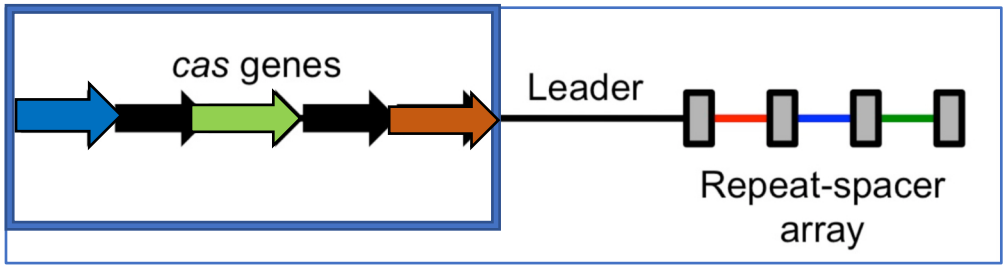
How does this genetic material in CRISPR locus then manage to kill bacteria ?

Simplified diagram of a CRISPR locus



CAS genes

The system can be slighty different in different types of bacteria but the best studies one is ***Streptococcus pyogenes*** so we will focus on that one

For the sake of simplicity lets focus on the 3 Cas genes (now colored arrows) most important for genetic engineering;

Codes for a **trans-activating CRISPR RNA (tracrRNA**) that will help in the process of ensuring the whole process only cuts bacteriophage DNA

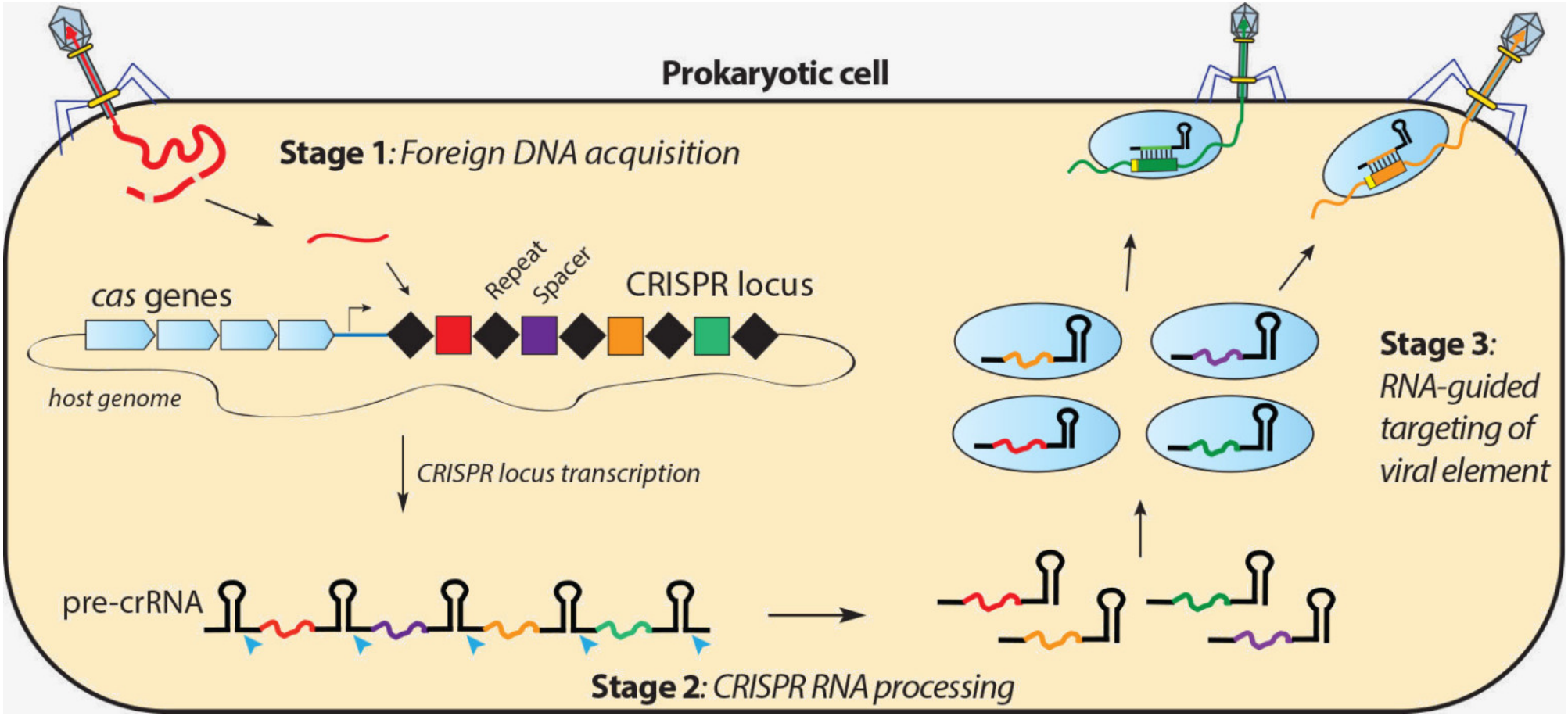
Codes **for a protein** that is a nuclease that cuts DNA but only if it is given a very specific set of signals to do so (otherwise it would potentially damage the bacteria's own DNA). The most common one used in genetic engineering approaches is called Cas9. ; additional Cas1 and Cas2 are responsible for spacer geration

Codes for a very specific piece of RNA (**crRNA or guide RNA**) that will help in the process of ensuring the whole process only cuts bacteriophage DNA

For now lets not worry about the other genes in the Cas locus

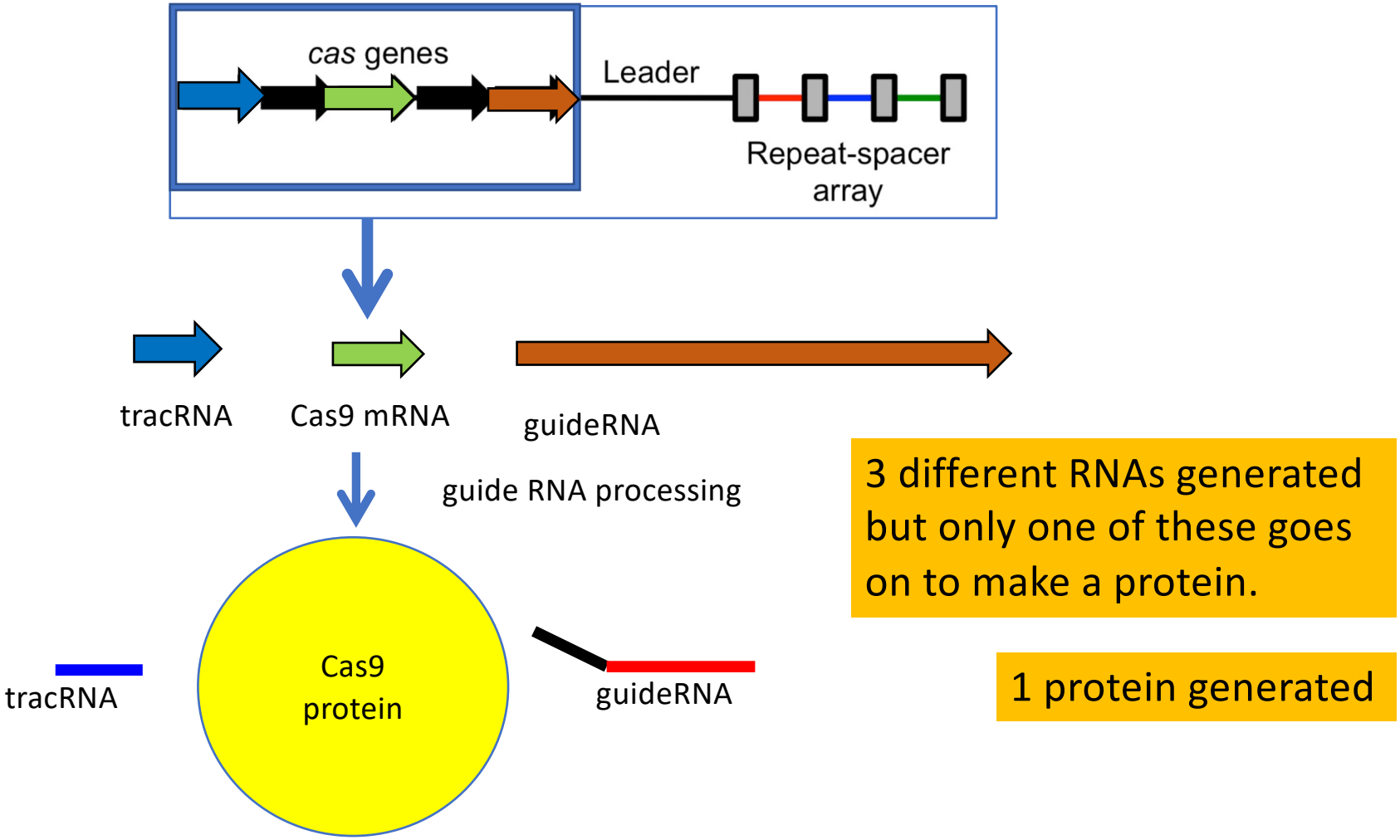
Acquisition of immunity

Adaptive immunity



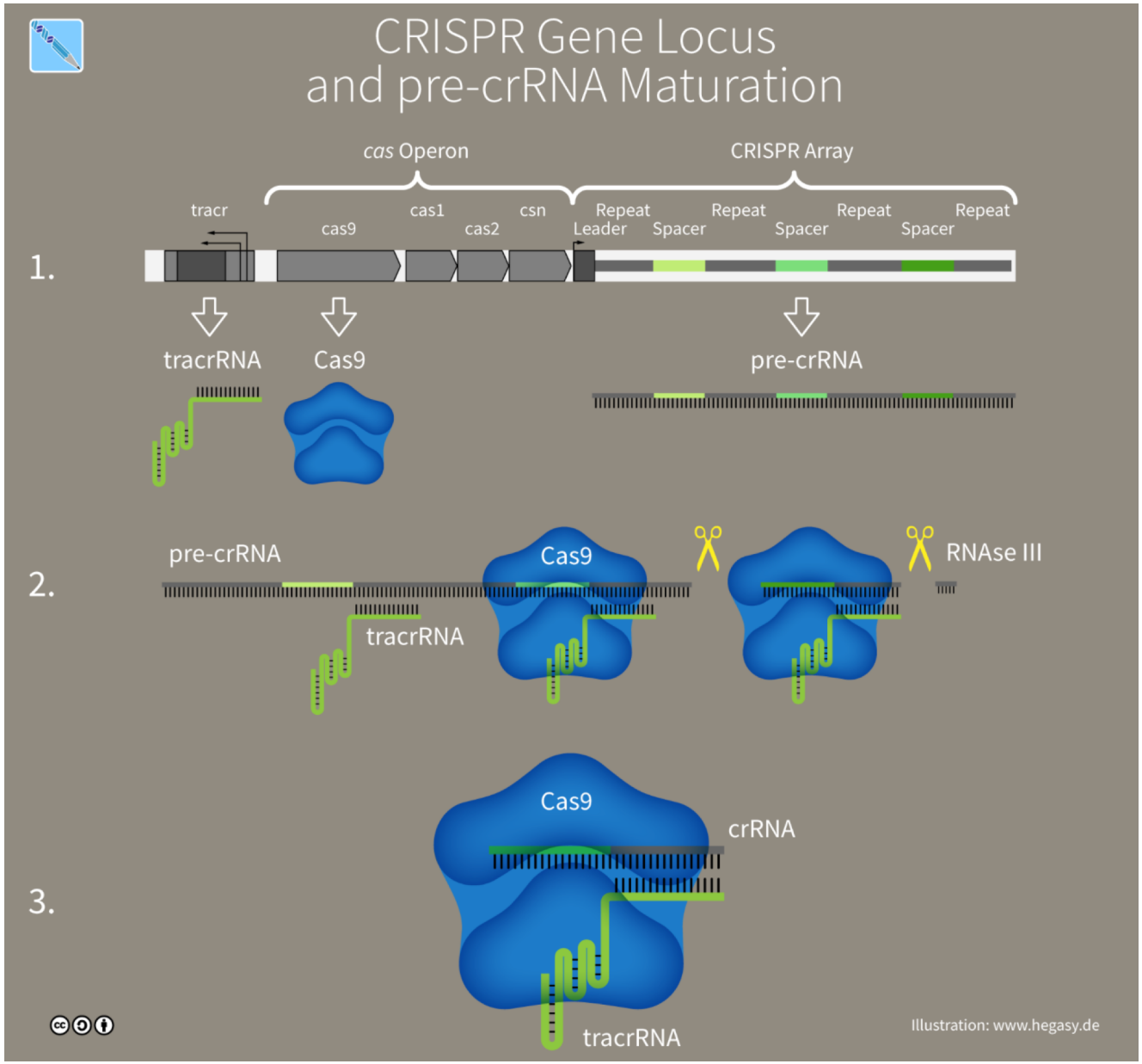
Cas1 and Cas2 are responsible for spacer generation: Bioinformatic analysis of regions of phage genomes that were excised as spacers (termed protospacers) revealed that they were not randomly selected but instead were found adjacent to short (3 – 5 bp) DNA sequences termed protospacer adjacent motifs (PAM).

How does this genetic material in CRISPR locus then manage to kill bacteria ?



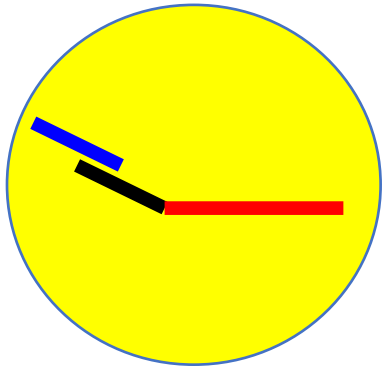
3 different RNAs generated but only one of these goes on to make a protein.

1 protein generated


CRISPR Gene Locus
and pre-crRNA Maturation
cas Operon
CRISPR Array
tracr
cas9
cas1
cas2
csn
Repeat
Leader
Spacer
Repeat
Spacer
Repeat
Spacer
Repeat
1.
tracrRNA
Cas9
pre-crRNA
2.
pre-crRNA
Cas9
tracrRNA
RNAse III
3.
Cas9
crRNA
tracrRNA
Illustration: www.hegasy.de

What is Cas9?

- Cas9 is an endonuclease that can cut double stranded DNA
- Cas 9 is only activated when the tracRNA and the guide RNA are associated with it (i.e it is a nucleoprotein). Imagine this a bit like the fail safe mechanism they use to prevent accidental launch of nuclear missiles where 2 people have to insert keys at exactly the same times
- In fact the tracRNA and the guide RNA have a short overlapping sequence that means they actually have to bind to each other in this complex for this to work properly



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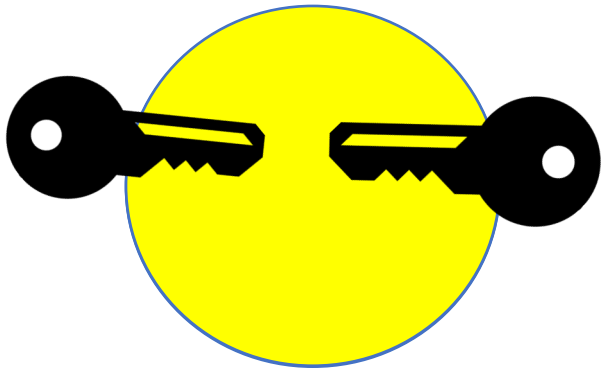
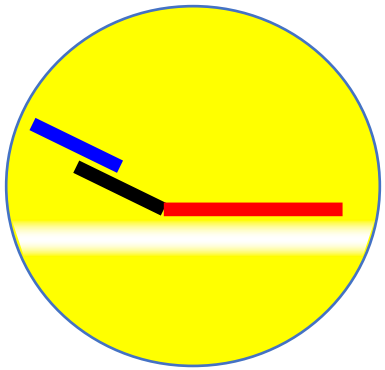


Active Cas9

How does Cas9 work?

- Cas9 has a channel that DNA can fit into.
- It scans the DNA looking for sequence that match the guide sequence



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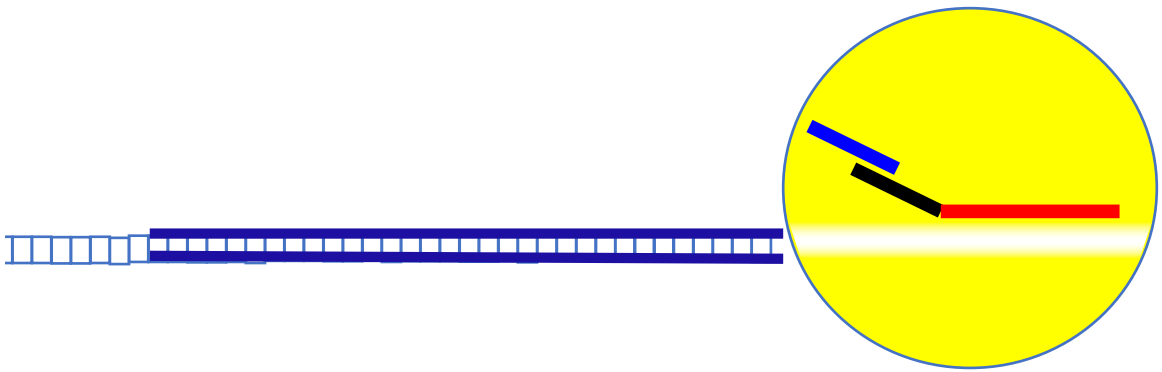

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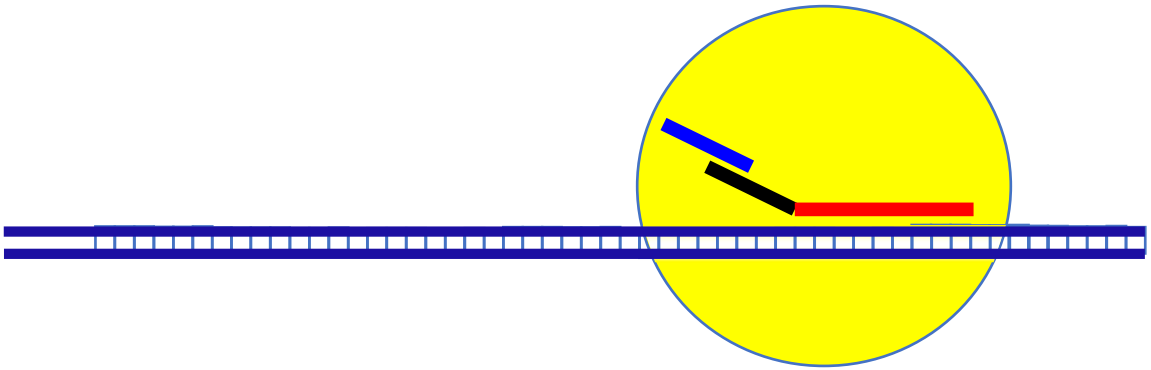
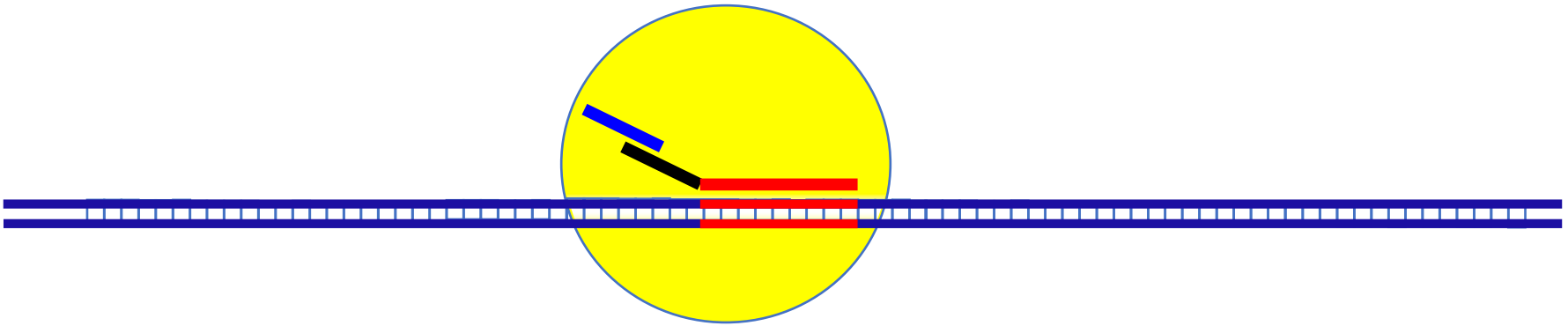


Active Cas9

How does Cas9 work?

- When a DNA sequence complementary to the guide RNA is found the scanning stops



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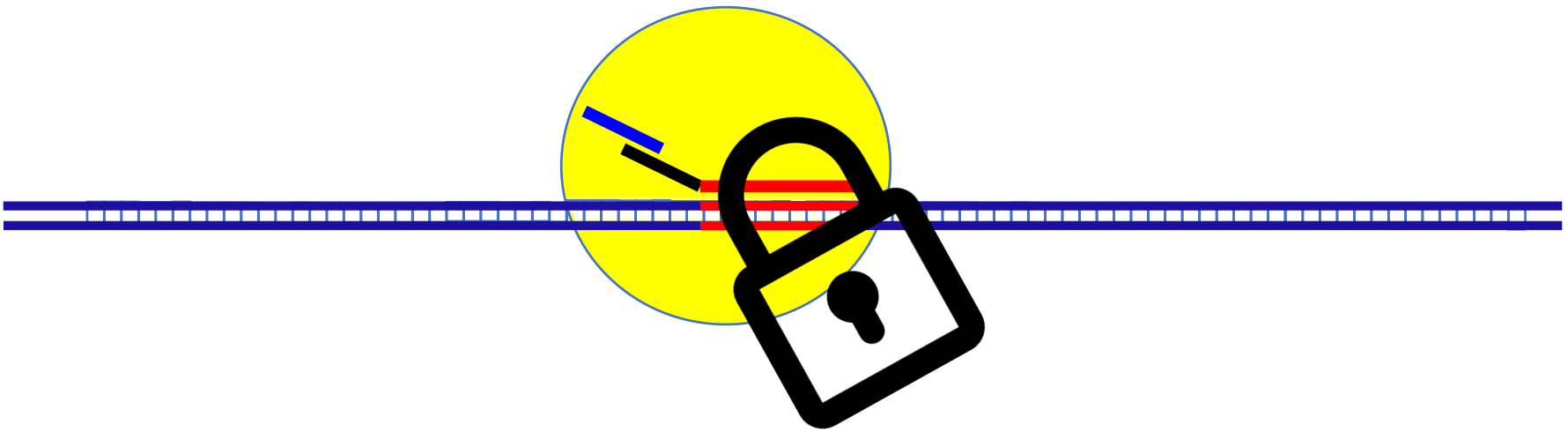
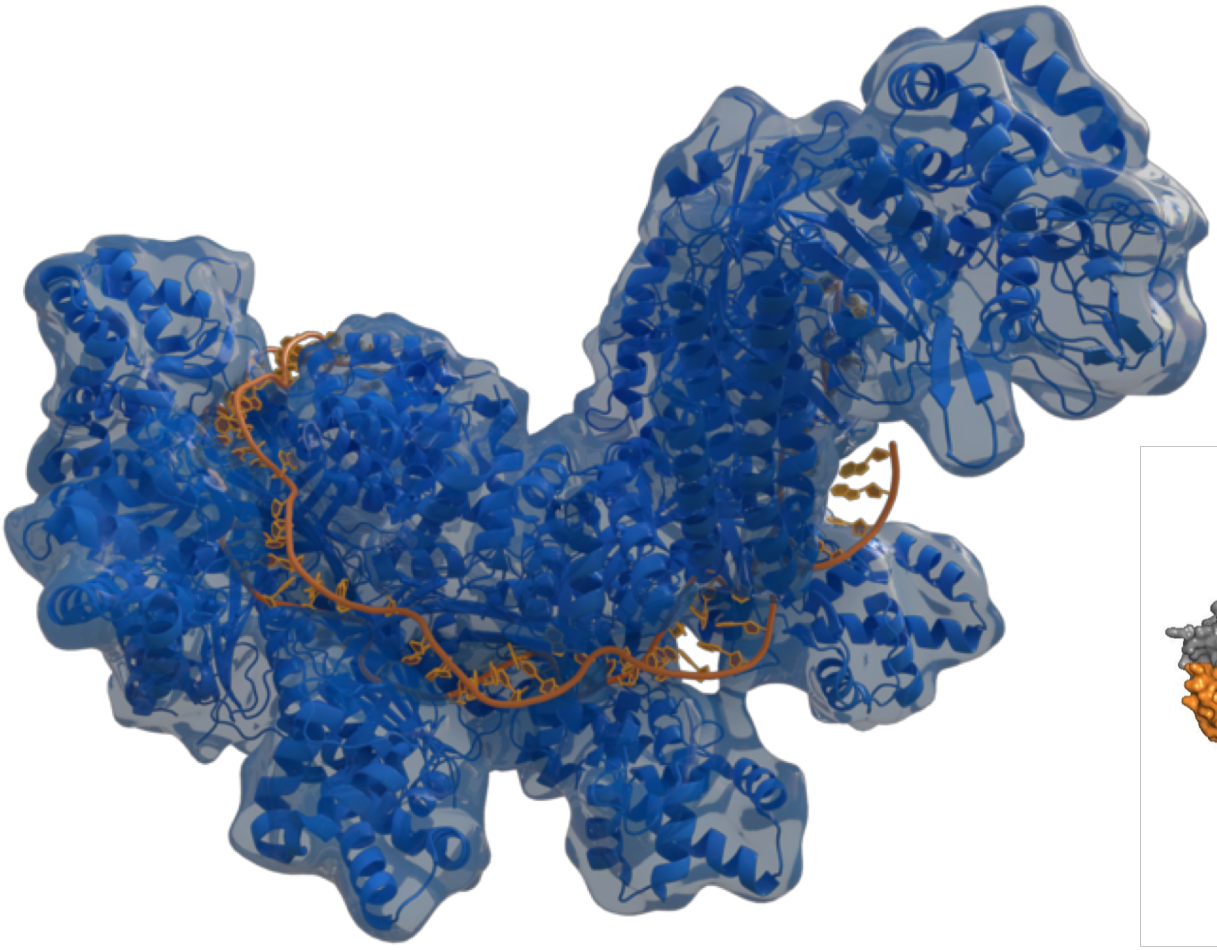
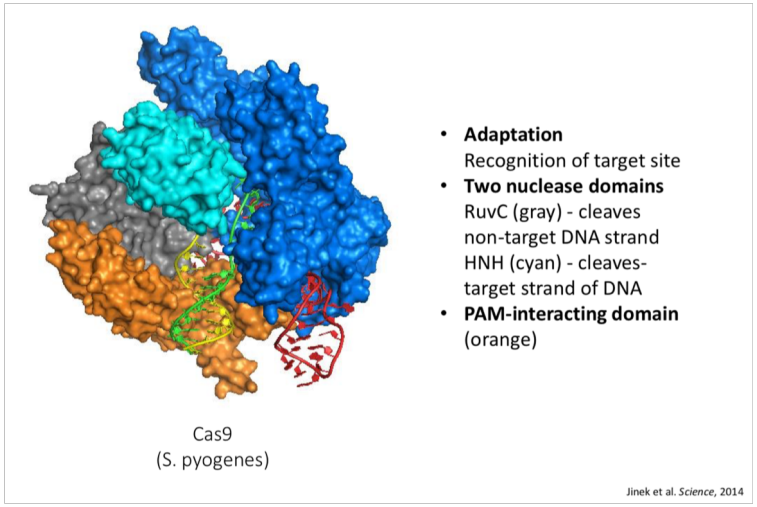


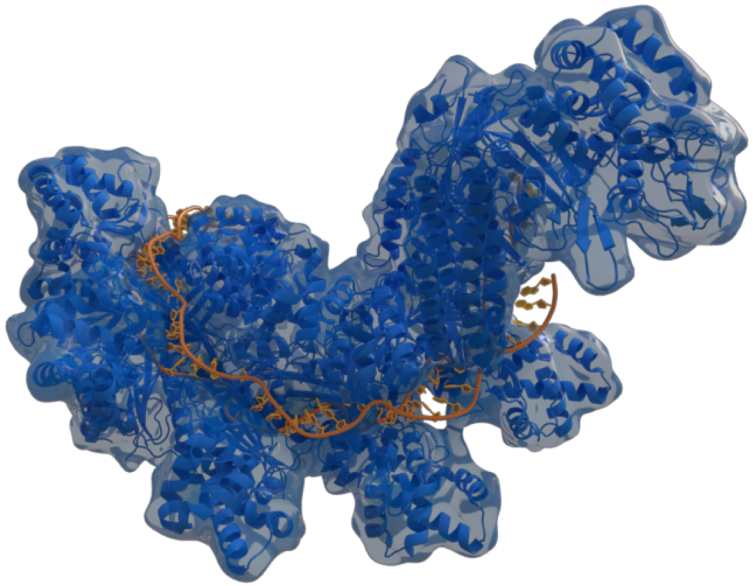
Structure of DNA bound to a Cas enzyme





Structure of DNA bound to a Cas enzyme

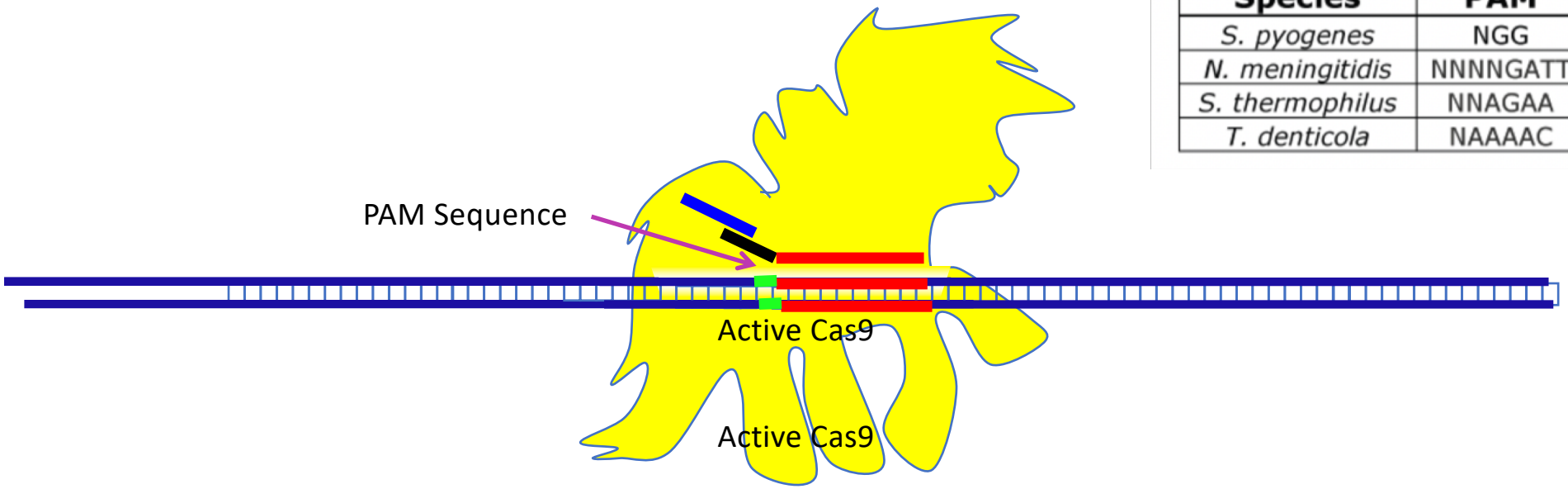
Completely irrelevant aside



How does Cas9 work?

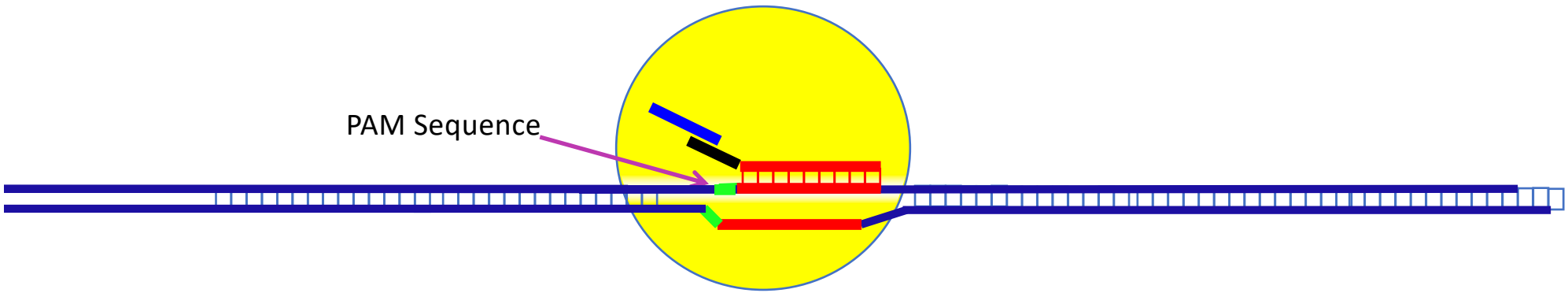
- There is one additional check
- In this control step the target site in the bacteriophage DNA needs to have the PAM sequence (**P**rotospacer **A**djacent **M**otif)
- PAM seqeunces DO NOT APPEAR in the bacterial genome
- PAM seqeunces are requried for Cas9 endonucease activity
- PAM sequences are specific for bacterial strains and protect the Cas locus from beeing cut by Cas9

Species	PAM
S. pyogenes	NGG
N. meningitidis	NNNNGATT
S. thermophilus	NNAGAA
T. denticola	NAAAAC



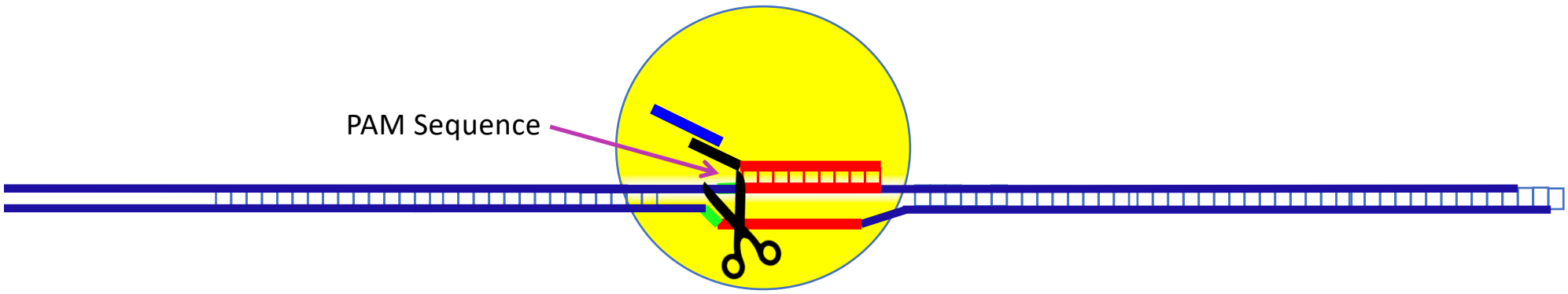
How does Cas9 work?

- Now the RNA binds to the complementary strand of the DNA and opens up the DNA helix



How does Cas9 work?

- Now the bacteriophages DNA gets cut very close to the PAM site



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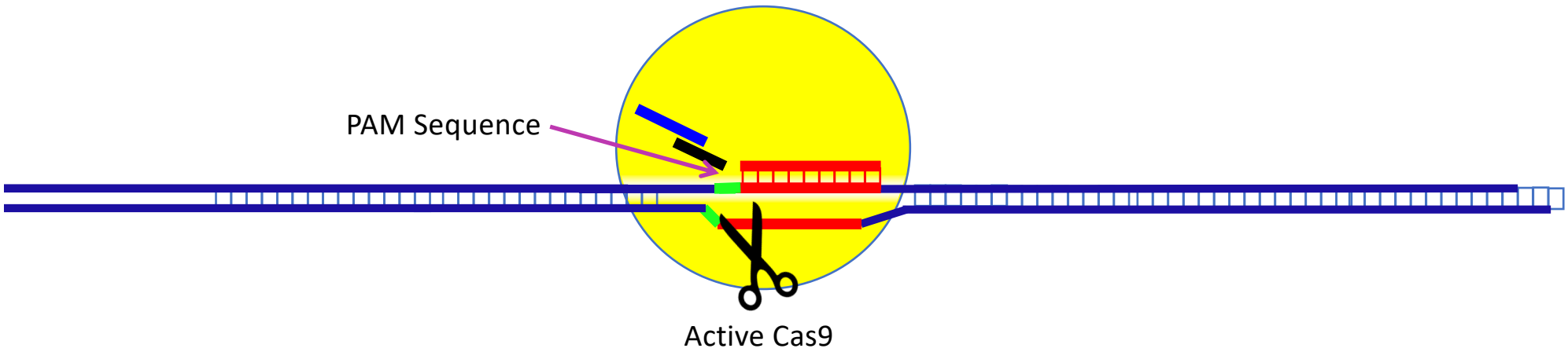
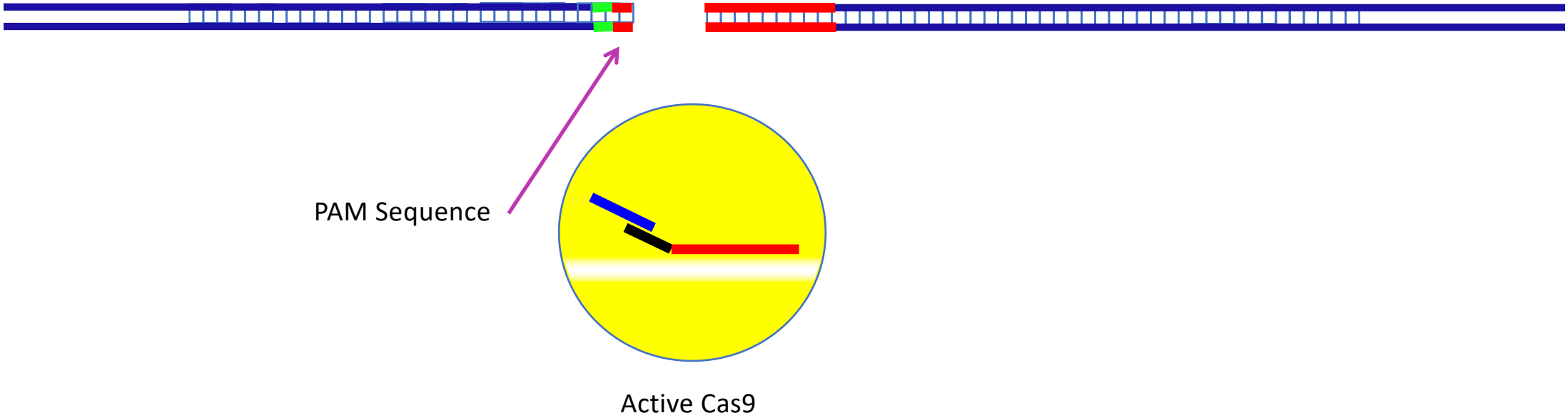
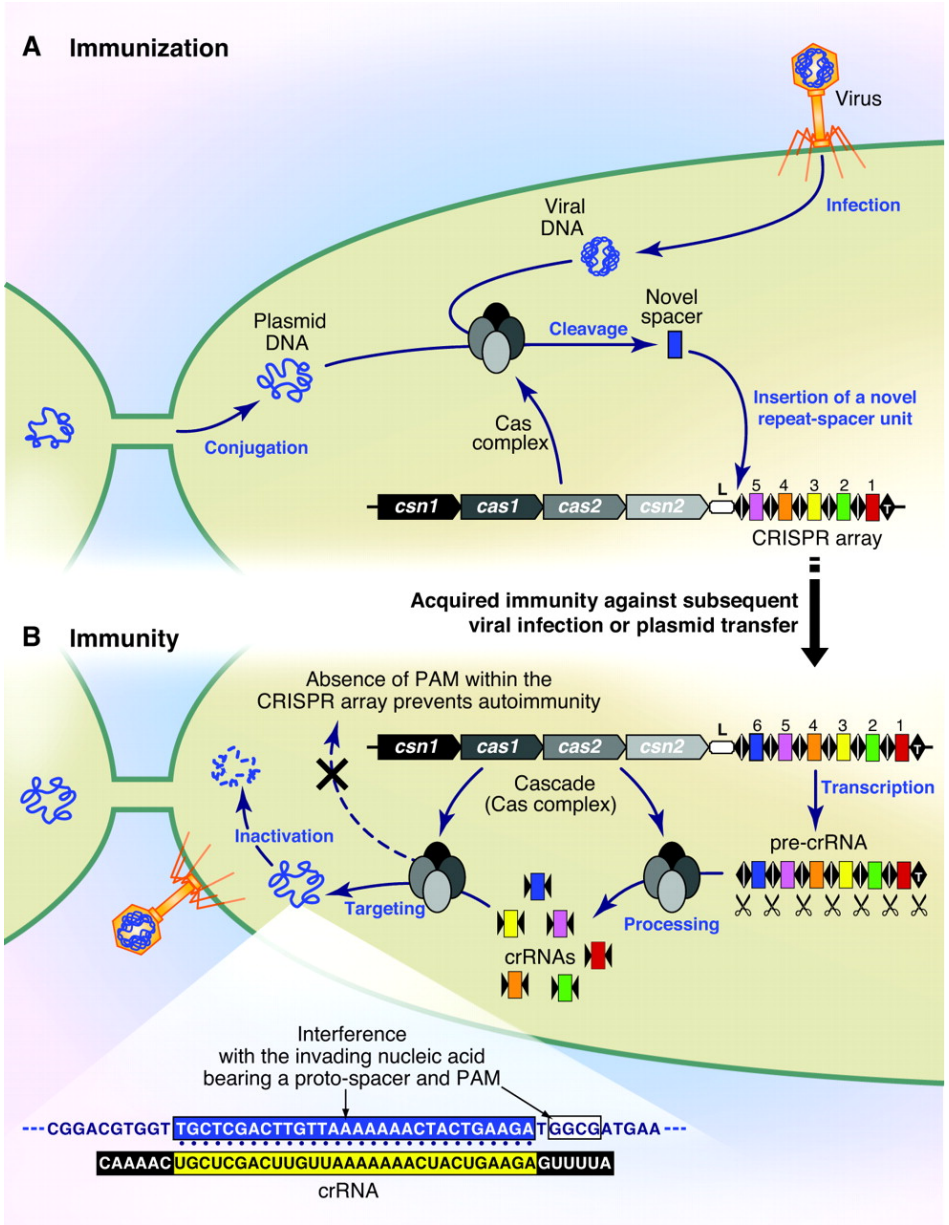


How does Cas9 work?

- Now the bacteriophages DNA gets cut very close to the PAM site, it looks like this and the bacteriophage is essentially inactivated

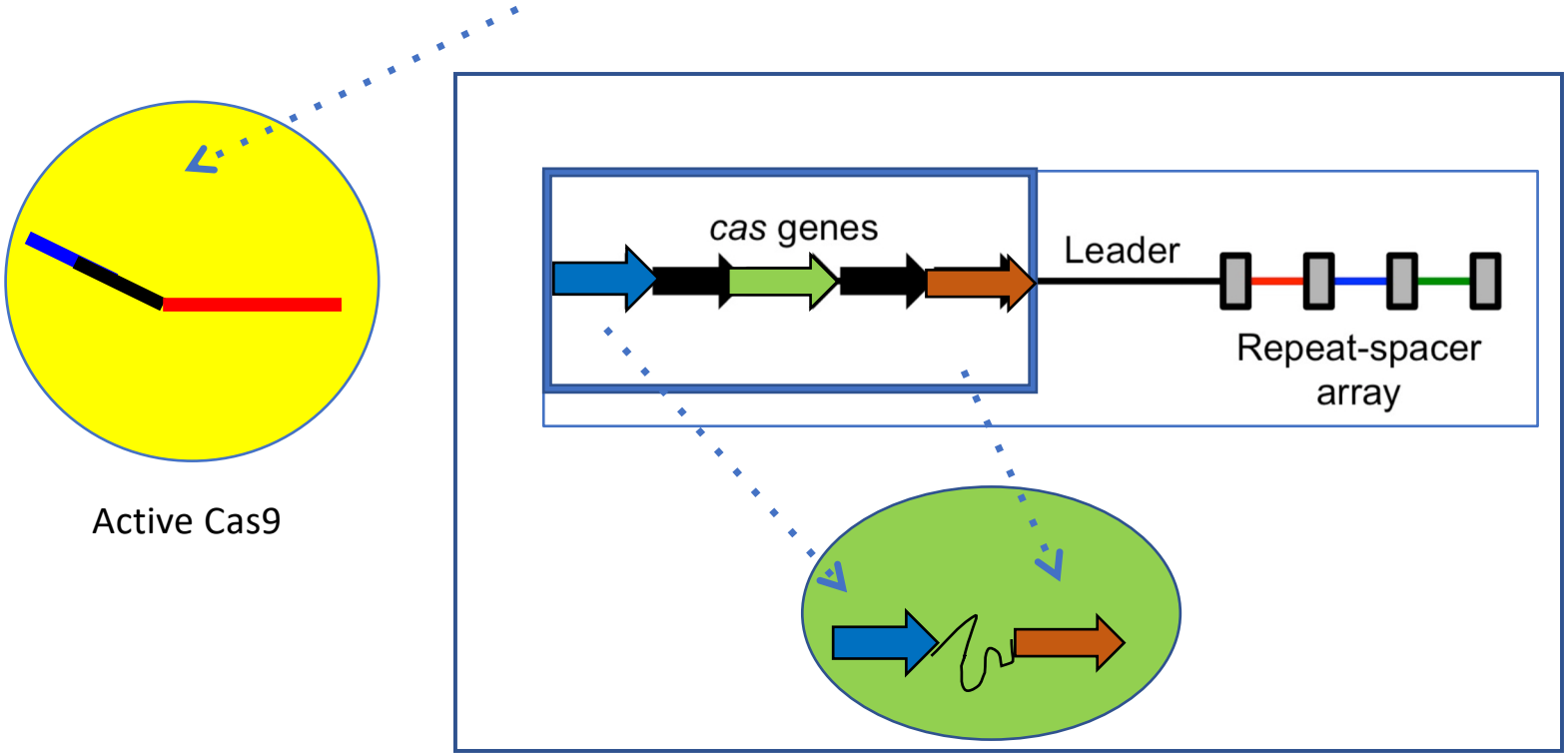




Protospacer adjacent motif (PAM) is a 2-6 base pair DNA sequence immediately following the DNA sequence targeted by the Cas9 nuclease in the CRISPR bacterial adaptive immune system.
PAM is a component of the invading virus or plasmid, but is not a component of the bacterial CRISPR locus. Cas9 will not successfully bind to or cleave the target DNA sequence if it is not followed by the PAM sequence.
PAM is an essential targeting component (not found in bacterial genome) which distinguishes bacterial self from non-self DNA, thereby preventing the CRISPR locus from being targeted and destroyed by nuclease.

How can we use CRISPR/Cas9 for genetic engineering?

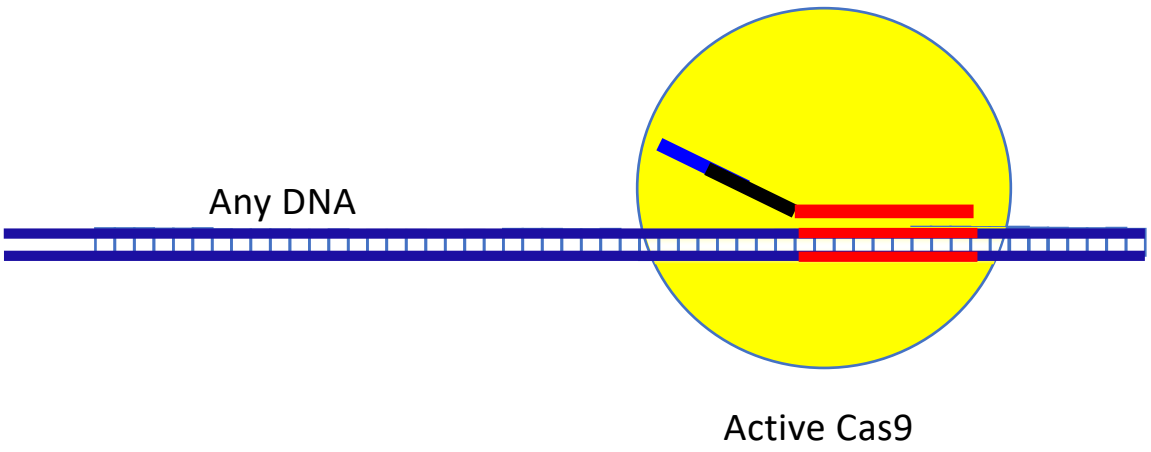
- Jennifer Doudna and Emmanuelle Charpentier re-engineered the Cas9 endonuclease into a more manageable two-component system by fusing the two RNA molecules tracRMA and guide RNA (or crRNA) into a "**SINGLE-GUIDE RNA**" (**sgRNA)** that, when combined with Cas9, could find and cut the DNA target specified by the guide RNA



Active Cas9

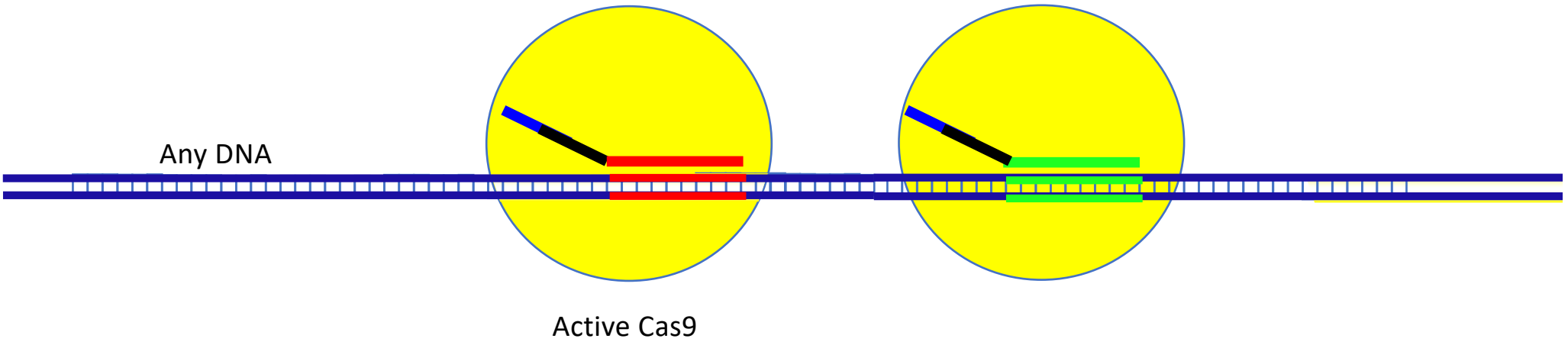
How can we use CRISPR/Cas9 for genetic engineering?

- This means we can artificially make a sgRNA that can be designed to target **any part of the genome** (as long as it has an appropriate PAM sequence nearby)
- **All we have to do is artificially express the Cas9 and the sgRNA together**



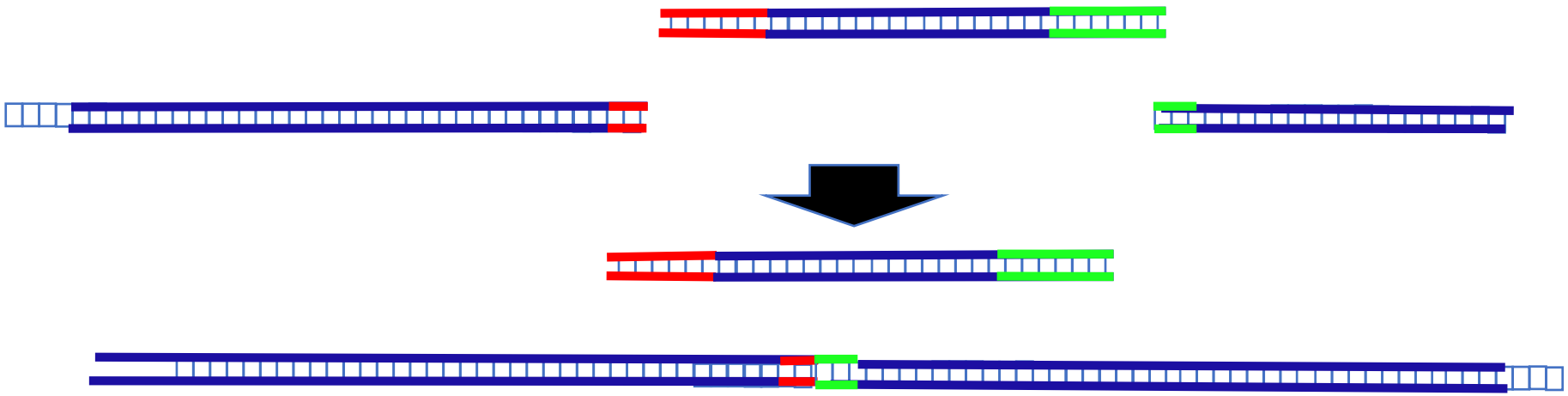
How can we use CRISPR/Cas9 for genetic engineering?

- We can put two different sgRNA into the same protein and cut at 2 places in the genome . → we can cut out large regions of DNA.



How can we use CRISPR/Cas9 for genetic engineering?

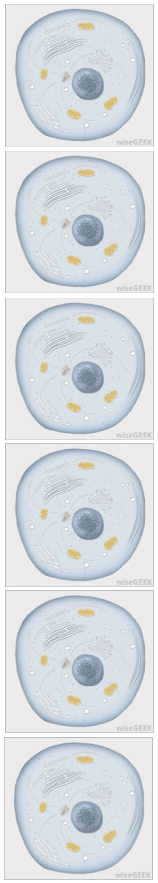
This allows us to selectively “knock out” regions of the genome



Just an example: Knockout of VEGF-a gene

1

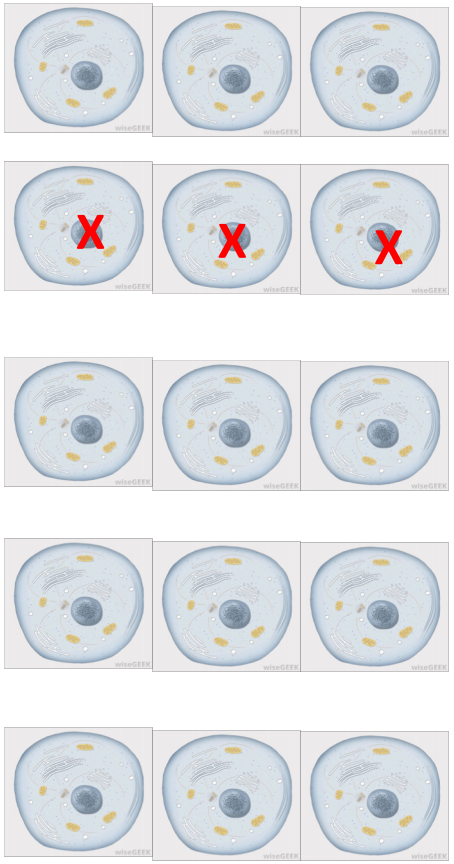
Take lots of cells and add the Cas9 protein plus 2 sgRNA that specifically bind to VEGFA gene



2

Isolate single cells (i.e select clones)

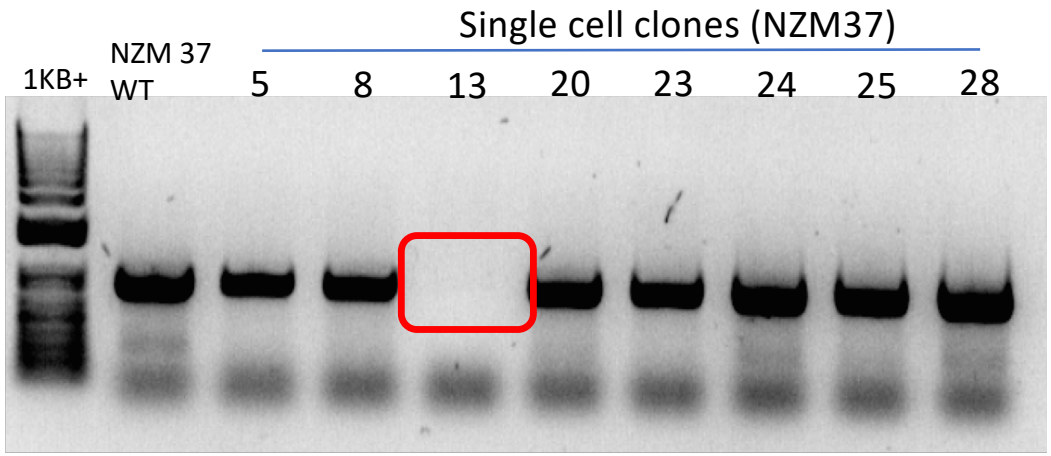
3 Grow cells



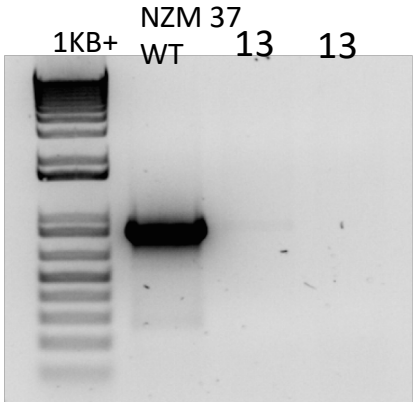
4

Isolate DNA from cells and find cells that have the gene knocked out

Just an example: Knockout of VEGF-a gene



Repeat PCR

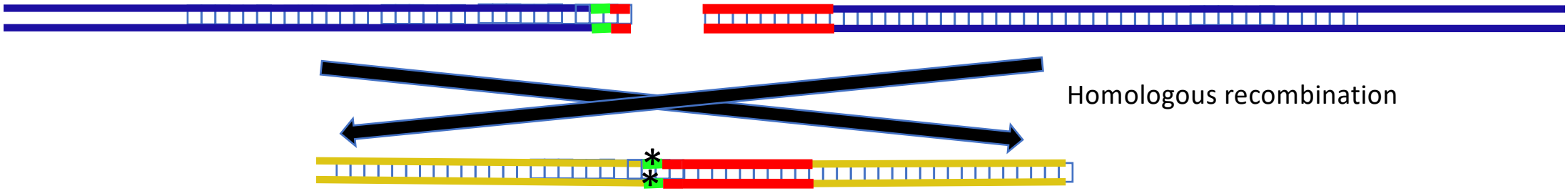


Here is an example of PCR of the VEGFA gene of melanoma cells where we have tried to use CRISPR to "knockout the VEGFA gene (achieved in clone 13)

Using CRISPR/Cas9 to “knockin” bits of DNA

- We can use an artificial piece of DNA that is identical to the cleaved region of DNA (with “corrected” sequence -∗-) ------
- when the cell tries to repair its own chromosomal DNA it will sometimes accidentally incorporate this into its own DNA by homologous recombination!

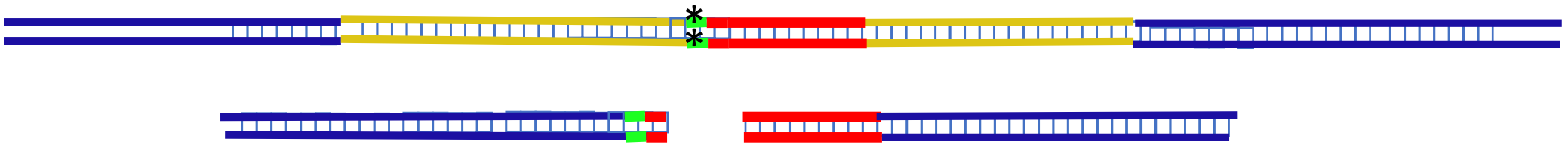
→ Bring in CRISP-Cas9 components to make a specific cut
→ Bring in a DNA fragement that contains the desired genetic alteration (wt→ mutant; mutant→wt)
→ Strand invasion by cut sequence and HR



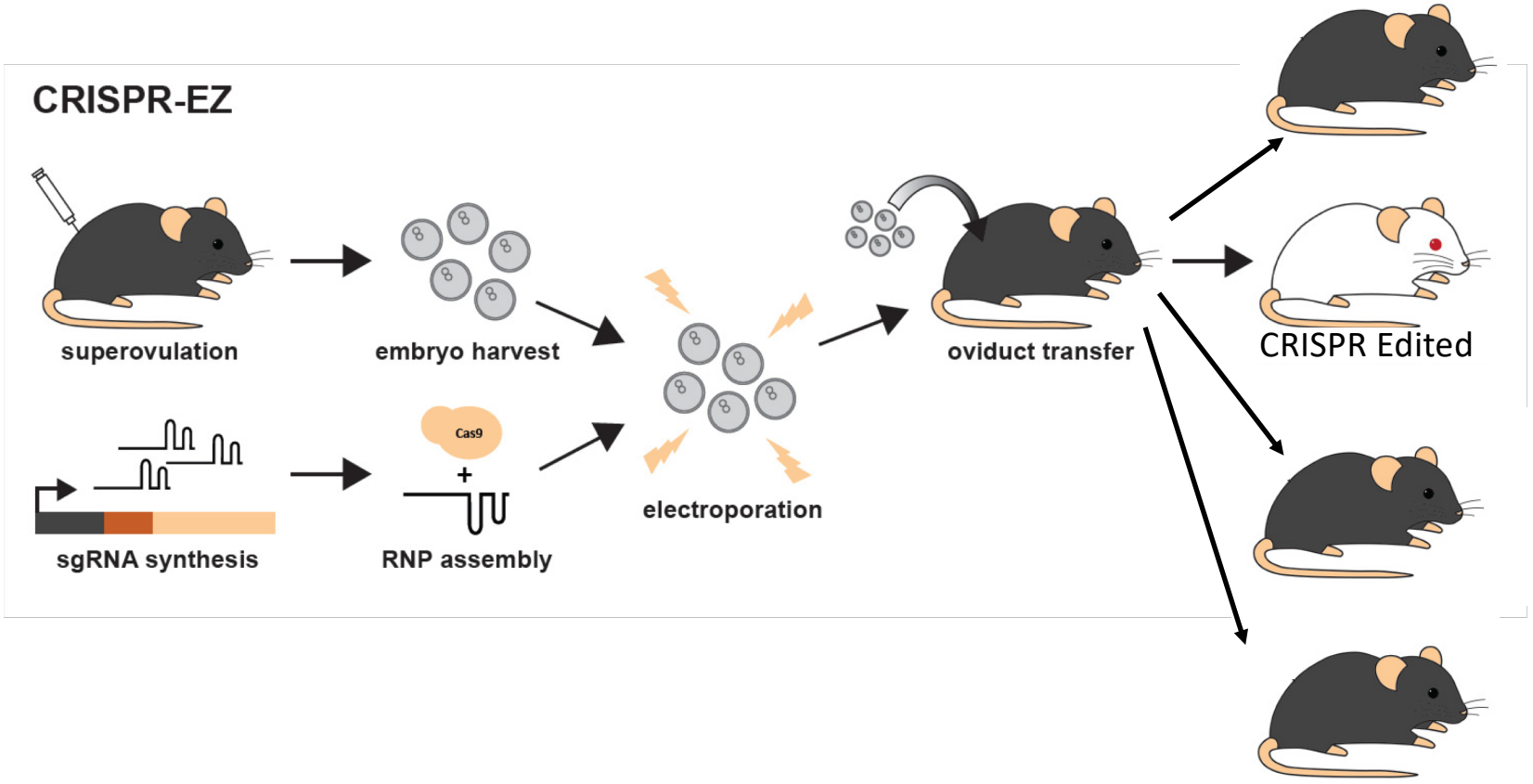
Homologous recombination

Using CRISPR/Cas9 to “knockin” bits of DNA

- Now the artificially produced piece of DNA is “knocked in” to the genome

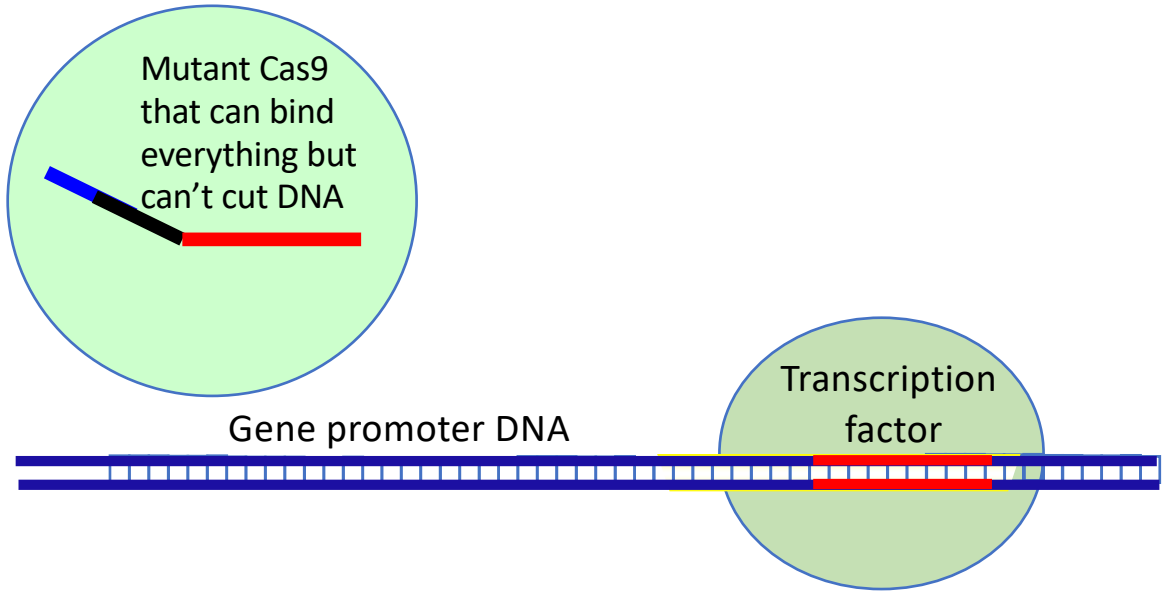


Making mice where genes are knocked out is easier and cheaper



CRISPR/Cas9 to SWITCH ON or OFF GENES

- Uses a mutant **Cas9 that can bind everything but can't cut DNA**
- This means it locks on tightly to the DNA that matches the guide sequence and stops on that region of DNA
- An example of how this can be used is by having a big Cas9 protein sitting at specific transcription factor binding site: we can block the transcription factor from coming into the gene promoter and therefore we switch off the expression of that specific gene controlled by TF in a highly targeted way.
- Negative aspect: keeps DNA in a short RNA:DNA hybrid state



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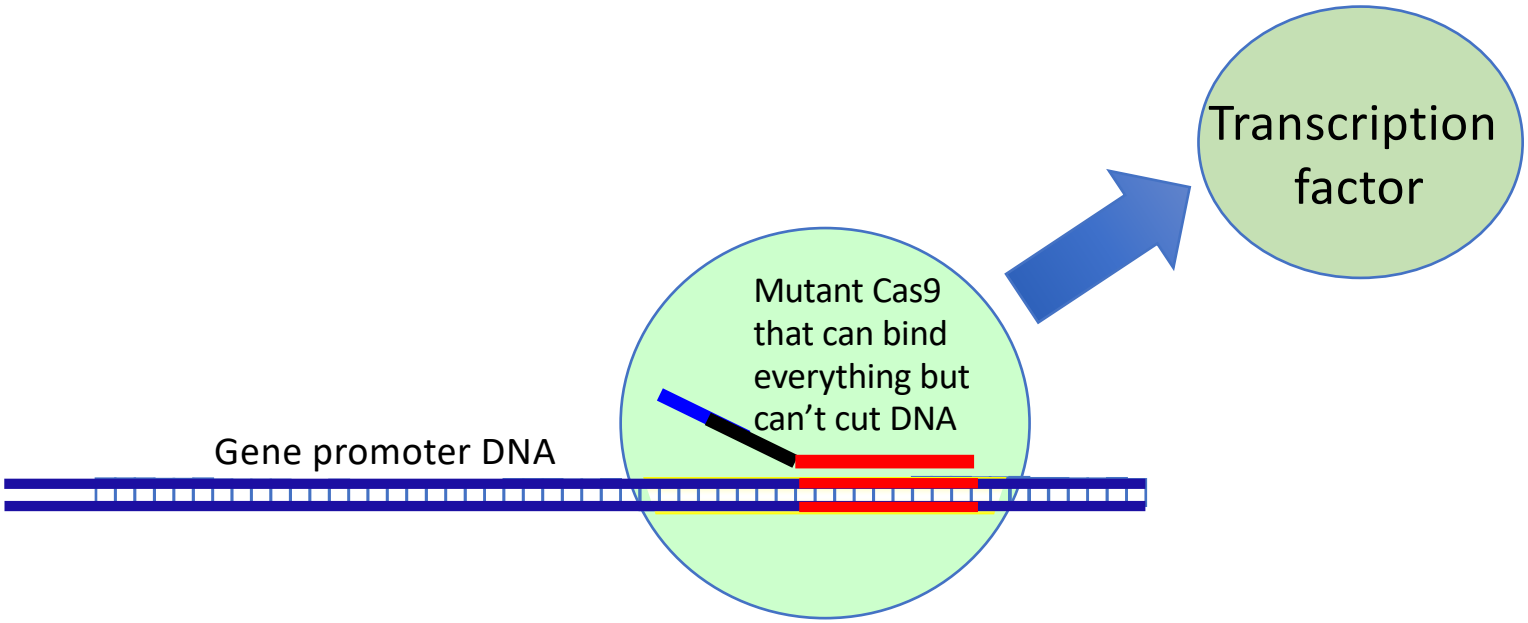
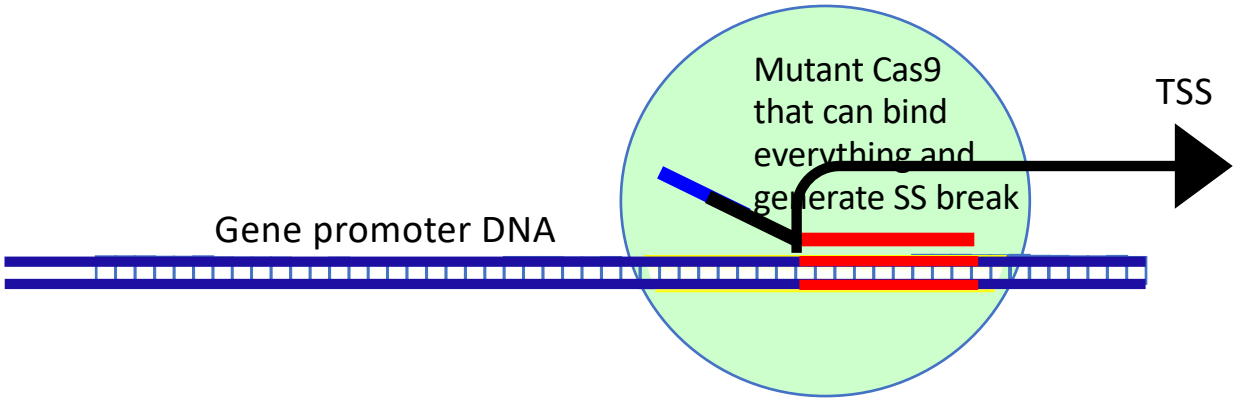


CRISPR/Cas9 to SWITCH ON or OFF GENES

- Alterantively we can use a mutant **Cas9 that can bind everything but generates a single strand cut DNA**
- This means it locks on tightly to the DNA that matches the guide sequence, stops and generate a single strand break that turn-off gene expression



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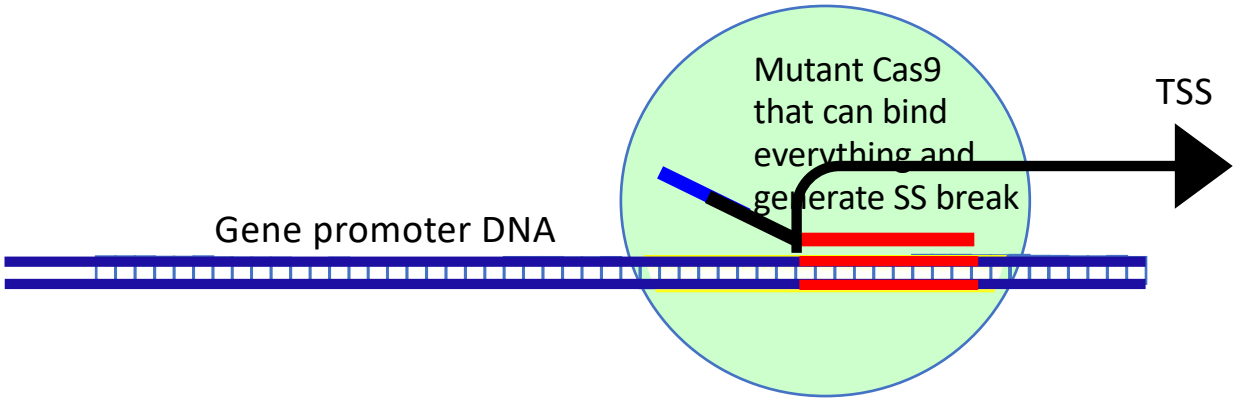

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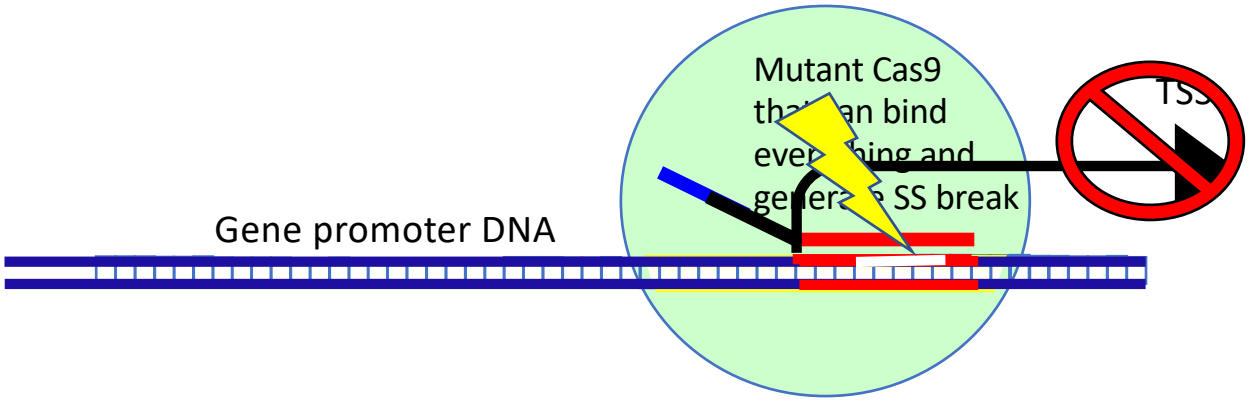

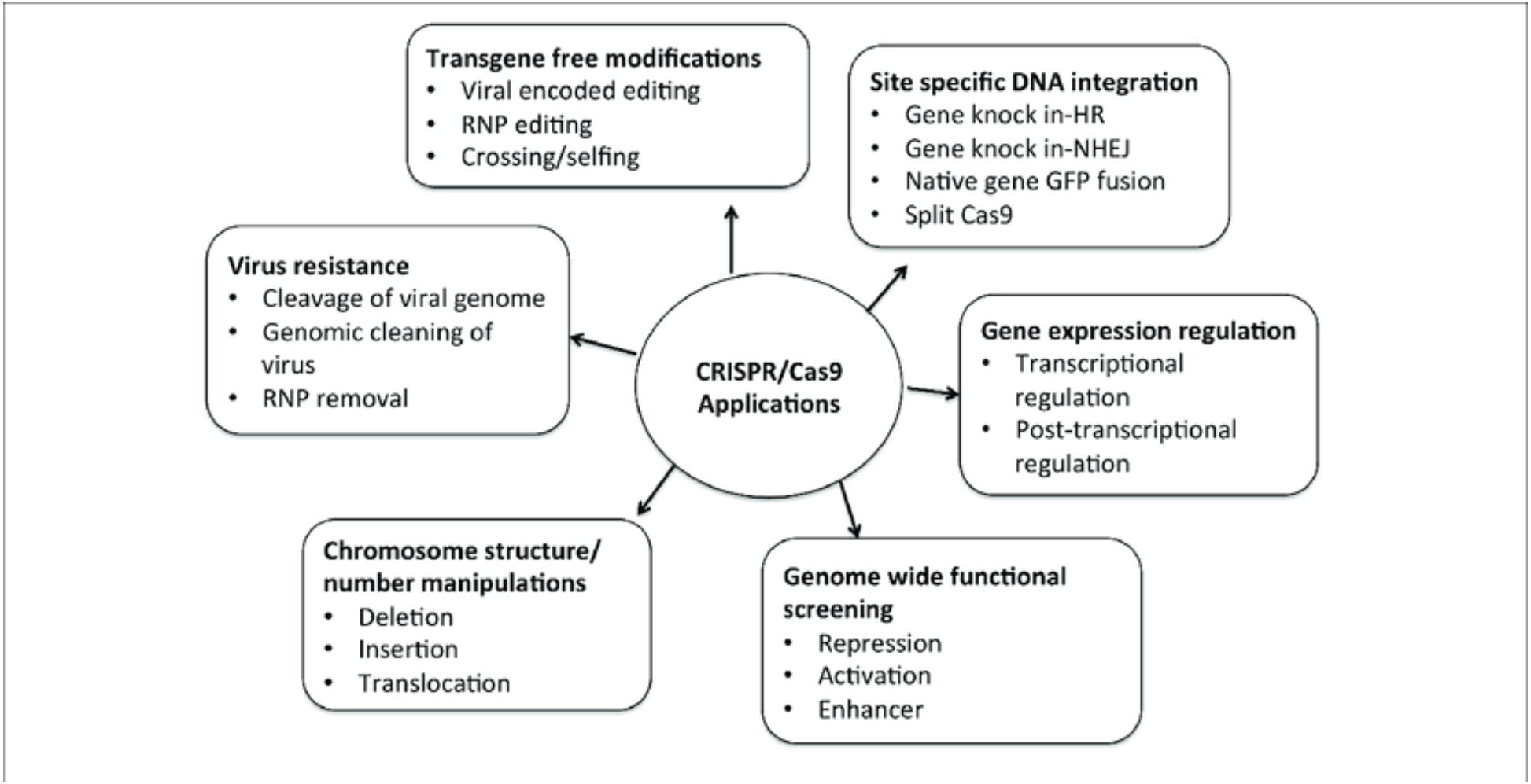
Transgene free modifications
• Viral encoded editing
• RNP editing
• Crossing/selfing
Site specific DNA integration
• Gene knock in-HR
• Gene knock in-NHEJ
• Native gene GFP fusion
• Split Cas9
Virus resistance
• Cleavage of viral genome
• Genomic cleaning of virus
• RNP removal
CRISPR/Cas9 Applications
Gene expression regulation
• Transcriptional regulation
• Post-transcriptional regulation
Chromosome structure/ number manipulations
• Deletion
• Insertion
• Translocation
Genome wide functional screening
• Repression
• Activation
• Enhancer

Using CRISPR a weapon to wipe out mosquitos

A gene drive is a genetic engineering technology that can propagate a particular suite of genes throughout a population. Gene drives can arise through a variety of mechanisms. They have been proposed to provide an effective means of genetically modifying specific populations and entire species.