UNIVERSITÀ
DEGLI STUDI DI TRIESTE

DIPARTIMENTO DI
SCIENZE DELLA VITA

Widespread RNA-based *cas* regulation monitors crRNA abundance and anti-CRISPR proteins

Liu, Chao et al. Cell Host & Microbe, 2023, Volume 31, Issue 9, 1481 - 1493.e6

Presented by Letizia Barbieri and Nora Costella

OUTLINE

- **INTRODUCTION**
- STUDY AIM
- RESULTS
- CONCLUSION

Prokaryotes VS Mobile Genetic Elements

Coevolution between **prokaryotes** and their **predators** has led to sophisticated **defence** mechanisms

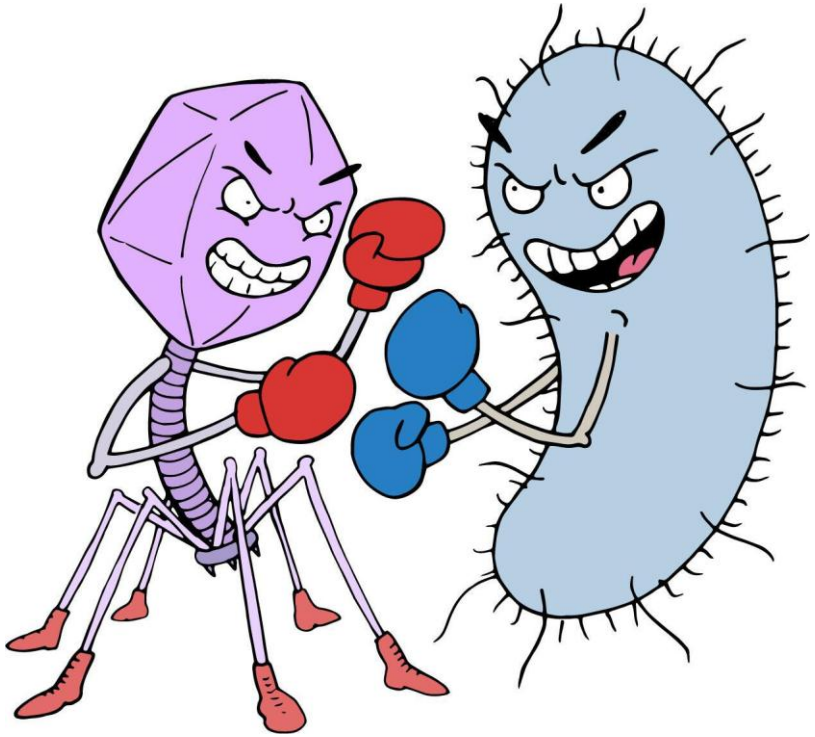
Prokaryotes employ:

Innate defence

- Preventing phage adsorption
- Restriction of invading DNA
- Abortive infection

Adaptive defence

- **CRISPR-Cas system**



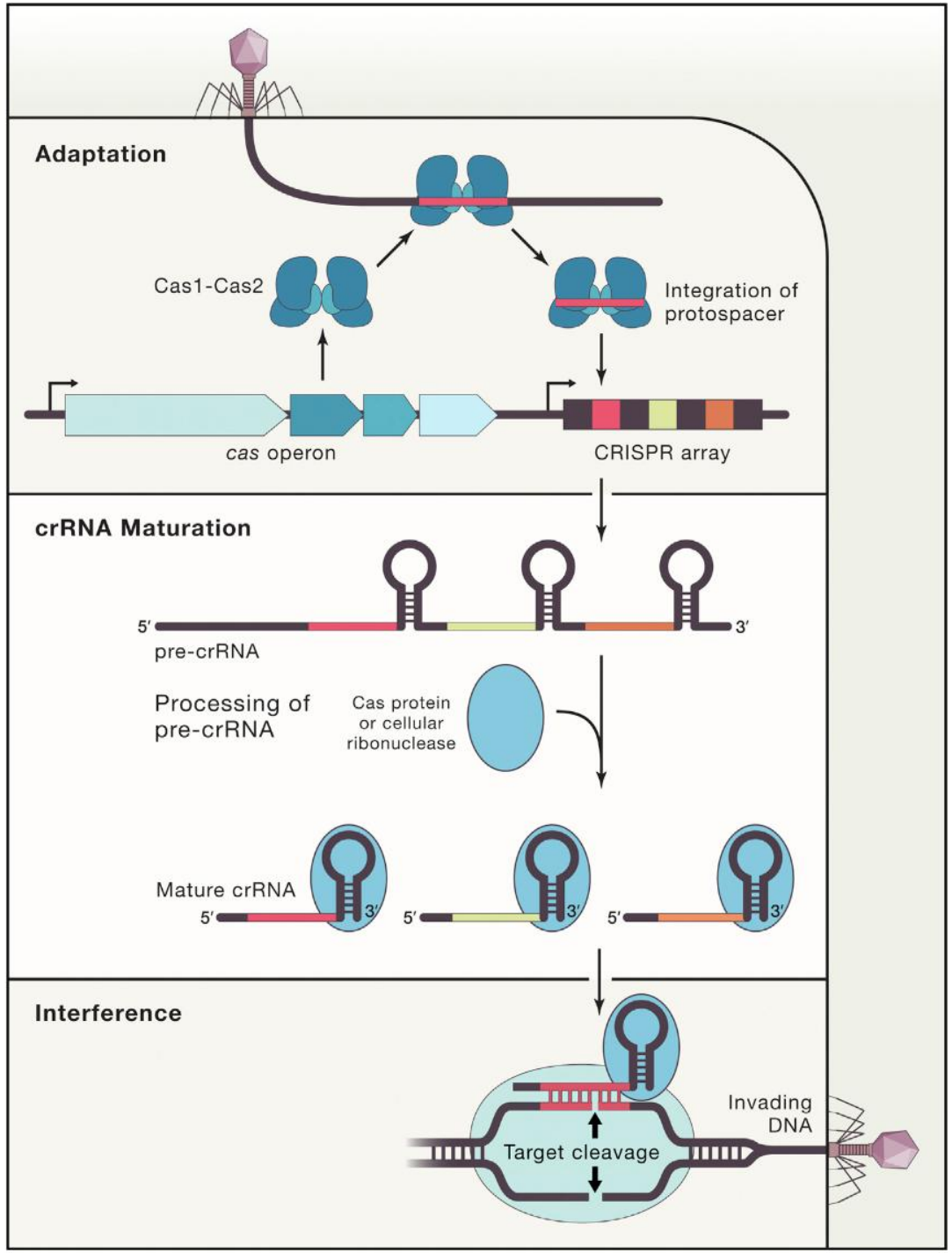
Stores **memory** of the past infections and, upon reinfection, uses **RNA-guided nucleases** to silence phage or other mobile genetic elements (MGEs)

CRISPR-Cas main actors

- **CRISPR array** - archive of previous infections
  Genomic locus:
  - **Repeats**: identical sequences recognized by Cas
  - **Spacers**: derived from integration of **protospacers** (= DNA fragments from past infections)

  Transcribed as **pre-crRNA** → processed to **crRNA**

- **Cas** - nuclease proteins with active role in:
  1. **Adaptation** (= spacer acquisition)
  2. **crRNA biogenesis**
  3. **Interference** → Cas use crRNA as guide to degrade foreign DNA



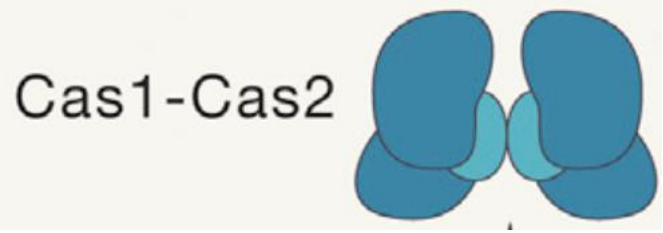
CRISPR-Cas systems Classification

	Class 1 multi-protein Cas complexes for interference			Class 2 Single Cas for interference		
Type	**Type I**	**Type III**	**Type IV**	**Type II**	**Type V**	**Type VI**
Effector	Cascade complex + **Cas3** ✂	Csm (III-A) or Cmr (III-B) + **Cas10** ✂	?	**Cas9** (HNH and RuvC domains) ✂	**Cas12** (only RuvC domain) ✂	**Cas13** (HEPN domain) ✂
Guide	crRNA	crRNA	?	crRNA + tracRNA	crRNA	crRNA
Target recognition	through PAM	5' repeat portion of crRNA unbound	?	through PAM	T-rich PAM	RNAs complementarity to crRNA
Cutting	ssDNA break intermedium first, then dsDNA break	DNA being transcribed and RNAs	?	blunt dsDNA break	dsDNA breaks (with overhangs) distal from PAM	ssRNA + collateral host ssRNAs

1. **Adaptation** – memory creation

Incorporation of protospacers from invading nucleic acids into the host's CRISPR array, generating spacers

Machinery (for almost all CRISPR-Cas systems in *E.Coli*)

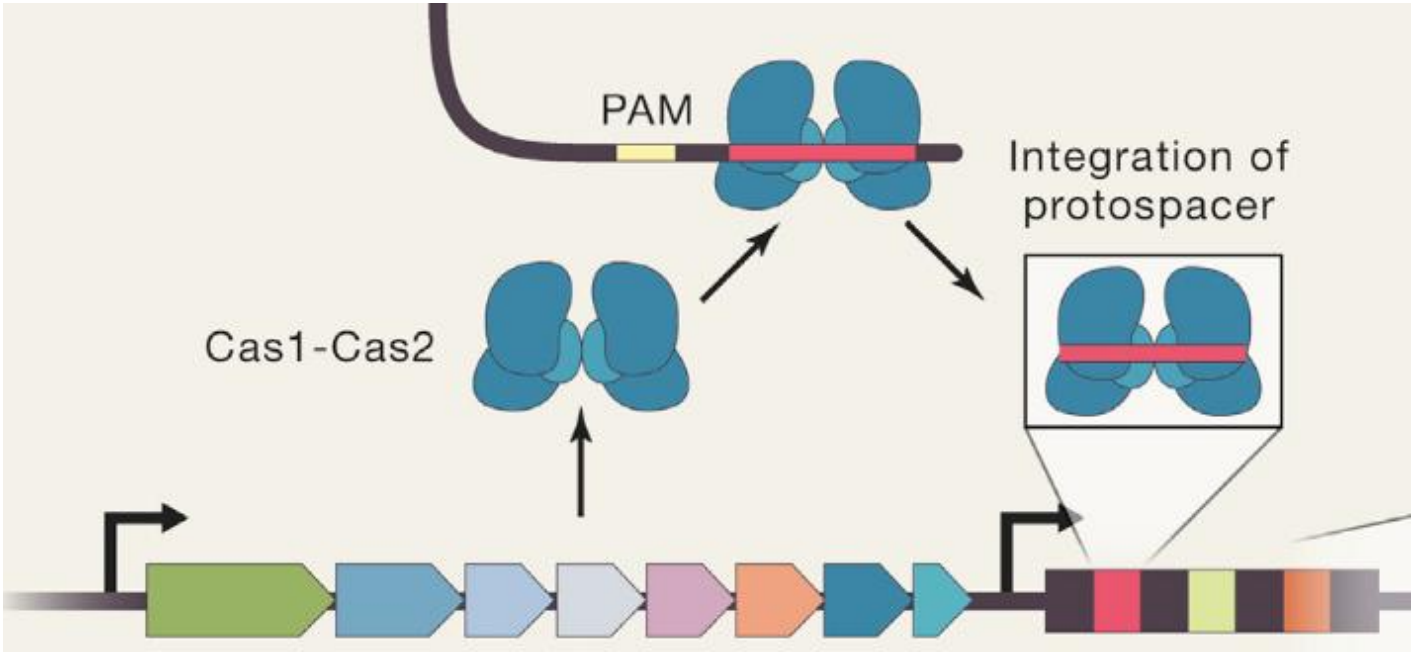


- Catalytic integrase
- Structural role

Adaptation steps:

- **Detection** of foreign genetic elements
- **Selection & processing** of protospacers for integration – e.g. type I & II recognize, and than remove, protospacers with PAM complementary to a Cas1 subunit
- **Integration** of spacers - preferentially at the leader end of the CRISPR array

Challenge: integrate ONLY invader genome to avoid lethal autoimmunity



2. **crRNA biogenesis** - memory retrieval

CRISPR array is transcribed in **long pre-crRNA**, his maturation leads to **crRNA** having:

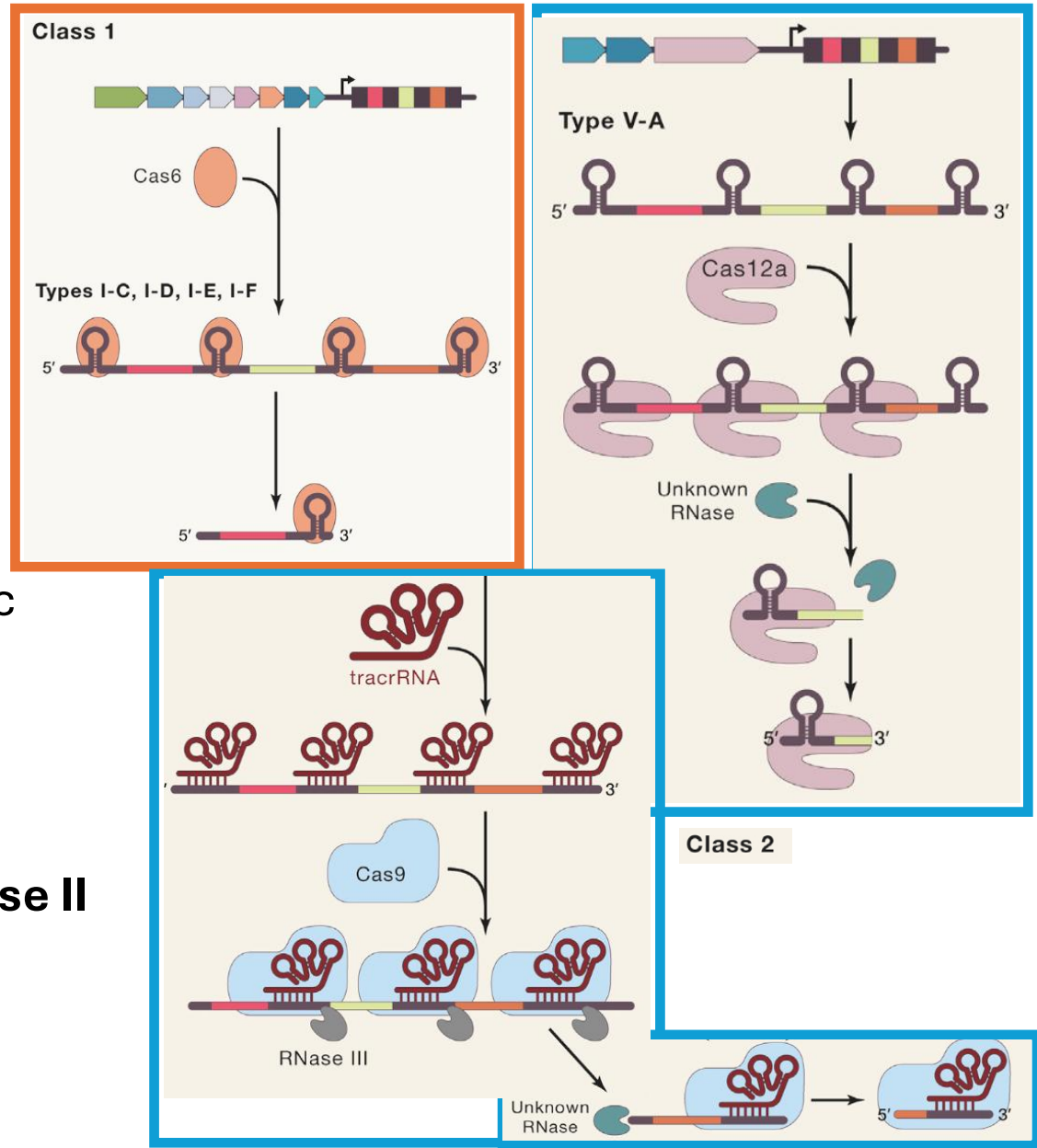
- **Repeats** → for Cas recognition
- **Spacer** → for target binding

→Class 1 crRNA maturation

Cas6 recognizes and cuts hairpin formed by palindromic sequences in repeats portion

→Class 2 crRNA maturation

- **Type II**: needs duplex **pre-crRNA:tracrRNA** and **RNase II**
- **Type V & VI**: effector proteins are sufficient



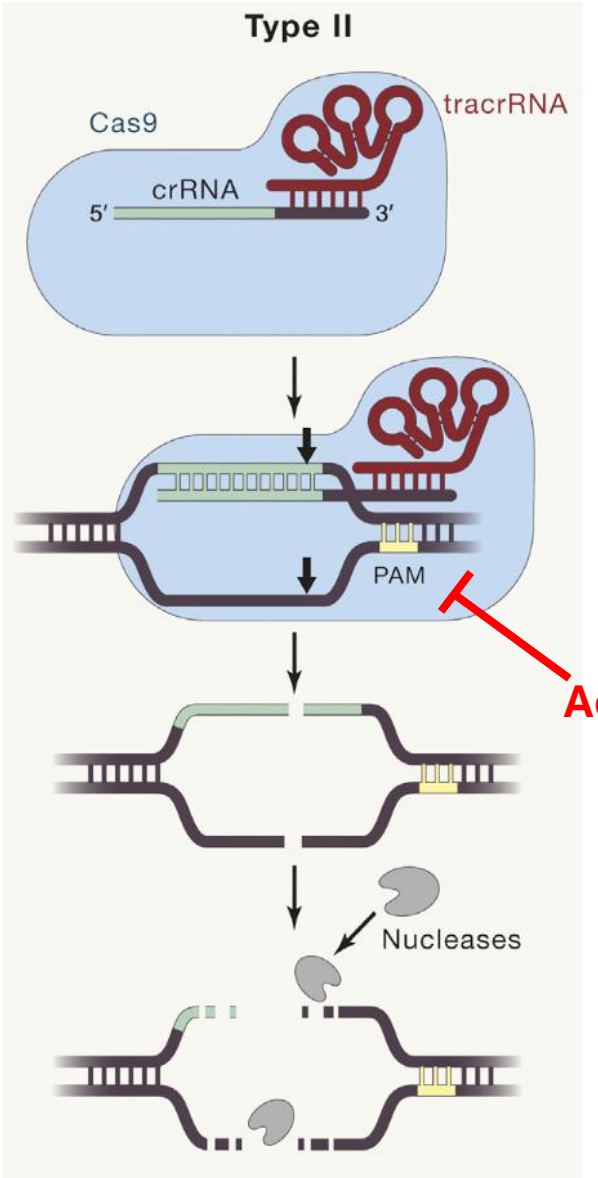
3. **Interference** - defence activation

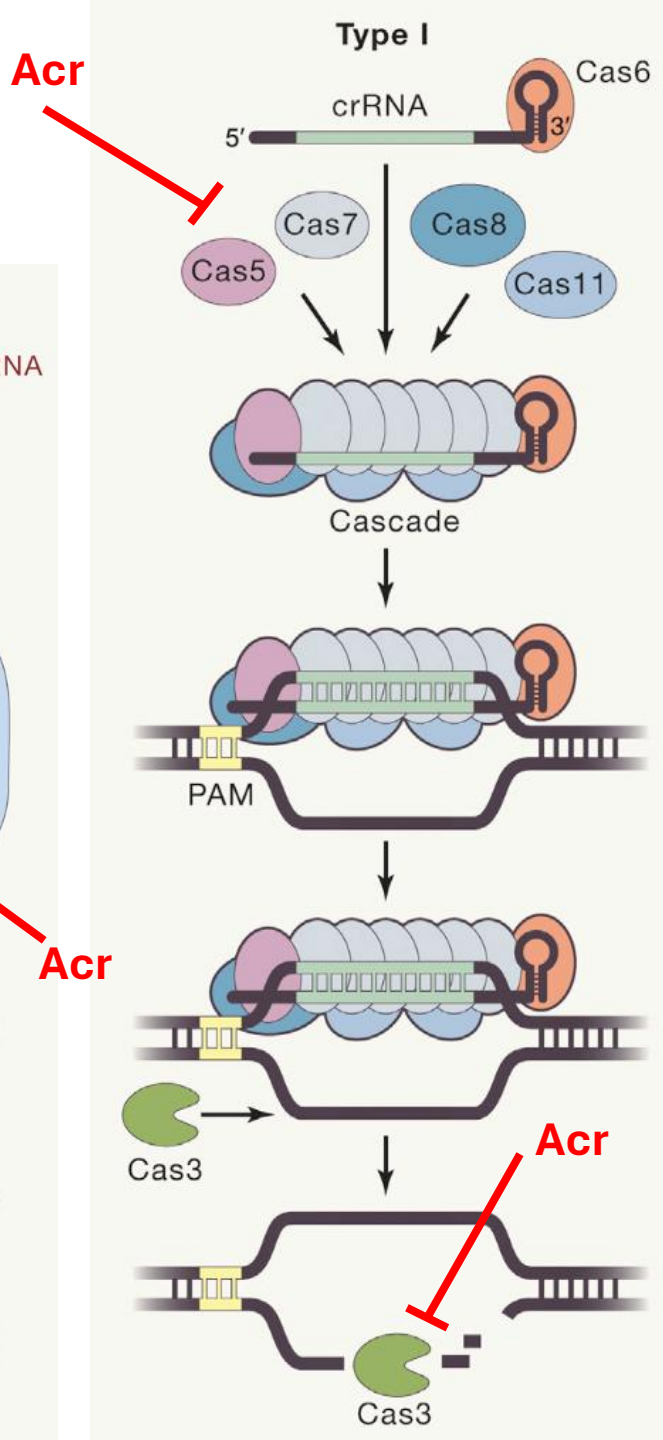
crRNA guides sequence-specifically the **effector machinery** to cleave invading nucleic acids

- **Crucial challenge** → **prevent autoimmunity**: cut only invading DNA and not CRISPR array
- **Solution** → **effectors recognize PAM**, which are ABSENT in CRISPR array

Phages defend themselves form CRISPR-Cas system

Acr (anti-CRISPR) proteins are a strategy employed by some **phages** to **circumnvent CRISPR-Cas** immunity





CRISPR-Cas comes with **fitness costs**

Limited adaptation
Acquisition of **beneficial MGE**-encoded traits is restricted by CRISPR-Cas systems

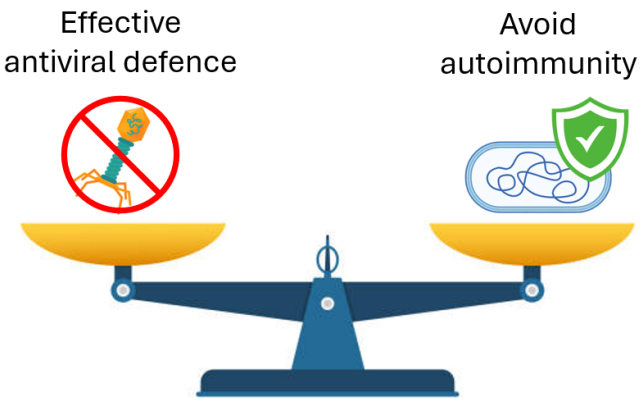
Autoimmunity
Adaptation machinery **can't distinguish self- vs non-self DNA**

Metabolic burden
Maintaining these complex systems is costly

How to **balance** these costs with the benefits of CRISPR-Cas systems?

Regulatory networks

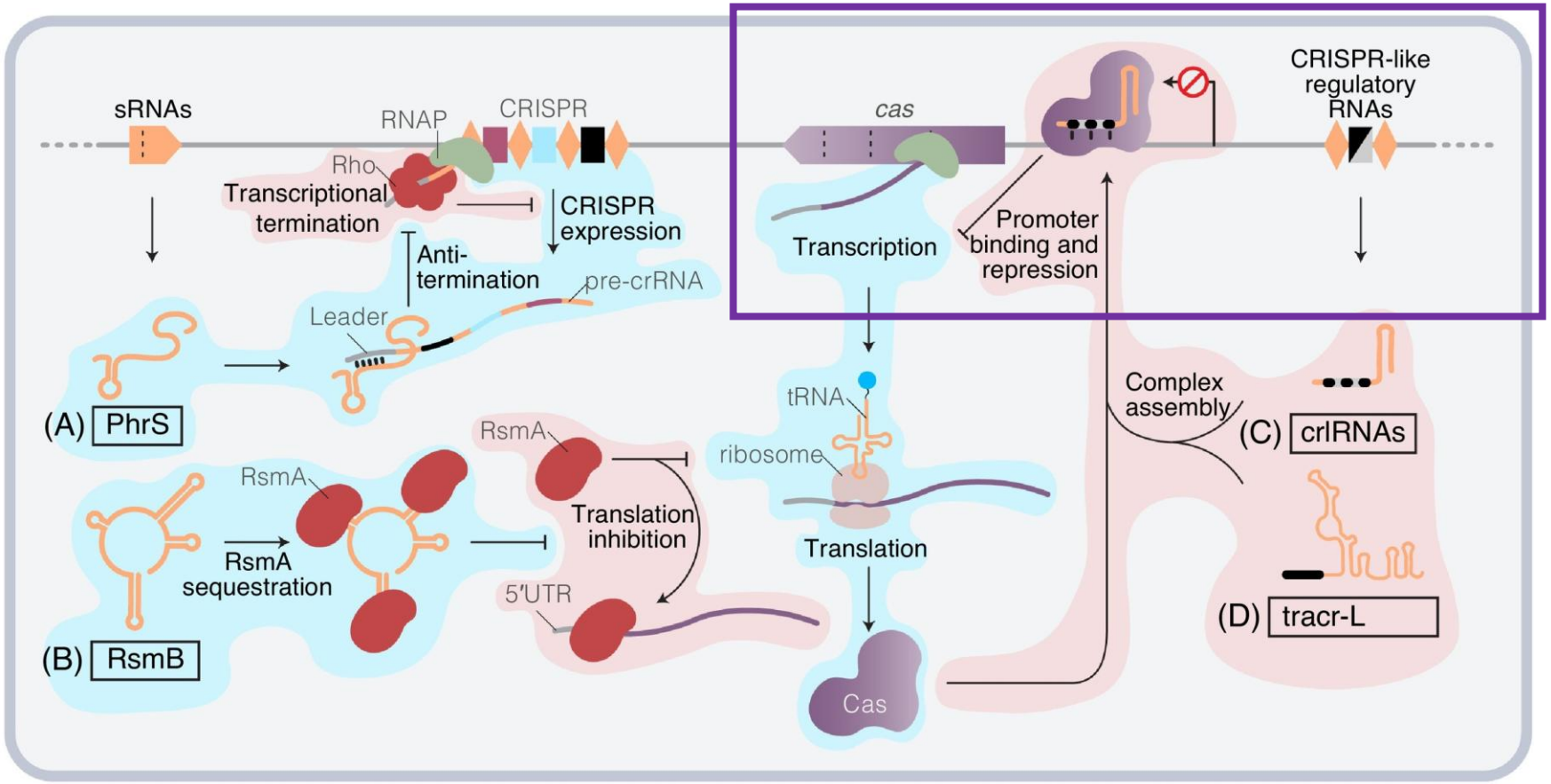
for **context-dependent induction** of CRISPR-Cas immunity



Careful regulation of immunity is a universal principle of cellular life!

→ ncRNA-based regulatory networks of CRISPR-Cas immunity

Diverse families of **ncRNA regulate crRNA or Cas production** acting on the transcriptional or post-transcriptional level

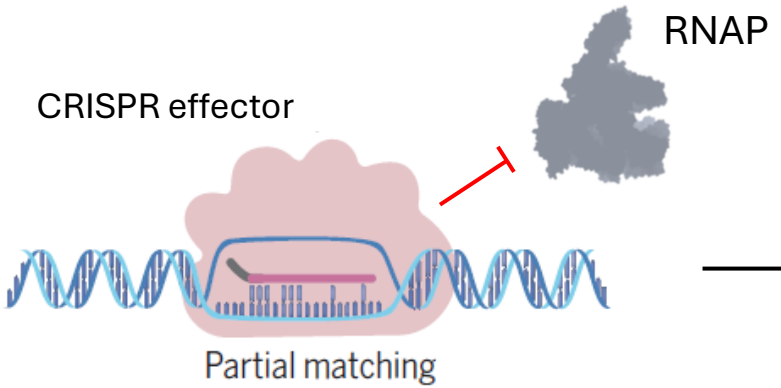


Some regulatory ncRNA act as non-canonical guides that **guide Cas to *cas* promoter** for transcriptional **autoregulation**

CRISPR effector is not just an immune effector but also a **gene regulator**!

→ **CRISPR effectors** as **gene regulators**

Transcriptional regulation is based on **PARTIAL or LIMITED spacer-protospacer complementarity**



NO cleavage of self DNA

→ **Repression** of target promoter
(protospacer)

Genes subjected to this kind of regulation:

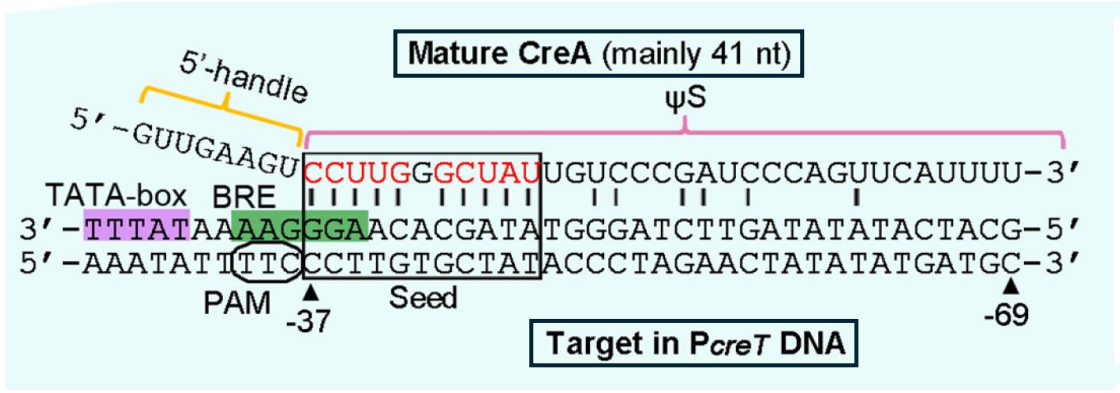
- *cas* genes (Cas autorepression)
- Virulence-related genes
- **Type VIII Toxin-Antitoxin** system
- …

Type VIII Toxin-Antitoxin system CreAT

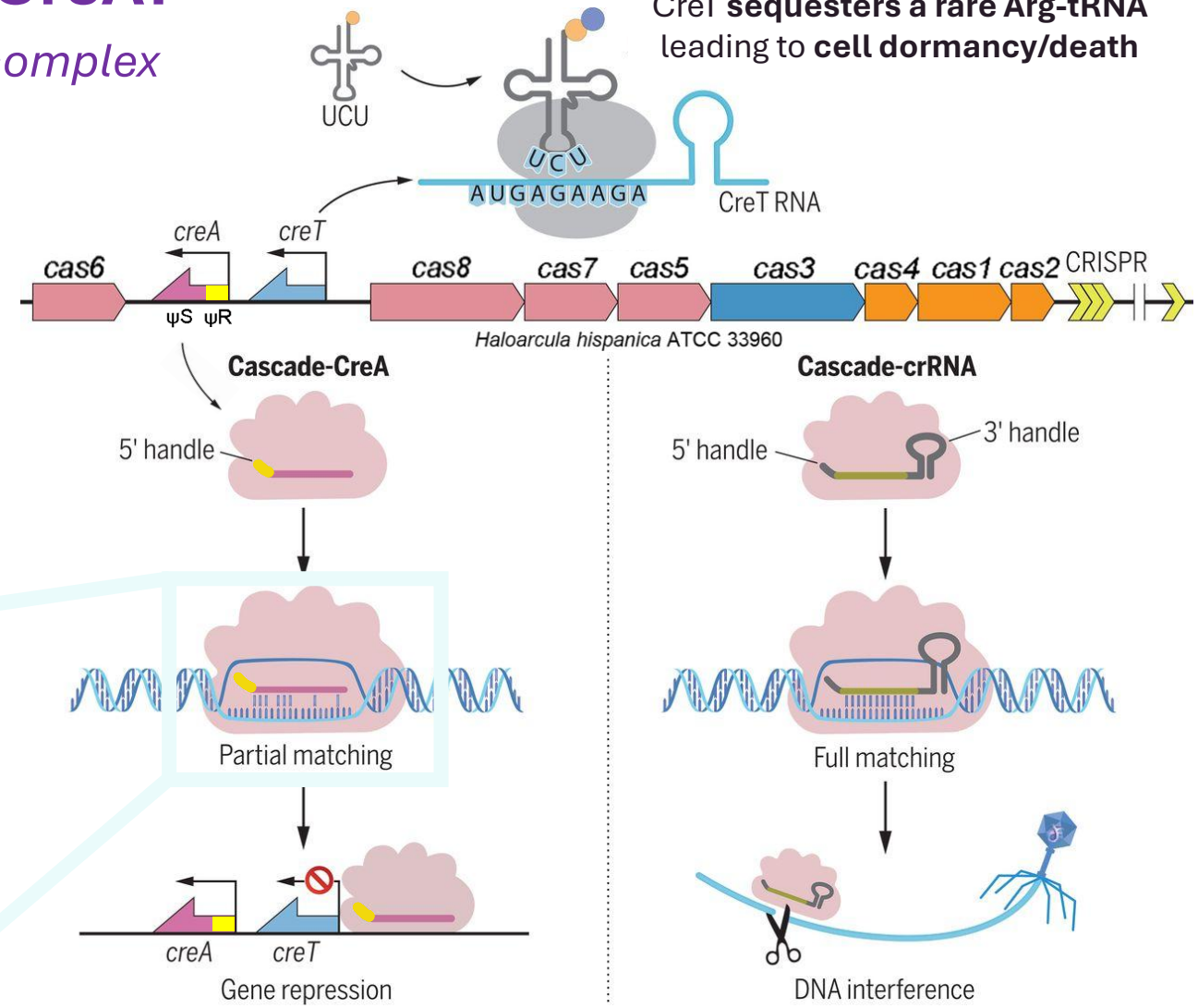
*The regulatory role of **type I-B** CRISPR effector complex*

CreA *(CRISPR repeat-like antitoxin)*:

- crRNA-like RNA (**crlRNA**) transcribed from a degenerated mini-CRISPR
- within **type I-B CRISPR-cas** loci of *Haloarcula hispanica*



CreT **sequesters a rare Arg-tRNA** leading to **cell dormancy/death**



<u>CreA antitoxin</u> reprograms **Cascade** to transcriptionally **repress** the RNA **<u>CreT toxin</u>**

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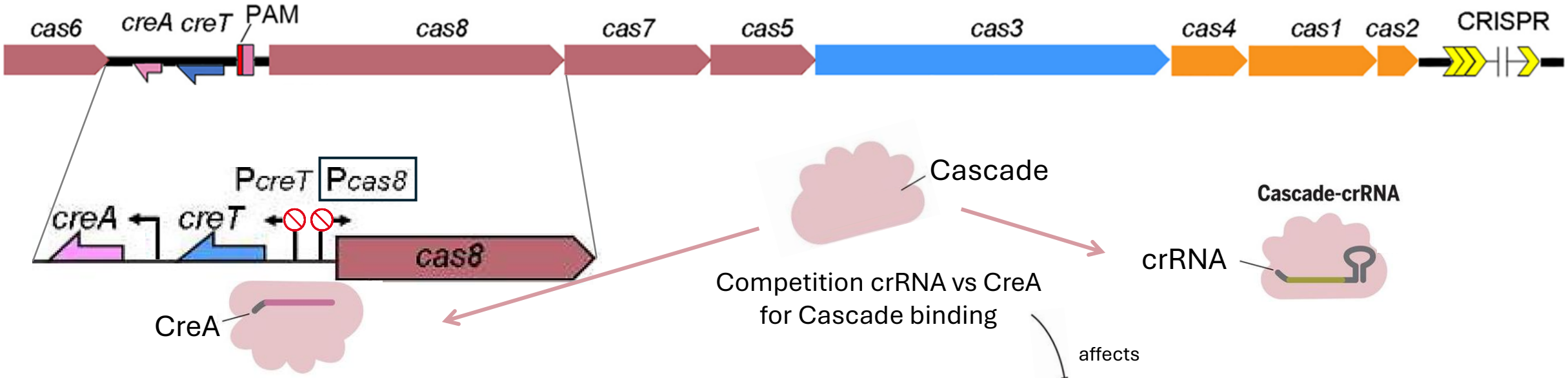
In this study:

SPOILER ALERT

Haloarcula hispanica **type I-B** CRISPR-cas loci



CreA target site includes a previously overlooked ***cas8* promoter**

CreA-Cascade synchronously represses *creT* and *cas*

A new **regulatory network** for **context-dependent *cas* expression** to **avoid autoimmunity**

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Article

Widespread RNA-based *cas* regulation monitors crRNA abundance and anti-CRISPR proteins

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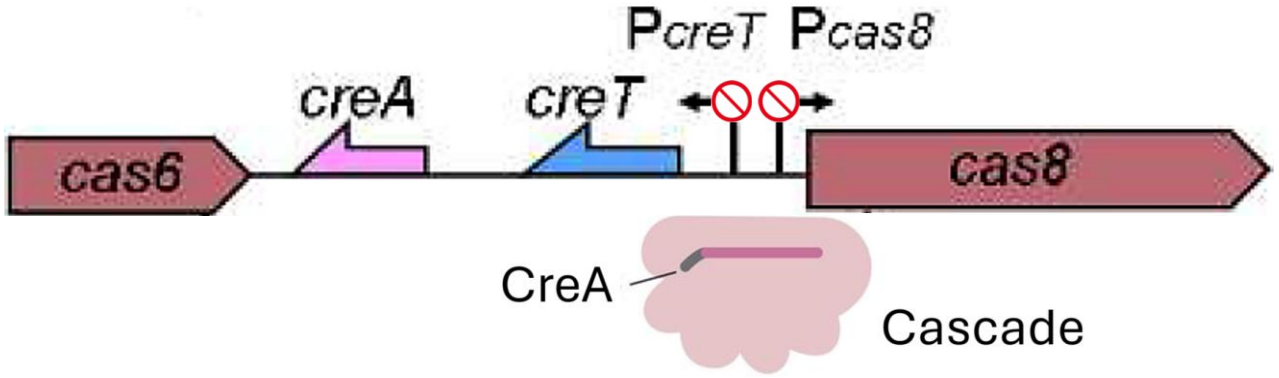
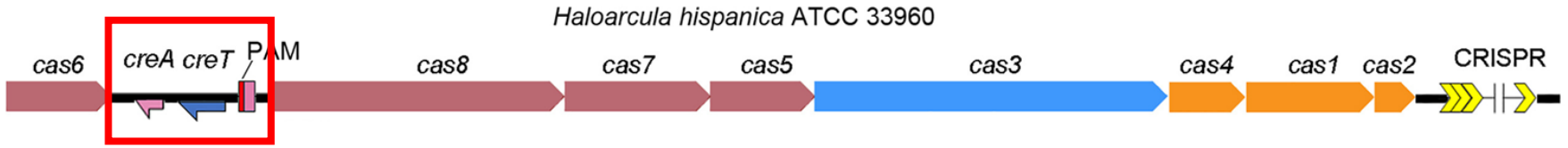
1. Demonstrating that *H. hispanica* **CreA** represses also ***cas*** transcription



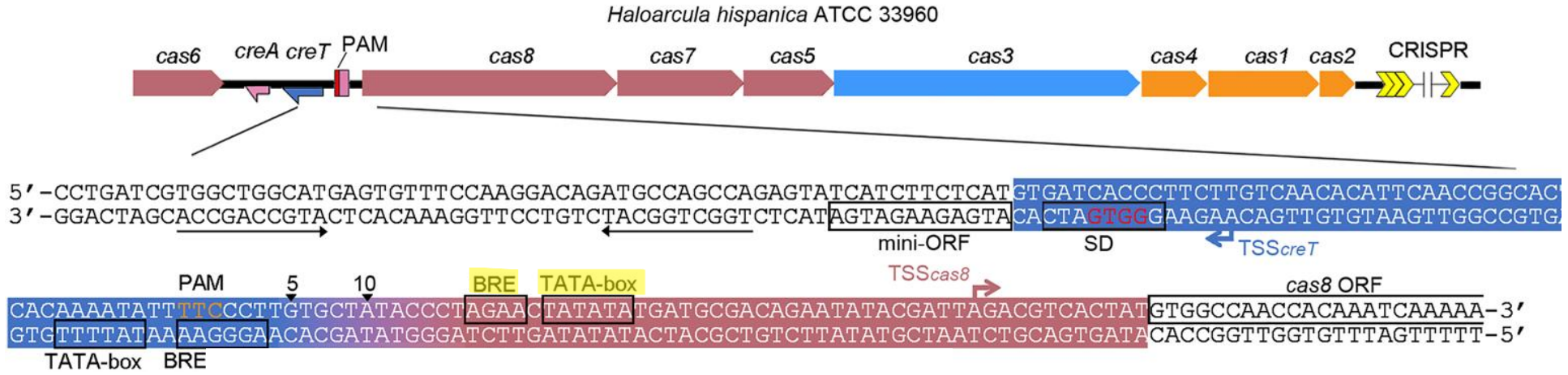
1. Demonstrating that *H. hispanica* CreA represses also *cas* transcription

Previous study: a ***H. hispanica* ΔTA** mutant showed **70-90% reduction of *cas* transcripts except for *cas6*** => An overlooked **promoter** precedes ***cas8***

~300 bp intergenic region
deleted in ΔTA

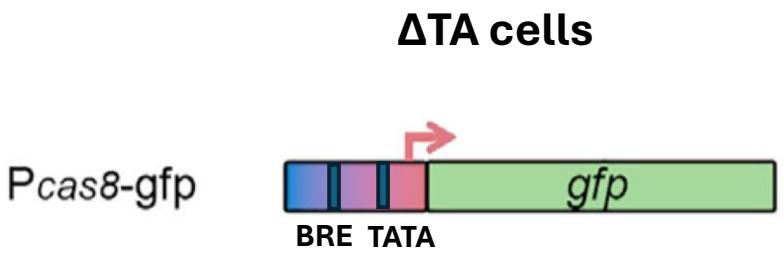


→ Reanalysis of previous RNA-seq data → ***cas8* TSS identification** 12 bp upstream *cas8* ORF
=> Prediction of archaeal promoter elements **BRE** and **TATA** box



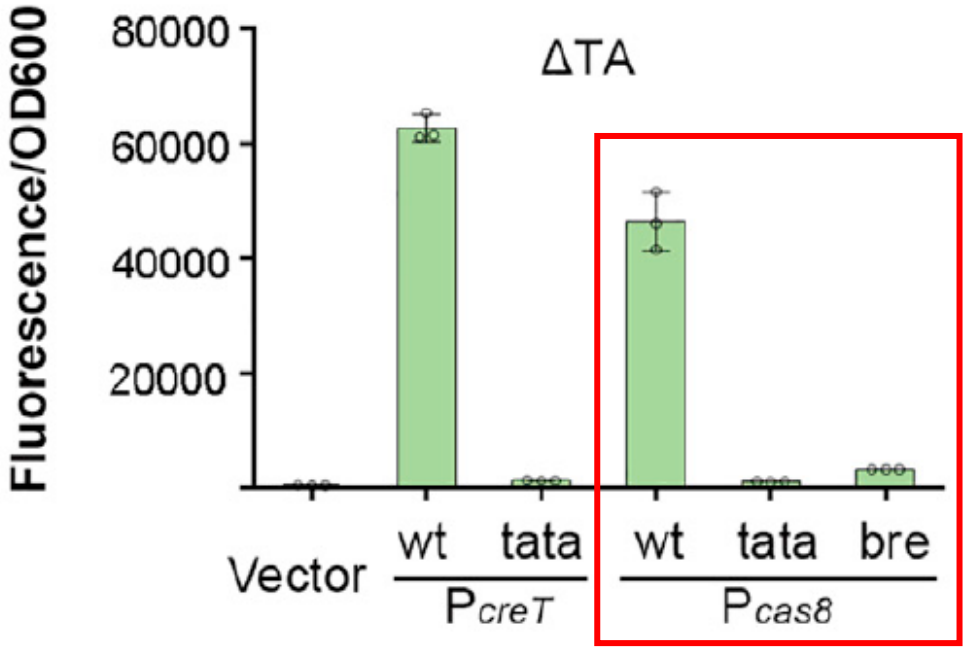
Validation of *cas8* promoter

***cas8* TSS identification** allowed to predict archaeal promoter elements **BRE** and **TATA** box
→ **VALIDATION**:



GFP-reporter system to evaluate the activity of:

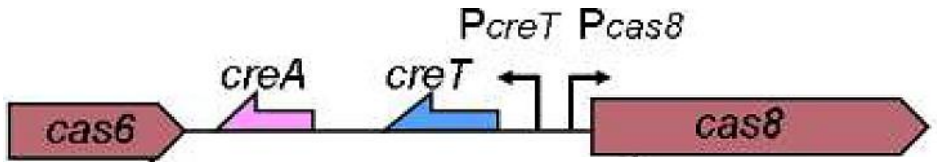
- **wt** P_{cas8}
- **TATA-mutated** P_{cas8}
- **BRE-mutated** P_{cas8}



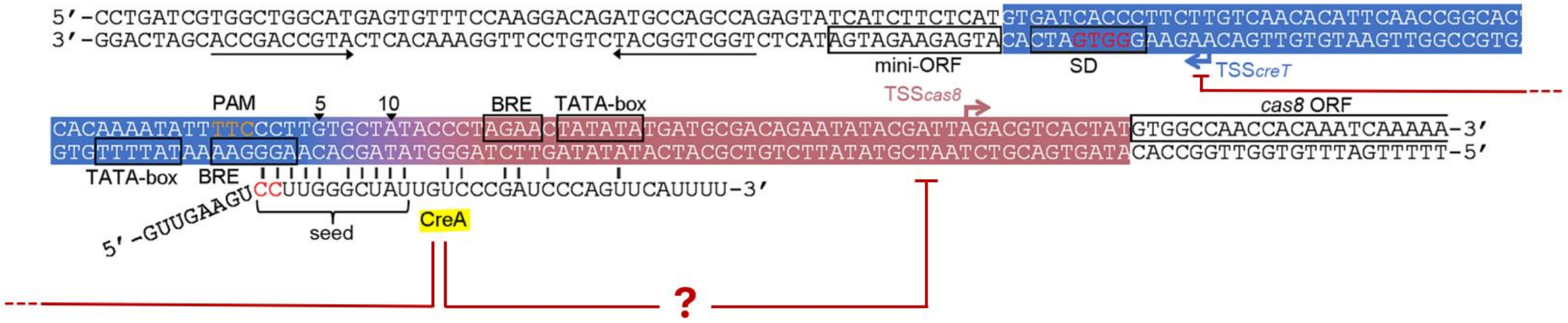
P_{cas8} activity **nullified** by mutating archaeal promoter elements sequences

1. Demonstrating that *H. hispanica* CreA represses also *cas* transcription

Validated *cas8* promoter



P_{cas8} and **P_{creT}** run divergently and tightly flank **CreA target site**



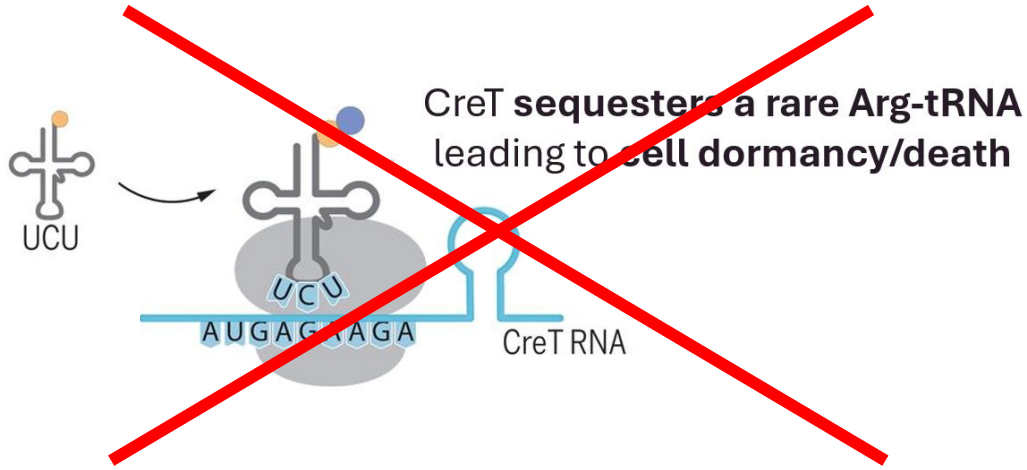
Is P_{cas8} also repressed by CreA?

1. Demonstrating that *H. hispanica* CreA represses also *cas* transcription

Is P_{cas8} also repressed by CreA?

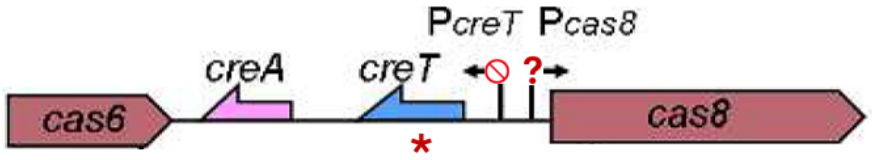
What happens if we **impair CreA binding** to its target site by **mutating CreA sequence**?

Problem: Abolishing CreA-mediated repression would lead to **CreT toxin expression**!



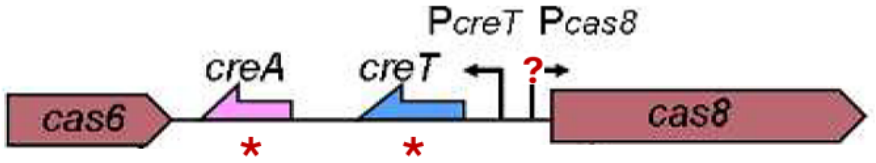
Solution: mutate **CreT** to make it **non-toxic**

Tm mutant cells - **CONTROL**



- ***creT* mutated** → **non-toxic** if expressed
- *creA* wt

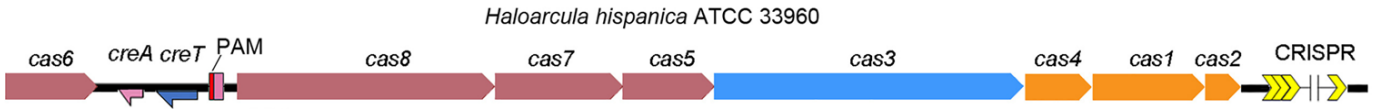
TA double mutant cells – **No CreA biding**



- ***creT* mutated** → **non-toxic** if expressed
- ***creA* mutated** → **impaired complementarity** to its target site => abolished repression

1. Demonstrating that *H. hispanica* CreA represses also *cas* transcription

Is P_{cas8} also repressed by CreA?



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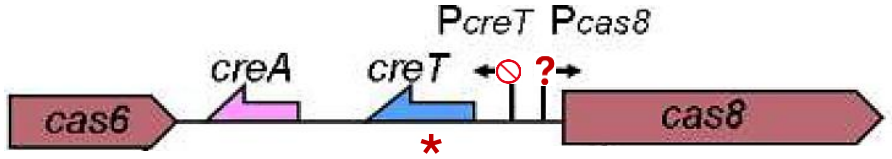

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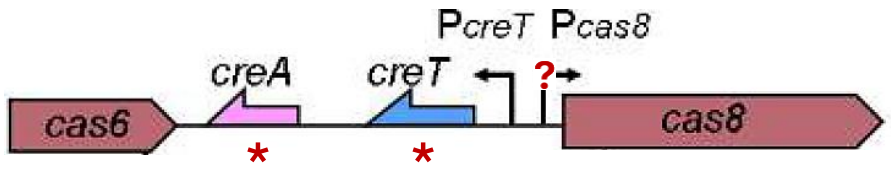
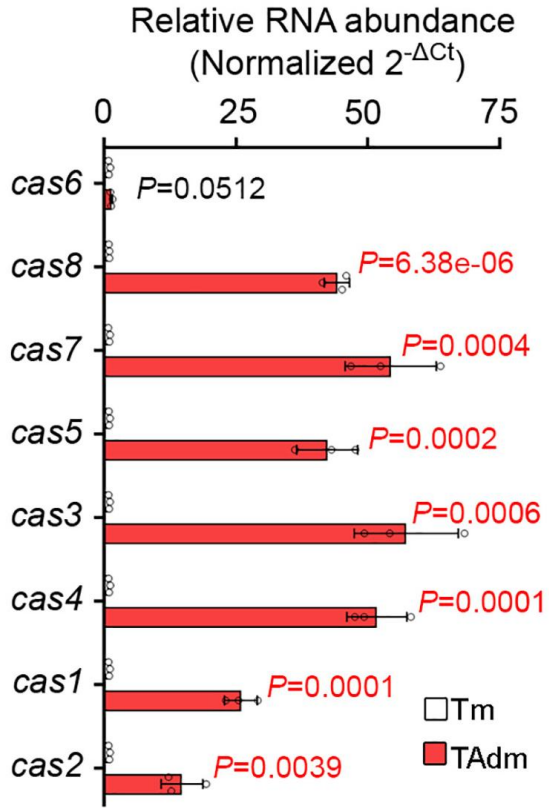


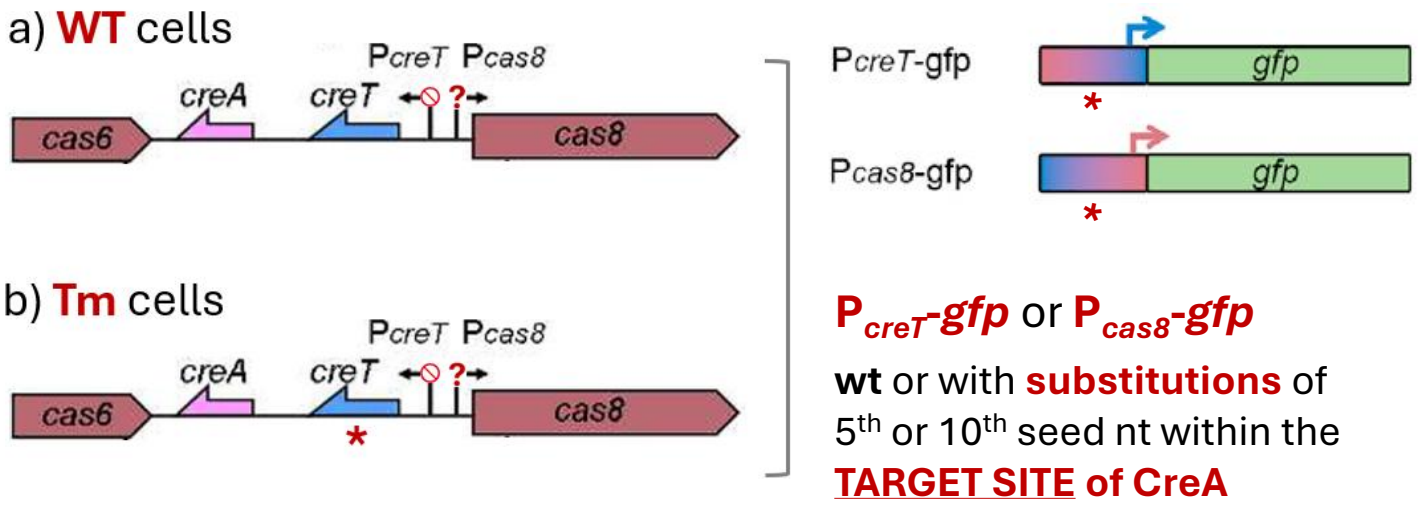
- ***creT* mutated** → **non-toxic** if expressed
- ***creA* mutated** → **impaired complementarity** to its target site => abolished repression



15- to 54-fold up-regulation of *cas8* and downstream *cas* in TAdm;
no significant changes for *cas6*

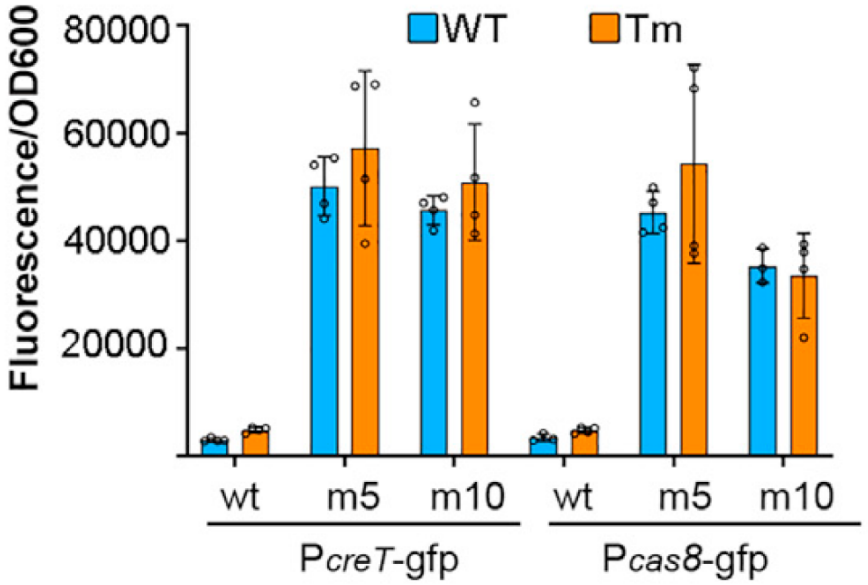
1. Demonstrating that *H. hispanica* CreA represses also *cas* transcription

Is P_{cas8} also repressed by CreA?



P_{creT}-*gfp* or **P_{cas8}-*gfp***

wt or with **substitutions** of 5th or 10th seed nt within the **TARGET SITE of CreA**



GFP-reporter assay to evaluate **P_{cas8}** and **P_{creT}** activity depending on **CreA capability to bind its target site**

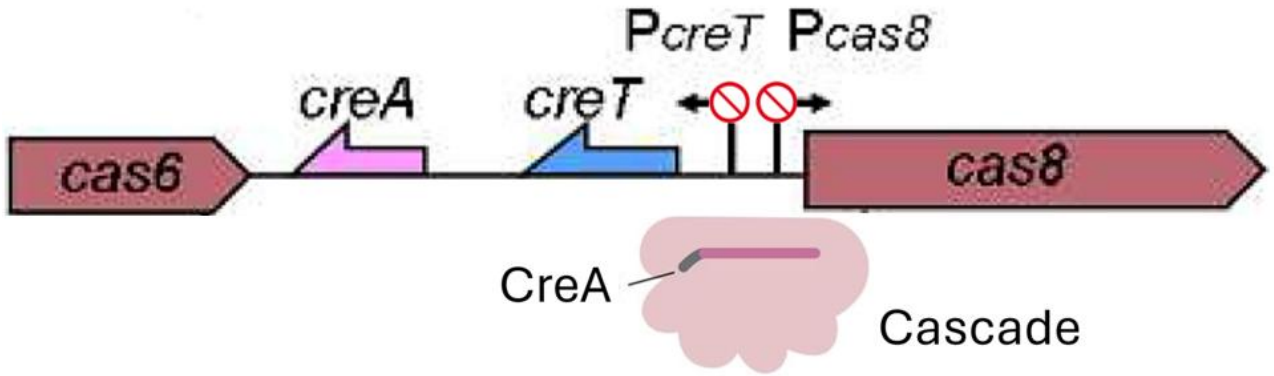
P_{cas8} and P_{creT} are **both de-repressed in m5 and m10** in WT and Tm cells when the **seed sequence is mutated**

1. Demonstrating that *H. hispanica* CreA represses also *cas* transcription

Is P_{cas8} also repressed by CreA?

↓

Conclusion: **P_{cas8}** and **P_{creT}** are **synchronously downregulated by CreA**

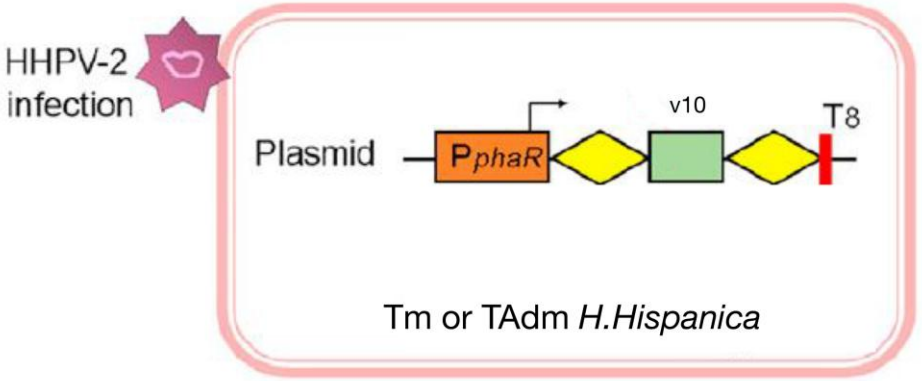


2. CreA-guided cas regulation shifts from **reducing autoimmunity** to **enhancing immunity against imperfect targets**

Can *cas* overexpression lead to a more proficient CRISPR immunity?

Model: **Tm** & **TAdm** + construct with **synthetic mini-CRISPR with v10 spacer** (targeting HHPV-2 virus) under the control of **P_{phaR} constitutive promoter**

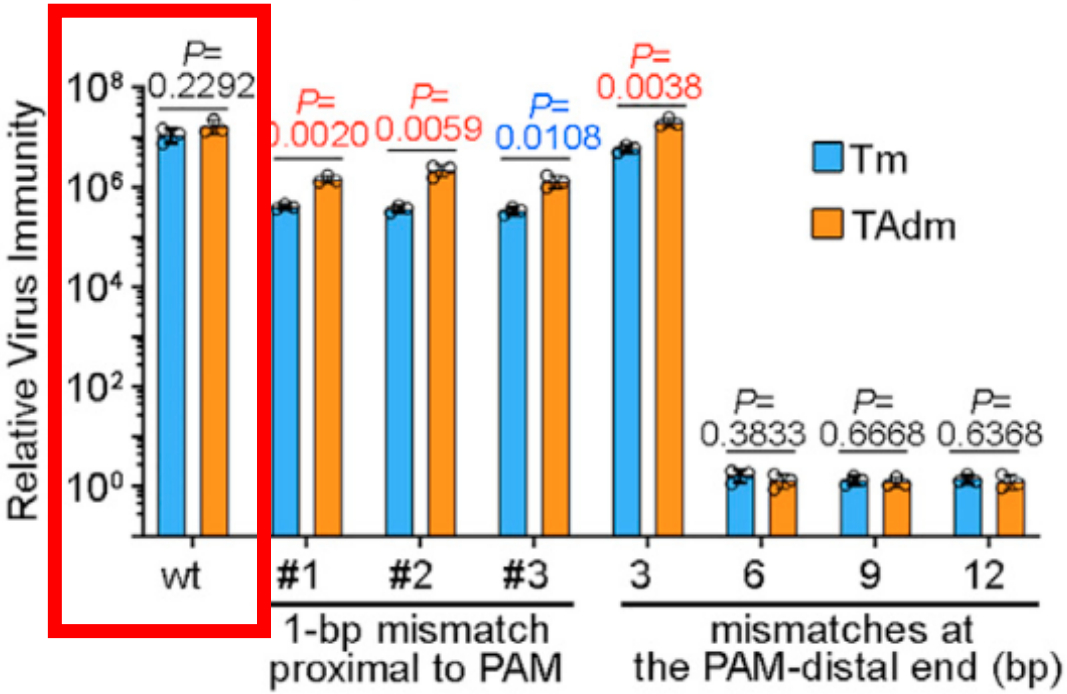
→ Cells are infected with **HHPV-2**



Methods: immunity level reveled by Plaque Assay

Result: **equivalent** viral immunity levels in both Tm and Tadm

→ Strong response also with wild levels of Cas



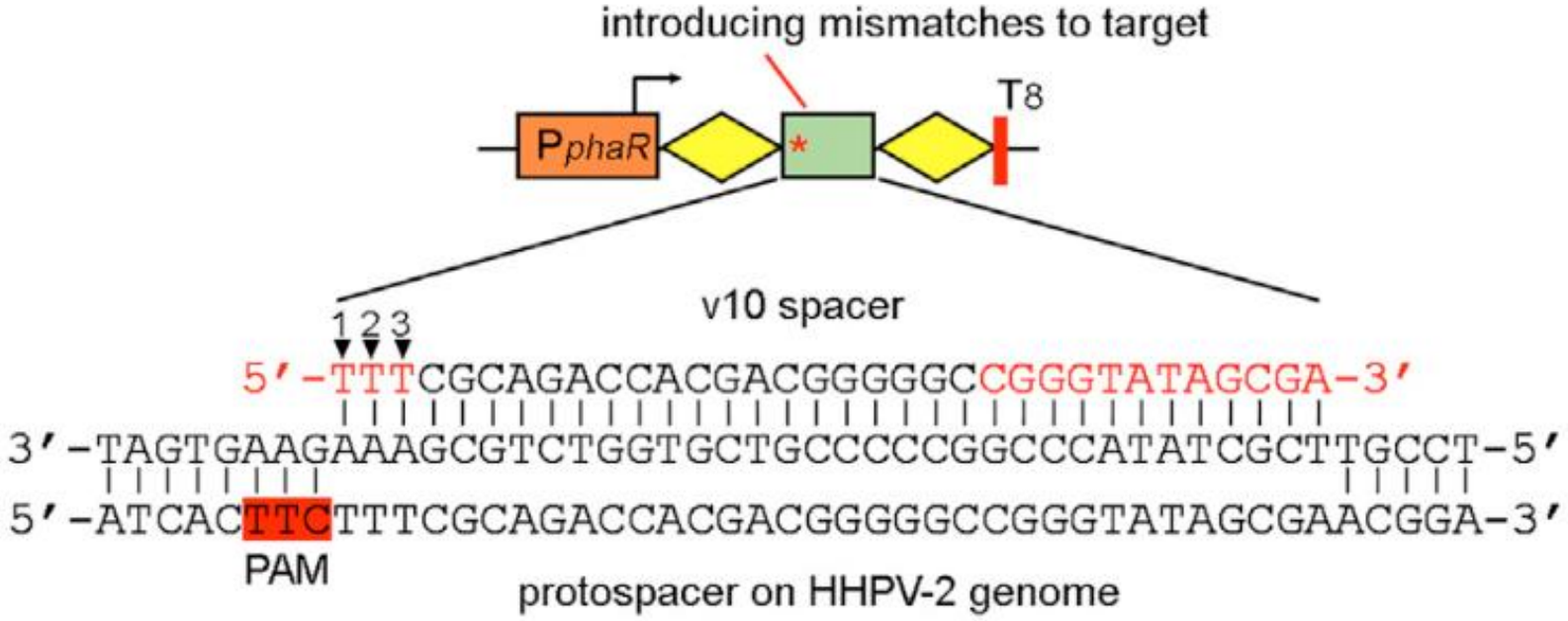
BUT viruses can escape CRISPR immunity by **mutating** the protospacer, generating imperfect targets

Can *cas* overexpression lead to more efficient immunity against imperfect targets?

Model: Tm & **TAdm** + construct with **synthetic mini-CRISPR with v10 spacer** (targeting HHPV-2 virus) with different **mutations** under the control of $\mathbf{P_{phaR}}$ constitutive promoter

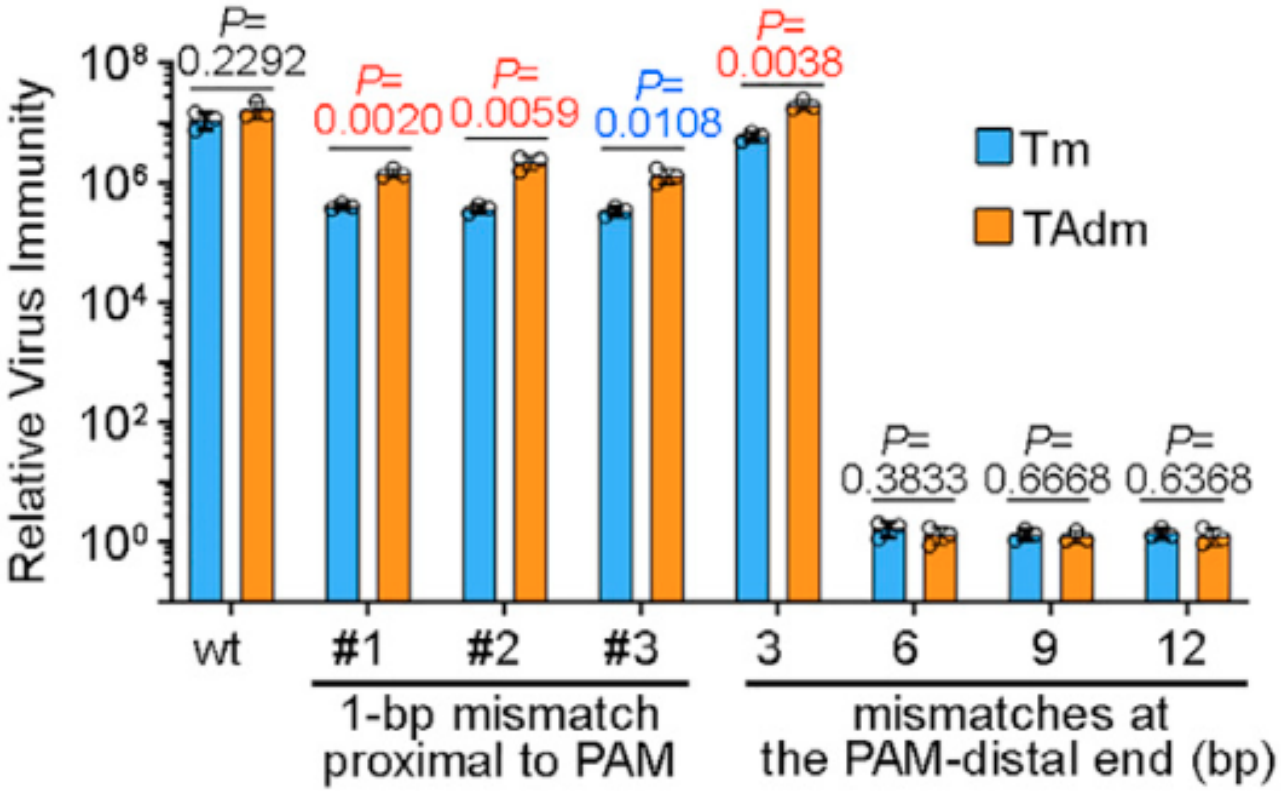
→ Cells are infected with **HHPV-2**

Methods: immunity level measured by Plaque Assay



Can *cas* overexpression lead to more efficient immunity against imperfect targets?

- Single nt mutation proximal to PAM
  Result: **TAdm higher** immunity level than Tm
- Little mismatches in the PAM-distal end
  Result: **TAdm higher** immunity level than Tm
- Big mismatch in the PAM-distal end
  Result: **neither** TAdm & Tm can activate CRISPR immunity



Interference step

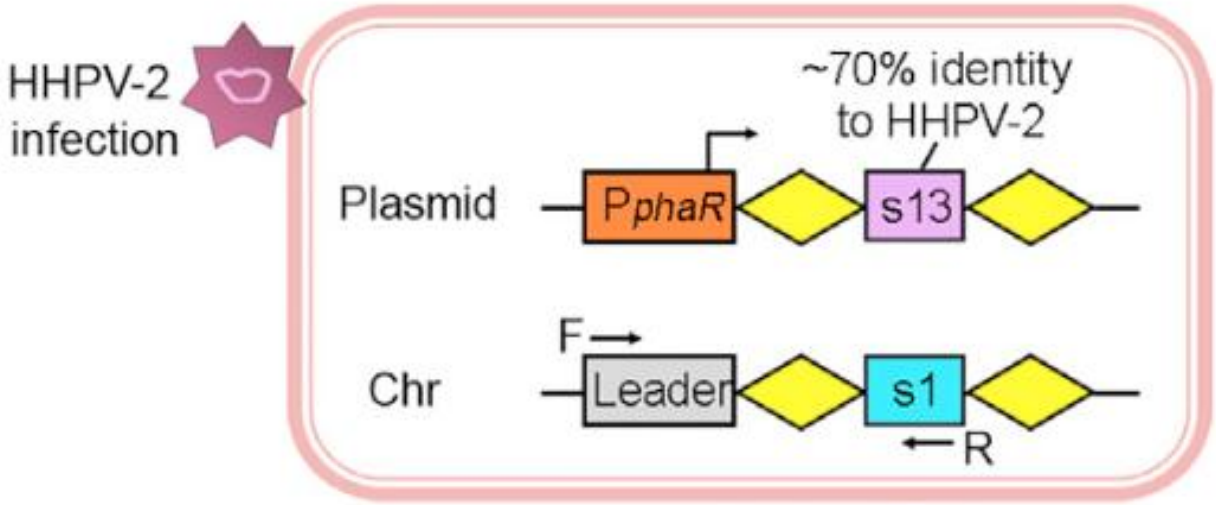
The overexpression of *cas* leads a **more proficient** CRISPR immunity, against **some escape mutants** that interact weakly with crRNA

As *cas* genes are involved in adaptation, can their up-regulation lead to more effective primed spacer acquisition?

Model: WT, Tm and TAdm + construct for overexpression of **crRNA s13**

crRNA s13 = a *H. hispanica* **spacer** that can **activate primed adaptation** (70% identity with HPPV-2)

→ Cells are infected with **HHPV-2 virus** in different quantity (multiplicity of infection = MOI)

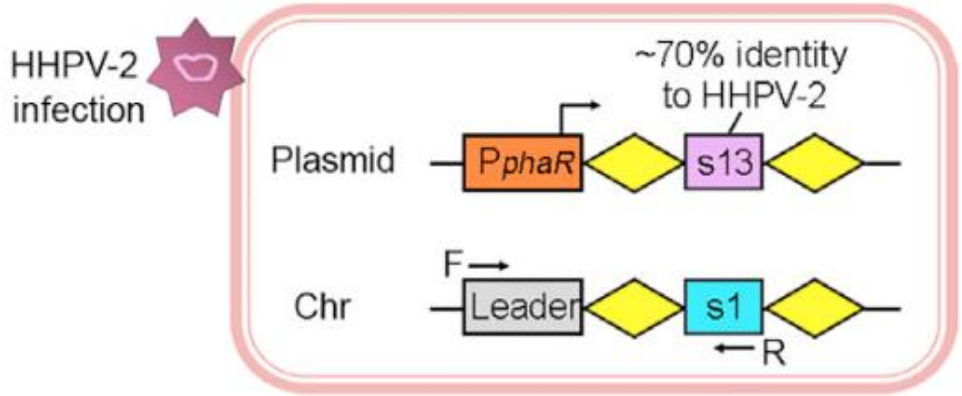


The s13-encoding plasmid makes **all cells express the same quantity of crRNA s13**
→ the results will reflect only the difference in *cas* expression

Knowing that *cas* genes are involved in adaptation, can their up-regulation lead to a more effective primed spacer acquisition?

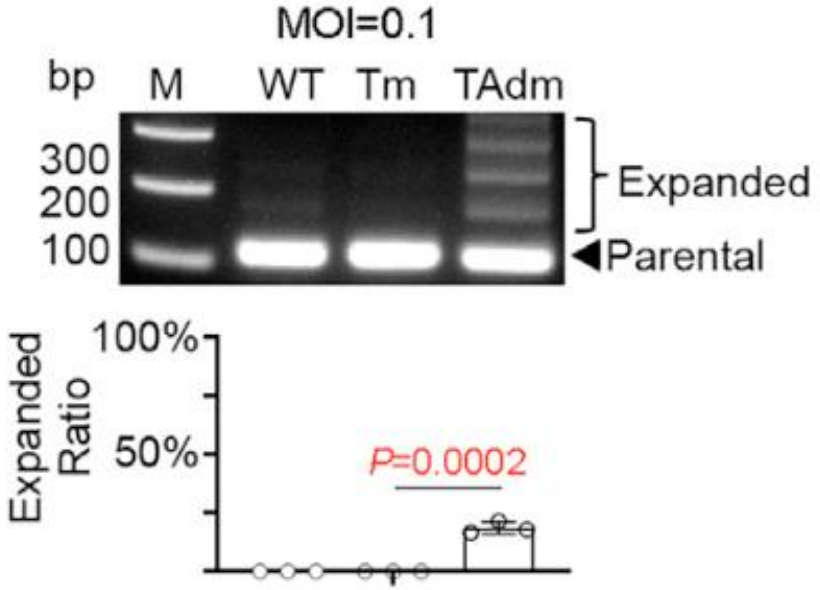
Methods: measure new spacers acquisition through PCR

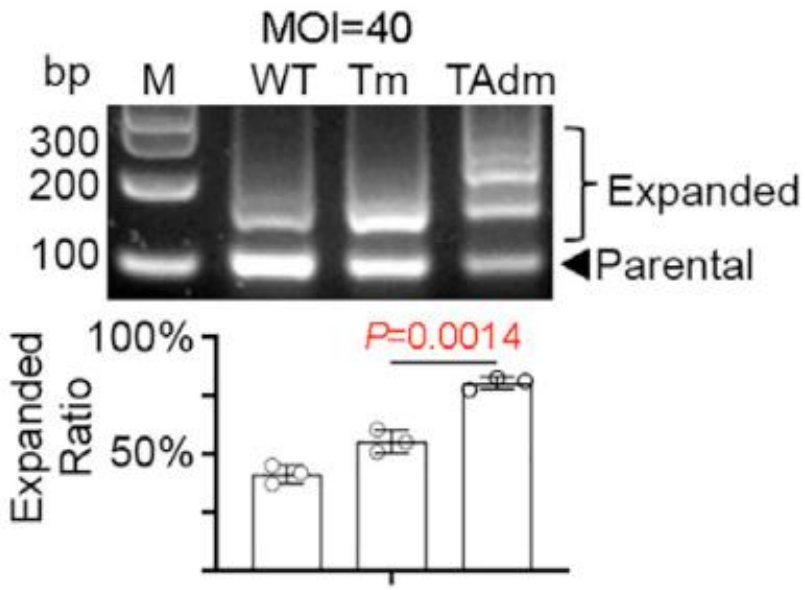
Result: **TAdm** show **higher frequency of incorporation** of new spacers in the CRISPR array compared to WT and Tm, in both low (0.1) and high (40) MOI



Adaptation step

The overexpression of *cas* leads a **more proficient primed spacer acquisition**



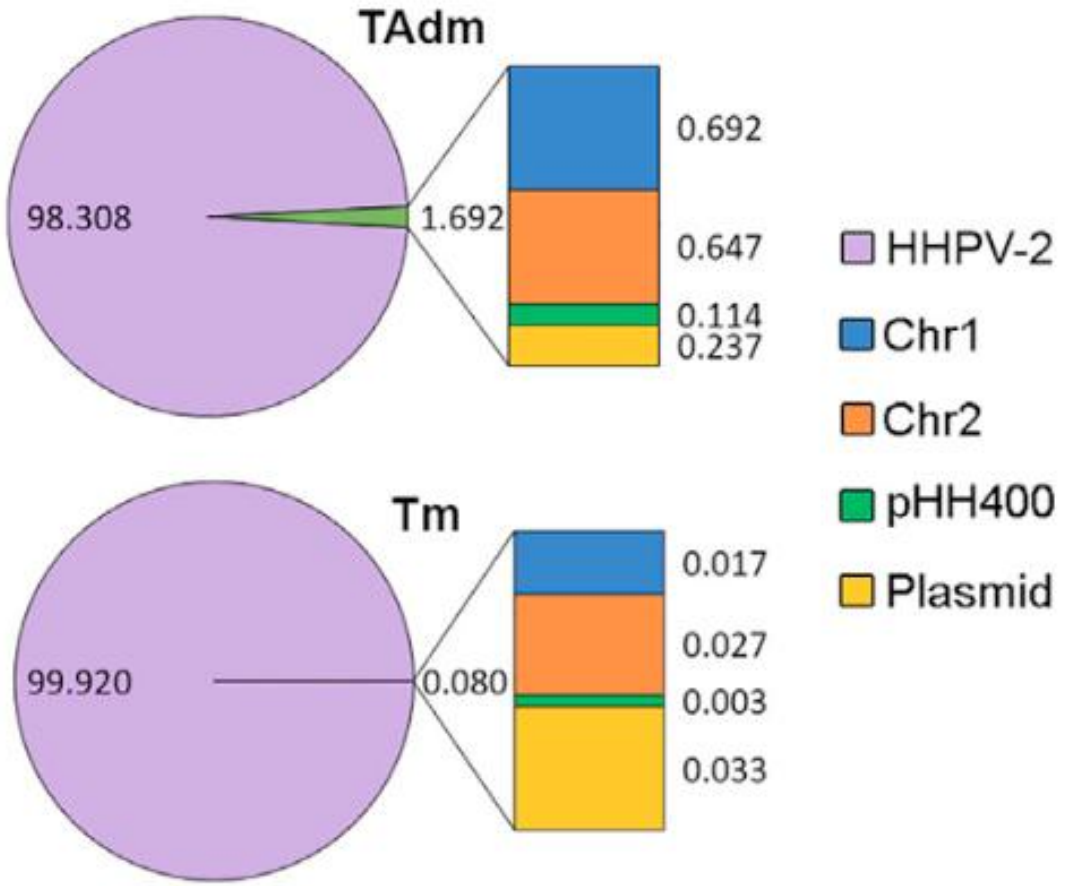


BUT

Can *cas* up-regulation lead to a higher risk of acquisition ALSO of self-derived spacers?

Result: Illumina seq data reveal → **TAdm** mistakenly acquires **self-derived spacers** at a higher frequency than Tm, leading to **autoimmunity**

TAdm 1.69% vs Tm 0.08%
self-derived spacers

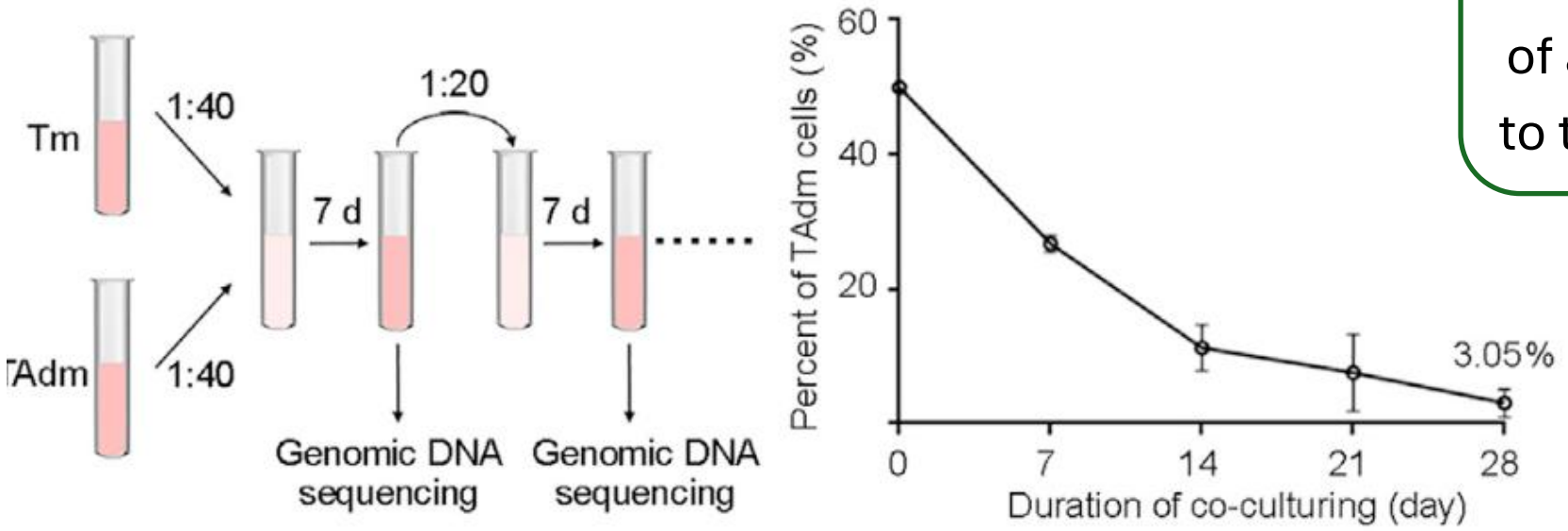


Does the accumulation of self-targeting spacers compromise the fitness?

Model: Tm & TAdm in long-term **co-culture**

Methods: measure cell percentage by high-throughput DNA seq

The **fitness** is mainly **compromised** because of **autoimmunity** related to the *cas* overexpression



Result: **Tm outcompeted** (= have a better fitness) **TAdm** over time, with proportion of TAdm dropping to 3.05%

2. CreA-guided *cas* repression reduces autoimmune risk

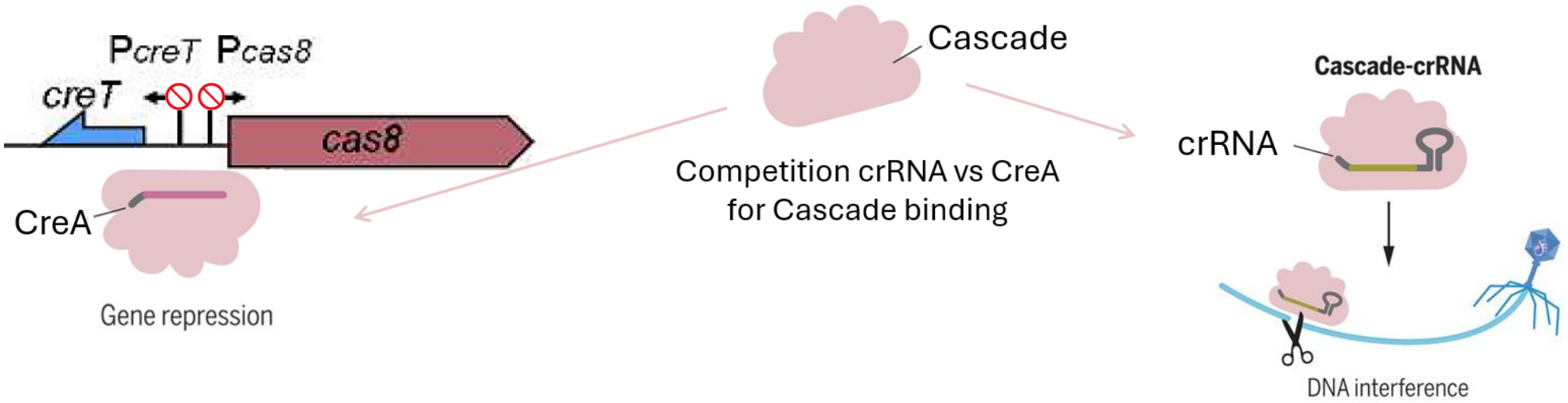
Conclusion: the repression of *cas* genes is necessary to balance viral immunity with the **prevention of autoimmunity**

3. The **entity of CreA-guided gene repression** depends on **crRNA levels**

crRNA and CreA <u>both bind Cascade</u>:

- **crRNA + Cascade** = immunity effector machinery
- **CreA + Cascade** = repression complex → suppression of $\mathbf{P}_{cas8}$

competition?

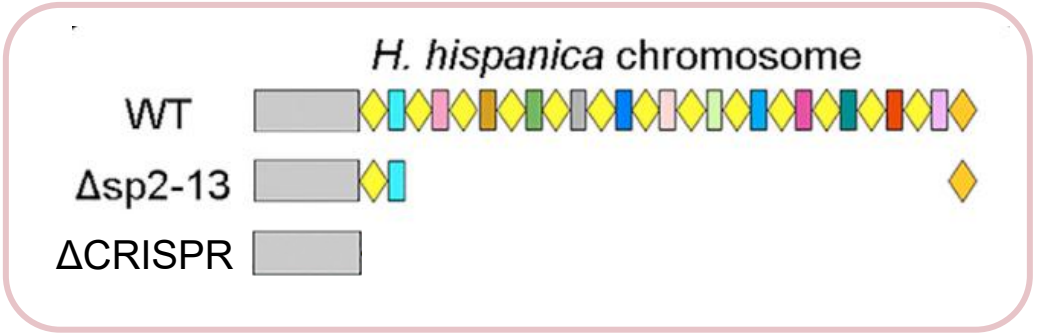


Does crRNA abundance affect CreA repression of P_{cas} ?

Hyp: **↓ crRNA** => ↑ CreA-mediated *cas* repression

Model:

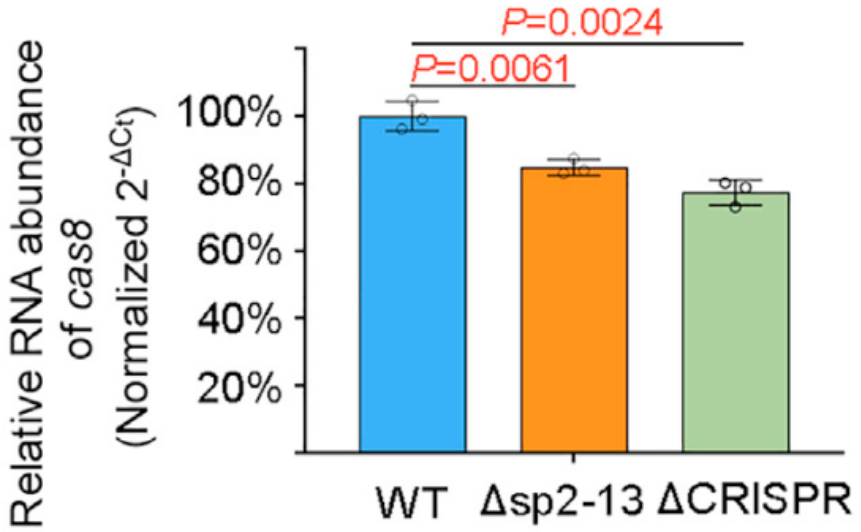
- WT cells
- **ΔCRISPR** cells
- **Δsp2-13** (= CRISPR array with only 1 spacer) cells



Methods: quantify ***cas8*** **transcription** by RT-qPCR

Result: in **ΔCRISPR & Δsp2-13** ***cas8*** **expression decreases by ~20%** compared to wt

(= more repression if less crRNA are present)



3. The entity of CreA-guided gene repression depends on crRNA levels

Does crRNA abundance affect CreA repression of P_{cas} ?

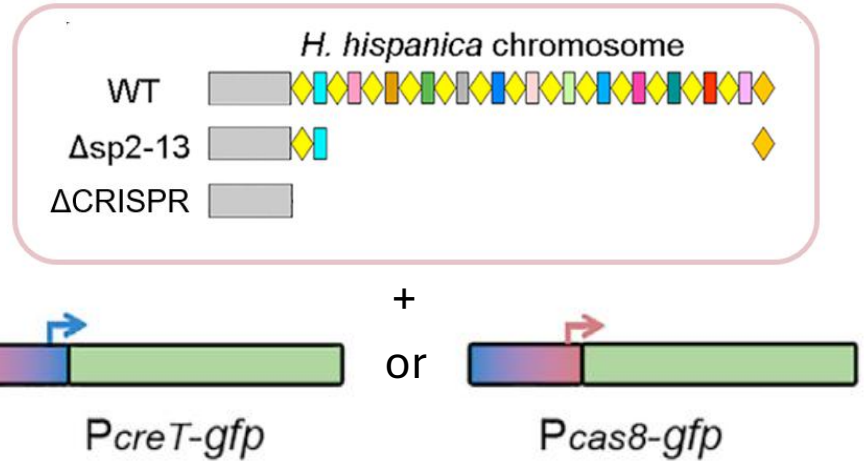
Hyp: ↓ crRNA => ↑ CreA-mediated *cas* repression

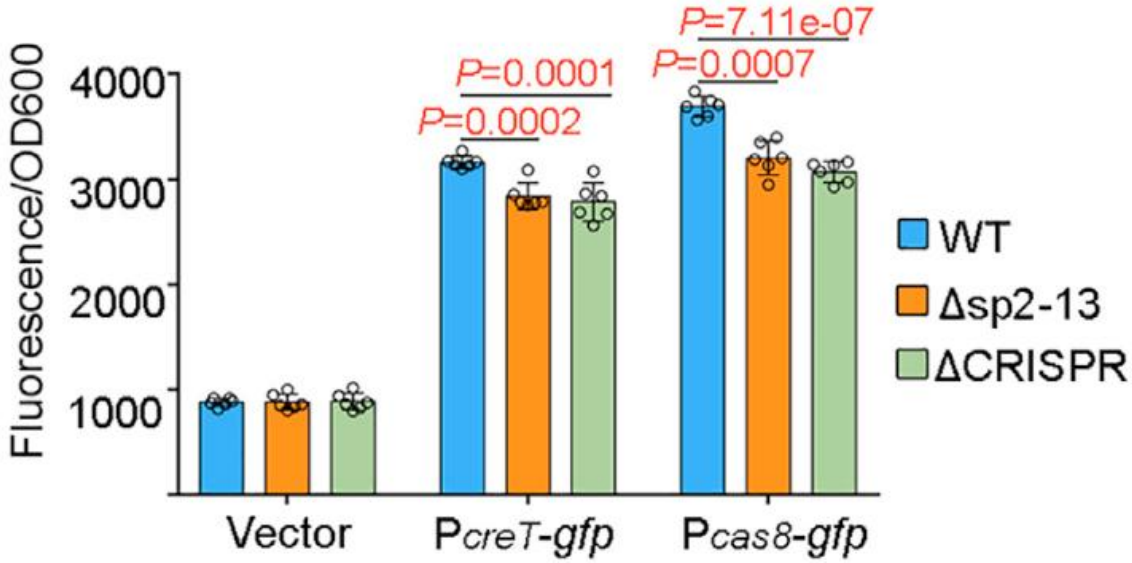
Model: WT, **ΔCRISPR** & **Δsp2-13** + P_{cas8}-*gfp* or P_{creT}-*gfp*

Methods: evaluate **P_{cas8} or P_{creT}** activity **at different crRNA levels** by *gfp*-reporter assay

Result: in ΔCRISPR & Δsp2-13 promoters P_{cas8} and P_{creT} **activity decreases by 10%-20%**

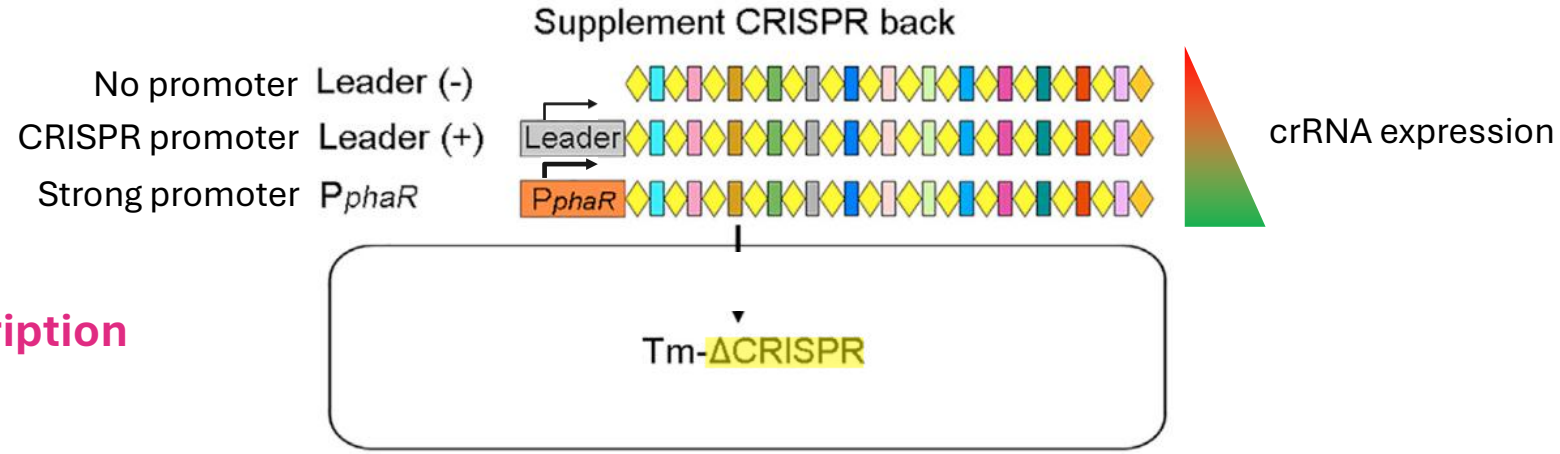
CreA tightly **suppresses P_{cas8}** & **P_{creT}** in case of **fewer** or **absence** of CRISPR **spacers** (crRNA)





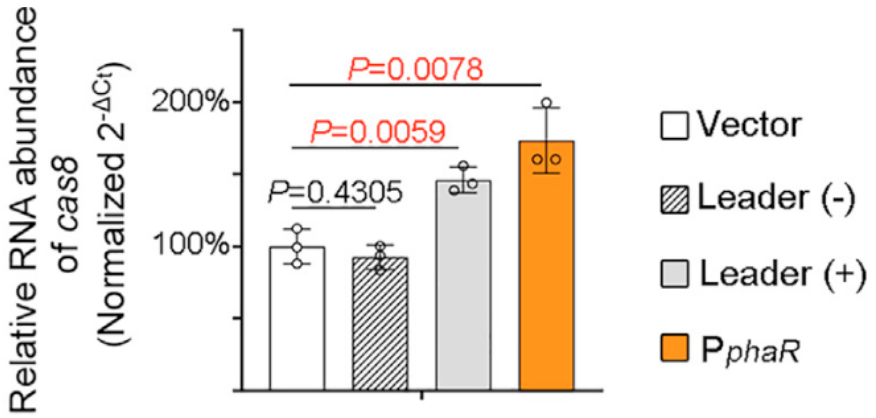
Does crRNA abundance affect CreA repression of P_{cas} ?

Hyp: ↑ crRNA => ↓ *cas* repression CreA mediated



Evaluate ***cas8* transcription** through **RT-qPCR**

Result: **crRNA expression** leads to almost **2-fold** **increase of *cas8* transcripts** in comparison with hosts having empty vector or leader-less array



CreA-guided gene **repression** can be **alleviated by expanding** the **crRNA** pool

Does the overexpression of CreA lead to tighter repression of P_{cas8}?

Hyp: ↑ *cas* repression CreA mediated => ↓ crRNA

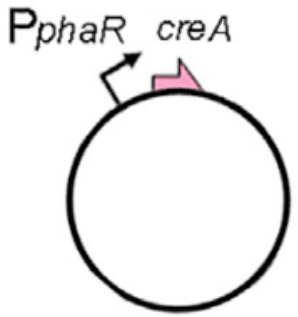
Model: WT + constructs:

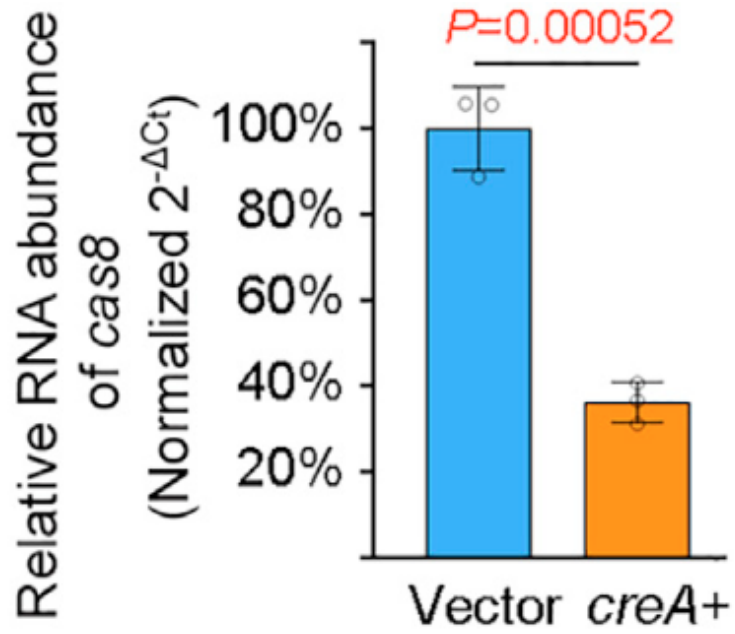
- Empty vector
- **Strong promoter** P_{phaR} upstream **CreA** → **overexpression of CreA**

Methods: quantify ***cas8* transcription** by RT-qPCR

Result: relative aboundance of **cas8 transcript decreases** by 60% in cells **overexpressing CreA**

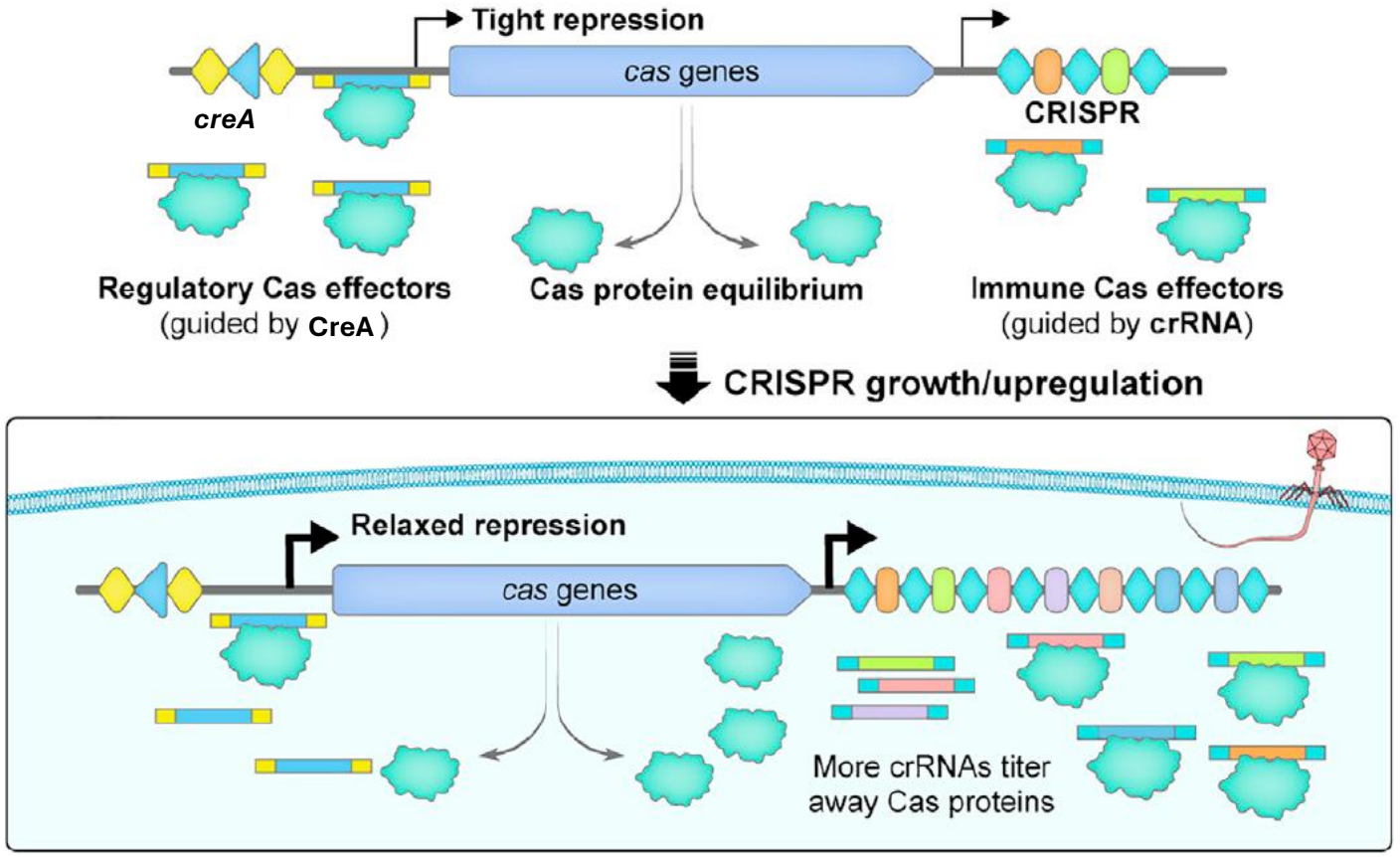
CreA overexpression leads to an **enhanced repression of *cas* genes**



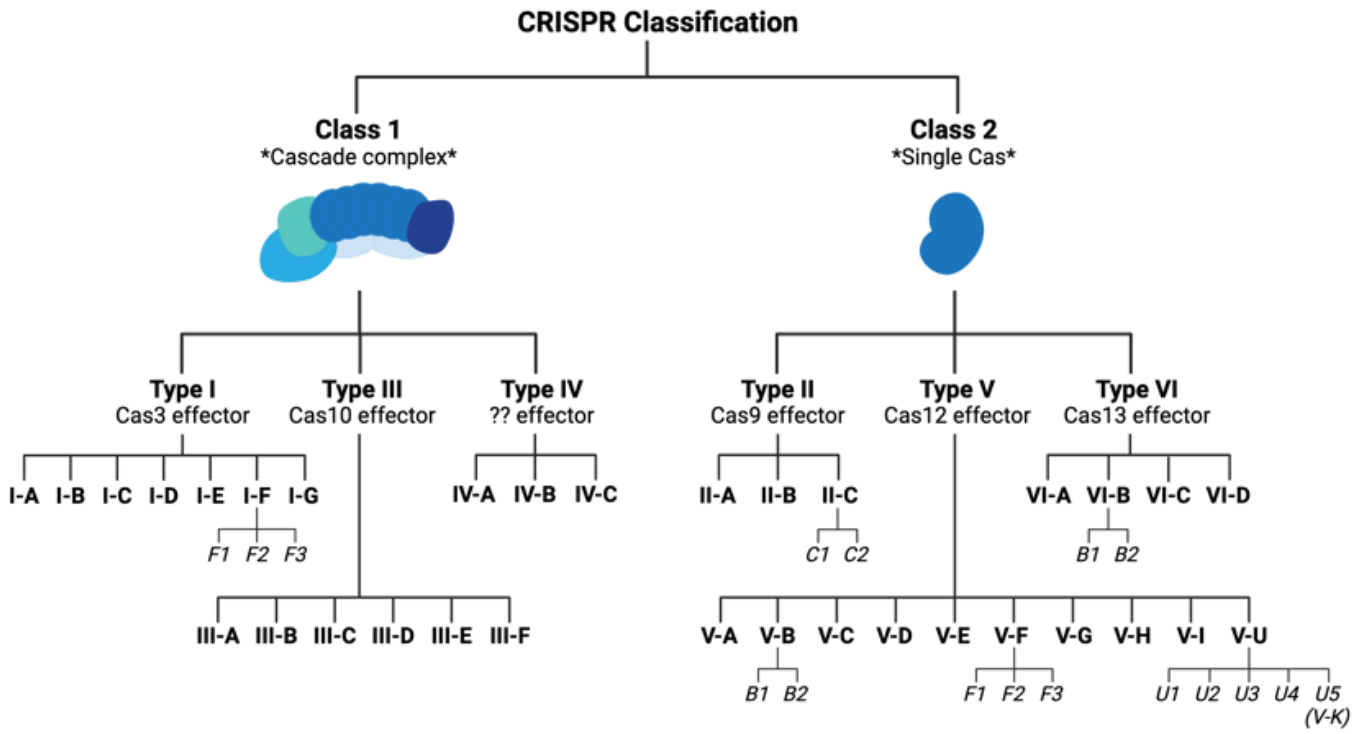


3. The entity of CreA-guided gene repression depends on crRNA levels

Conclusion: ***cas* autorepression** can be **fine-tuned** by altering the relative transcripts **abundance** of the defensive RNA guides **crRNA** and regulatory **CreA**



4. Inspecting the wider **distribution** of CreA in different CRISPR-Cas systems

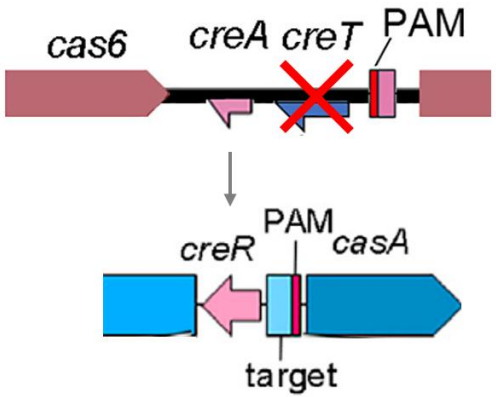


4. Inspecting the wider distribution of CreA

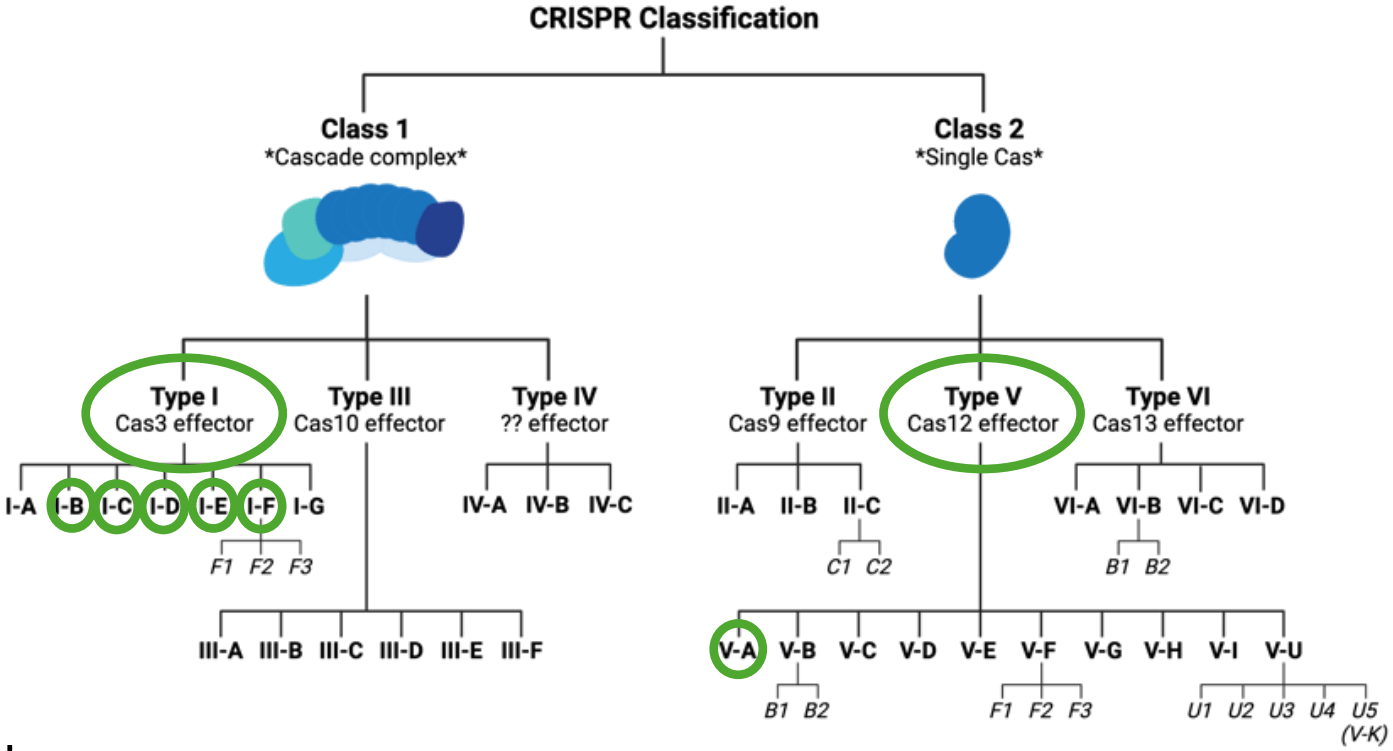
Sequence analysis of more CRISPR-Cas systems revealed ***creA*-like** elements also in: various **type I** systems (Cascade complex) & dozens of **type V-A** systems (Cas12a)

In most cases **CreA targeted** the **promoter of *cas* genes**

Many of these *creA*-like elements **DO NOT co-locate with a toxic gene**!



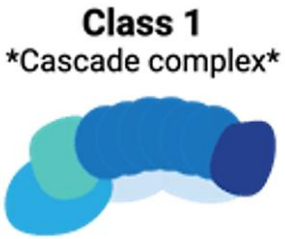
«standalone» *creA* genes will be referred as ***creR*** (*cas*-regulating RNA)



Cas **autoregulation** directed **by CreA or CreR** appears to be a **general mechanism** of both **class 1** and **class 2** CRISPR systems

Experimental validation of **CreR** regulatory role in directing the **autorepression circuit**:

5. CreR in **class 1** type **I-E** CRISPR system



6. CreR in **class 2** type **V-A** CRISPR system

Conclusion: Cas **autoregulation** directed **by CreA or CreR** is a **general mechanism** of both **class 1** and **class 2** CRISPR systems and **monitors crRNAs** abundance in the cell

7. Phage **Acr** proteins can **relieve or subvert** CreR-guided **Cas autorepression**

***cas* autoregulation circuit** can surveil phage attack

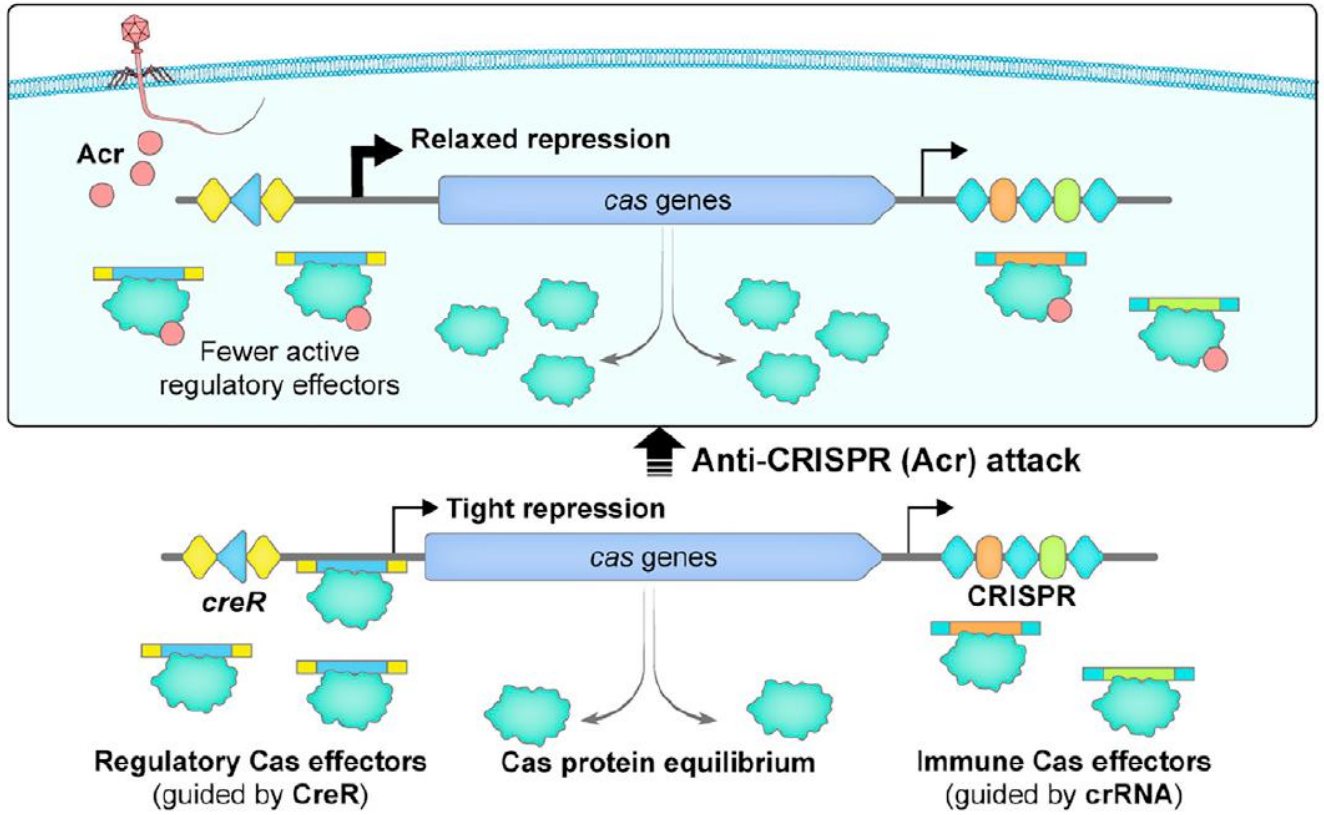
<u>Anti-Anti-CRISPR effect</u>:

Arc proteins can **relieve** or **subvert** the **CreR repression** leading to the increase of **new Cas production** for phages defeat

7. Phage Acr proteins can relieve or subvert CreR-guided Cas autorepression

Arc proteins bind & repress

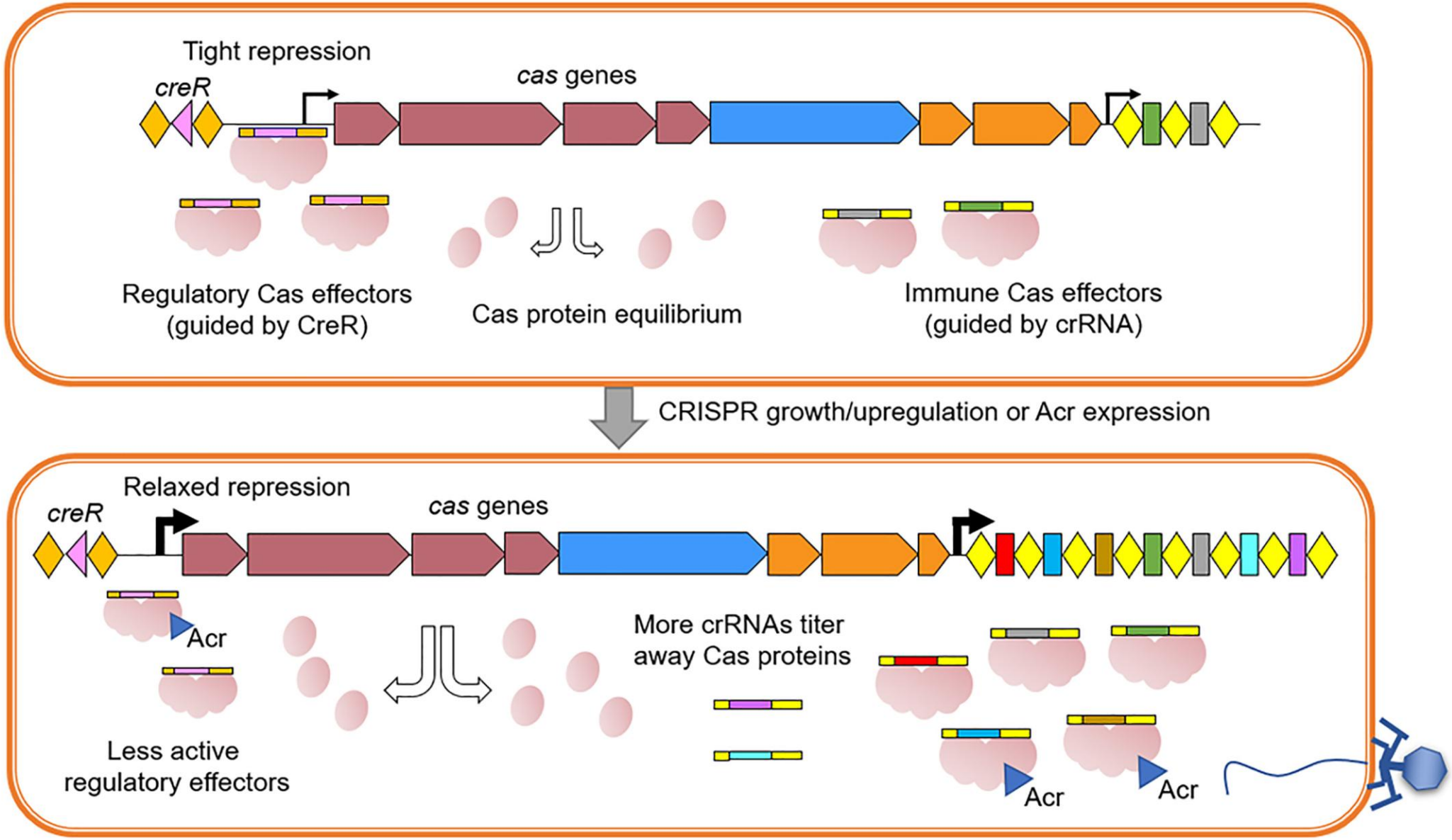
- Cas effectors + crRNA → inhibit the CRISPR-Cas system → **NO interference**
- Cas effectors + CreR → inhibit the CreR repression of *cas* genes → expression of *cas* → **Enhanced CRISPR-Cas immune response**



OUTLINE

- INTRODUCTION
- STUDY AIM
- RESULTS
- **CONCLUSION**

Model of CreA or CreR-guided Cas autorepression



Cas autoregulation circuit directed by crlRNA CreA or CreR:

Balances benefits and downsides of CRISPR-Cas

Might represent a distinct **anti-anti-CRISPR** strategy

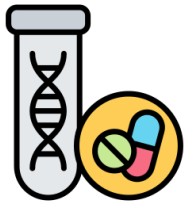
With CreT, might have **promoted persistence of CRISPR-Cas** in prokaryotes

Exploring ncRNA in bacterial immunity and phages counterattack

Revealing molecular parallels to **conserved eukaryotic antiviral pathways**

Developing novel **biotechnologies**

- **Control** strategies for CRISPR-Cas biotechnologies
- Improving **antimicrobial** phage therapy efficacy



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Complementary slides

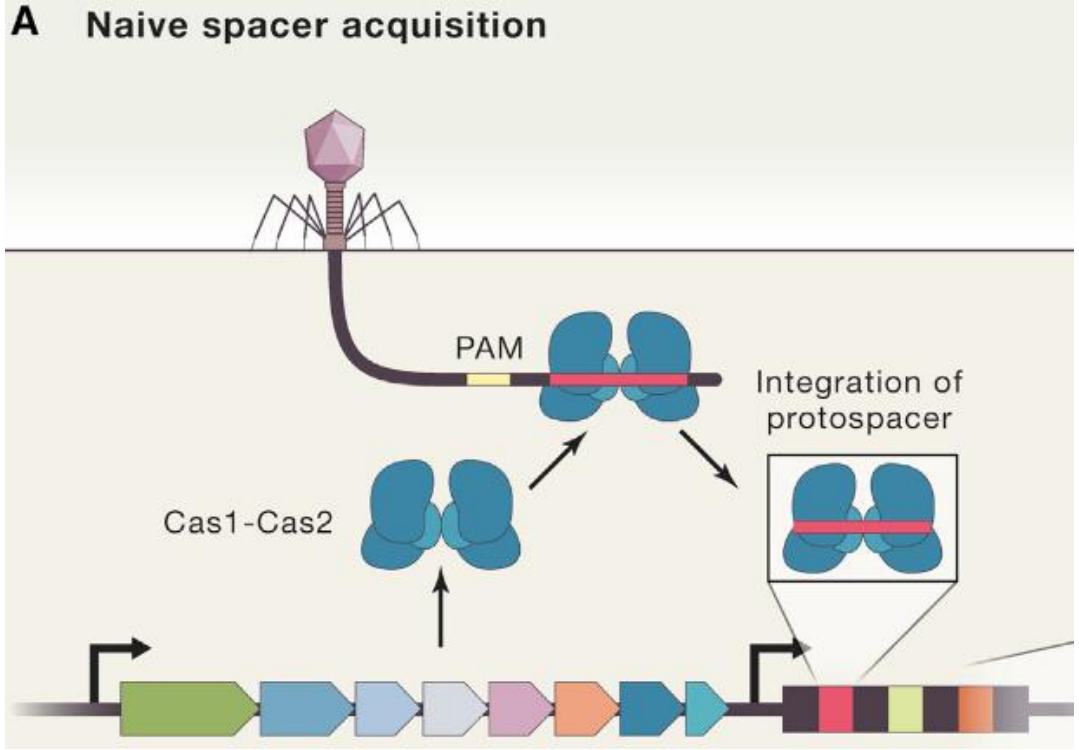
1. Adaptation

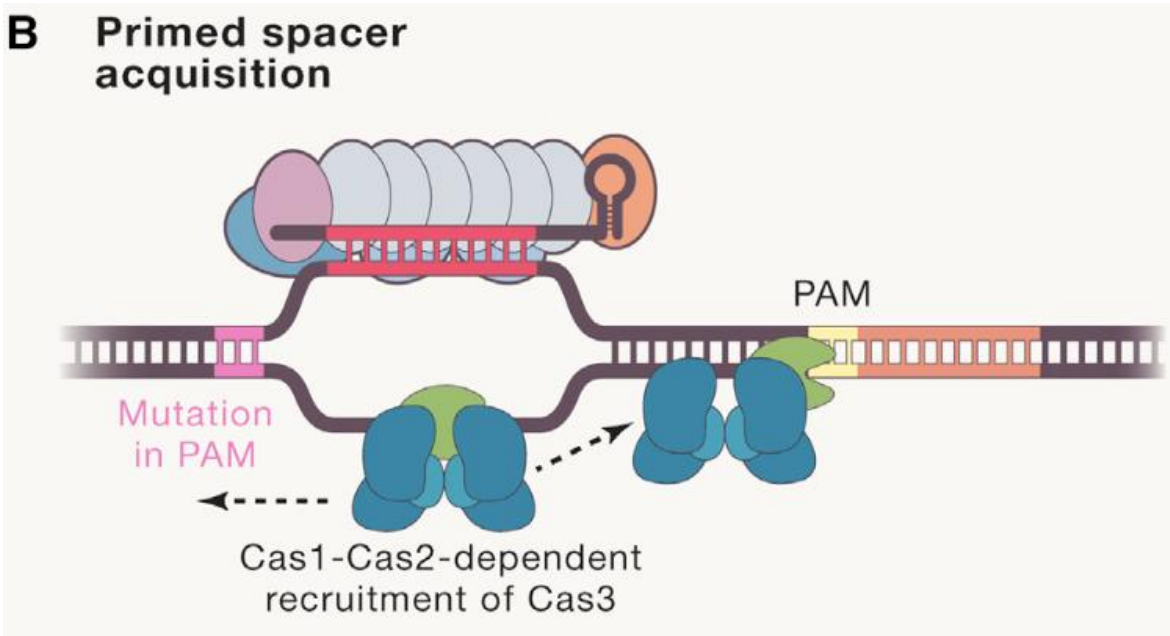
Methods spacer acquisition:

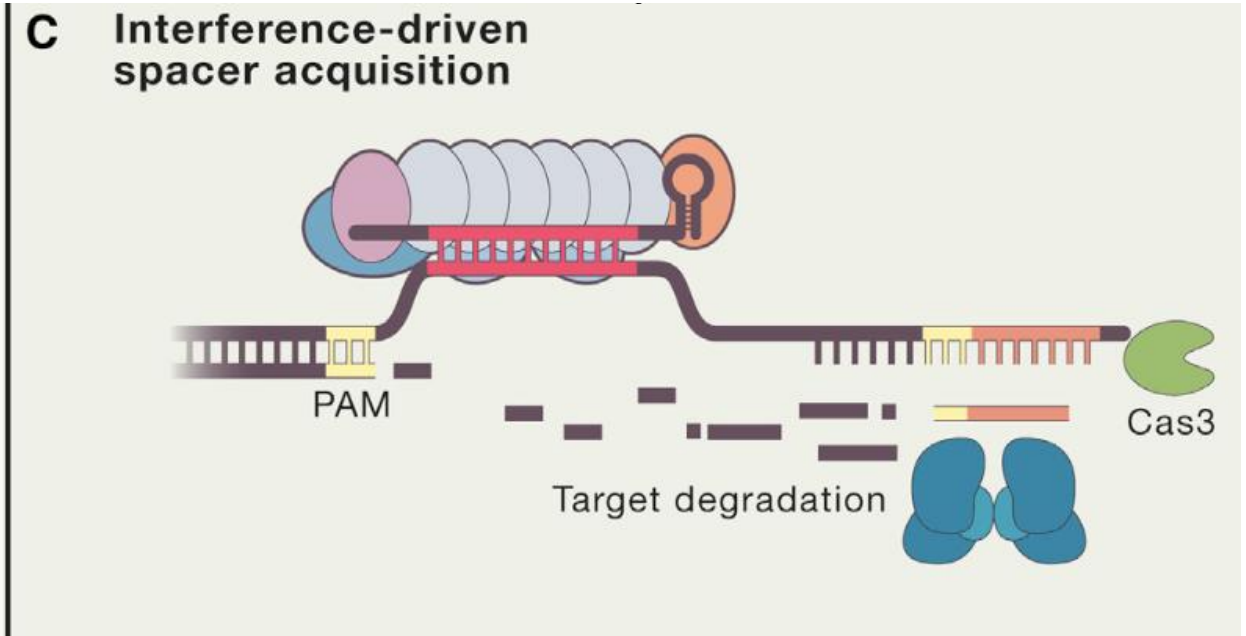
A. **Naïve**: from **previously unconfronted** MEGs

B. **Primed**: from **pre-existing spacers partially complementary** to previously encountered MEGs

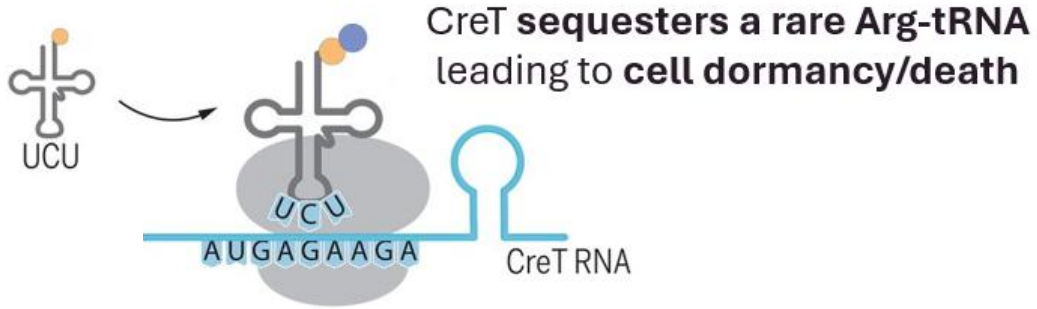
C. **Interference-driven**: from **interference degraded** MEGs

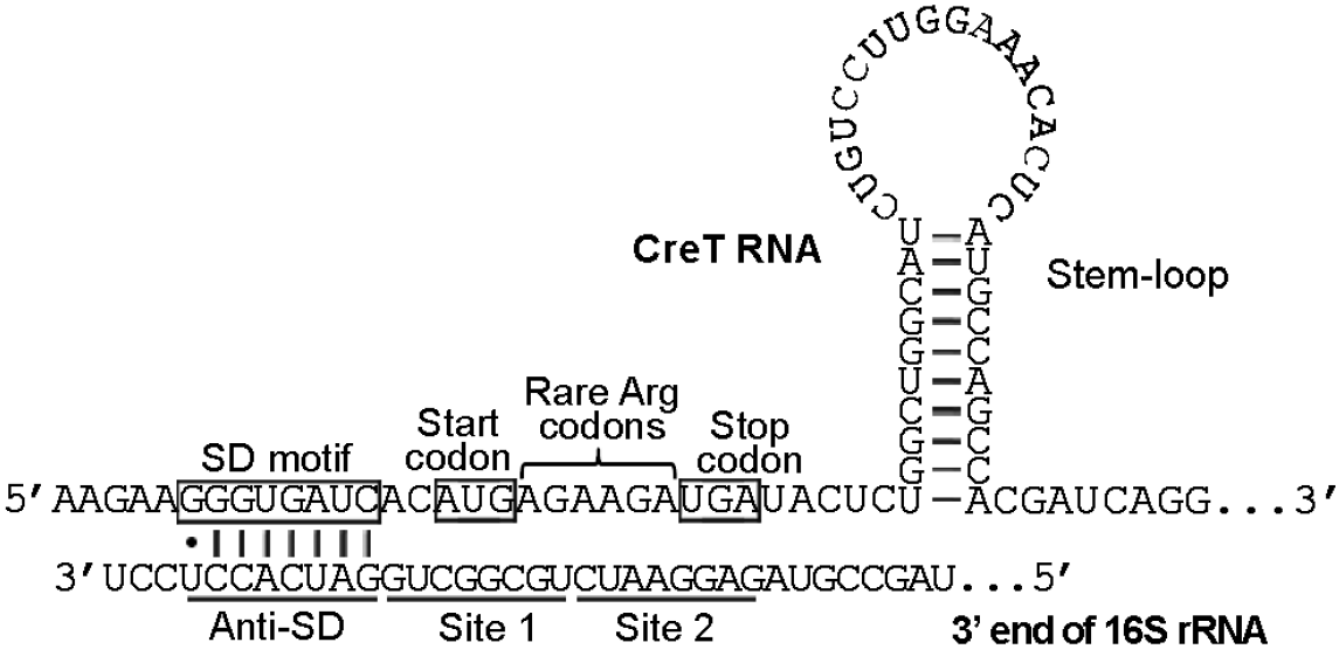






CreT toxic RNA





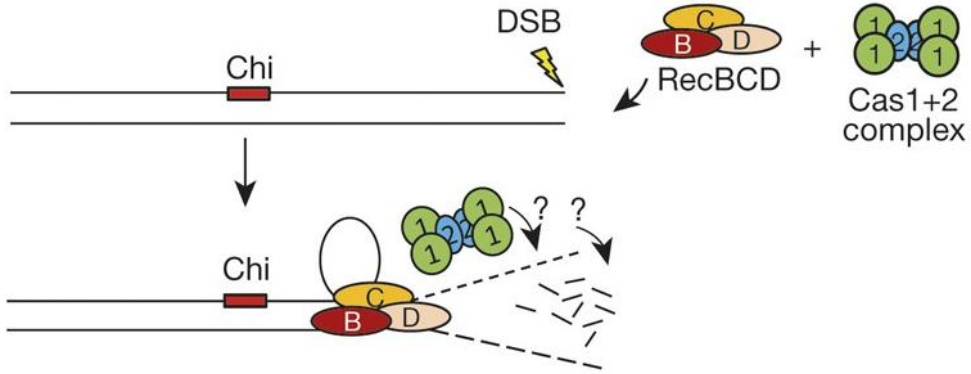
Adaptation machinery lacks intrinsic ability to distinguish self- and non-self DNA → autoimmunity risk

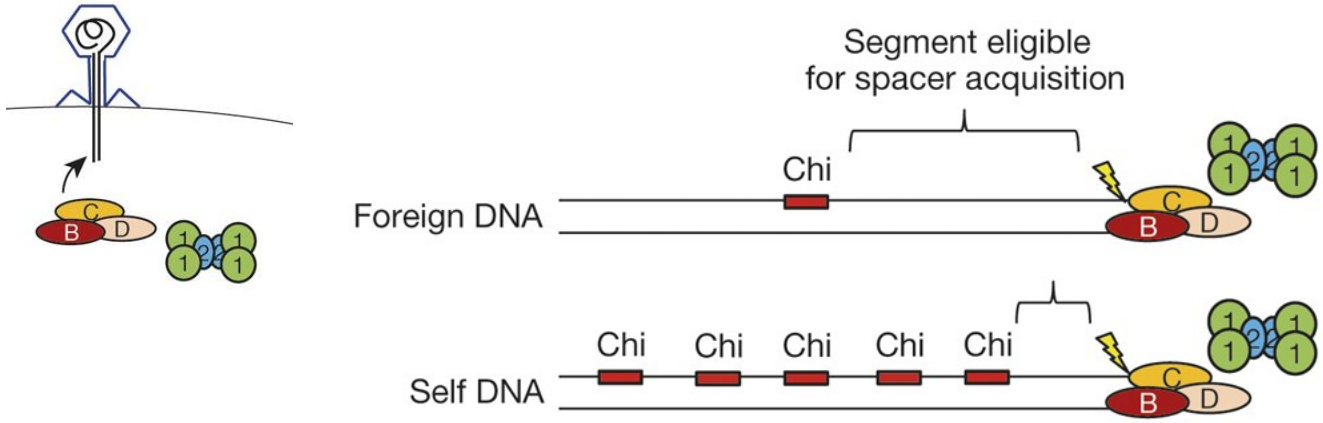
E. coli:

DSB/linear DNA => **RecBCD** indiscriminately unwinds/**degrades** **DNA until the nearest Chi site**

↓

Generated DNA fragments = **substrates for spacer acquisition** by Cas1–Cas2



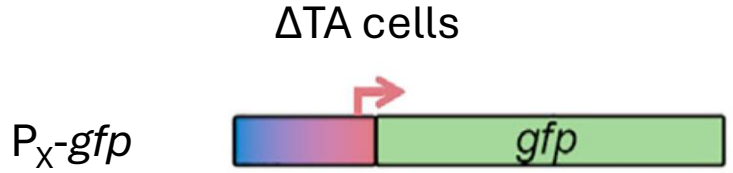


↓ Chi sites density on foreign DNA = **more extensive RecBCD processing**
=> **more** substrate for spacer **acquisition**

↑ Chi sites density on chromosome = less RecBCD processing
=> **reduced** self-derived spacer **acquisition**

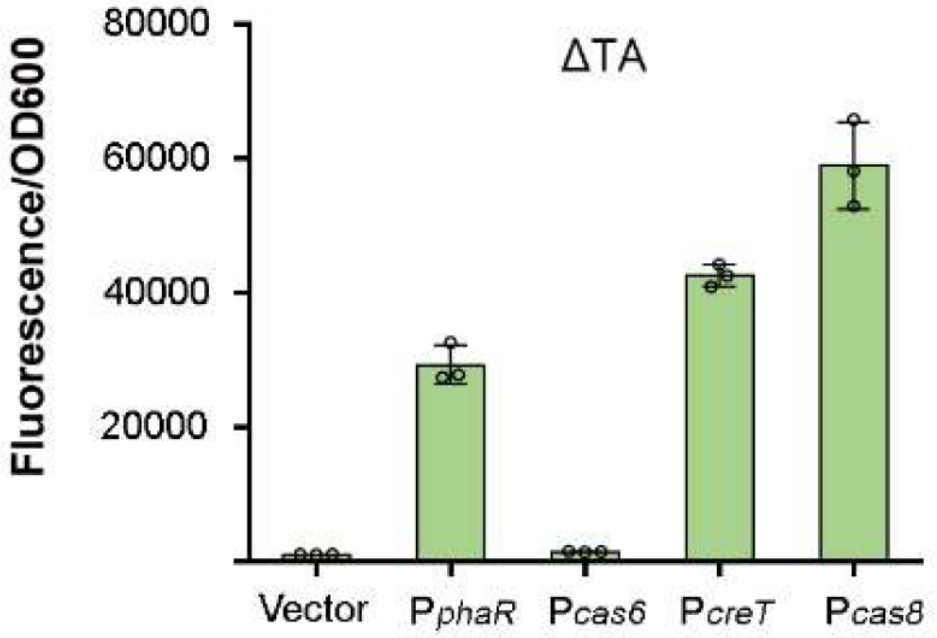
A matter of «**preferential acquisition**» rather than «recognition» of non-self DNA

Validation and characterization of *cas8* promoter



GFP-reporter system to **compare** the activity of:

- **P_{cas8}**
- P_{cas6}
- P_{creT}
- P_{phaR} (strong constitutive promoter)



P_{cas8} exhibits **135 times** the activity of **P_{cas6}** and outperforms P_{creT} and P_{phaR} by 1.4 and 2.0 times

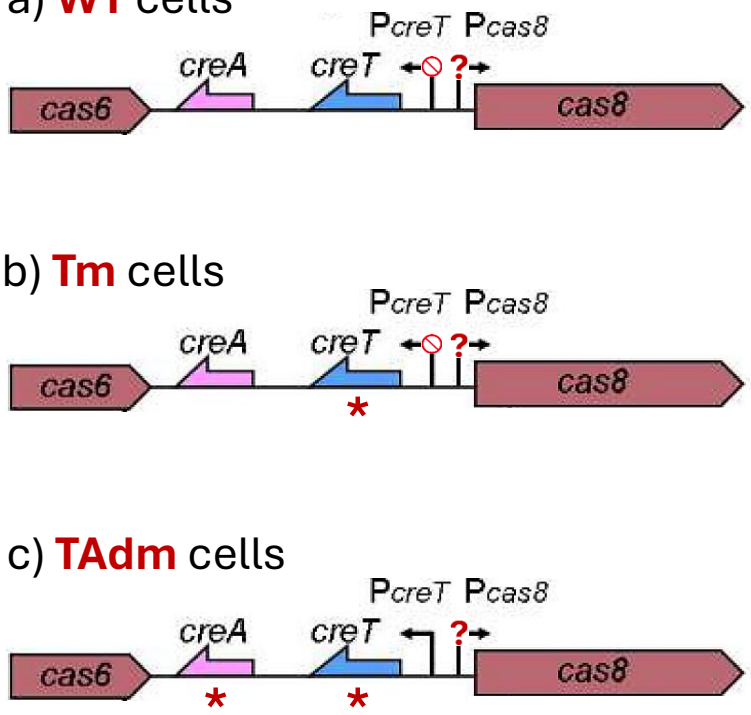
1. Demonstrating that *H. hispanica* CreA represses also *cas* transcription

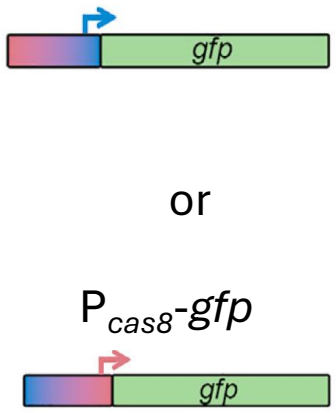
Is P_{cas8} also repressed by CreA?

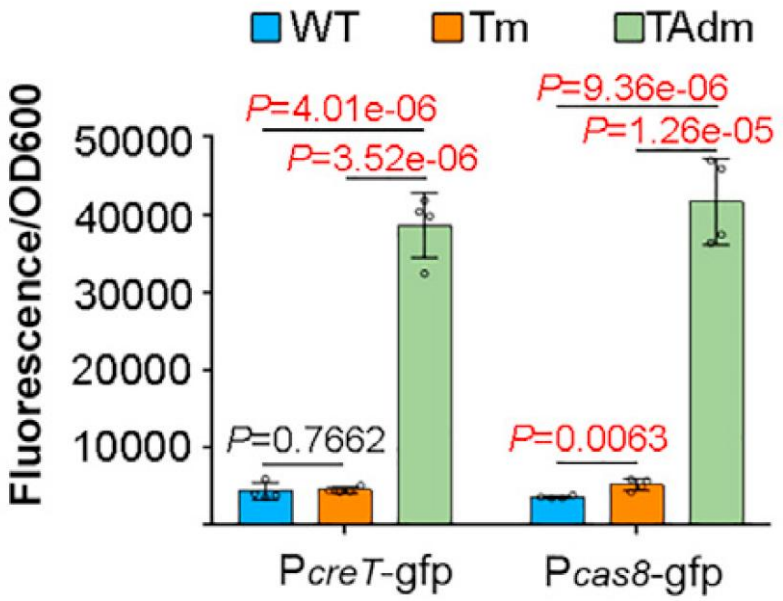
a) **WT** cells

b) **Tm** cells

c) **TAdm** cells







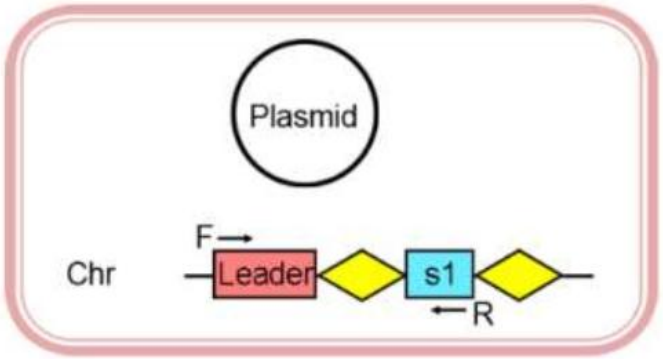
P_{cas8} and P_{creT} are **both >10-fold more active in TAdm** compared to WT and Tm

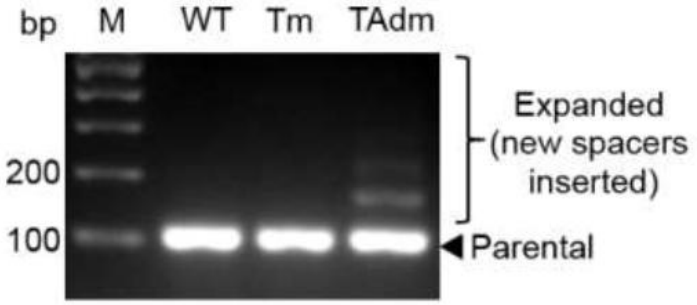
GFP-reporter assay to evaluate **P_{cas8}** and **P_{creT}** activity depending on **CreA capability to bind its target site**

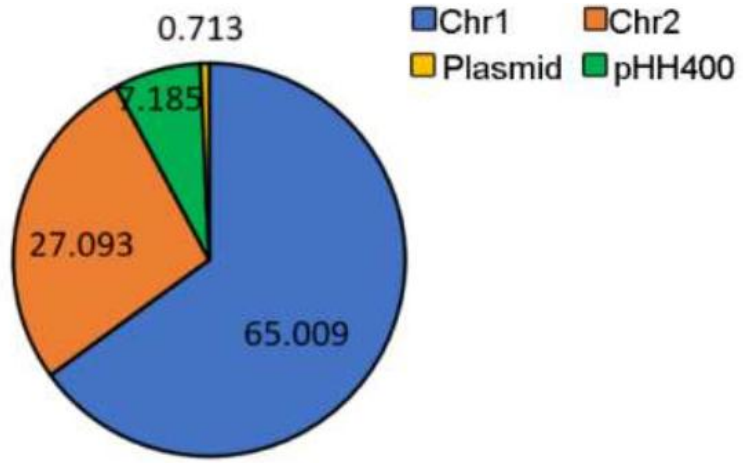
Are self-derived spacers gained through primed or naïve acquisition?

Model: WT, Tm & TAdm + empty vector

Methods: measure new spacers acquisition through PCR







Result: Without virus infection TAdm show **inefficient** acquisition of **endogenous** spacers

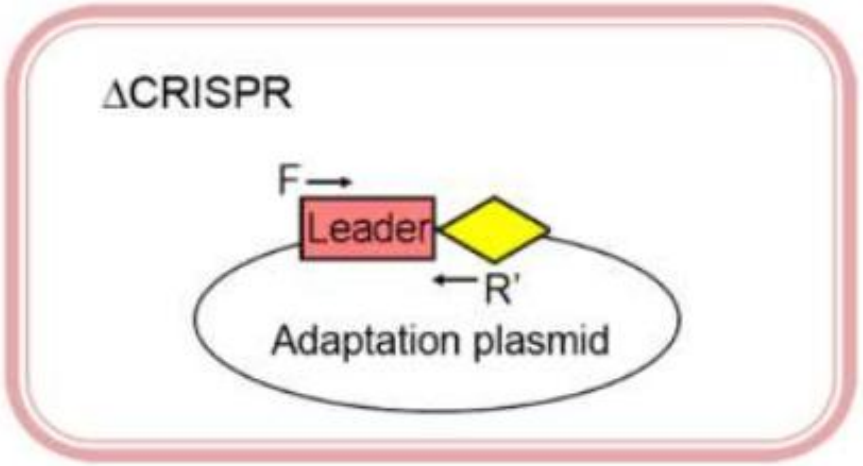
Maybe the **13 pre-existing spacers in the CRISPR array partially match the endogenous DNA**

Are self-driven spacers gained through primed or naïve acquisition?

Model: WT, Tm-**ΔCRISPR** & TAdm-**ΔCRISPR**

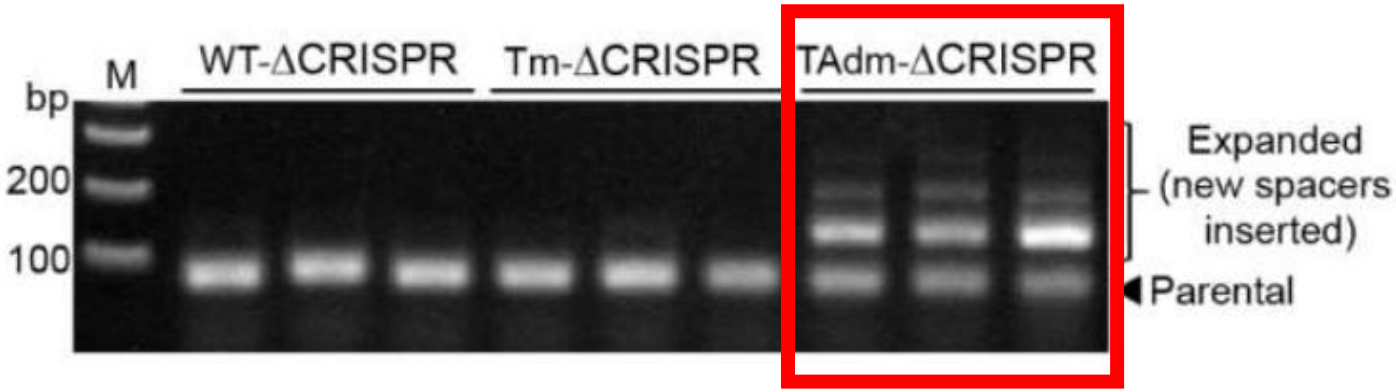
= **no possibility of primed acquisition of new spacers**

Methods: measure new spacers acquisition through PCR



Result: TAdm-ΔCRISPR has **new spacers without infection** through **naïve acquisition**

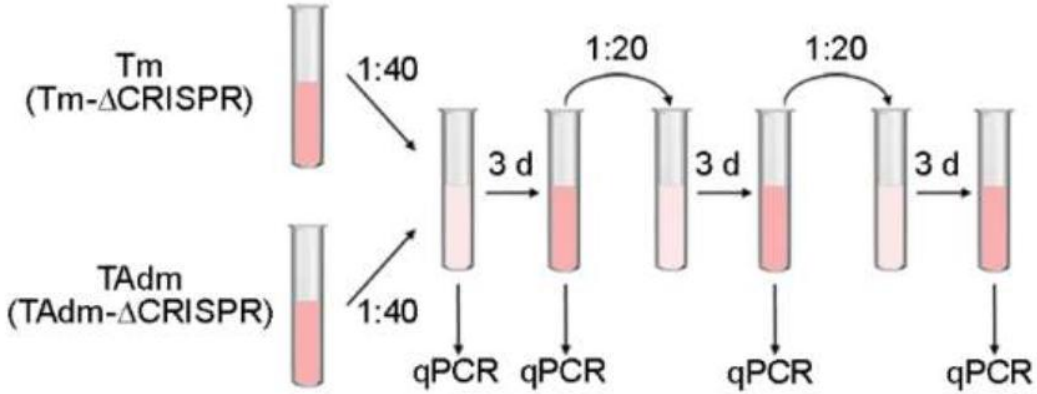
cas up-regulation increases the adaptation frequency but with a **higher risk of self- targeting** through **naïve spacer acquisition**



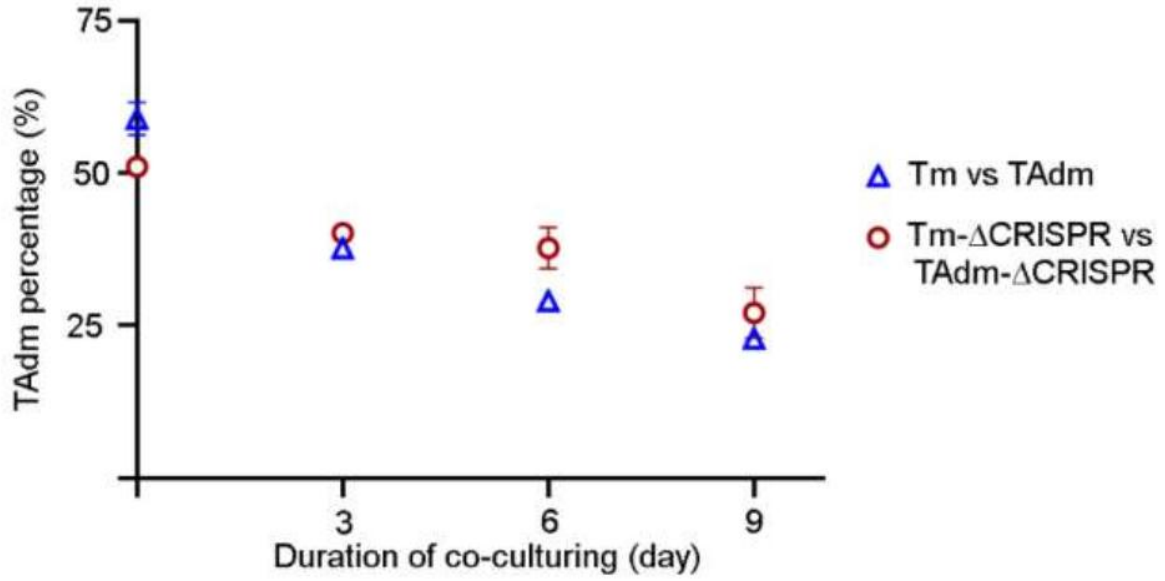
Does the accumulation of self-targeting spacers compromise the fitness?

Model: Tm-**ΔCRISPR** & TAdm-**ΔCRISPR** in long-term co-culture

Methods: measure cell percentage by qPCR



Result: **Tm-ΔCRISPR outcompeted TAdm-ΔCRISPR** over time, but with a **slower decrease**



The fitness is **mainly** compromised because of **autoimmunity**, but also **other metabolic disadvantages** related to the *cas* overexpression

CONFIRMED

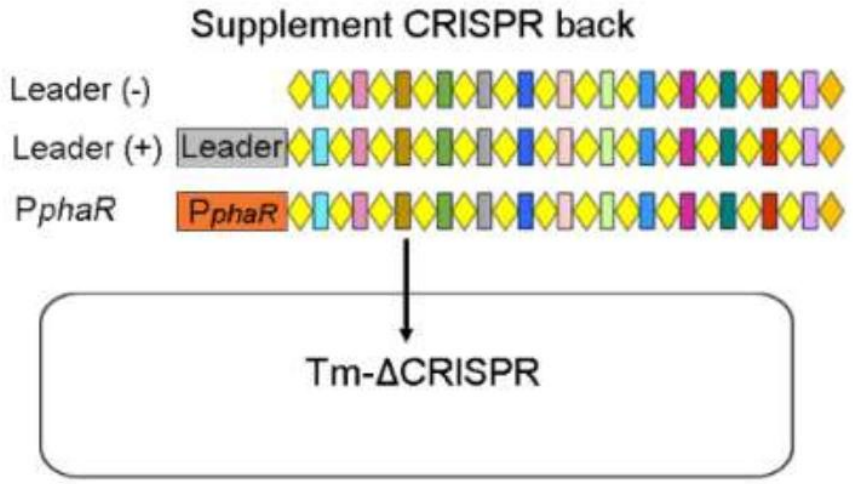
Does the shown result relay on CRISPR-array expression that leads crRNA transcript increase?

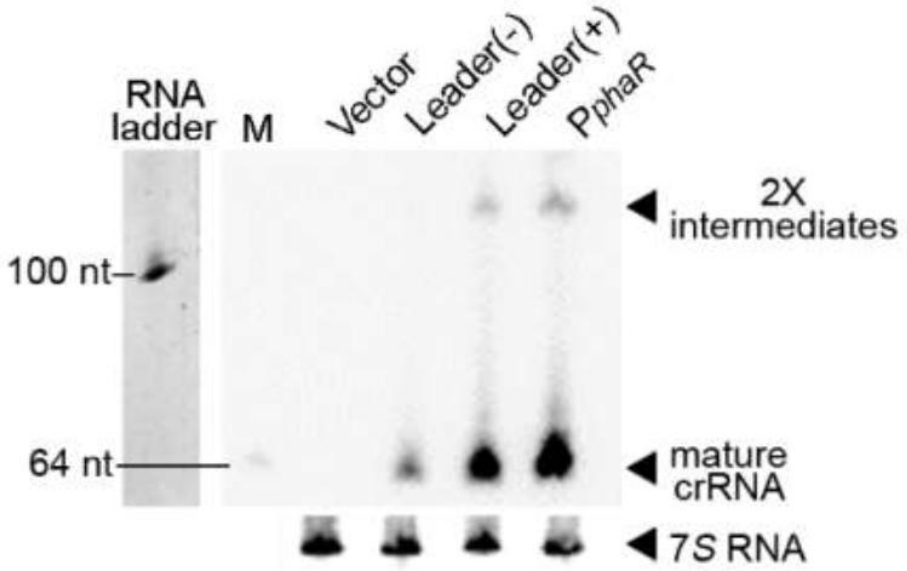
Model: Tm-**ΔCRISPR** + same plasmids

Methods: measure crRNA transcription by Northern Blot assay

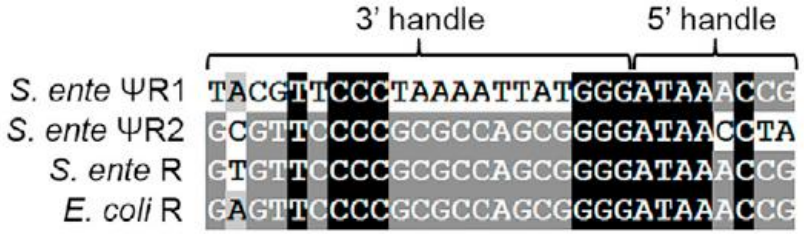
Result: **CRISPR-expressing** cells DO produce significantly **more crRNAs** than the controls

CreA-guided gene **repression** can be **alleviated by expanding** the **crRNA** pool





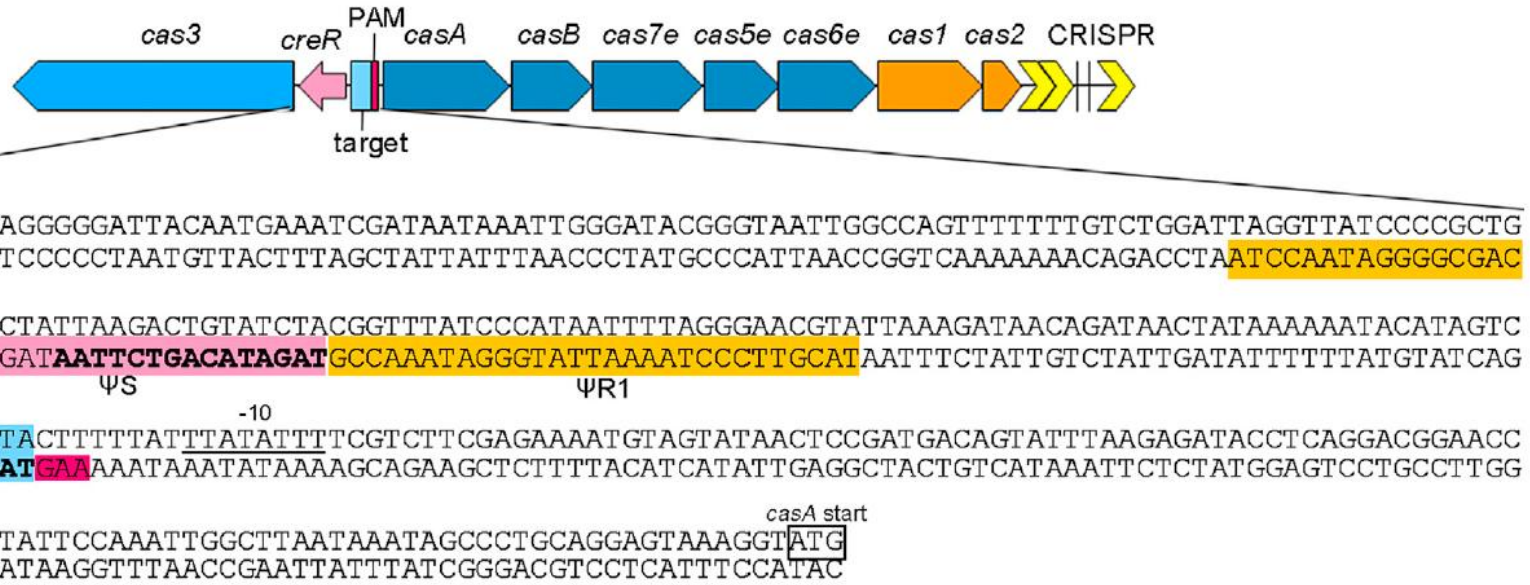
Testing *cas* repression effect of *Salmonella enterica* CreR (SeCreR) in *cas*-expressing *E. coli* MG1655 Δ*hns* strain



High sequence similarity of the **CRISPR repeat** between *S. enterica* ATCC 51960 and *E. coli* MG1655

=> ***E. coli* Cas** probably recognises ***S. enterica* CreR**

Salmonella enterica
I-E CRISPR-Cas



Phages have developed a range of Acr proteins to protect themselves against CRISPR-Cas

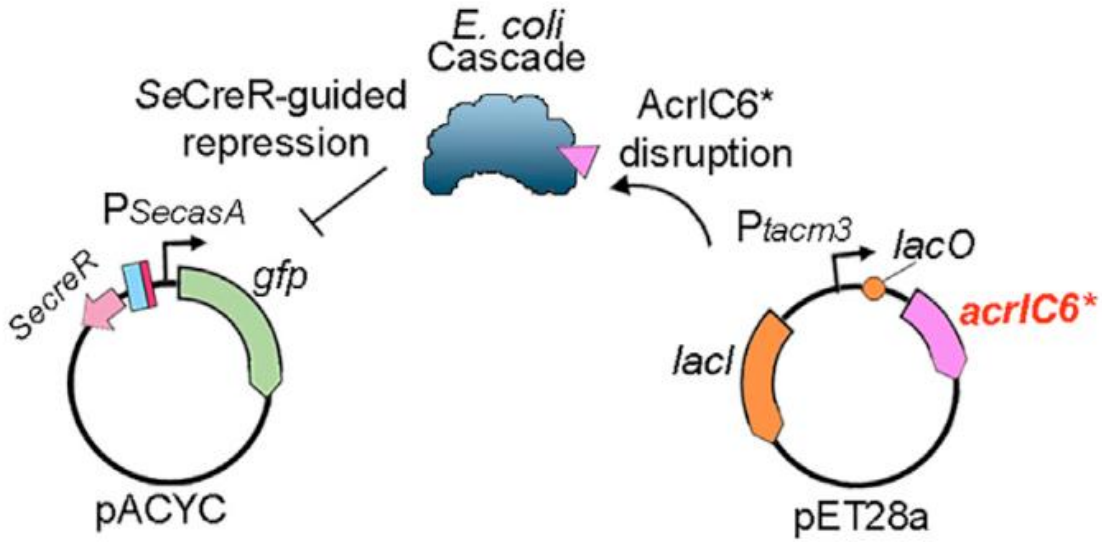
How does Cas autorepression respond to the action of Acr proteins?

Looking at ArcIC6* targeting cas system I-E in *E. coli*

What's the effect of ArcIC6* on P_{secasA} known for being repressed by Cascade-CreR in *S. enterica*?

Model: **Δ*hns*** *E. coli* cells + constructs:

- IPTG **inducible promoter** *tacm3* upstream ***acrIC6****
- *S. enterica* ***creR*** & **P_{secasA}-*gfp***



→ Treated with increasing dosage of **IPTG**

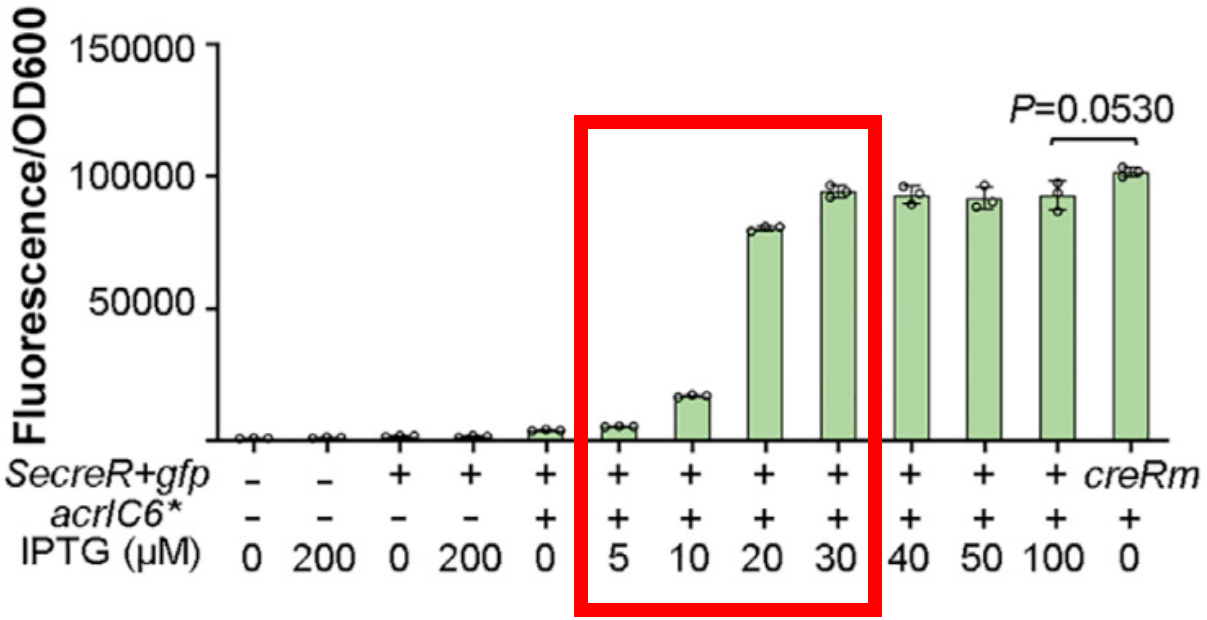
What's the effect of ArcIC6* on P_{secasA} known for being repressed by Cascade-CreR in *S. enterica*?

Methods: measure **P_{secasA}** activation detecting fluorescence by ***gfp*-reporter assay**

Result: induction of *arcIC6** with **5-30 µM IPTG increases fluorescence** by a factor of 1.37-24.51, but **higher dosage does not result in further increases**

Even **low levels of ArcIC6*** can **relieve** or **subvert** the **repression effect on P_{secasA}**

→ I-E Cas autorepression circuit can respond to Acr, stimulating late mass **production of new Cas effectors**



Studying ArcVA1-5 targeting cas system V-A in *M. bovoculi*

↓

Conclusion: analogously to what demonstrated for I-E Cas autorepression circuit by AcrIC6*, **autoregulation circuit of MbCas12a responds to vary AcrVA proteins**