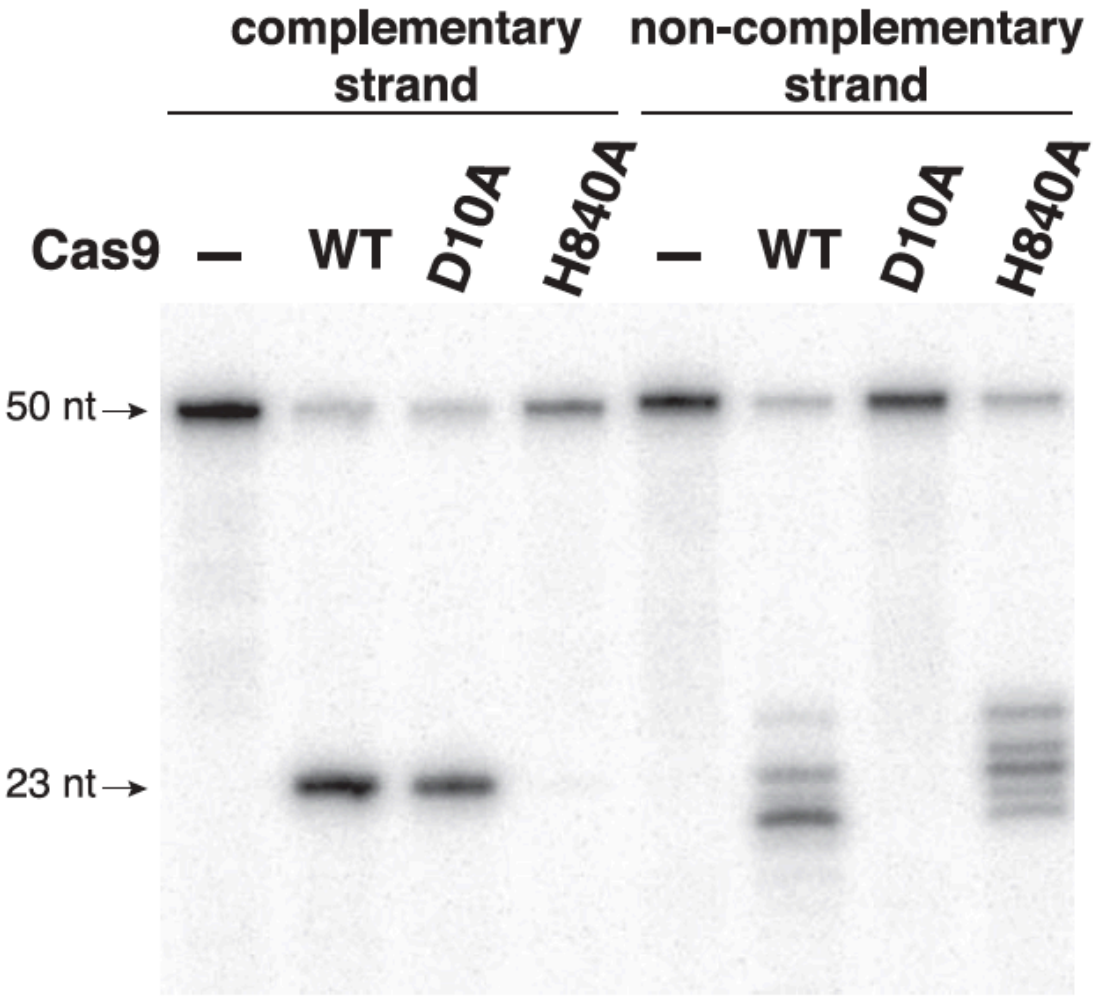
Cleavage requires HNH and RuvC domains





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

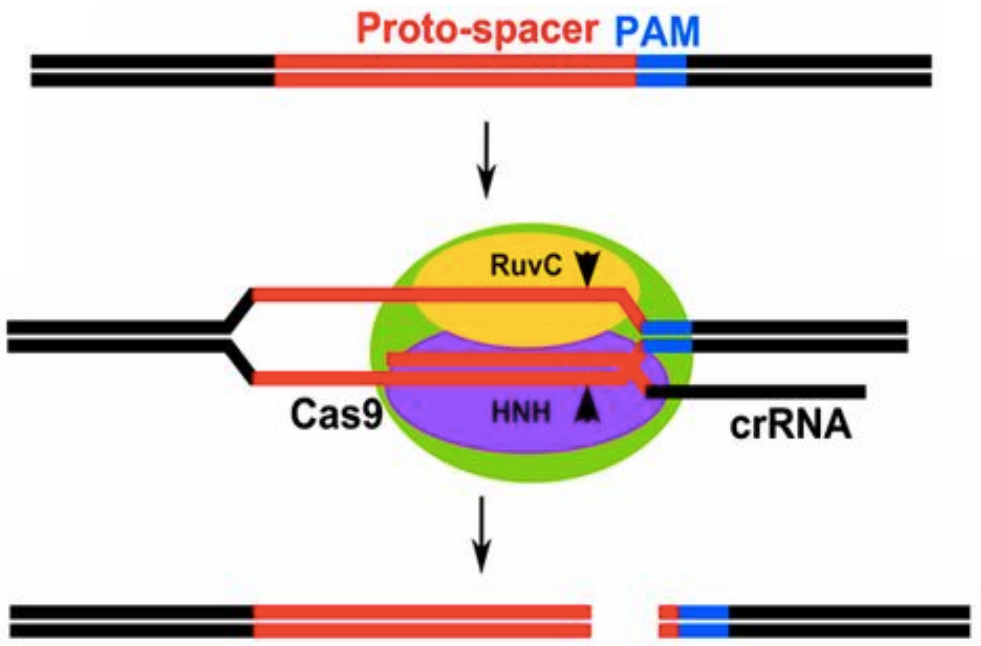
HNH and RuvC domains target specific DNA strands



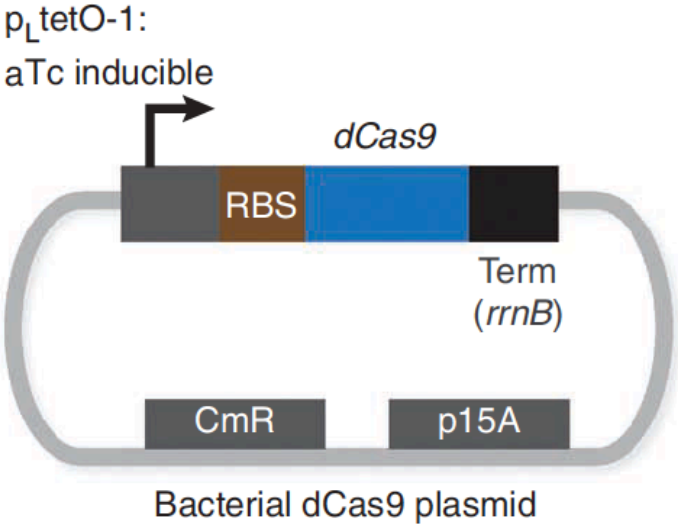
Jinek et al. (2012) *Science*. 337:816-820.

Schematic of Cas9 DNA cleavage

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

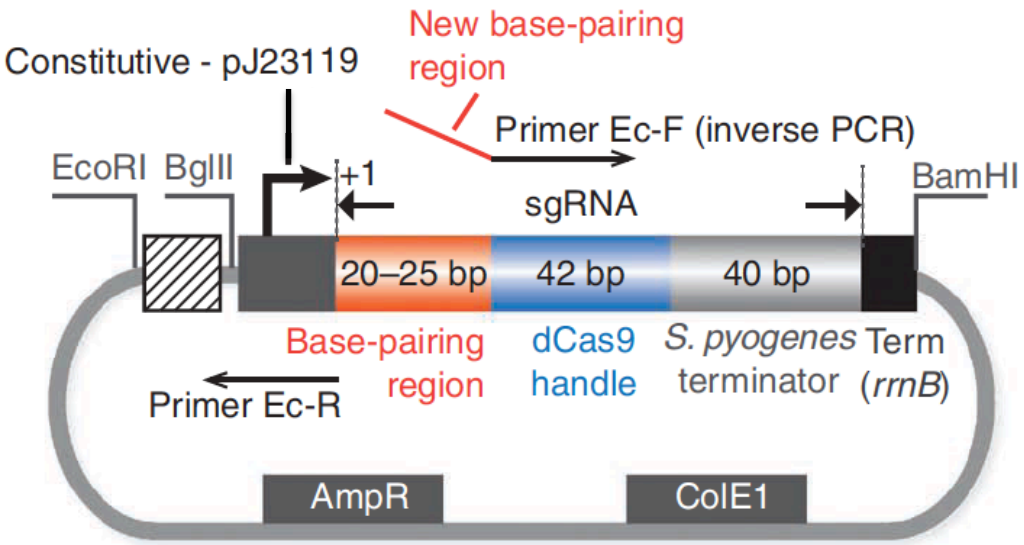


Closer look at pgRNA and pdCas9



Bacterial dCas9 plasmid

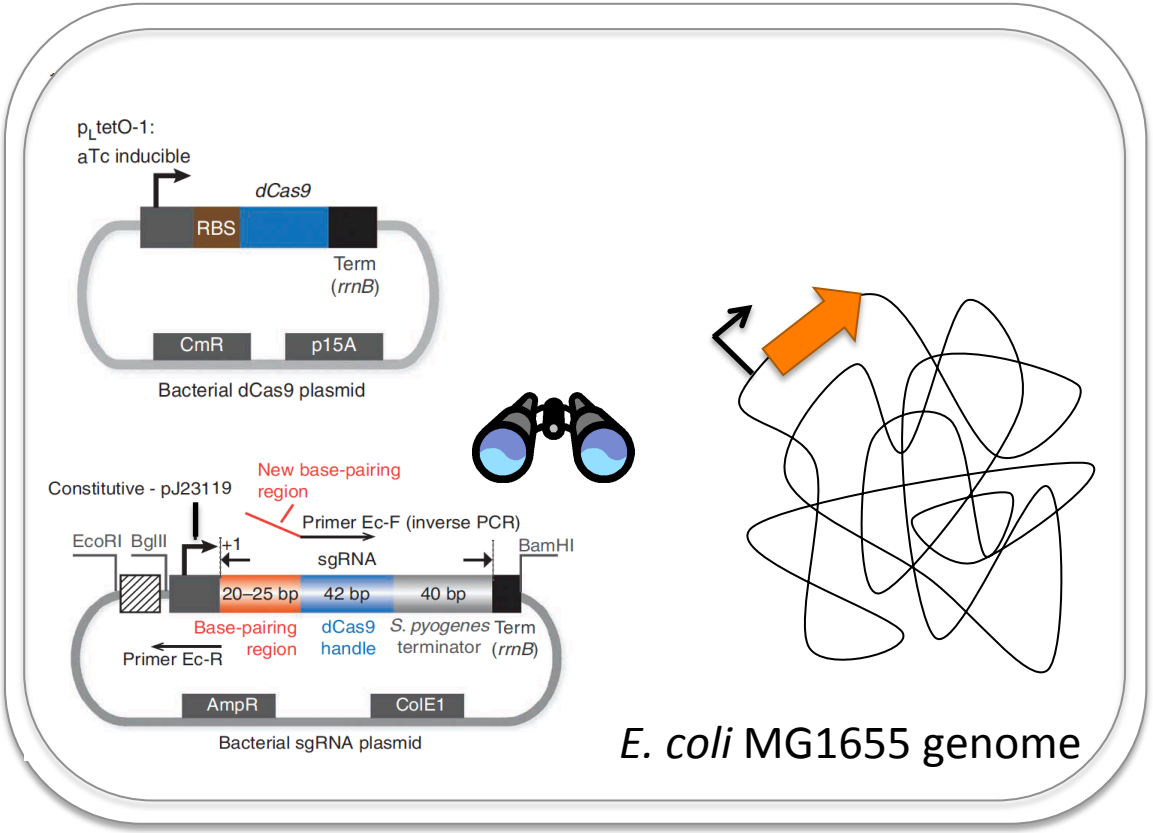
- Confirmation digest prepared on D1



Bacterial sgRNA plasmid

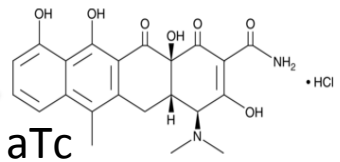
- Insert (gRNA target sequence) designed on D2

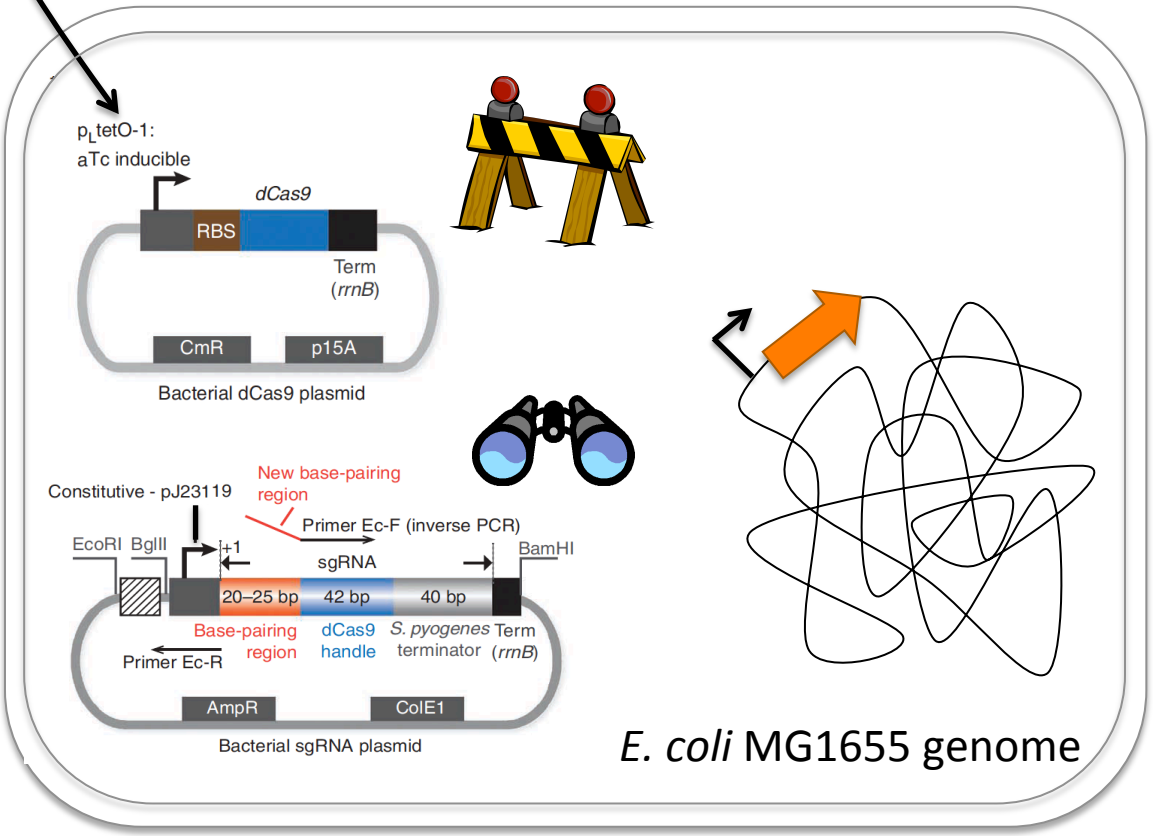
CRISPRi 'inactive' in absence of inducer



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

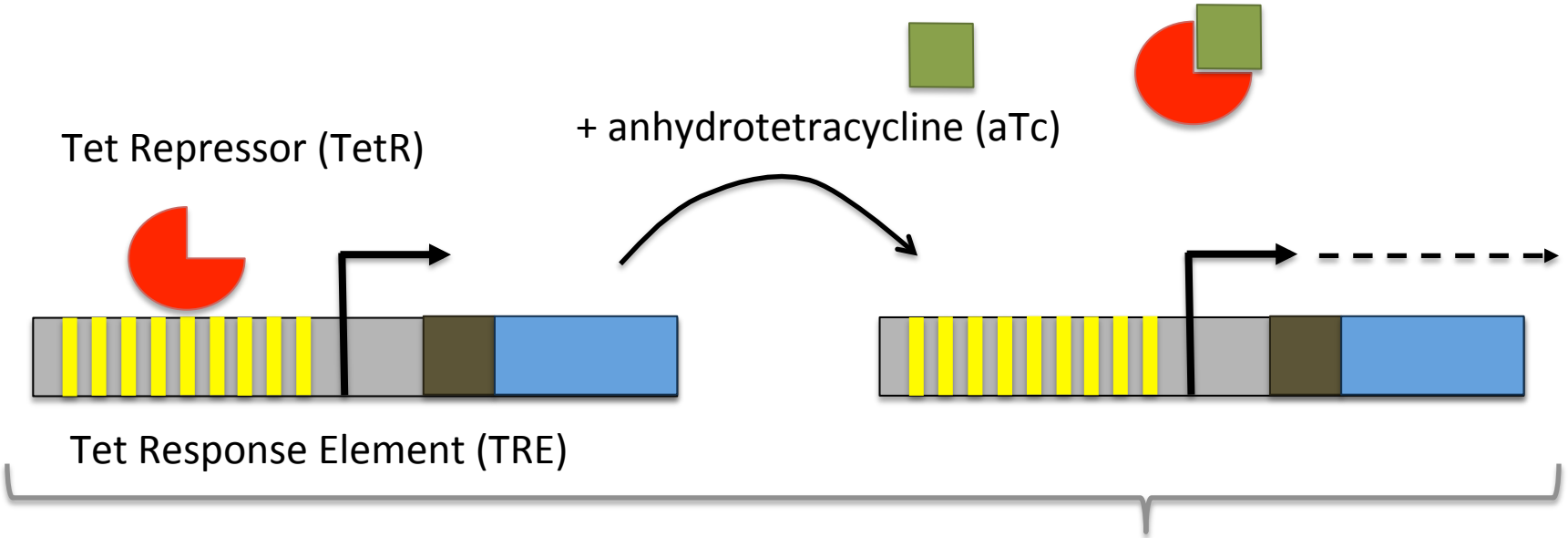
CRISPRi ‘blocks’ gene expression in presence of inducer



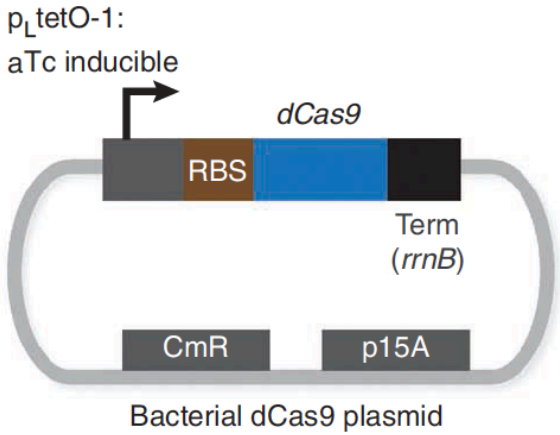


- pdCas9 expressed when aTc added
  - When transcribed associates with pgRNA_target / target gene

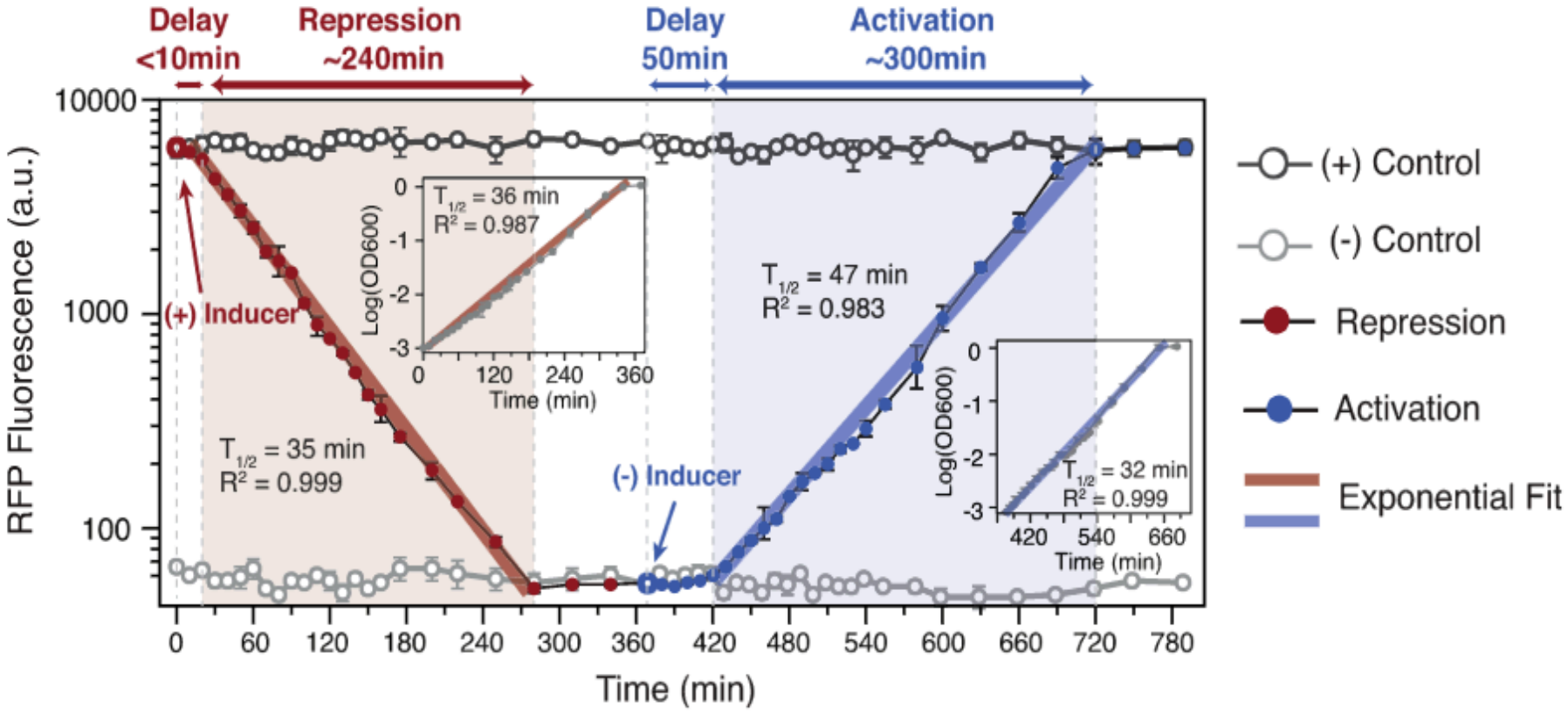
Closer look at aTc induction of pdCas9



- Tet promoter regulates expression of dCas9 gene

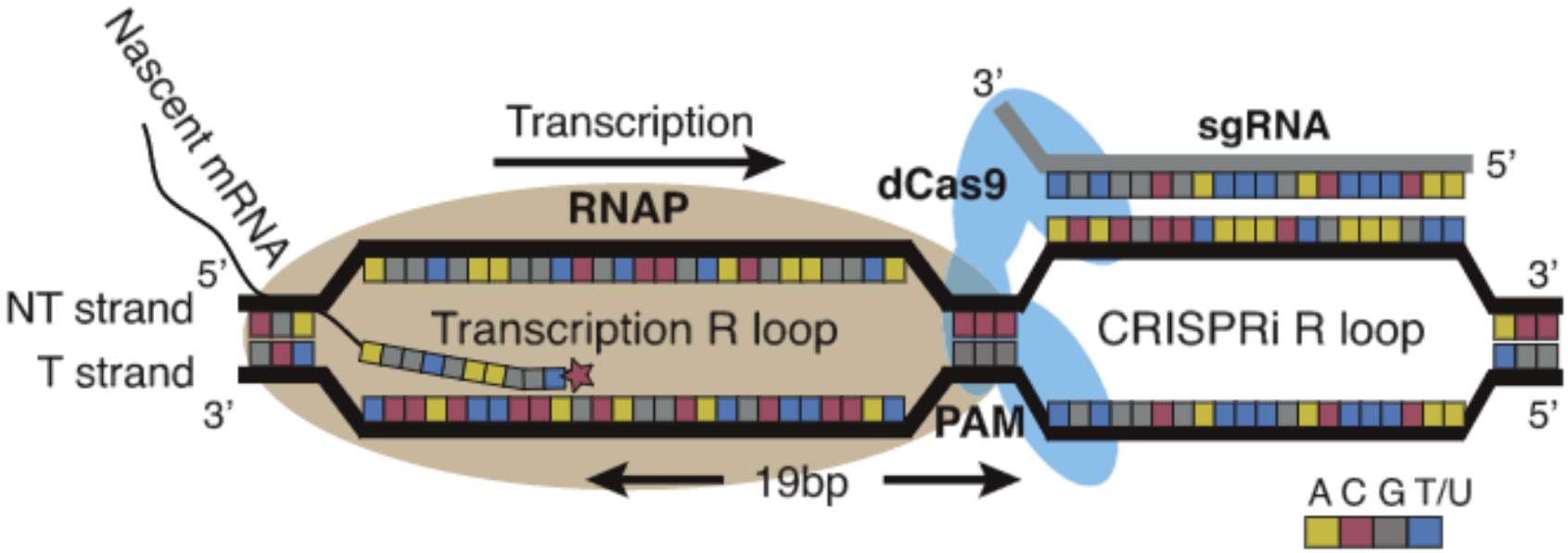


Inducible promoter can be used to control dCas9-mediated gene expression



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

CRISPRi collision model



In the *laboratory*…

Meet at 1p in 16-336 for Journal Club presentations