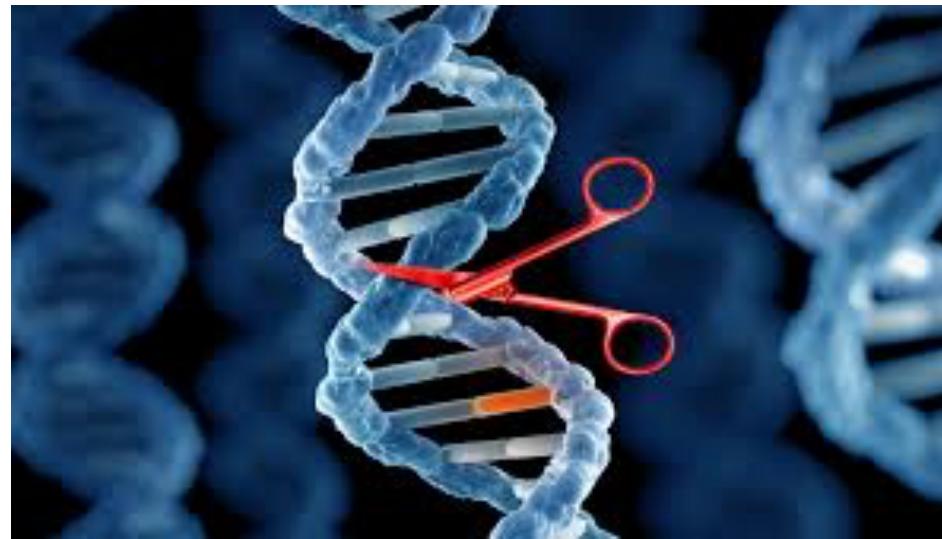


# Gene Editing



ENABLING DISCOVERY  
THROUGH GENOMIC TOOLS  
PROGRAM

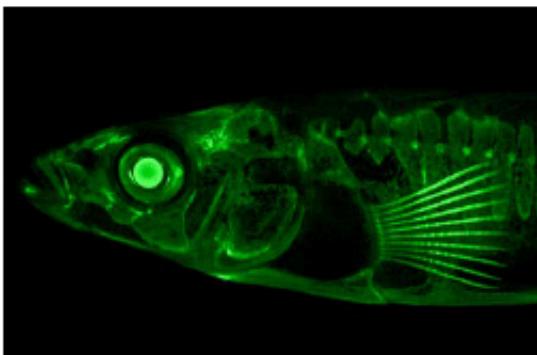
1st Annual Investigator Meeting



National Science Foundation

# Expanding Functional Genetics in Threespine Stickleback to Place Genomics Into Its Natural Context

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A stable transgenic adult threespine stickleback fish showing GFP expression in skeletal tissues.

## Project Objectives

Threespine stickleback present a massively-replicated natural evolutionary experiment. Marine stickleback repeatedly and independently colonized freshwater watersheds in postglacial environments. Their (partly) parallel adaptation to disparate freshwater habitats provides an opportunity to investigate the genetic basis of adaptive evolution in many disparate organismal traits: development, immunity, neurobiology, and more, set within a well-understood historical and ecological setting. Extensive genomic data provides a valuable foundation for this genetic research, but key tools remain underused or unavailable, hindering progress at functionally characterizing candidate genes. We seek to expand the functional genetics toolkit for stickleback, by optimizing methods for CRISPR-Cas9 editing of embryos by microinjection, adult brain tissue by viral transformation, and *in vitro* cell culture by nucleoporation. In support of these tools, we are also establishing a stickleback stock center where researchers globally can order fish eggs, adults, cryopreserved sperm, or cryopreserved cell cultures. Lastly, we are developing a gene expression atlas to improve gene function annotation needed to guide experimental design.

## Progress to Date

We are developing several parallel lines of functional genetics tools for threespine stickleback:

Craig Miller's lab has established that CRISPR-Cas9 homology-directed repair is feasible in stickleback embryos with minimal genetic scarring. They have shown that mutations in *Hps5* result in albino larvae and fish, making a simple tool for scoring Cas9 editing efficiency, as well as better visualization of fluorescent transgenes. They have also been optimizing transgenesis in sticklebacks to study enhancer and gene function.

Dan Bolnick's lab generated fibroblast cell cultures from multiple source populations. They developed protocols to cryogenically preserve cultures, and measured population differences in cell function. They are testing *in vitro* gene editing protocols.

Kathryn Milligan-Myhre's lab has surveyed the stickleback research community to determine demand for stock center materials and initiated a breeding program at the University of Alaska.

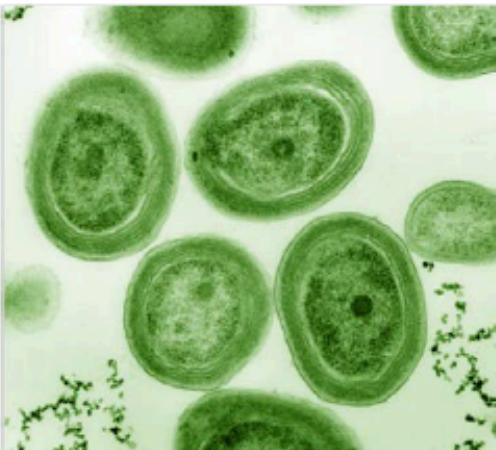
Mike White's lab has improved the reference genome sequence and annotation of coding and regulatory regions. Long-read sequencing characterized full length transcripts and isoforms across the genome. ATAC-seq profiled regions of open chromatin across multiple tissues and developmental stages.

Alison Bell's lab has successfully altered brain gene expression in adult stickleback brains using the HSV1 derived virus with both a short-term (mCMV, peak expression by 4 days post-injection) and longer-term (hCMV) promoter.

# Development of Genetic Tools for the Dominant Phototroph in the Oceans

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TEM image of *Prochlorococcus*,  
the smallest and most abundant oxygenic phototroph on Earth

## Project Objectives

The goal of this project is to develop an efficient transformation method and a panel of functional genomics tools for *Prochlorococcus*, the numerically dominant phototroph in the oceans.

The first aim was to develop an efficient way to deliver DNA inside *Prochlorococcus* cells without compromising the cell viability. To that end a collaboration was established with co-PI, Cullen Buie, who has designed electroporation microfluidic technology specifically for this purpose. The second aim has been to develop transposon genetic tools that will allow us to perform global mutagenesis experiments and provide a way to link genes to their phenotype in a genome-wide manner. The third aim is to develop targeted mutagenesis tools - using the CRISPR method - to allow specific gene knockout to answer more focused biological questions.

In terms of broader impacts, *Prochlorococcus* is an amazing vector to foster public interest on topics such as ocean ecology, climate change, biodiversity and genetics. It attracts the attention of science journalists, and serves as an 'ambassador' for microorganisms, drawing young scientists into the field.

## Progress to Date

We have developed an electroporation protocol for *Prochlorococcus*. We are now able to successfully deliver DNA inside cells while keeping cells viable.

We have designed and tested several transposon tools using various antibiotic resistance genes. We have attempted to transform these elements in several strains of *Prochlorococcus*, without success so far.

We have identified and characterized small mobile genetic elements within *Prochlorococcus* genomes which are promising targets to repurpose for genetic engineering. They are small, inducible, integrative and replicative elements that we are currently modifying to carry a selectable marker.

We have established new collaborations on the project that promise to advance our work significantly. With David Kehoe we are leveraging his success in developing a CRISPR system with *Synechococcus*. With Christopher Johnston we are drawing on his expertise to avoid bacterial defenses to exogenous DNA.

Finally, we have been working with colleagues at the U. Cordoba, Duke, and Roscoff Marine Station (CNRS) to organize a Symposium and Workshop focused on marine picocyanobacteria. The Workshop will be funded in part by this grant with supplemental funds from private foundations and will be focused on distributing tools developed under the auspices of this grant. The goal is to train the next generation of researchers on the nuances of working with recalcitrant microbes like *Prochlorococcus*, that have only recently been domesticated.

# Generating Tools to Study Spiralian Development

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Ventral views of large female and small male black-footed slipper limpets, *Crepidula atrasolea*. Rack aquarium system shown in the background.

## Project Objectives

**1) Design and construct an automated closed-system marine aquarium for rearing filter-feeding marine organisms.**

- design and modify aquarium system with Iwaki Aquatics
- design and develop automated feeding and monitoring systems

*This system is designed to be self-sufficient and run for days to weeks at a time without human intervention, reducing the challenges of rearing marine animals.*

**2) Raise *Crepidula atrasolea*, a model marine lophotrochozoan (spiralian), for biological study, including transgenic lines.**

- reduce generation time and increase reproductive output to produce sufficient numbers of animals for stable supplies of wildtype and transgenic animals
- develop SnailTrail App for tracking and reporting biological parameters
- use improved delivery methods (e.g., injection, electroporation) to generate transgenic animals (e.g., CRISPR/Cas9, plasmid-based integration, etc.)

**3) Broaden awareness of *Crepidula atrasolea* as a model marine system:**

- publication and online distribution of all plans, data, instructional videos, etc.
- solicitation of spiralian community input and feedback for future directions.

## Progress to Date

Completed conversion of a commercially available (Iwaki) freshwater rack aquarium system for use as a fully automated marine aquarium system, with delivery in April 2019. We have made excellent progress with the design and construction of accessory components, including the syringe-pump driven automated feeding system; real-time sensor to monitor feeding and adjust levels of phytoplankton, allowing for automated changes in response to consumption rates; and incorporation of an automated bypass loop to remove mechanical, biological and carbon filtration during feeding.

We have also designed the first version of the SnailTrailApp using FileMaker Pro. This interactive database system allows users to track the physical parameters of the aquarium system and uses relational databases to connect this information to biological outputs. We are measuring animal growth, sexual transitions, and reproductive output (brood frequency and brood size). Data will be available via a web-based portal. Incorporation of Bluetooth LE enabled devices such as a 2-D barcode scanner and digital calipers are being used to increase the efficiency of data collection from the system.

We have also developed electroporation protocols and had success in producing F0 transgenic animals via this method as well as by traditional micro-injection. We are currently using plasmid-based promoters to drive fluorescent marker expression and CRISPR/Cas9 to label specific cell types.

## Highlights

Design of automated feeding system for near-continuous feeding, including sensors to monitor and regulate food levels and consumption.

# Creation of a Genetically Tractable Cephalopod Model Using The Hawaiian Bobtail Squid

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(508) 289-7253



Adult specimen of *Euprymna scolopes*

## Project Objectives

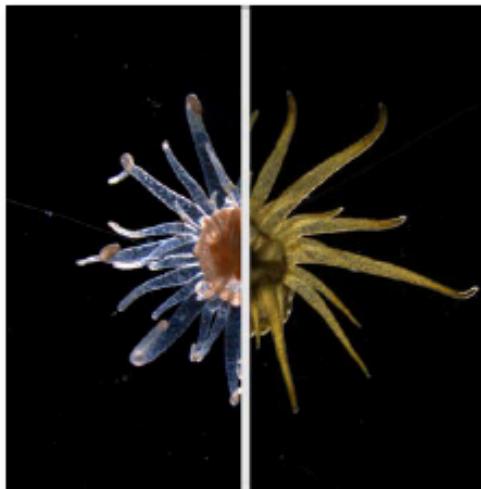
The coleoid cephalopods exhibit remarkable biological innovation. Their brain size and behavioral sophistication rival those of higher vertebrates. For example, the octopus's brain has ~ 4 orders of magnitude more neurons than non-cephalopod molluscs, and ~ 4 times the number of neurons as a rat. Besides their brains, they offer a wealth of other features that could stimulate broad areas of research. In spite of these opportunities, the cephalopod research community remains small. This is because it lacks a genetically tractable model. We propose to overcome a key bottleneck in cephalopod biology by creating the first transgenic cephalopod model using the *E. scolopes*. Cephalopods are difficult to culture in captivity, and this has been the primary impediment to developing a genetic model. We propose to create a life-cycle culture facility for *E. scolopes* and then use it as a resource to create transgenic lines using CRISPR-Cas9. The long-term goal is to create a resource center at the Marine Biological Laboratory that will provide animals, transgenic lines, and refined protocols to the research community. By creating the first transgenic cephalopod model, we will open new avenues to study these organisms on a mechanistic basis.

## Progress to Date

Our award started in September 2018. Since that time, we have made significant progress towards our two major aims: 1) establishing an *E. scolopes* culture facility and 2) creating transgenics using CRISPR-Cas9. Currently there are 490 animals in our facility, and this number has kept steady at close to ~500 animals over the past 6 months. Our facility has produced greater than 6000 embryos, and thousands of these have been used for microinjections. We have cultured animals for three generations within the facility. The initial brood stock was captured in Hawaii and we continue to capture wild animals to add to our facility. Currently, the greatest bottleneck for life-cycle culture has been low survivorship during the first 2-3 weeks after hatching. We have also established a culture of *E. berryii*, a highly similar species from Okinawa, Japan with many favorable characteristics for model development. For our gene knockout studies, we are focusing on Tryptophan Dioxygenase (TDO), an enzyme that catalyzes the first committed step in ommochrome pigment synthesis in the retina and chromatophores. Over the past 6 months we have been injecting embryos at the 1-8 cell stages with Cas9 and CRISPR guides to TDO. Needles, injection volumes and reagent concentrations have been optimized. Thus far, a total of 657 embryos have been injected. ~25% survive to hatching. Of these, 42% show clear lack of pigmentation in the eyes. All embryos that were injected at the 1-2 cell stages exhibit the eye phenotype. After hatching, animals were sacrificed for genotyping. Both a T7 nuclease assay and MiSeq sequencing on a small subset of animals have confirmed the presence of indels at the CRISPR gRNA directed sites.

# Functional Genomics Tools for Cnidarian-Dinoflagellate Symbiosis

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(541) 737-4359



Aposymbiotic and symbiotic *Exaiptasia pallida* (Aiptasia)

## Project Objectives

Overall goal is to develop effective genetic methods in the sea anemone *Aiptasia* and its algal symbiont *Breviolum minutum* (Family Symbiodiniaceae), a rapidly developing model system for the study of coral-algal symbiosis.

Aim 1: Genetic manipulation of *Breviolum minutum*.

Aims 2 and 3: Development of methods for gene knockdown, knockout, tagging, and overexpression in *Aiptasia*.

Aim 4: Achieving settlement and metamorphosis of *Aiptasia* larvae.

## Progress to Date

### *Aiptasia*:

- Regular spawning year-round.
- Plasmid-based expression of fluorescent proteins driven by *Aiptasia* promoters delivered by microinjection and electroporation of zygotes.
- Successful expression of capped mRNA delivered via microinjection of zygotes.
- Testing different settlement cues is ongoing with no consistent metamorphosis or settlement observed.
- Initial attempts to electroporate pedal lacerates and are currently optimizing this using capped mRNA and recombinant fluorescent proteins.

### Symbiodiniaceae:

- Attempted delivery of both Cas9 RNPs and CRISPR/Cas9 expression constructs via electroporation, biolistics, glass bead abrasion, lithium acetate transformation, freeze-thaw, and nanostraw methods for mutagenesis of *URA3*, *DHC1*, and *nitrate reductase* genes.
- No mutants have been obtained, but low-level editing activity was observed in *URA3* using Cas9 RNPs delivered through electroporation.
- We are optimizing the isolation of nuclei from *Breviolum minutum* for ATAC-seq. We expect the information on genome-wide chromatin accessibility will be useful in future editing attempts.

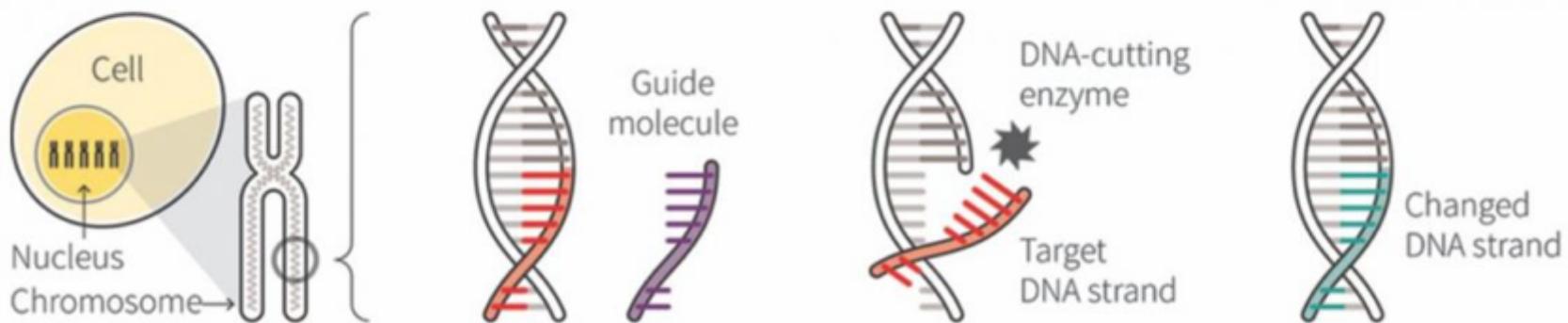
## Highlights

# What is it?

## Gene editing

A DNA editing technique, called CRISPR/Cas9, works like a biological version of a word-processing programme's "find and replace" function.

### HOW THE TECHNIQUE WORKS



A cell is transfected with an enzyme complex containing:  
■ Guide molecule  
★ DNA-cutting enzyme

A specially designed synthetic guide molecule finds the target DNA strand.

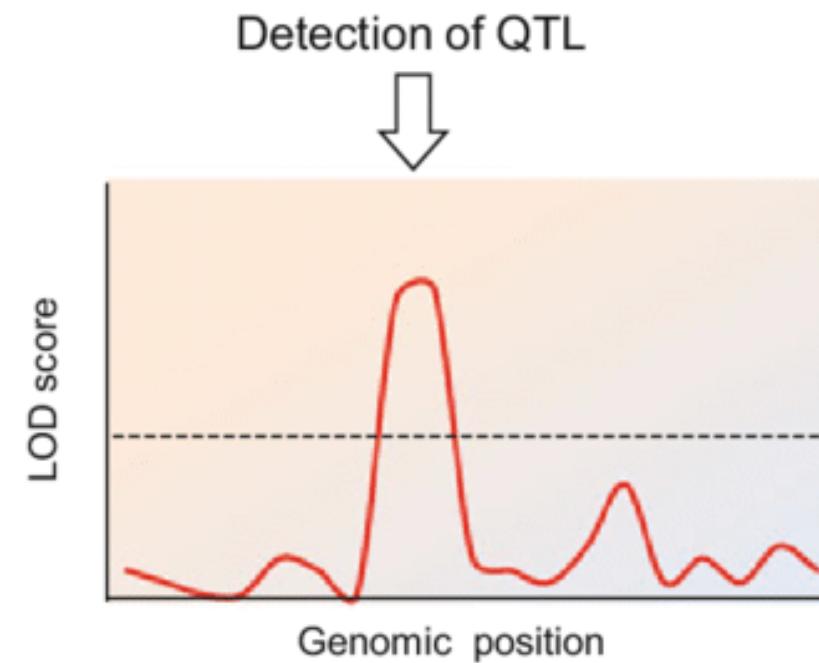
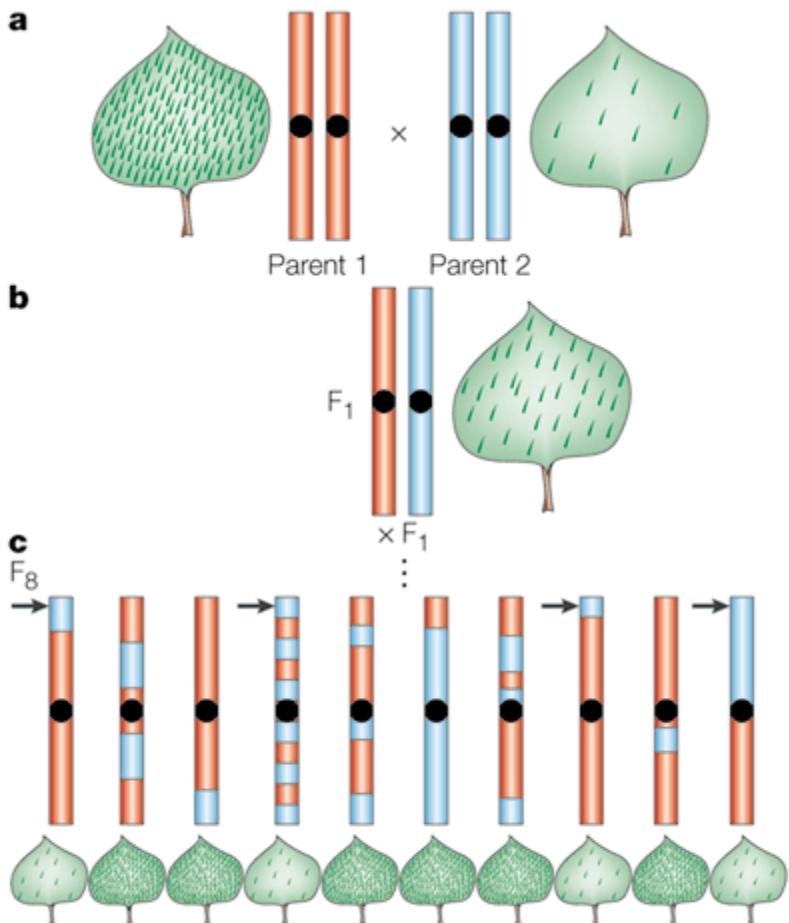
An enzyme cuts off the target DNA strand.

The amended DNA strand repairs itself.

# Why do it?

# Why do it?

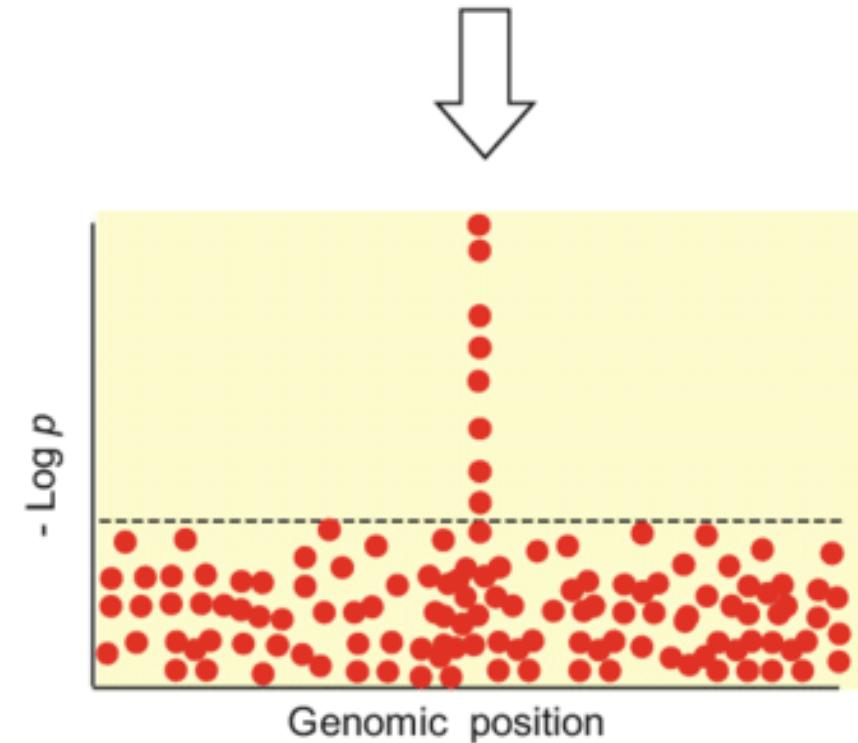
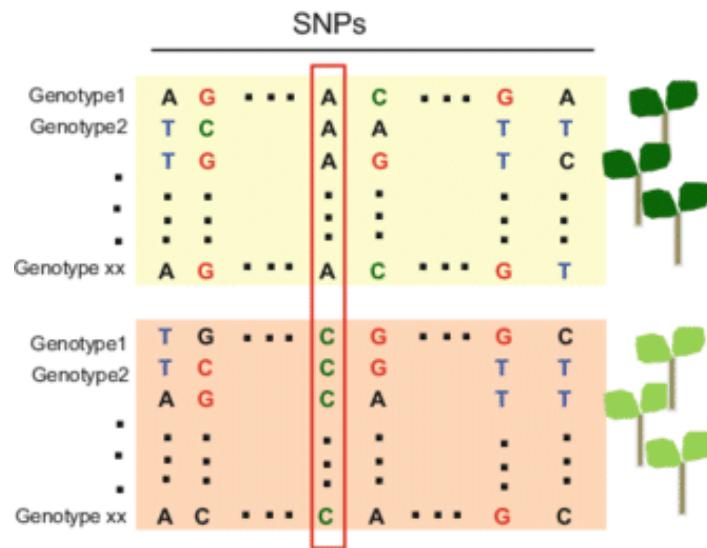
Confirm suspected effect of gene X on trait Y



# Why do it?

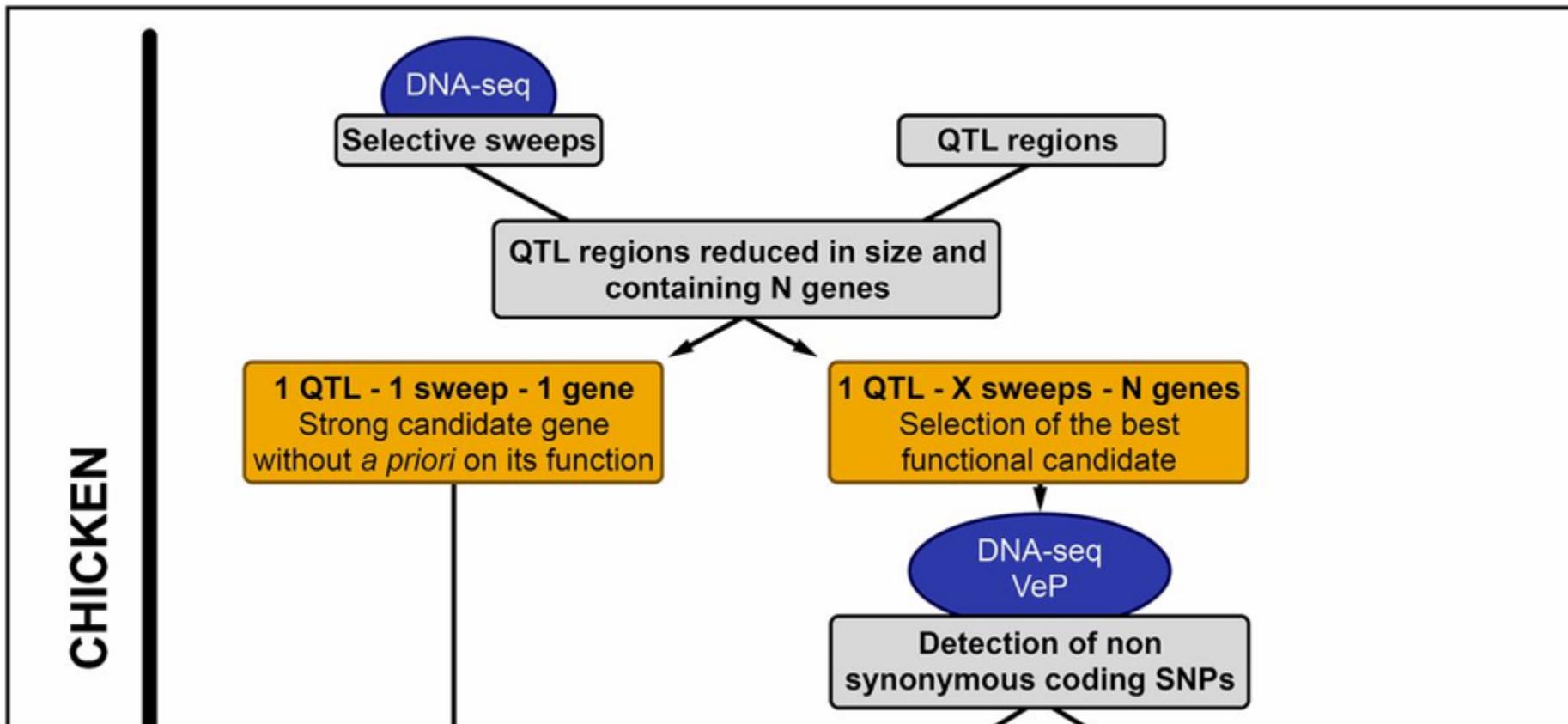
Confirm suspected effect of gene X on trait Y

Genome-Wide Association studies  
GWAS: natural population



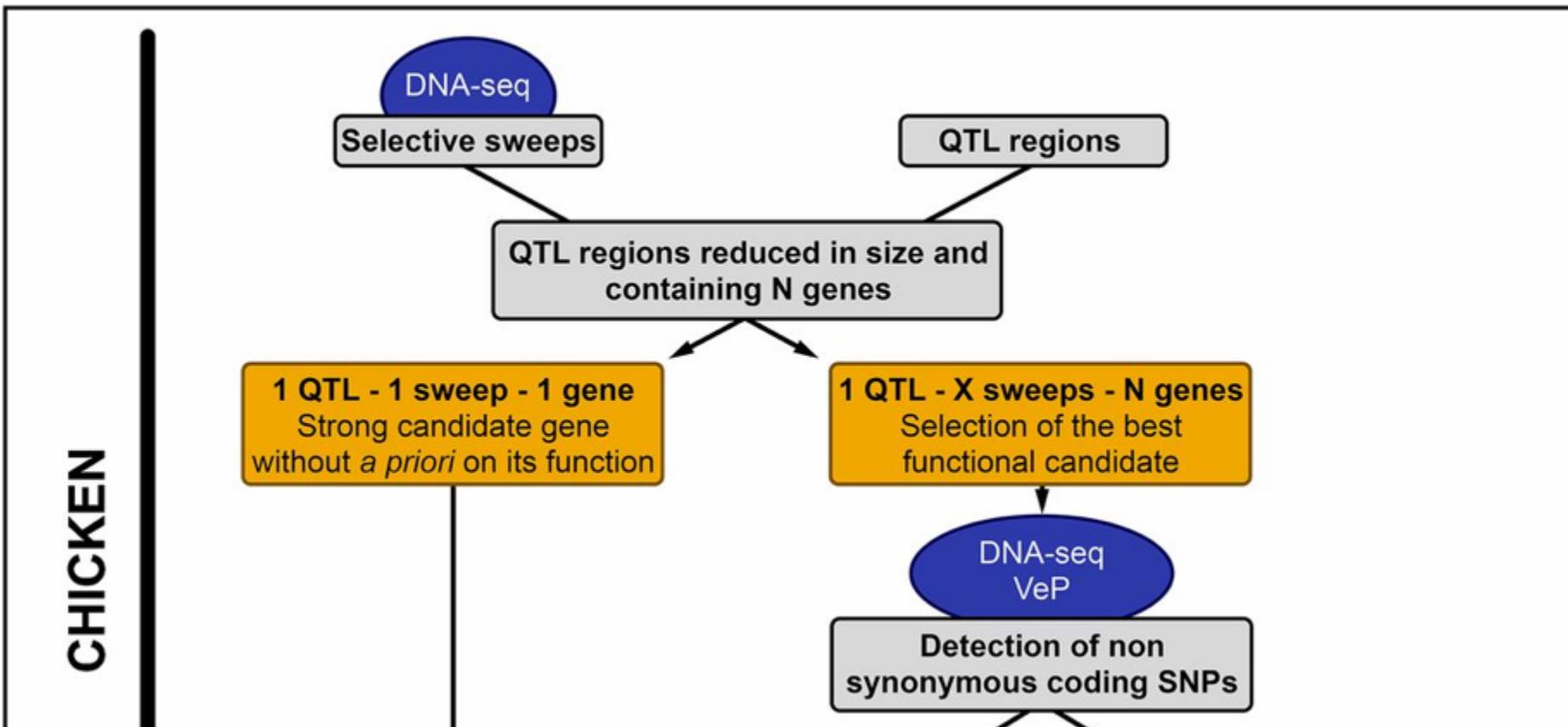
# Why do it?

Confirm suspected effect of gene X on trait Y



# Why do it?

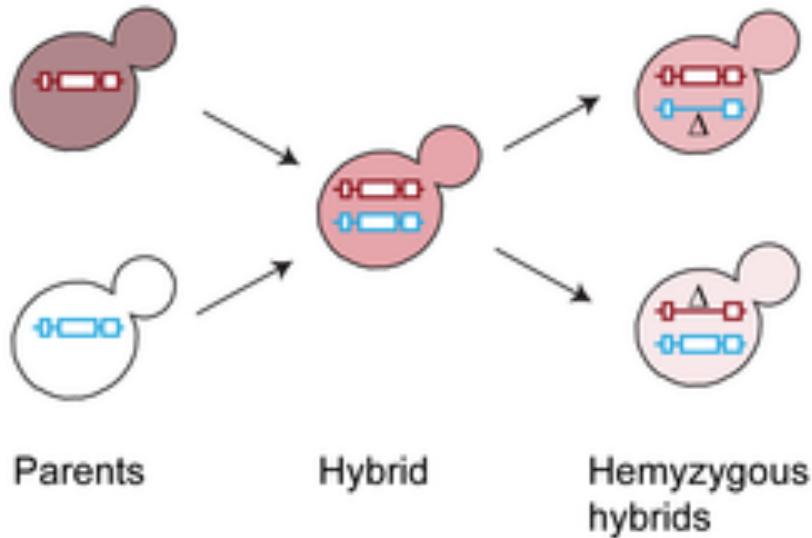
Confirm suspected effect of gene X on trait Y



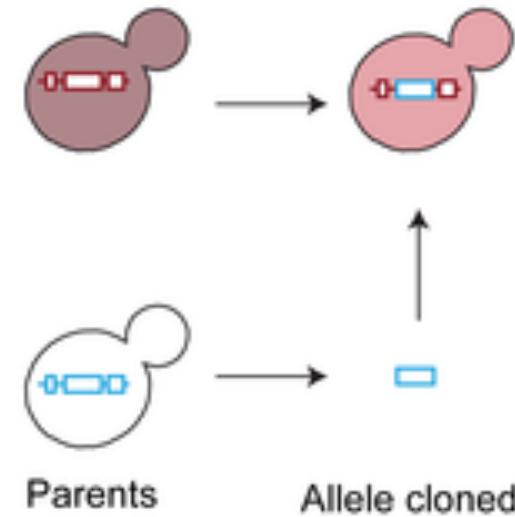
# Why do it?

Confirm suspected effect of gene X on trait Y

## A Reciprocal hemizygosity



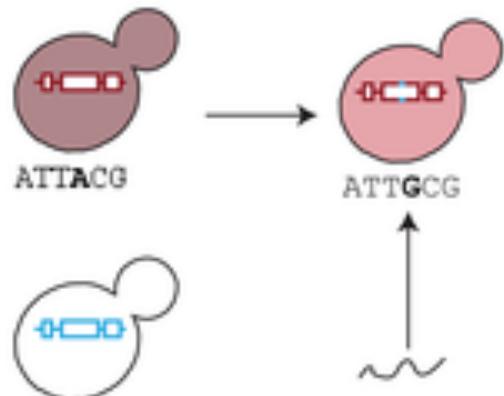
## B Allele swapping



# Why do it?

Confirm suspected effect of gene X on trait Y

## C Site directed mutagenesis

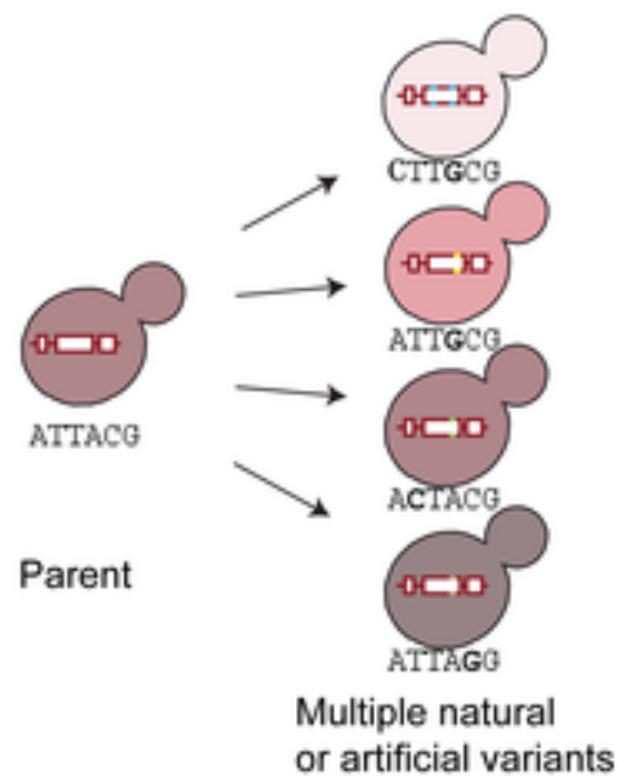


Parents

Single bp  
change



## D Synthetic biology



Parent

Multiple natural  
or artificial variants

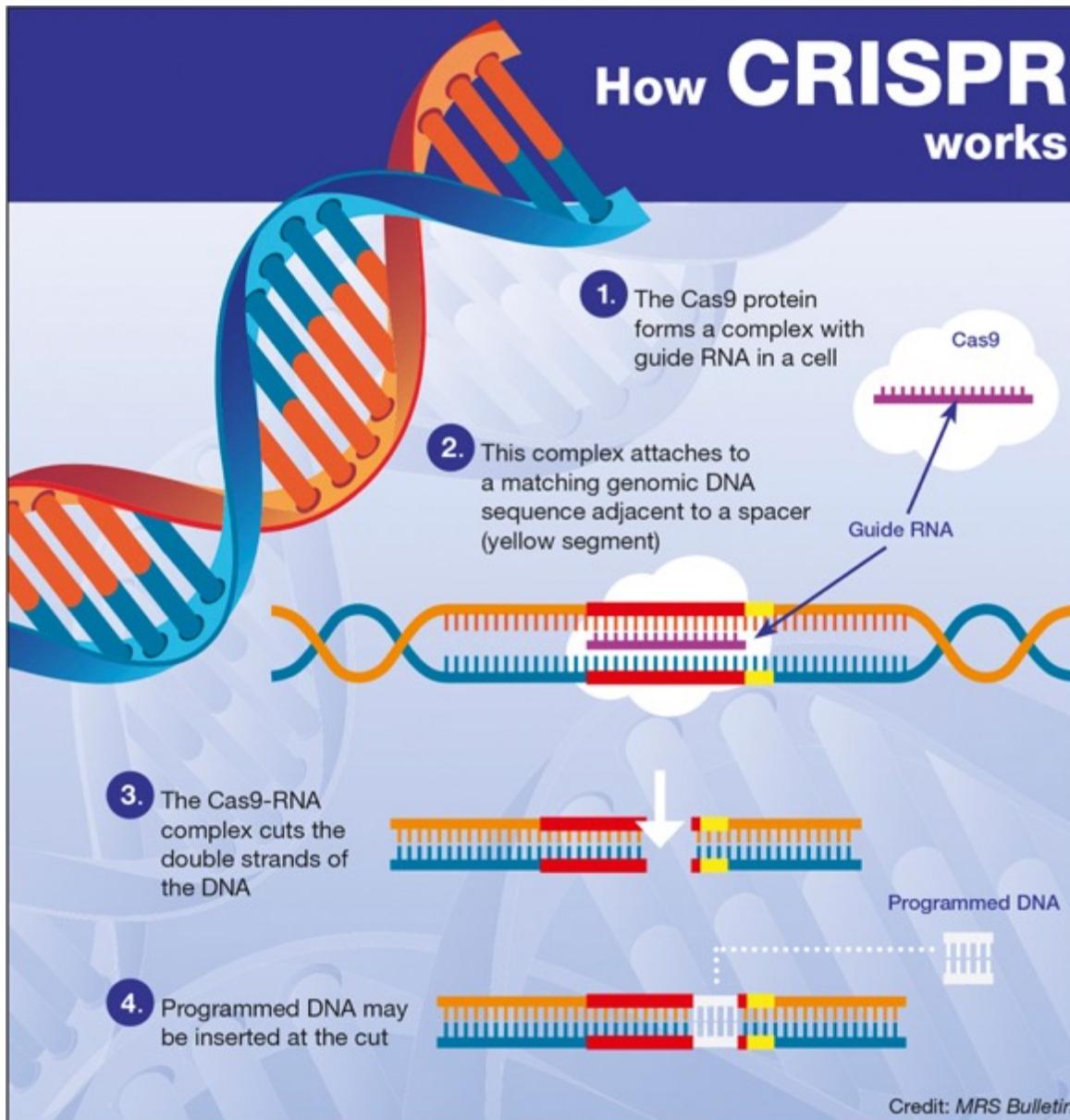
# Why do it?

Create transgenic population with useful properties

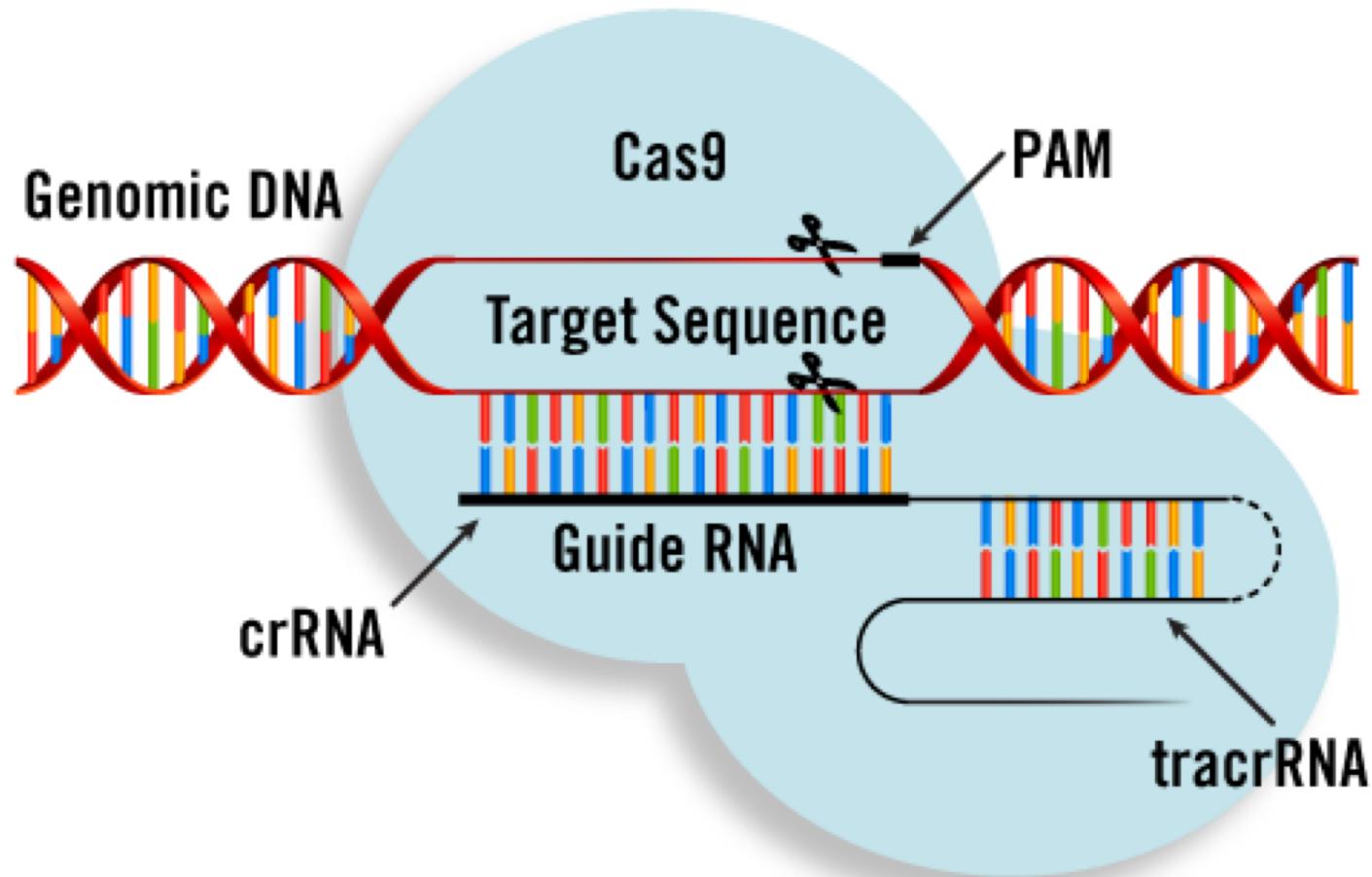


Disease-resistant transgenic American Chestnut

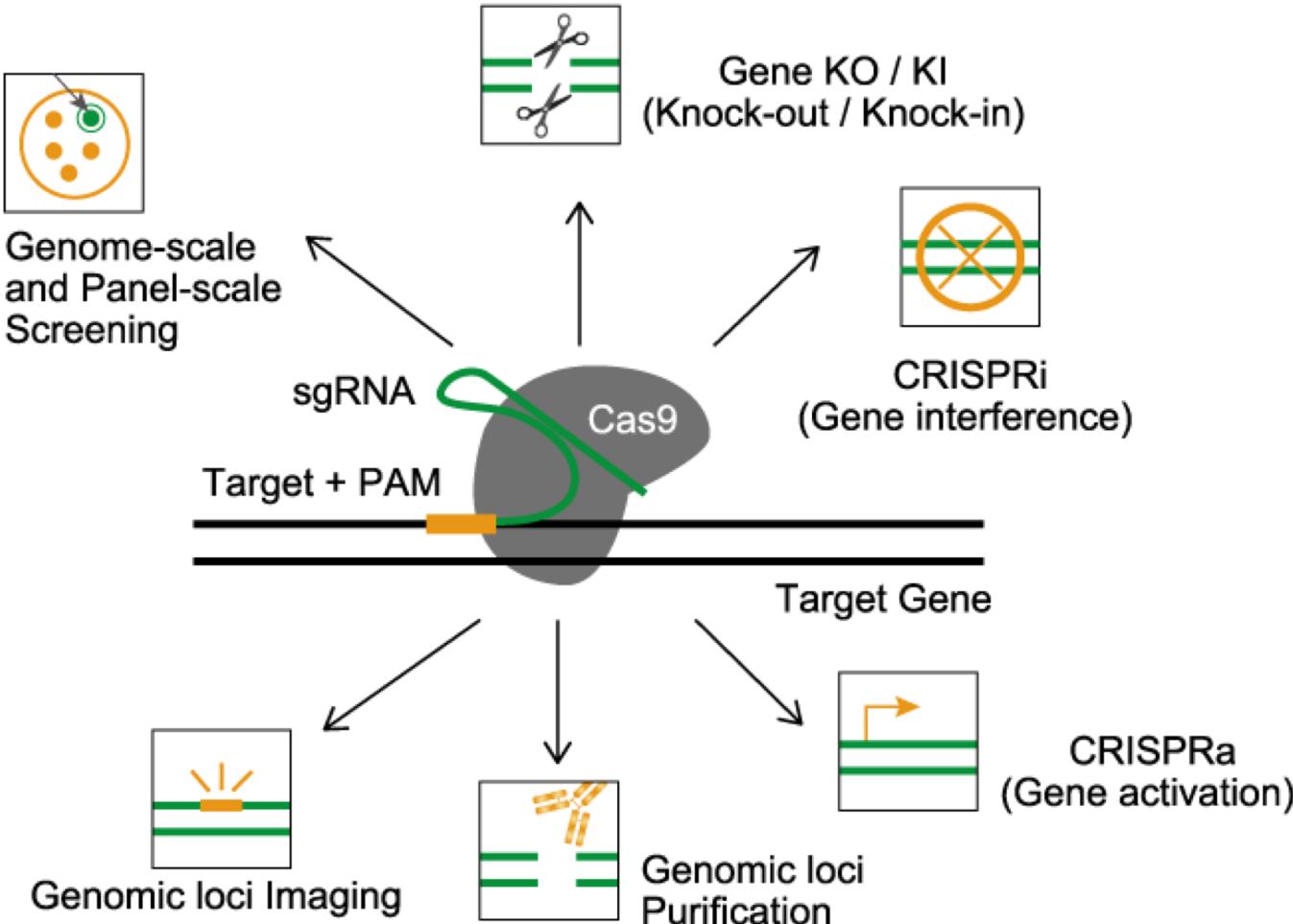
# How does it work?



# How does it work?



# More than just cutting



# Hurdles

## CRISPR Cas9

## Basic Considerations

GC Content

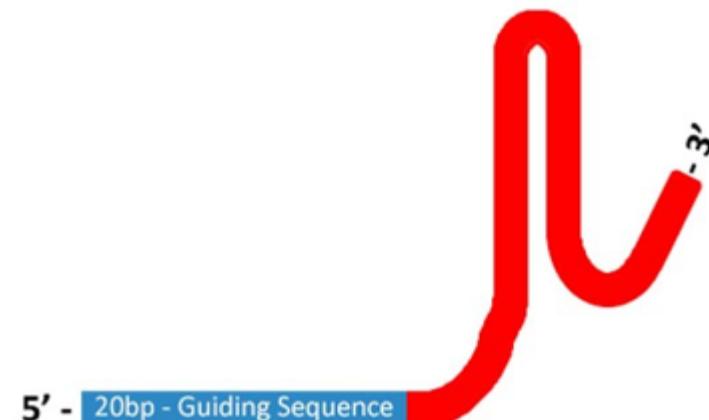
40%-80%

Length

17-24 Base Pairs

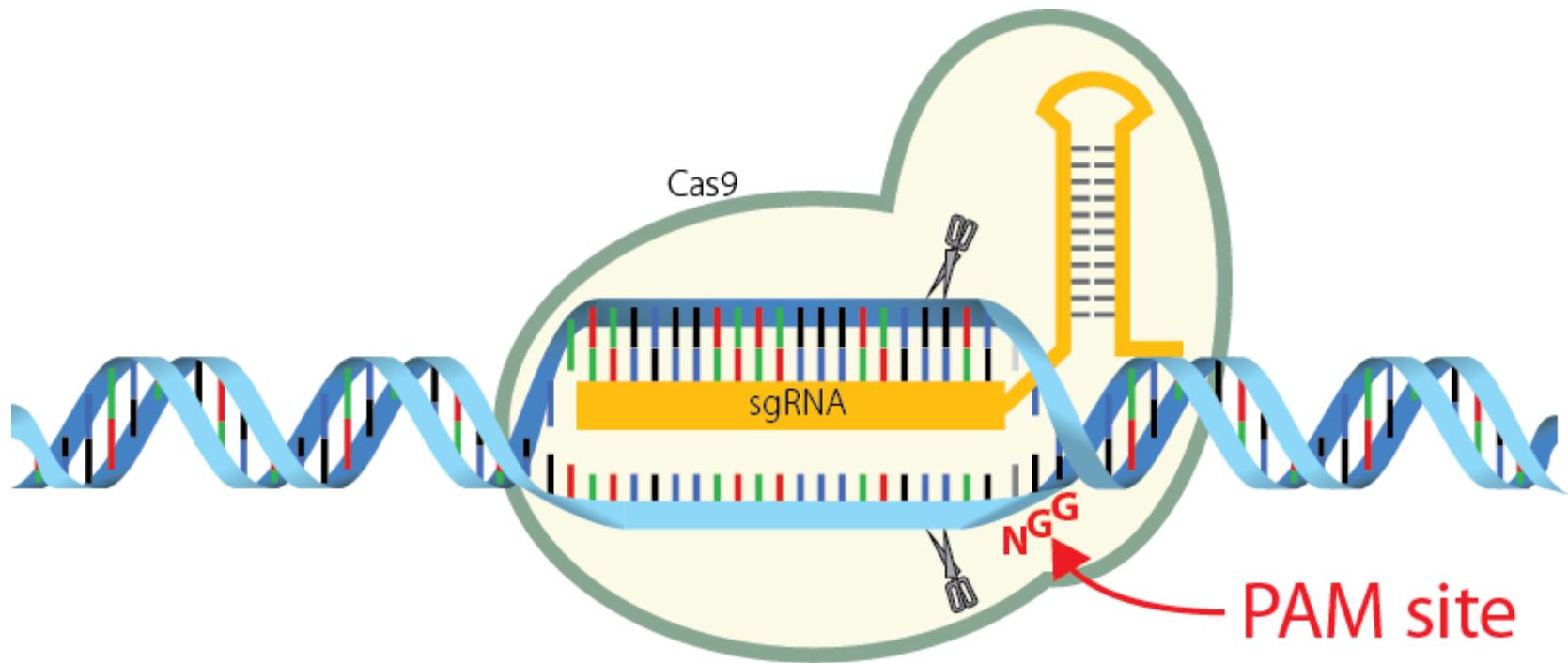
Potential Off-Target Effects

17-24 Base Pairs



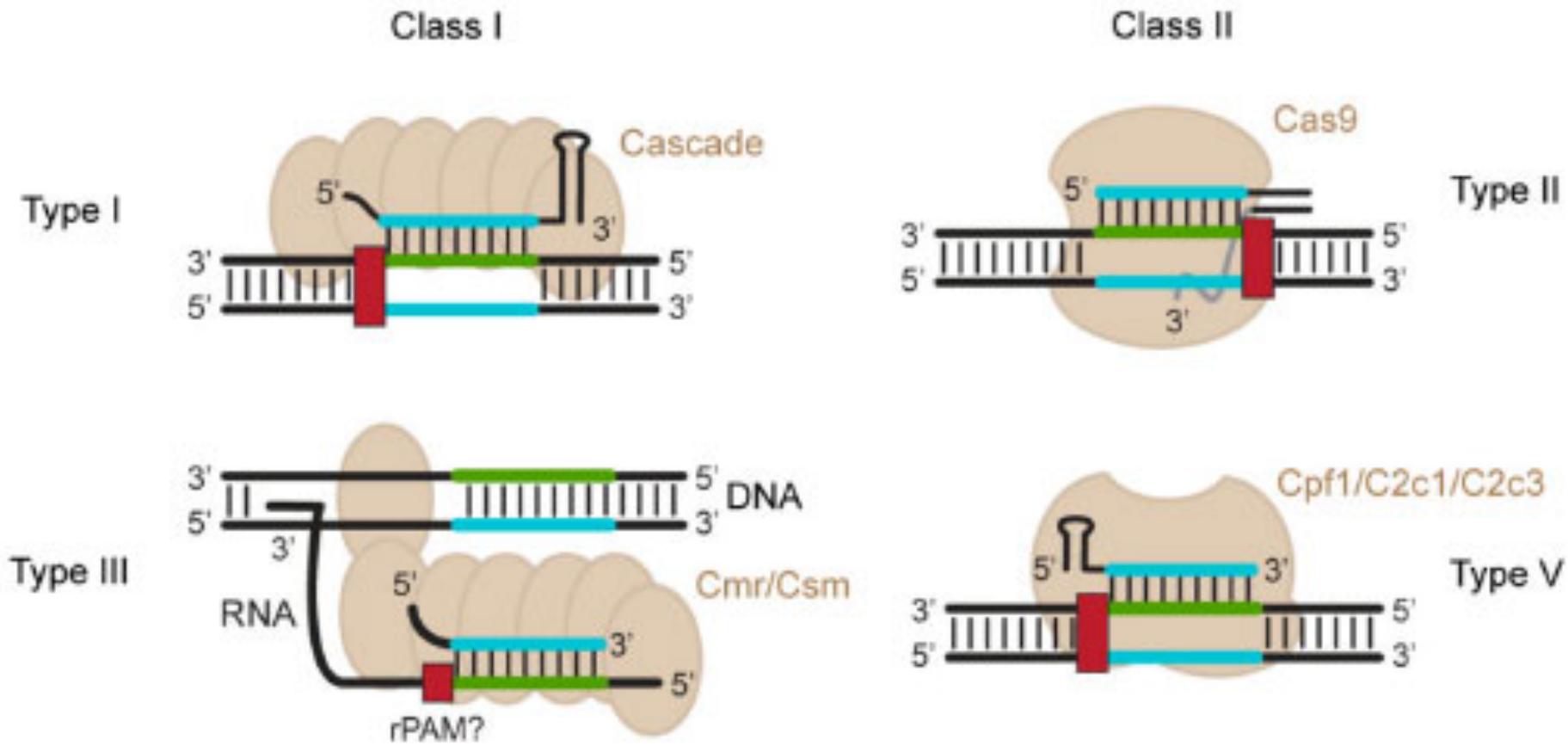
Off-target effects

# Hurdles



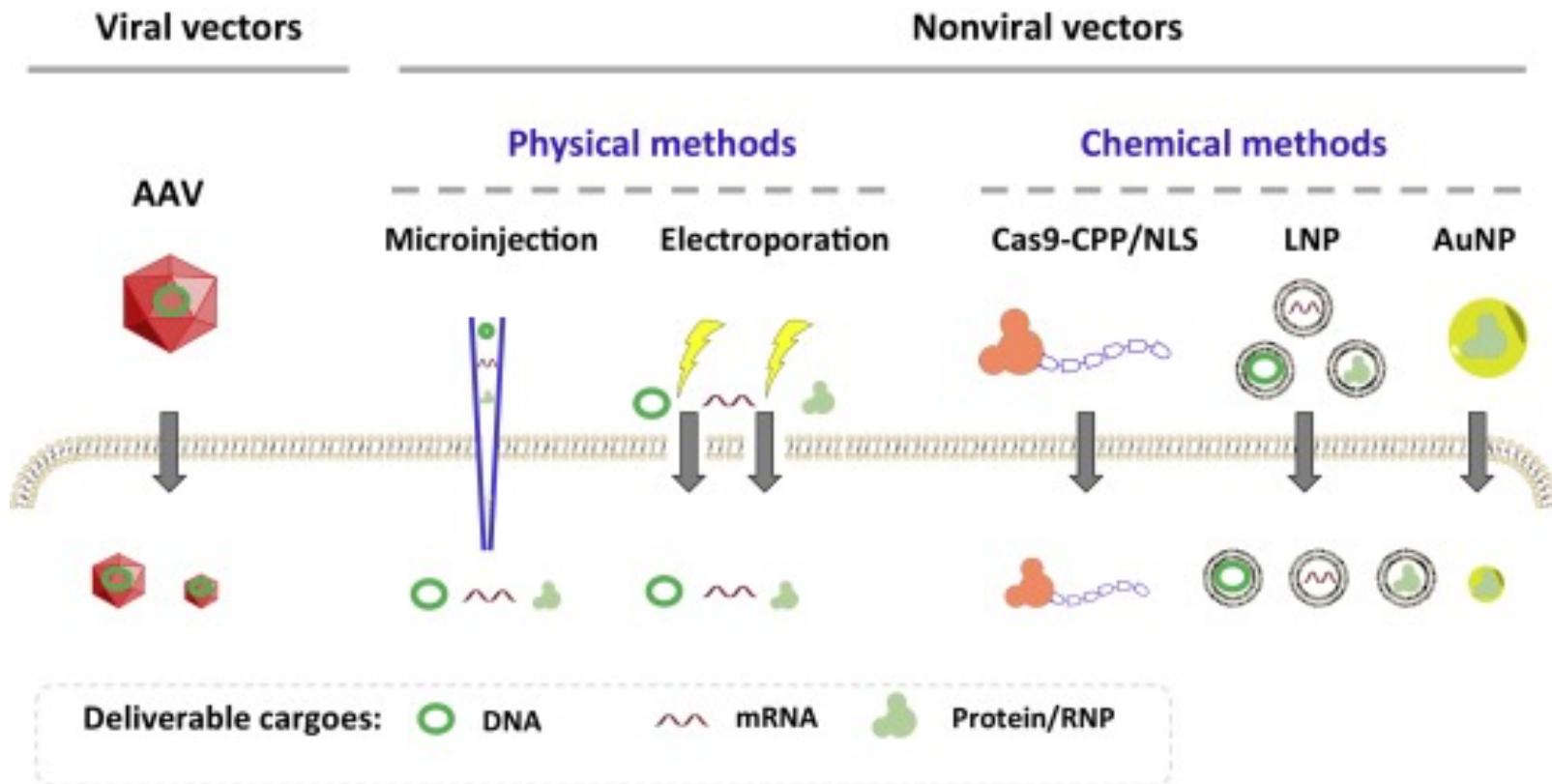
Need the correct PAM site

# Hurdles



Diverse cas proteins with different PAM sequences

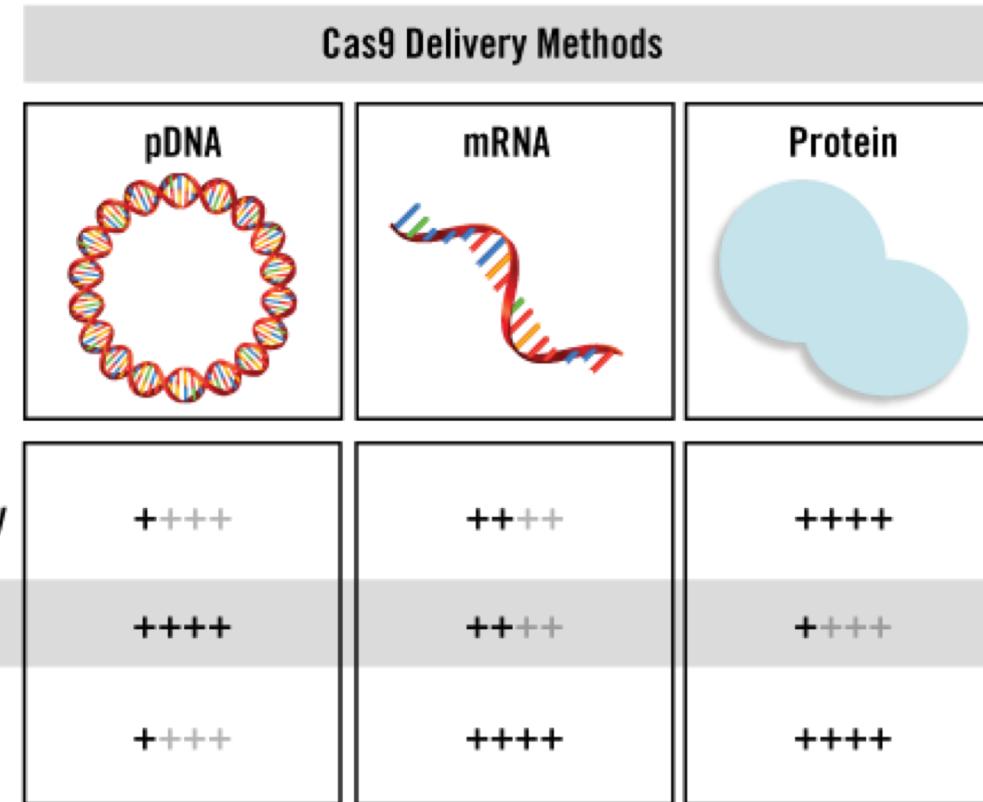
# Hurdles



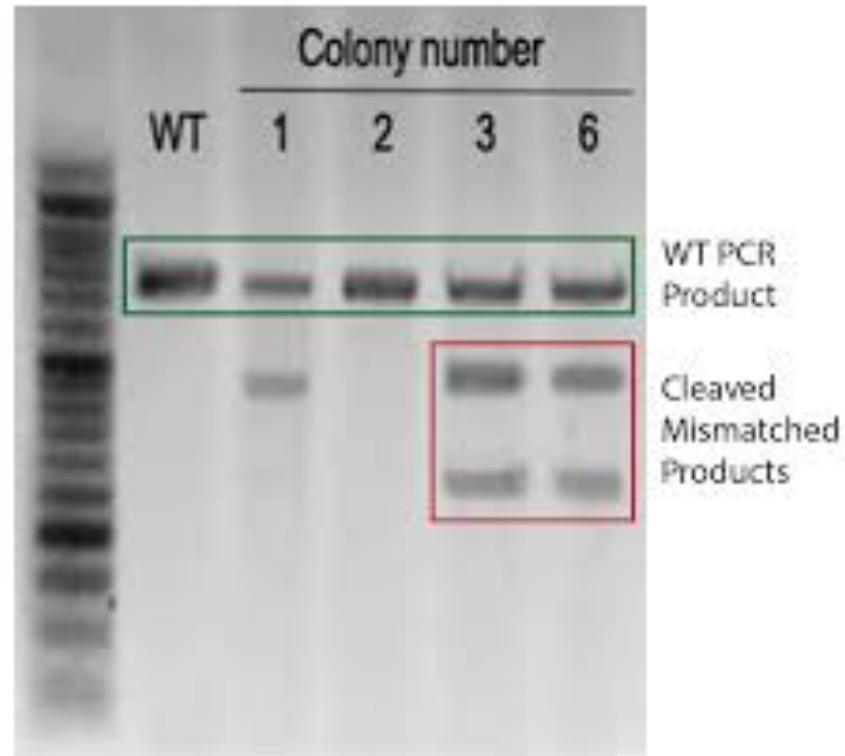
Trends in Biotechnology

Delivery

# Hurdles

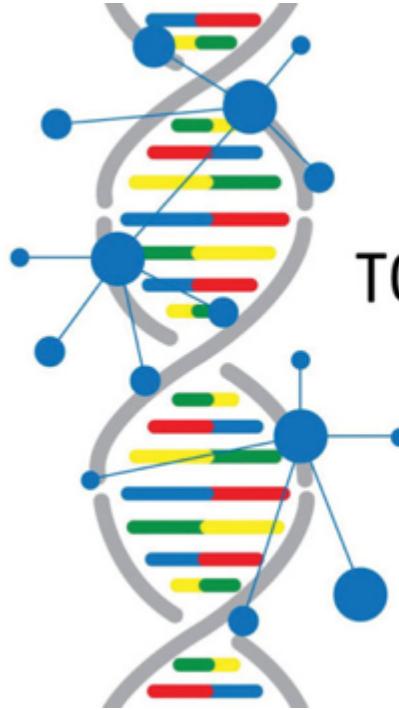


# Hurdles



Confirmation

# Hurdles



## TO CRISPR OR NOT TO CRISPR?

Examining the ongoing ethical debate surrounding embryonic gene editing



- Genome editing for basic research, including embryos
- Somatic gene editing to treat serious diseases
- Germline editing to treat serious genetic diseases where no reasonable alternative exists
- Somatic or germline editing for enhancement purposes

Ethics