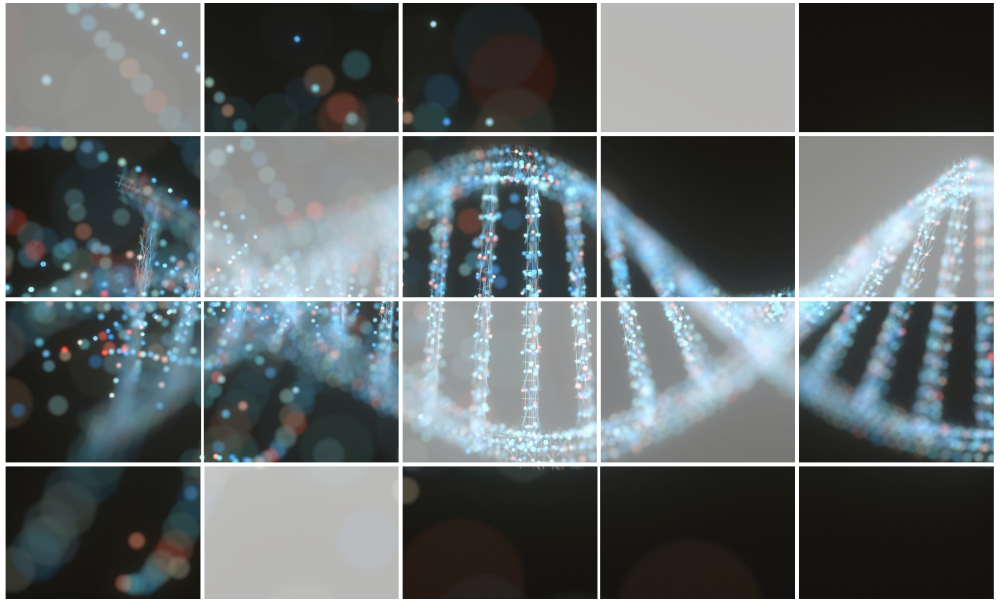
Hipolit's Biotech Breakdown:
Gene Editing 101 & CRISPR-Cas9



Hipolit Cichocki 2022.08
Dragon Gate Investment Partners LLC

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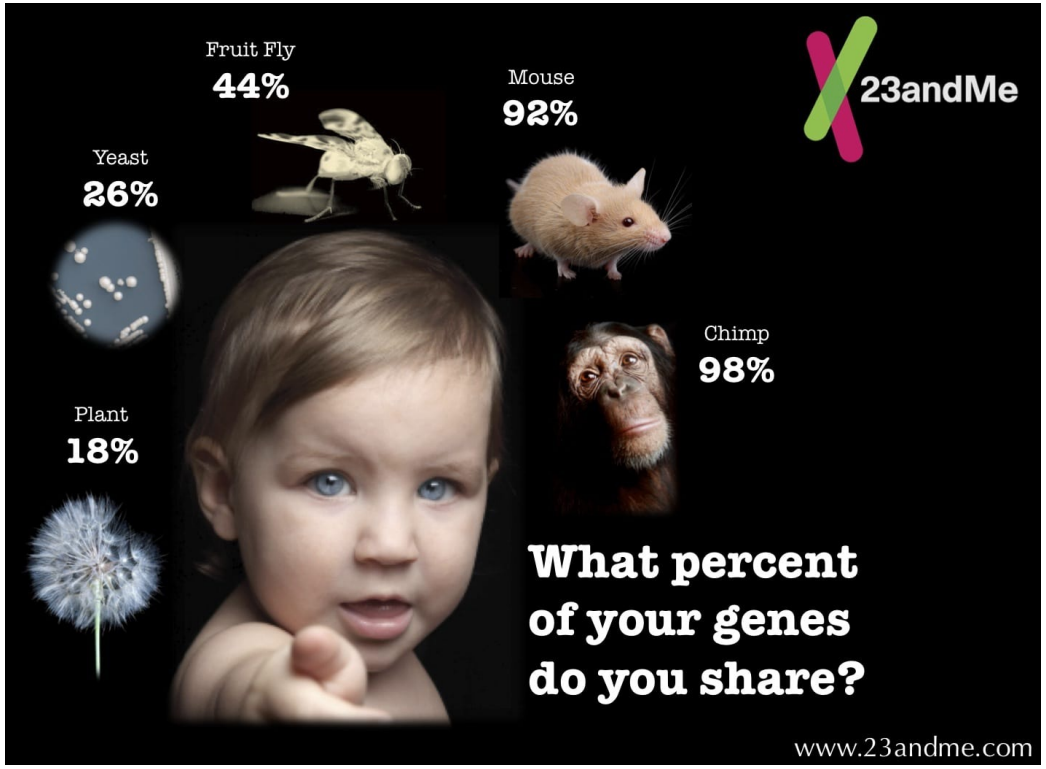
Gene Editing Overview

Every Living Thing Has Genes

- **What do genes and DNA do?**
  - **DNA**- deoxyribonucleic acid contains the biological information that makes every living thing unique
  - **Genes are made of DNA,** which carries the instructions for us to develop, survive, reproduce, and make proteins which do most of the work in our bodies.
  - **Genes are a basic unit of inheritance.**

- **Can our DNA change over time?**
  - As we age, some of our genes experience epigenetic changes or chemical variations that can turn genes off or on without changing information.
  - Over long periods of time our DNA can change through mutation and natural selection.

- **Is it possible to edit DNA?**
  - **Scientists are** now **able to make changes in DNA** to solve problems.



Source: 23andMe.

Source: "Deoxyribonucleic Acid (DNA) Fact Sheet", https://www.genome.gov/about-genomics/fact-sheets/Deoxyribonucleic-Acid-Fact-Sheet. Accessed Jul. 15, 2022.; "Does Human DNA Change With Time?", https://www.scienceabc.com/pure-sciences/does-human-dna-change-with-time.html. Accessed Jul 15, 2022.; "GENE", https://www.genome.gov/genetics-glossary/Gene. Accessed Jul. 27, 2022.

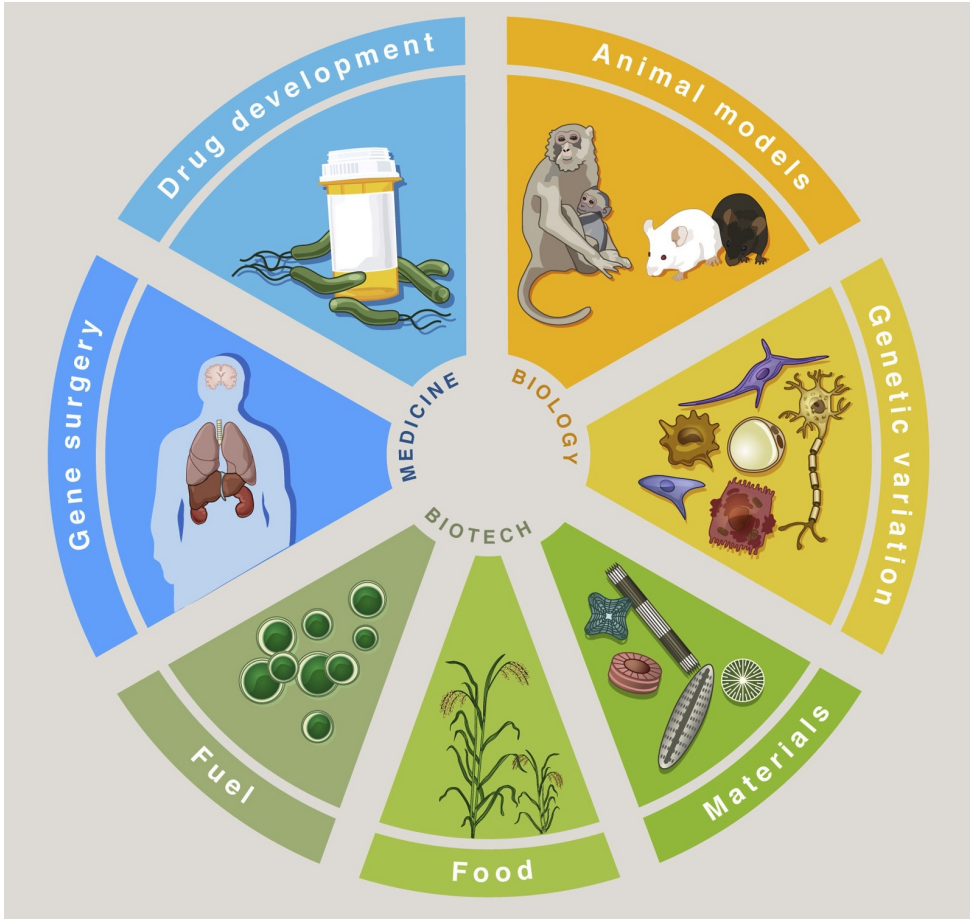
The World of DNA

- **Why Does DNA Matter?**
  - **Understanding DNA and gene editing is important.** It allows us to better understand what we already have and have the knowledge needed to help shape the growing field.

- **What can be done with DNA?**
  - **DNA has become something that scientists are able to decipher, identify specific traits carried within it, and even edit.**

- **Potential Impacts**
  - **Cell and gene therapies**
  - **Diagnostics**
  - **Agriculture**
  - **Bioenergy**

"You could turn someone into a freakin butterfly"– Elon Musk



Source: Cell: Hsu et al 2014.

Source: "The Ultimate Guide To CRISPR: Mechanism, Applications, Methods & More", https://www.synthego.com/learn/crispr. Accessed Jul. 6, 2022.; "Captain Marvel's Powers: Could CRISPR Make Them a Reality?", https://www.synthego.com/blog/crispr-make-captain-marvel-powers. Accessed Jul. 28, 2022.

Return of the Wooly Mammoth

- **Can we bring back extinct species?**
  - **Through gene editing, it is possible to bring back extinct species** such as the Wooly Mammoth

- **The Wooly Mammoth project**
  - **George Church,** a geneticist at Harvard and MIT**, and his team have raised $15M to bring back the wooly mammoth.**
  - **Church was optimistic that he could** rewrite the Asian elephant's DNA to **produce something that looks and behaves like a mammoth through CRISPR gene editing.**
  - **By tweaking certain genes** to produce denser hair or a thicker layer of fat, **the team hopes to create an animal with mammoth-like characteristics**.
  - They are optimistic this can be done within the next few years and hope to have a complete woolly mammoth within the decade.

Source: Flying Puffin via Wikicommons.

Source: "These Scientists Plan to Fully Resurrect a Woolly Mammoth Within the Decade", https://www.smithsonianmag.com/smart-news/these-scientists-plan-to-fully-resurrect-a-woolly-mammoth-within-the-decade-180978655/. Accessed Jul. 8, 2022.

Reading Our DNA: The Human Genome Project

Key Definitions (Human Genome Project)

Human Genome Project

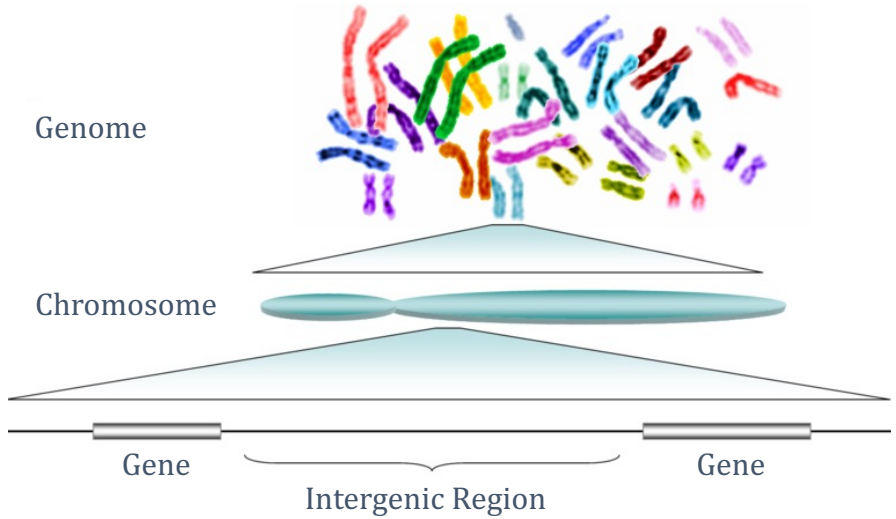
- The Human Genome Project (HGP) was an international, collaborative research program whose goal was the complete mapping and understanding of all human genes.

Dimer

- A molecule composed of two identical, simpler molecules.

Genome

- A genome is an organism's complete set of deoxyribonucleic acid (DNA), a chemical compound that contains the genetic instructions needed to develop and direct the activities of every organism.



Source: SITN: Harvard University.

Source: "What is the Human Genome Project?", https://www.genome.gov/human-genome-project/What, Accessed Jul. 18, 2022.; "Human Genome Project FAQ", https://www.genome.gov/human-genome-project/Completion-FAQ. Accessed Jul 18, 2022.; "CHROMOSOME", https://www.genome.gov/genetics-glossary/Chromosome, Accessed Jul. 18, 2022.; " GENE", https://www.genome.gov/genetics-glossary/Gene, Accessed Jul. 18, 2022.; "dimer", https://www.dictionary.com/browse/dimer. Accessed Jul. 18, 2022.

Key Definitions Continued (Human Genome Project)

Chromosomes

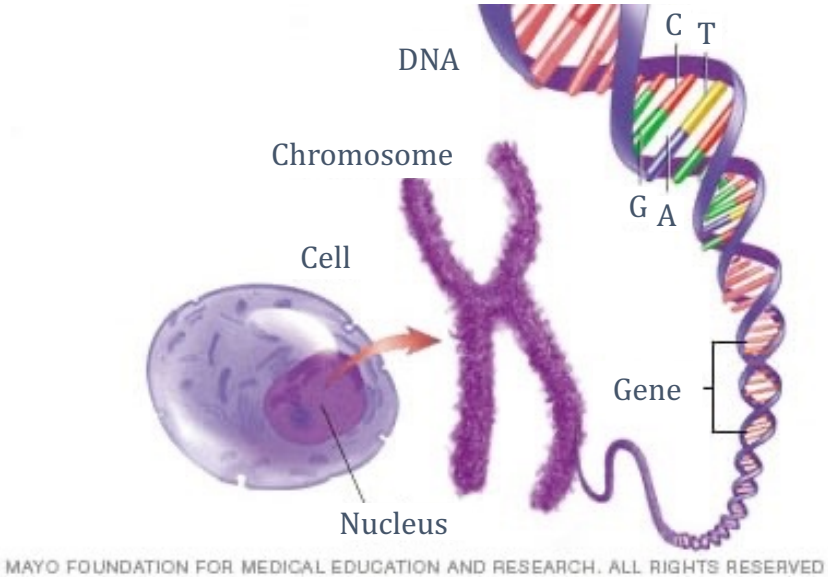
- Threadlike structures made of protein and a single molecule of DNA that serve to carry the genomic information from cell to cell. Humans have 22 pairs of numbered chromosomes (autosomes) and one pair of sex chromosomes (XX or XY), for a total of 46.

Genes

- The gene is considered the basic unit of inheritance. Genes are passed from parents to offspring and contain the information needed to specify physical and biological traits. Most genes code for specific proteins or segments of proteins, which have differing functions within the body.

Nucleotide bases

- Nucleotides are the four chemical units that make up the strands in a DNA molecule. The bases are adenine (A), thymine (T), guanine (G) and cytosine (C). Bases on opposite strands pair specifically; an A always pairs with a T, and a C always with a G.



Source: Mayo Clinic.

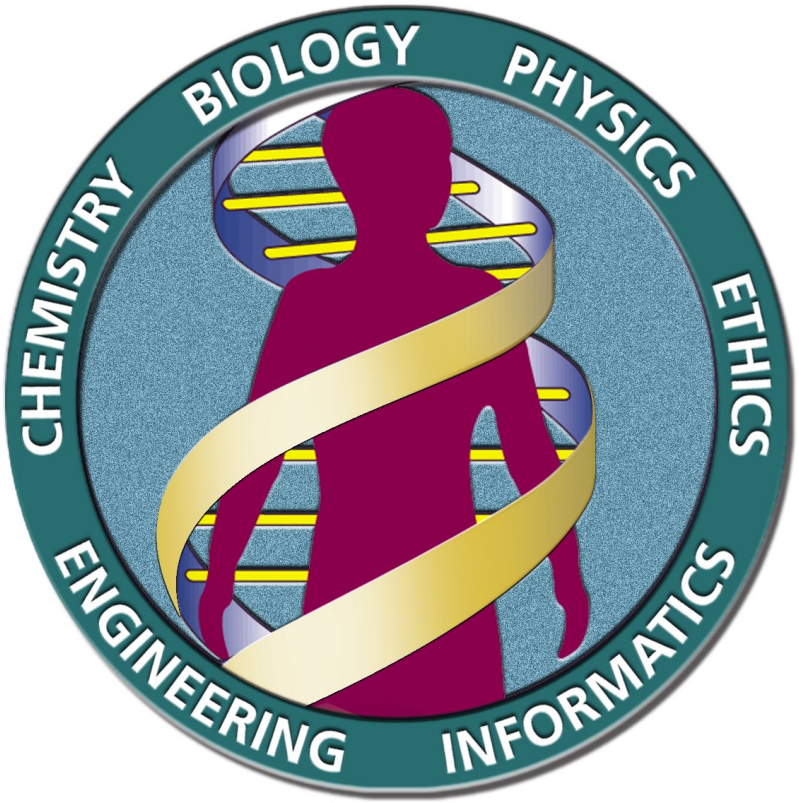
Source: "What is the Human Genome Project?", https://www.genome.gov/human-genome-project/What, Accessed Jul. 18, 2022.; "Human Genome Project FAQ", https://www.genome.gov/human-genome-project/Completion-FAQ. Accessed Jul. 18, 2022.; "CHROMOSOME", https://www.genome.gov/genetics-glossary/Chromosome, Accessed Jul. 18, 2022.; " GENE", https://www.genome.gov/genetics-glossary/Gene, Accessed Jul. 18, 2022.; "dimer", https://www.dictionary.com/browse/dimer. Accessed Jul. 18, 2022.

Human Genome Project

- **Project mission**
  - Decipher the chemical sequence of the entire genome, identify all 50,000 to 100,000 genes contained within the genome, and provide the research tools needed to analyze all this genetic information.

- **Isolating and analyzing the genetic material**
  - At the time, this study could potentially provide scientists with powerful new approaches to understand disease development and to create new strategies for their prevention and treatment.

- **A great scientific undertaking**
  - This international effort to sequence the 3 billion DNA letters in the human genome is considered one of the most ambitious scientific undertakings of all time, even compared to splitting the atom or going to the moon.



Source: U.S. Department of Energy, Human Genome Project.

Source: "Human Genome Project Results", https://www.genome.gov/human-genome-project/results. Accessed Jul. 11, 2022.; "The Human Genome Project", https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6875757/. Accessed Jul 11, 2022

Timeline

1984	Early meetings **assess** the **feasibility of a Human Genome Project**.
1988	The **main goals** of the Human Genome Project were **first articulated by a special committee of the U.S. National Academy of Sciences**, and later adopted through a detailed series of five-year plans jointly written by the National Institutes of Health (NIH) and the Department of Energy (DOE).
1990	The **initial planning stage was completed** with the publication of a joint research plan, "Understanding Our Genetic Inheritance: The Human Genome Project, The First Five Years, FY 1991-1995." This initial research plan set out specific goals for the first five years of what was then projected to be a 15-year research effort.
1995	Human Genome Project **researchers publish a physical map of the human genome**.
1999	The Human Genome Project **successfully completes the pilot phase of sequencing the human genome**. Human Genome Project **researchers decode the DNA sequence of the first human chromosome**.
2003	The Human Genome **Project is completed**.
2004	The **International Human Genome Sequence Consortium publishes their finished human genome sequence**.

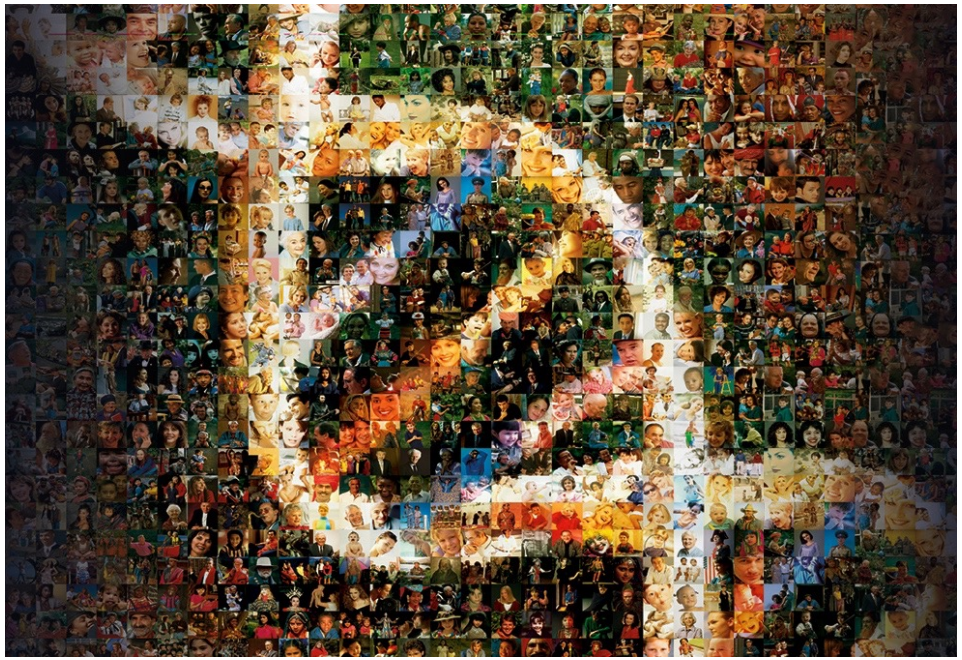
Source: "What is the Human Genome Project?", https://www.genome.gov/human-genome-project/What. Accessed Jul. 6, 2022.; "Human Genome Project Timeline of Events", https://www.genome.gov/human-genome-project/Timeline-of-Events. Accessed Jul. 6, 2022.

Human Genome Project Contributors and Findings

- **Project Contributors**
  - Contributors include the NIH, DOE, and numerous universities and research centers throughout the US, UK, France, Germany, Japan, and China.

- **What was accomplished?**
  - The finished sequence covers about 99 percent of the human genome's gene-containing regions has been sequenced to an accuracy of 99.99 percent.
  - In 2003, an accurate and complete human genome sequence was finished and made available to scientists and researchers two years ahead of the original schedule and under the original estimated budget.

- **Completing the last 8%**
  - About 8% of our genome was not sequenced due to technological limitations. The T2T (Telomere 2 Telomere) team took on this challenge and recently completed it, with 6 papers being posted on Science in 2022.



Source: NIH National Human Genome Research Institute.

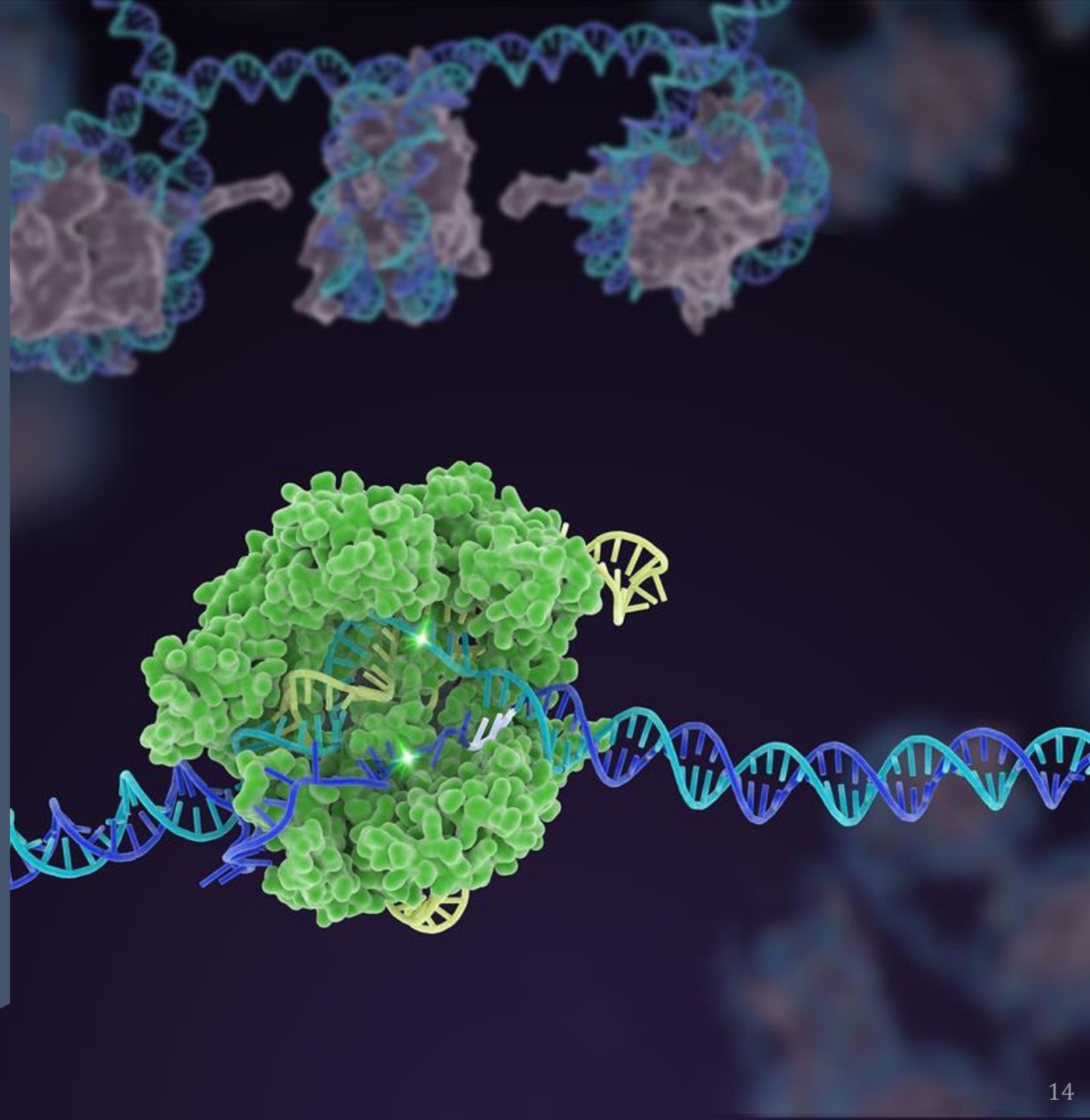
Source: "Human Genome Project Results", https://www.genome.gov/human-genome-project/results. Accessed Jul. 11, 2022.; "The complete sequence of a human genome", https://www.science.org/doi/10.1126/science.abj6987. Accessed Jul. 12, 2022.; "Telomere-to-Telomere (T2T)", https://www.genome.gov/about-genomics/telomere-to-telomere. Accessed Jul. 12, 2022.

Accessing the Information

- The information is accessible on the NIH website: https://www.ncbi.nlm.nih.gov/projects/genome/guide/human/

Source: "Human Genome Resources at NCBI", https://www.ncbi.nlm.nih.gov/projects/genome/guide/human/. Accessed Jul. 13, 2022.

CRISPR-Cas9: Genetic Scissors



Definitions (CRISPR-Cas9)

CRISPR-Cas9

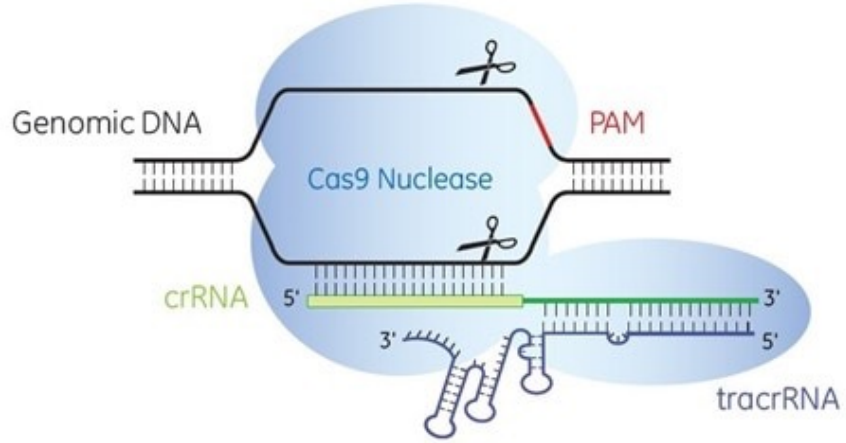
- CRISPR-Cas9 is a gene editing tool that was adapted from a naturally occurring genome editing system that bacteria use as an immune defense.

Guide RNA

- A two-piece molecule that Cas9 binds and uses to identify a complementary DNA sequence. Composed of the CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). Cas9 uses the tracrRNA portion of the guide as a handle, while the crRNA spacer sequence directs the complex to a matching DNA sequence.

Endonuclease

- An enzyme that breaks down a nucleotide chain into two or more shorter chains by cleaving the internal covalent bonds linking nucleotides.



Source: horizon A PerkinElmer company.

Source: "RNA", https://www.cancer.gov/publications/dictionaries/cancer-terms/def/rna. Accessed Jul.18, 2022.; "GLOSSARY Guide RNA (gRNA)", https://innovativegenomics.org/glossary/guide-rna/. Accessed Jul. 18, 2022.; "What are genome editing and CRISPR-Cas9", https://medlineplus.gov/genetics/understanding/genomicresearch/genomeediting/. Accessed Jul. 18, 2022.; "Endonuclease", https://www.merriam-webster.com/dictionary/endonuclease. Accessed Jul. 20, 2022.

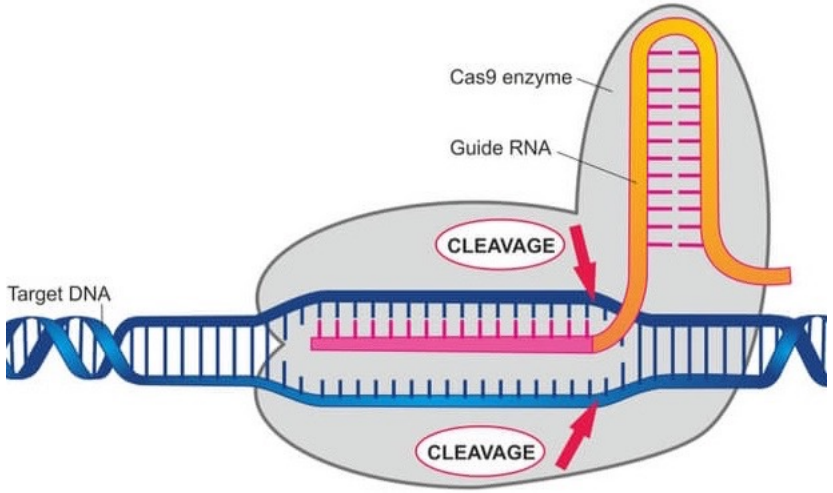
Definitions Continued (CRISPR-Cas9)

Enzyme

- A substance that acts as a catalyst in living organisms, regulating the rate at which chemical reactions proceed without itself being altered in the process.

RNA

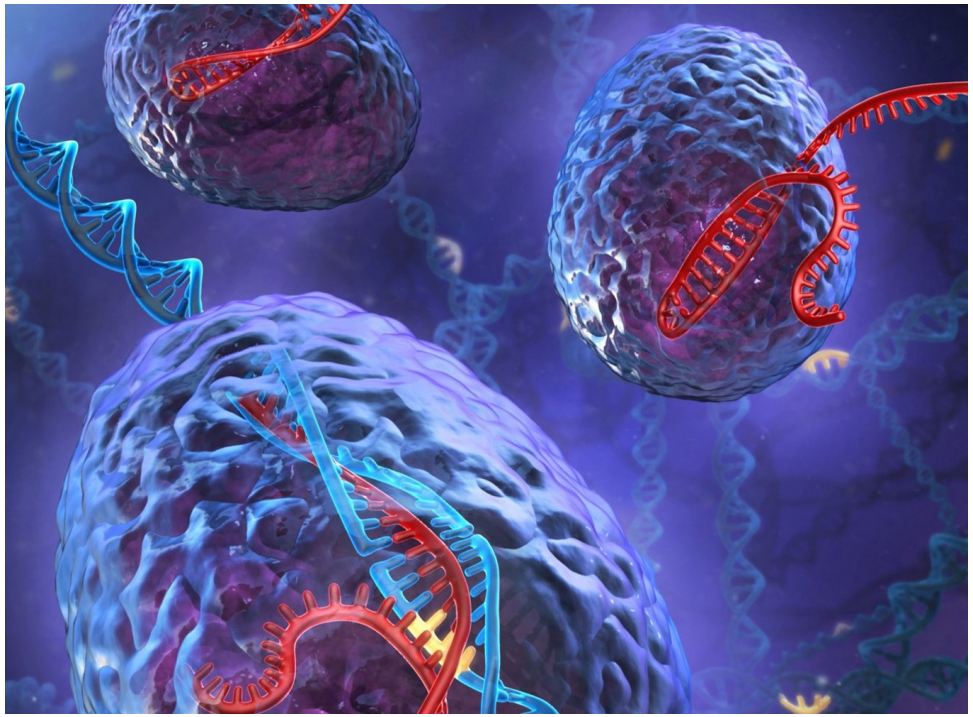
- Ribonucleic acid contains information that has been copied from DNA. Cells make several different forms of RNA, and each form has a specific job in the cell. Many forms of RNA have functions related to making proteins. RNA is also the genetic material of some viruses instead of DNA.



Source: Cell: LABIOTECH.eu.

Source: "RNA", https://www.cancer.gov/publications/dictionaries/cancer-terms/def/rna. Accessed Jul.18, 2022.; "GLOSSARY Guide RNA (gRNA)", https://innovativegenomics.org/glossary/guide-rna/. Accessed Jul. 18, 2022.; "What are genome editing and CRISPR-Cas9", https://medlineplus.gov/genetics/understanding/genomicresearch/genomeediting/. Accessed Jul. 18, 2022.; "Endonuclease", https://www.merriam-webster.com/dictionary/endonuclease. Accessed Jul. 20, 2022.

CRISPR-Early Discovery



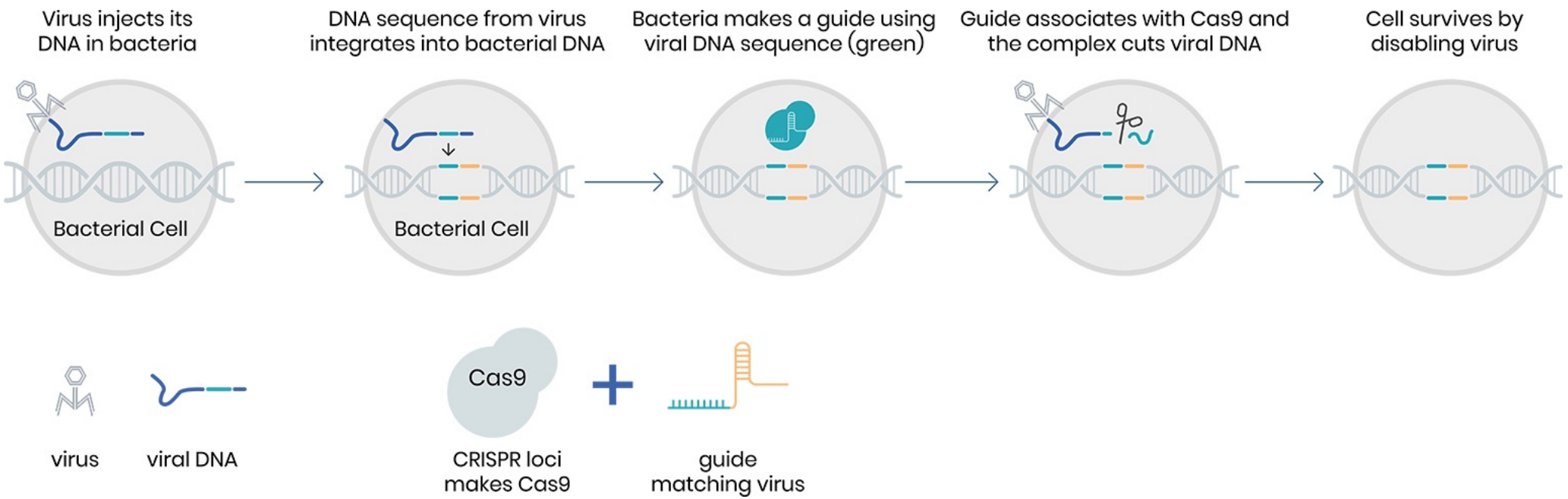
Source: Stephen Dixon.

- **CRISPR was first observed in 1987.**
  - Researchers in Japan noticed a weird, repeating sequence **in the DNA of E. coli bacteria**."
- **Later studies found repeating segments of DNA in other microbial species.**
  - These mysterious repeats consisted of a **short sequence of genetic code and a similar sequence in reverse**. This peculiar **palindrome pattern was dubbed CRISPR** — "clustered regularly interspaced short palindromic repeats."
  - **Further research led to the discovery of CRISPR-associated (Cas) genes, which produce Cas enzymes that can slice through DNA.**
  - Scientists eventually realized that bacteria have been using CRISPR-Cas complexes for billions of years to attack and destroy enemy viruses, and that this ancient bacterial immune system could be adapted for use in genetic engineering.
- **International recognition**
  - In 2020, Emmanuelle Charpentier and Jennifer Doudna were awarded the **Nobel Prize** in Chemistry for their work on CRISPR-Cas9.

Source: "Target, delete, repair CRISPR is a revolutionary gene-editing tool, but it's not without risk", https://stanmed.stanford.edu/2018winter/CRISPR-for-gene-editing-is-revolutionary-but-it-comes-with-risks.html. Accessed Jul. 6, 2022.; "Nobel Prize 2020 in Chemistry honors CRISPR: a tool for rewriting the code of life", https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7782372/. Accessed Jul. 28, 2022.

How Does CRISPR Work?

- **Bacterial immune system**
  - In bacteria, **CRISPR-Cas9 is a naturally occurring genome editing system that bacteria use as an immune defense.**
  - The patterns of integrated viral DNA are known as CRISPR arrays and help bacteria remember the virus, in case of another attack. **Researchers adapted this immune defense system to edit DNA**.

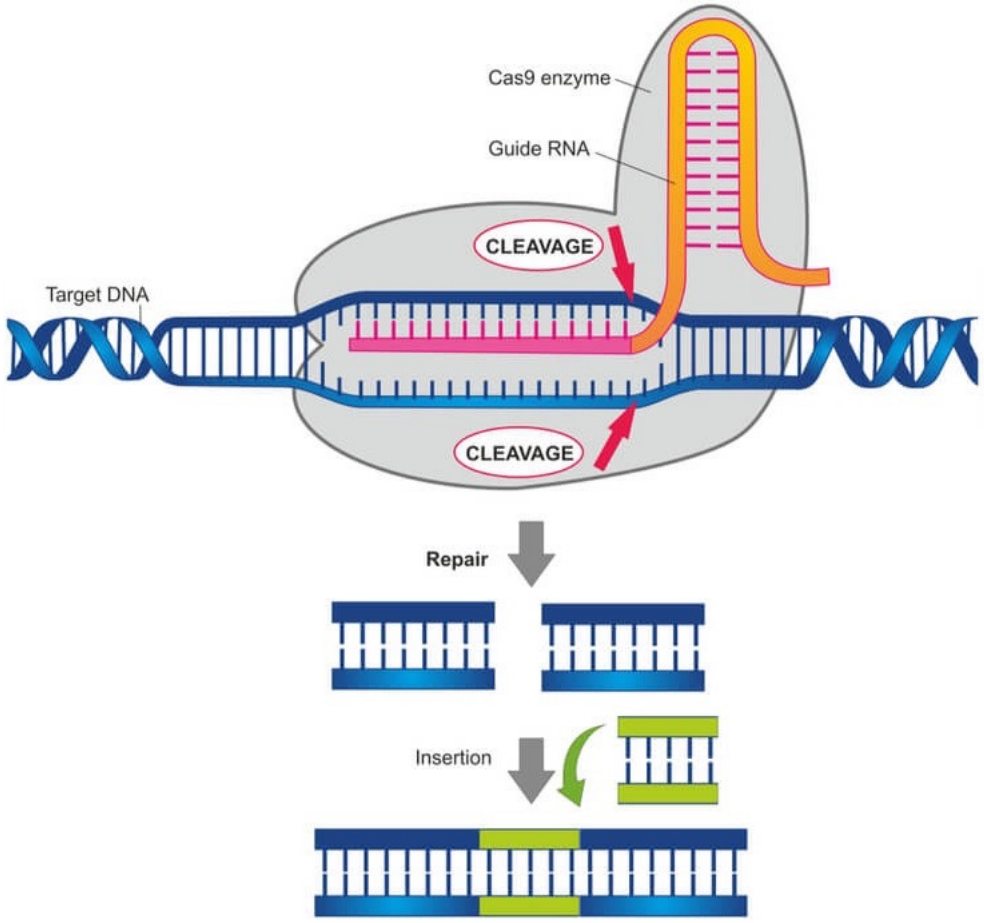


Source: Intellia Therapeutics.

Source: "What are genome editing and CRISPR-Cas9?", https://medlineplus.gov/genetics/understanding/genomicresearch/genomeediting/. Accessed Jul. 7, 2022

CRISPR-Cas9 Gene Editing Overview

- **Design an RNA molecule**
  - **The molecule matches the mutated DNA sequence in the gene.**
- **Combine the RNA with a Cas9 enzyme**
  - Cas9 can cut through DNA like scissors.
- **The cut is made**
  - The **RNA** acts like a very fast GPS — it **guides the Cas9 enzyme to the mutated DNA sequence. The enzyme then binds to the sequence and deletes it."**
- **The final repair is made**
  - Benign virus is engineered to deliver and insert the correct DNA sequence into the edited gene.
  - Use the cell's own DNA repair machinery to add or delete pieces of genetic material, or to make changes to the DNA by replacing an existing segment with a customized DNA sequence



Source: Cell: LABIOTECH.eu.

Source: "Target, delete, repair CRISPR is a revolutionary gene-editing tool, but it's not without risk", https://stanmed.stanford.edu/2018winter/CRISPR-for-gene-editing-is-revolutionary-but-it-comes-with-risks.html. Accessed Jul. 6, 2022.; "What are genome editing and CRISPR-Cas9?", https://medlineplus.gov/genetics/understanding/genomicresearch/genomeediting/. Accessed Jul. 7, 2022

CRISPR vs Past Gene Editing Techniques

Feature	CRISPR	TALEN's	ZFN's
Construction	22bp + pam sequence	30-40bp	9-18bp
Target sequence recognition	sgRNA, RNA-DNA interactions	Repeat variable diresidues (RVDs) repeats, protein-DNA interactions	Zinc fingers protein, protein-DNA interactions
Endonuclease	Cas	FokI	FokI
Endonuclease construction	sgRNA synthesis or cloning	8-31RVD repeats	3-4 Zinc fingers domains
Delivery	sgRNA complementary to the target sequence with CAS protein	2 TALENs around the target sequence required	2 ZFNs around the target sequence
DNA sequence recognition size	17-20bp +NGG *1	(8-31bp) *2	(9 or12bp) *2
Targeting efficiency	High	Moderate	Low
Affordability	Highly Affordable	Affordable	Resource intensive
Timeliness	Rapid	Time consuming	Time Consuming

Definitions

- **ZFN**- Zinc-finger nucleases are fusions of the nonspecific DNA cleavage domain from the FokI restriction endonuclease with zinc-finger proteins. ZFN dimers induce targeted DNA DSBs that stimulate DNA damage response pathways.
- **TALEN**- Transcription activator-like effector nucleases are fusions of the FokI cleavage domain and DNA-binding domains derived from TALE proteins. TALENs induce targeted DSBs that activate DNA damage response pathways and enable custom alterations.

Source: "Various Aspects of a Gene Editing System—CRISPR–Cas9", https://www.researchgate.net/publication/347668474_Various_Aspects_of_a_Gene_Editing_System-CRISPR-Cas9. Accessed Jul. 15, 2022.; "ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering", https://www.cell.com/trends/biotechnology/pdf/S0167-7799(13)00087-5.pdf. Accessed Jul. 18, 2022.

CRISPR Gene Editing Procedures

Step 1: Determine the Genetic Tool to Use

Gene Knockout	• NHEJ- non homologous end joining
Gene Editing	• HDR-homology directed repair • Base editing • Prime editing
Gene Activation and Inhibition	• CRISPRa- CRISPR activation • CRISPRi- CRISPR inhibition

Source: "How to Design Your gRNA for CRISPR Genome Editing", https://blog.addgene.org/how-to-design-your-grna-for-crispr-genome-editing. Accessed Jul. 12, 2022.

Step 2: Identify the Target Sequence

Direct Sequencing

- Identify each individual base pair, in sequence, and compare the sequence to a normal gene.

DNA Hybridization Models

- Use of the strong binding of a lone DNA strand to a strand whose sequence is the perfect complement of the first. Binding can differentiate between normal and mutated DNA.

Restriction Enzyme Digestion or Hybridization

- Restriction enzymes are specialized enzymes that recognize and cut the DNA wherever they encounter a specific, very short sequence.

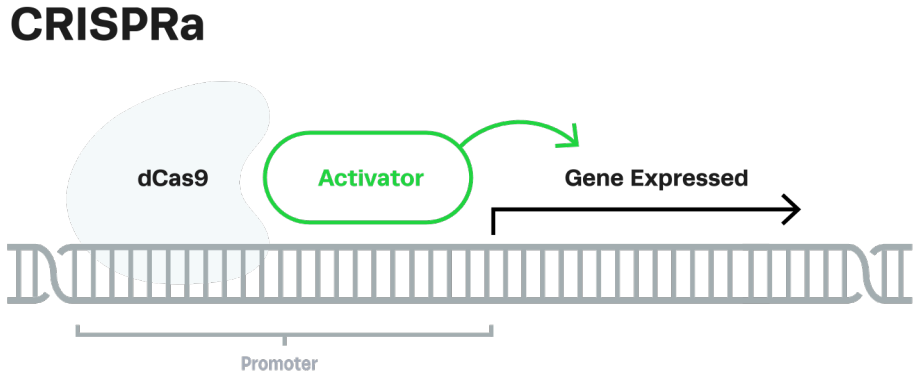
Source: "Genetics 101: detecting mutations in human genes", https://www.ncbi.nlm.nih.gov/pmc/articles/PMC117476/. Accessed Jul. 12, 2022.

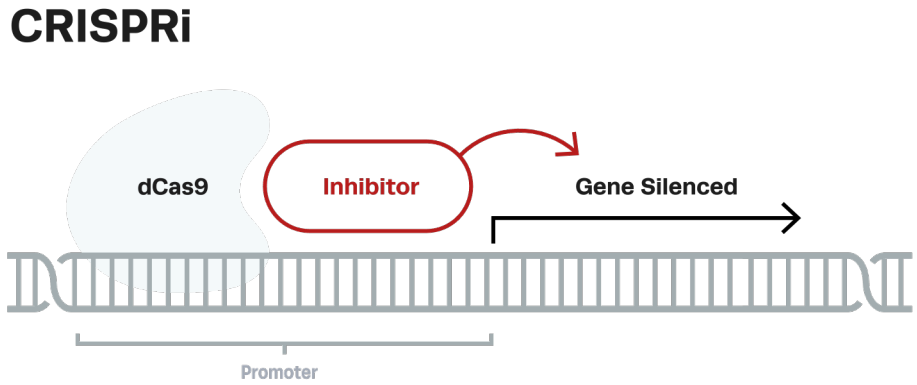
Step 3: Create the Guide RNA for (CRISPRa & CRISPRi)

Note: Perfect gRNA does not exist.

Designing Guide RNAs for CRISPRa & CRISPRi

- The critical parameters for gRNA design are different for CRISPRa and CRISPRi.
- **These tools involve gene activation or inhibition by targeting the promoter of the target gene.**
- **The location target DNA range for guide RNA is therefore quite narrow**, requiring a balance of complementary sequence and optimized location while designing.





Source: Synthego.

Source: "CHAPTER 01 How To Design Guide RNA for CRISPR", https://www.synthego.com/guide/how-to-use-crispr/design-grna-crispr. Accessed Jul. 12, 2022.

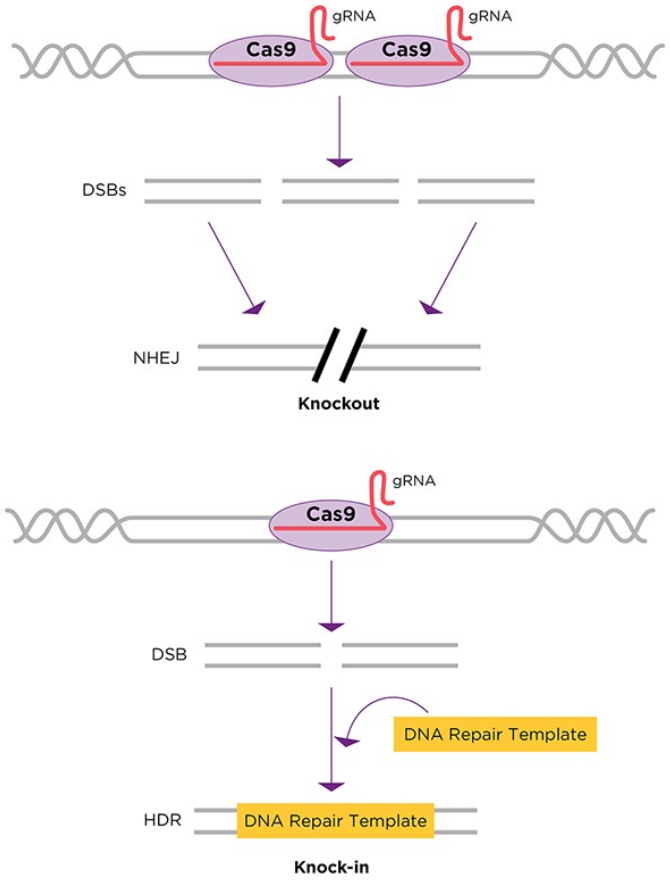
Step 3: Create the Guide RNA for (CRISPR Knockouts & Knock-ins)

Designing gRNA for Gene Knockouts

- **CRISPR-mediated knockouts are commonly done through the non-homologous end joining (NHEJ) repair pathway.**
- In this method, the Cas creates a double-strand break (DSB). The DSB is fixed by NHEJ.
- **This pathway** often results in insertion or deletion (indel) of one or more nucleotides at the break, which **leads to frameshift mutations that completely knock out gene function.**

Guides for CRISPR Knock-ins

- **A knock-in is done by inserting a new DNA fragment into the genome.**
- **Researchers use** a more sophisticated repair pathway, known as the homology-directed repair (**HDR**). In HDR, the cell copies the sequence of donor DNA template to fill the broken piece accurately.
- During knock-ins, researchers supply a surplus of template DNA containing the desired genomic change, improving the chance that the cell will pick this DNA for repair.

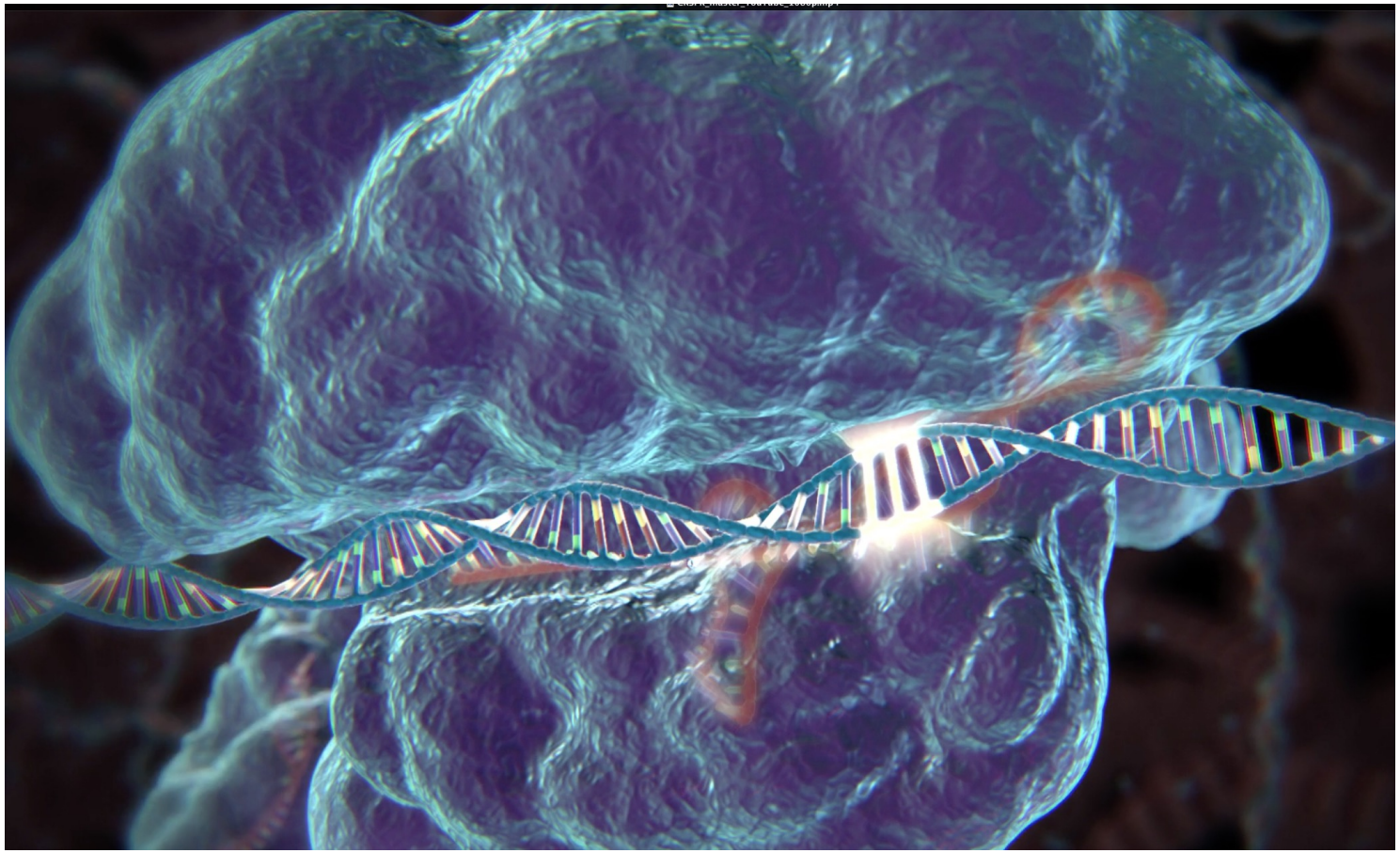


Source: Taconic Biosciences.

Source: "CHAPTER 01 How To Design Guide RNA for CRISPR", https://www.synthego.com/guide/how-to-use-crispr/design-grna-crispr. Accessed Jul. 12, 2022.

Step 4: Choose a Tool to Design Guide RNA (Tool List)

Geneious	Benchling
Guides	Broad Institute GPP
Horizon Discovery	CasOFFinder
IDT	CHOPCHOP
Off-Spotter	CRISPOR
Synthego	Deskgen
TrueDesign	E-CRISP

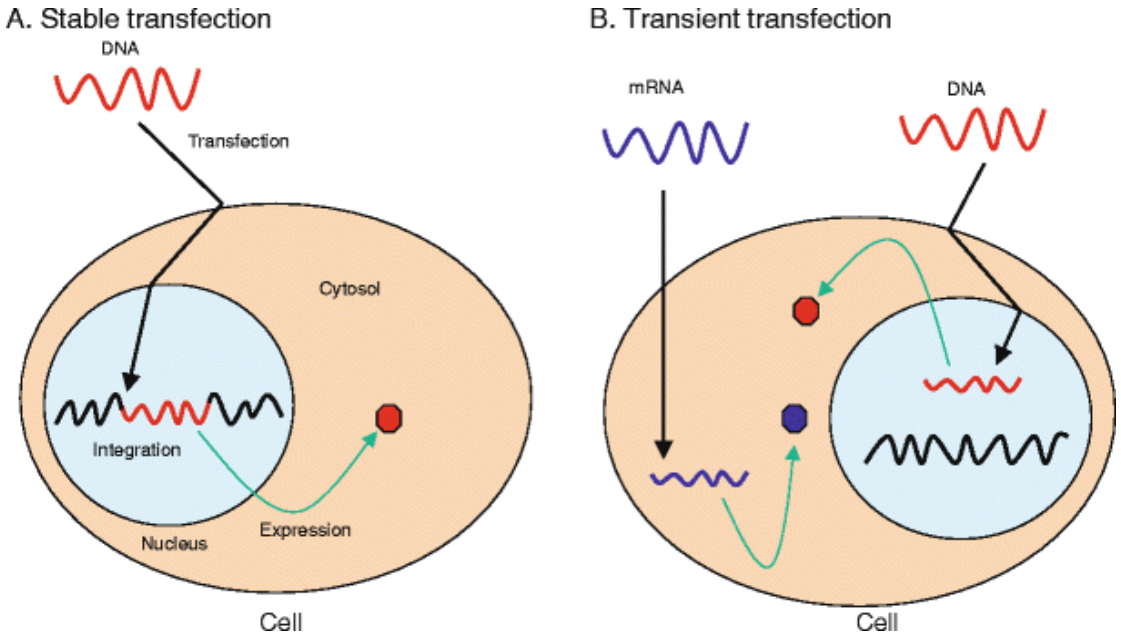


Source: McGovern Institute.

Source: "Guide Design Resources", https://zlab.bio/guide-design-resources, Accessed Jul. 12, 2022.

Step 5: Insert CRISPR to the Target

- **Transient vs. Stable Transfection**
  - The method depends if the goal is permanent or temporary expression of Cas9 and/or the gRNA in cells.
  - **Transient transfection**: **CRISPR components are introduced into the cell, but no DNA encoding gRNA or Cas9 are incorporated into the cell's genome**.
    - CRISPR-Cas9 can only cleave the DNA for a limited time.
  - **Stable transfections: most effectively achieved by using a viral vector but can occur at low frequency after introducing plasmid DNA.**
    - Typically, DNA encoding Cas9 (but not guide RNA) is transfected to produce a Cas9-expressing cell line. Then, gRNAs can be introduced to Cas9-expressing cells through transient transfection.
- **Format of CRISPR Components-** Format of CRISPR components may affect which transfection method to use.
  - **DNA**
  - **RNA**
  - **Ribonucleoprotein complex (RNP)**



Source: Springer Link: Mammalian cell transfection: the present and the future.

Source: "CHAPTER 06 How to Select the Best CRISPR Transfection Protocol", https://www.synthego.com/guide/how-to-use-crispr/transfection-protocols. Accessed Jul. 14, 2022.

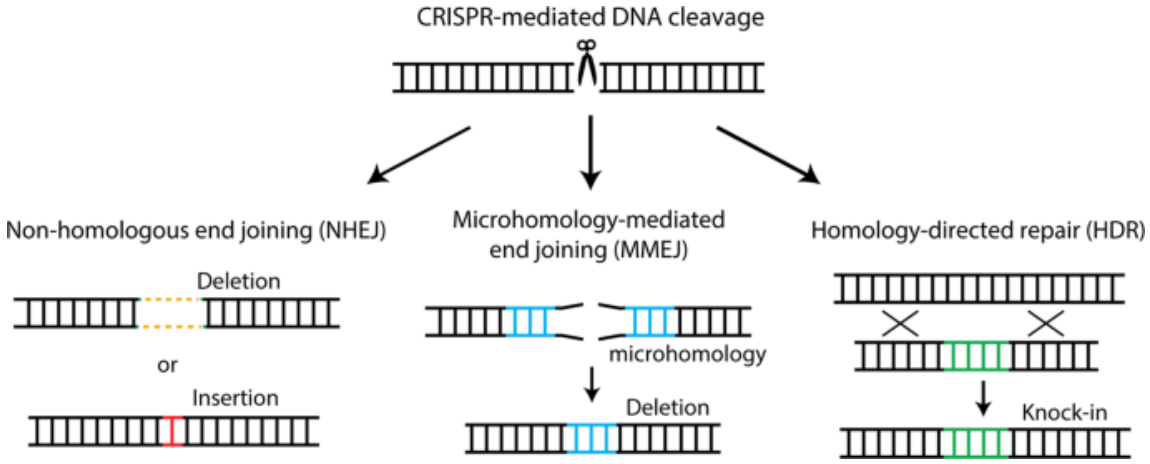
Step 5 Continued: Physical Transfection of CRISPR

	Lipofection	Electroporation	Nucleofection	Microinjection	Virus
Principle	Lipid complexed with genetic material fuses with cell membrane	Electric pulse forms pores in cell membrane for entry of DNA/RNA.RNP	An electrorotation-based optimized for nuclear delivery, pre-optimized for each cell type	Microneedle injects CRISPR components inside cells, oocytes, or zygotes	DNA/RNA is packaged into infectious particles and introduced into cells
Advantages	Cost-effective, high throughput	Easy, fast, high efficiency	Easy, fast, high efficiency	High efficiency	High efficiency
Limitations	Less efficient	Requires optimization	Requires reagents and equipment	Time-consuming, technically demanding, low throughput	Time-consuming, safety requirements, expensive
Cell Types	Few	Numerous	Numerous	Few	Numerous

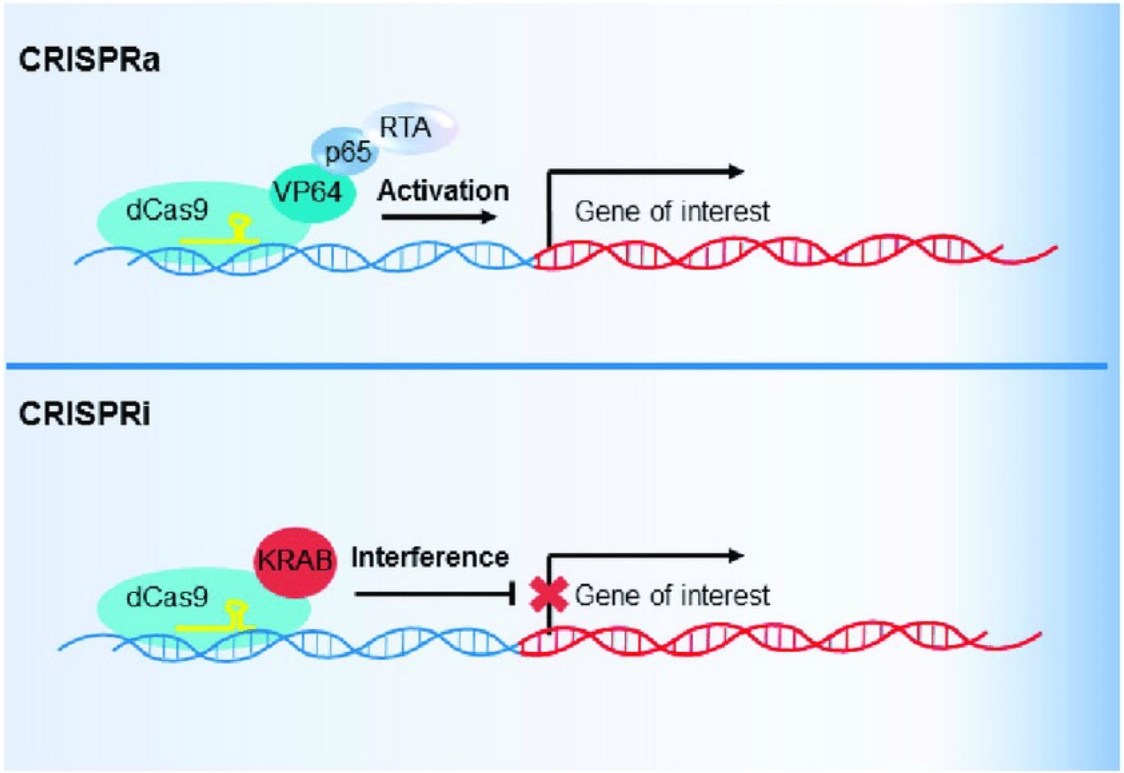
Source: "CHAPTER 06 How to Select the Best CRISPR Transfection Protocol", https://www.synthego.com/guide/how-to-use-crispr/transfection-protocols. Accessed Jul. 14, 2022.

Step 6: Fixing the Gene

- **Once the gene is cut, the gene error is resolved through**
  - A knockout
  - Edit
  - Activation or inhibition



Source: Nature: Current trends in gene recovery mediated by the CRISPR-Cas system.



Source: Researchgate: CRISPR–Cas9 Genetic Analysis of Virus–Host Interactions.

Step 7: Check the Results

CRISPR Analysis Using Next-Generation Sequencing (NGS)

- Pros-Gold standard, extremely sensitive for detecting editing outcomes, high-throughput sequence-based data provides a comprehensive view of the indels (insertions or deletions) generated
- Cons- Time and labor intensive, expensive, best with large samples

Inference of CRISPR Edits (ICE) from Synthego

- Pros- New, user-friendly, online tool that uses Sanger sequencing data to determine the relative abundance and levels of indels due to CRISPR editing.
- Cons- Deletion and insertion detection limitations, Sanger sequencing affects sensitivity, assumes edits use spCas9

Tracking of Indels by Decomposition (TIDE)

- Pros- Lower cost alternative to NGS, Sanger sequencing, like ICE, TIDE software aligns your sgRNA sequence to unedited and edited samples and conducts a comparative analysis
- Cons: modifying parameters in model can be difficult for user

T7 Endonuclease 1 (T7E1) Assay

- Pros- Does not use DNA sequencing to determine CRISPR editing efficiency, takes advantage of the T7 endonuclease, which preferentially cleaves mismatched DNA, the small fragments can be viewed by agarose gel electrophoresis.
- Cons: not quantitate, no info on different indel sequences generated

Source: "CHAPTER 07 How to Pick the Best CRISPR Data Analysis Method for Your Experiment", https://www.synthego.com/guide/how-to-use-crispr/analysis-methods. Accessed Jul. 22, 2022.; "ICE Limitations", https://www.synthego.com/help/ice-limitations. Accessed Aug. 4, 2022.

Minimizing Off-Target Effects

Ensure On-Target Activity of Guide RNA

- **Doench et al.** analyzed the specificity of thousands of guide RNAs while creating genome-wide mice and human libraries. Using sophisticated computational biology tools, the team **established scoring rules to predict the on-target activity of gRNAs**, and the "Doench rules" are now implemented in several online gRNA design tools.

Minimize gRNA Off-Target Effects

- The **Doench study** also **yielded** another useful metric: the **off-target activity score**. This **score of a designed gRNA indicates how likely it is that the gRNA will bind non-intended targets**.

Multiple gRNAs to Improve CRISPR KO

- **Multiple gRNAs targeting the same gene have been shown to improve editing efficiency and greatly increase the chance of generating a knockout.**

Choose the Best CRISPR Design Tool

- Details explained in pg. 26

Source: The Scientist Magazine.

Source: "CHAPTER 01 How To Design Guide RNA for CRISPR", https://www.synthego.com/guide/how-to-use-crispr/design-grna-crispr. Accessed Jul. 12, 2022.

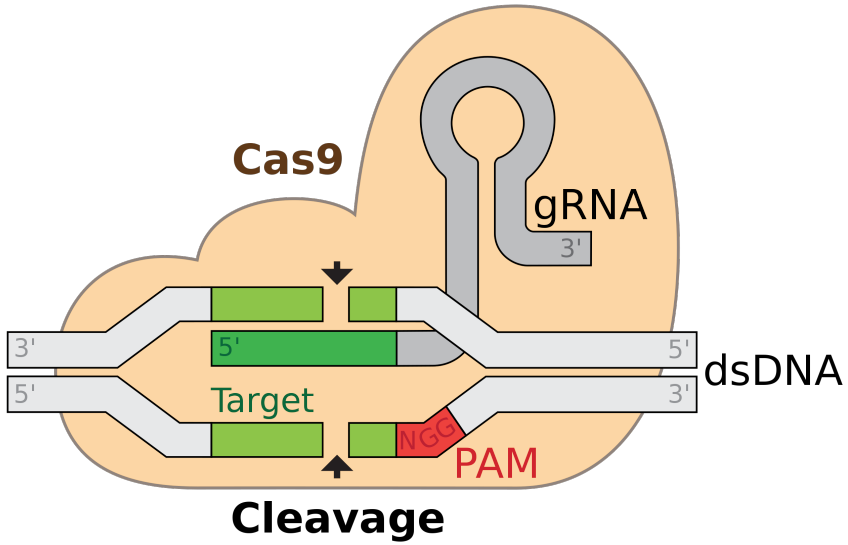
Guide RNA, PAM, & CAS

Guide RNA

- **Cas9 is guided** to its target sites with **by two RNAs: tracrRNA and crRNA**
- crRNA defines the genomic target for Cas9 and tracrRNA links the crRNA to Cas9 and facilitates processing of mature crRNAs from pre-crRNAs derived from CRISPR arrays**.**
- **In most CRISPR systems, two RNAs have been condensed into one RNA known as the guide RNA (gRNA) or single guide RNA (sgRNA).**

CAS Endonuclease

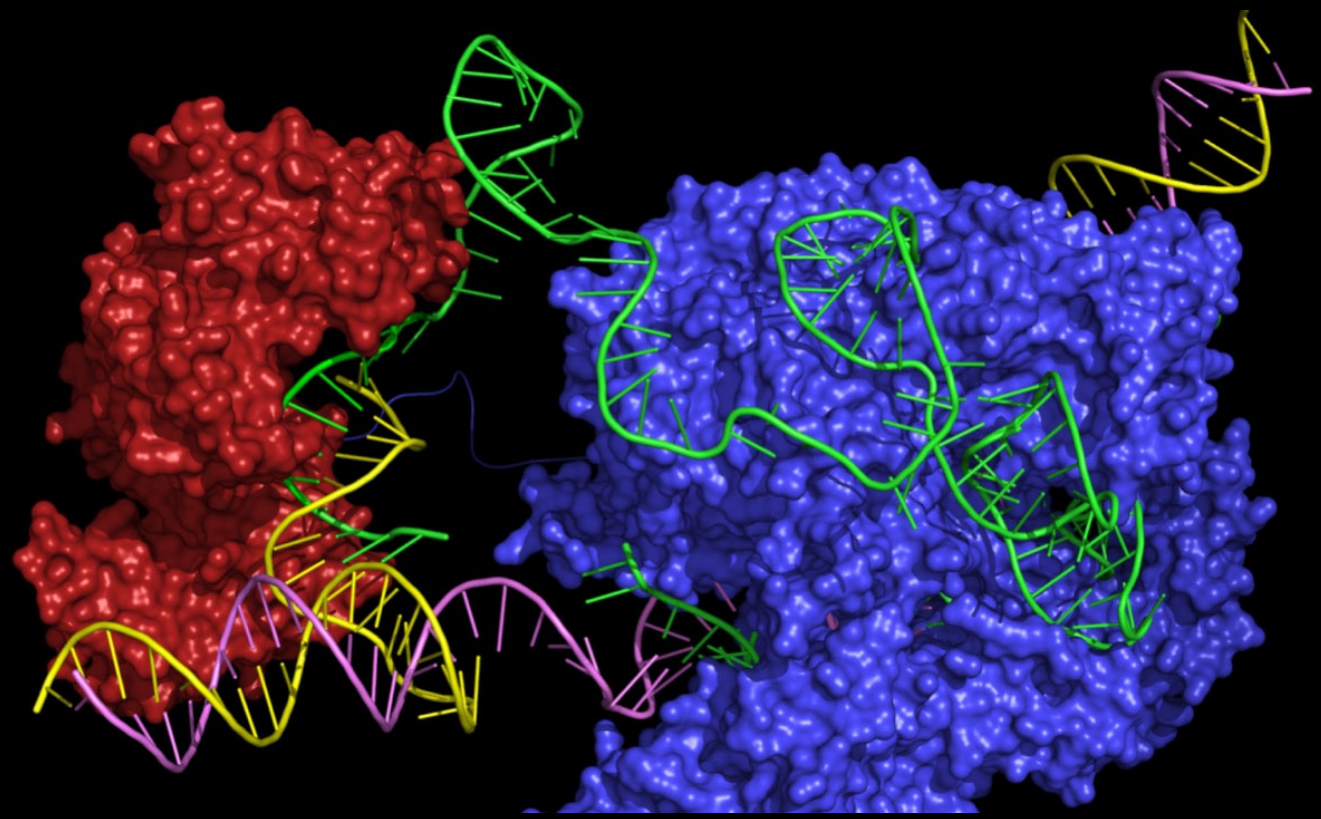
- **Cas9 binding to the targeted is mediated both by the gRNA and a 3-base pair sequence known as the Protospacer Adjacent Motif or PAM.**
- For DNA to be cut by Cas9, it must contain a PAM sequence immediately downstream (3') of the site targeted by the gRNA. In the absence of either the gRNA or PAM sequence, Cas9 will neither bind nor cut the target.
- Depending on the type of editing tool and PAM sequence, the appropriate endonuclease is chosen.



Source: Wikipedia: Marius Walter.

Source: "Components of CRISPR/Cas9", https://blog.addgene.org/components-of-crispr/cas9-our-new-crispr-101-ebook. Accessed Jul. 14, 2022.

Other Gene Editing Techniques



Base Editing vs CRISPR vs Prime Editing

Feature	CRISPR Nuclease	Base Editing	Prime editing
Size	~4 kilobase (kb)	~5kb	~6kb
PAM Dependence	High	High	Low
Endonuclease	Cas	d/nCas9	Cas
Breaks	Double strand break (DSB)	Single strand nick	Single strand nick
Ideal Use	Very large DNA insertions or deletions	Correcting point mutations	Multi letter mutations and somewhat longer sequences
Target Sequence Recognition	sgRNA, RNA-DNA interactions	sgRNA, RNA-DNA interactions	pegRNA, RNA-DNA interactions

Definitions

- **Base editing-** CRISPR-Cas9-based genome editing technology that allows the introduction of point mutations in the DNA without generating DSBs. Two major classes of base editors have been developed: cytidine base editors or CBEs allowing C>T conversions and adenine base editors or ABEs allowing A>G conversions.
- **Prime editing-** The more versatile prime editing (PE) system contains a prime editing extended guide RNA (pegRNA)-guided reverse transcriptase instead of a deaminase. The development of PE was a breakthrough as it requires no PAM sequence adjacent to the target site and it can accomplish not only all 12 types of point mutations, but also insertions (of up to 44 bp) and deletions (of up to 80 bp), or even combination of substitutions, insertions and deletions.

Source: "ARK INVEST BIG IDEAS 2022", https://research.ark-invest.com/hubfs/1_Download_Files_ARK-Invest/White_Papers/ARK_BigIdeas2022.pdf?hsCtaTracking=217bbc93-a71a-4c2b-9959-0842b6fe301c%7C2653a4d0-af35-42f0-853a-c5f90f002abb#page=82. Accessed Jul. 20, 2022.; "Base and Prime Editing Technologies for Blood Disorders", https://www.frontiersin.org/articles/10.3389/fgeed.2021.618406/full. Accessed Jul. 21, 2022.; "Super-precise new CRISPR tool could tackle a plethora of genetic diseases", https://www.nature.com/articles/d41586-019-03164-5. Accessed Jul. 21, 2022.

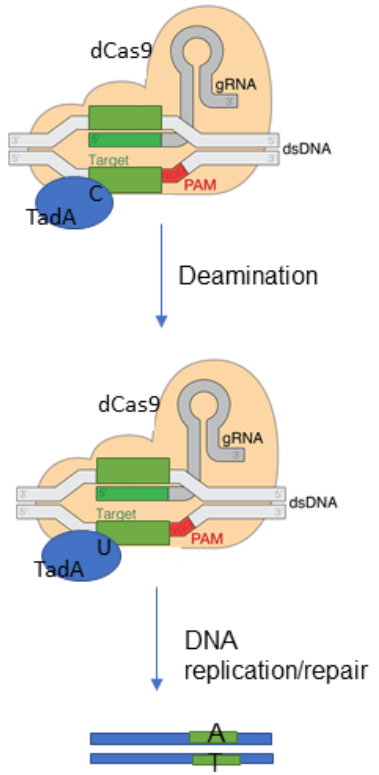
How Does Base Editing Work?

DNA base editors (BEs) are made **of fusions between a catalytically impaired Cas nuclease and a base-modification enzyme that operates on a single strand DNA**.

Upon binding to its target in DNA, **base pairing between the guide RNA and target DNA strand leads to displacement of a small segment of single-stranded DNA in an "R-loop"**.

DNA bases within this single-stranded DNA bubble are modified by the deaminase enzyme.

To improve efficiency in eukaryotic cells, the nuclease **also generates a nick in the non-edited DNA strand, inducing cells to repair the non-edited strand using the edited strand as a template.**



DNA base editing

Source: MATER METHODS 2019;9:2800.

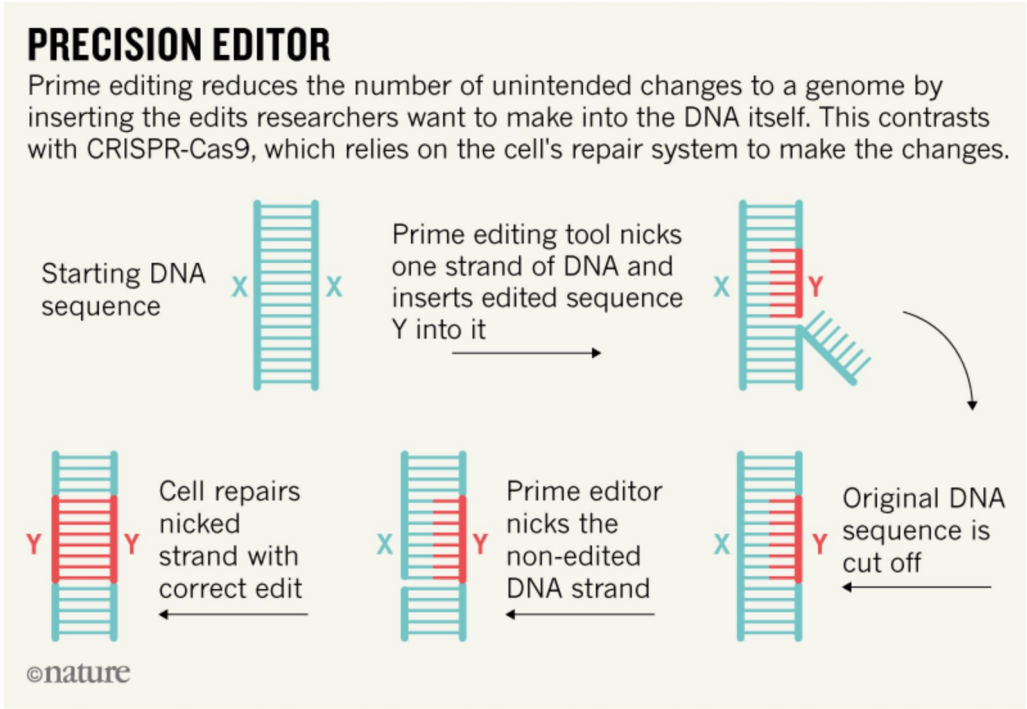
Source: "Base editing: precision chemistry on the genome and transcriptome of living cells", https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6535181/. Accessed Jul. 21, 2022.

How Does Prime Editing Work?

Prime Editing Works by nicking DNA at a specific point in the genome

Although it also **uses Cas9** to recognize specific DNA sequences — just like CRISPR-Cas9 does — the **Cas9 enzyme in Prime editing is modified to nick only one DNA strand.**

Then, a second enzyme called reverse transcriptase, guided by a strand of RNA, makes the edits at the sight of the cut.



Source: "Super-precise new CRISPR tool could tackle a plethora of genetic diseases", https://www.nature.com/articles/d41586-019-03164-5. Accessed Jul. 6, 2022.

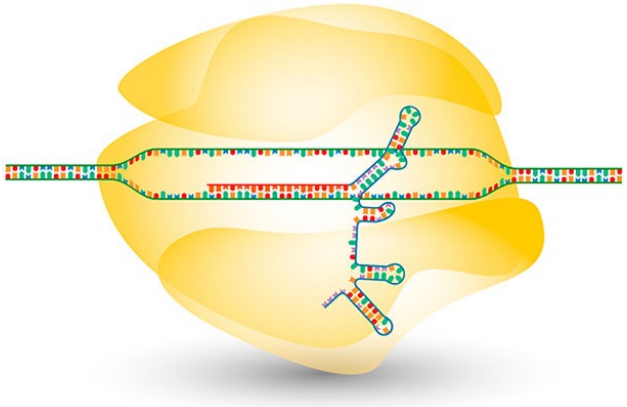
Prime Editing Advancements

Prime shows promise

- **Prime editing has shown promise in reducing 'off-target' effects that are a key challenge for some applications of the standard CRISPR–Cas9 system.**
- This **could make prime** gene therapies **safer for use in people**.

Prime editing seems capable of making a wider variety of edits.

- One day, it can be used to treat the many genetic diseases that have so far stumped gene-editors.



Source: tebubio.

Source: "Super-precise new CRISPR tool could tackle a plethora of genetic diseases", https://www.nature.com/articles/d41586-019-03164-5. Accessed Jul. 6, 2022.

Where Prime Prevails

Crispr drawbacks

- **CRISPR–Cas9 breaks both strands of DNA and then relies on the cell's own repair system** to patch the damage and make the edits, however, **that repair system is unreliable.**
- It can insert or delete DNA letters at the points where the genome was cut potentially leading to an uncontrollable mixture of edits that vary between cells.
- Although researchers **include a template** to guide how the gene is edited, the **DNA repair system in most cells is far more likely to make small, random insertions or deletions rather than adding a specific DNA sequence to the genome**.
- That makes it difficult — and in some cases, nearly impossible — for researchers to use CRISPR–Cas9 to overwrite one piece of DNA with a sequence of their choosing.

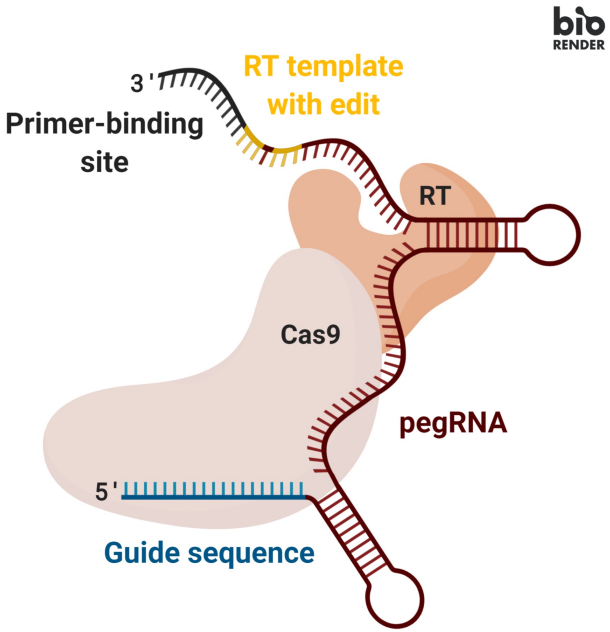
Source: Lily Padula / The New York Times.

Source: "Super-precise new CRISPR tool could tackle a plethora of genetic diseases", https://www.nature.com/articles/d41586-019-03164-5. Accessed Jul. 6, 2022.

Where CRISPR Prevails

Prime editing drawbacks

- **"Prime editing may not be able to make the very large DNA insertions or deletions that CRISPR–Cas9 is capable of.**
- Prime is unlikely to completely replace the well-established editing tool," says molecular biologist Erik Sontheimer at the University of Massachusetts Medical School in Worcester.
- This is due to edits being encoded in a strand of RNA. The longer that strand gets, the more likely it is to be damaged by enzymes in a cell.



Source: Wikipedia: Ldinatto.

Source: "Super-precise new CRISPR tool could tackle a plethora of genetic diseases", https://www.nature.com/articles/d41586-019-03164-5. Accessed Jul. 6, 2022.

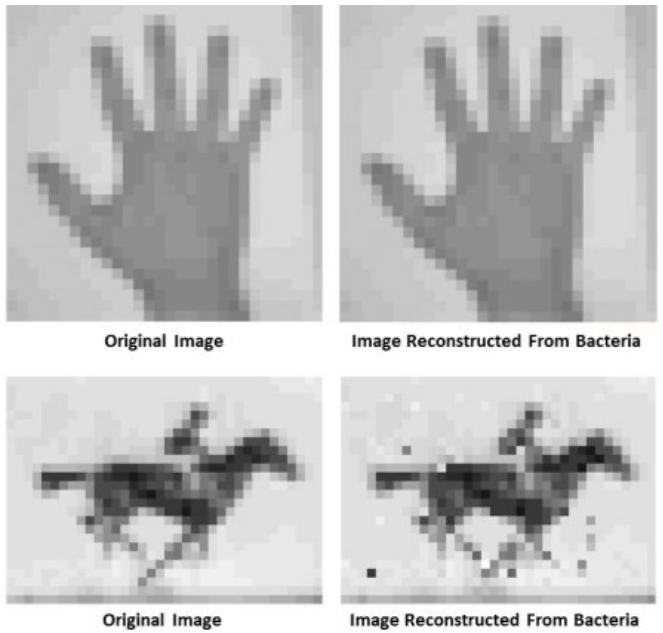
CRISPR Achievements and Developments

Present: What Has Been Done?

CRISPR progress

- Aside from being a noble prize-winning tool, CRISPR has many achievements.
- Scientists inserted a short-animated image in the E. coli DNA, which could eventually be used as a tiny hard drive.
- Gene editing techniques have shown potential to fight superbugs.
- Crispr has shown potential in extracting HIV from a living organism
- In 2018, the first gene edited babies announced by a Chinese Scientist who was convicted for this in 2019.

Images stored in bacterial DNA



Source: Seth Shipman, Harvard Medical School, Boston.

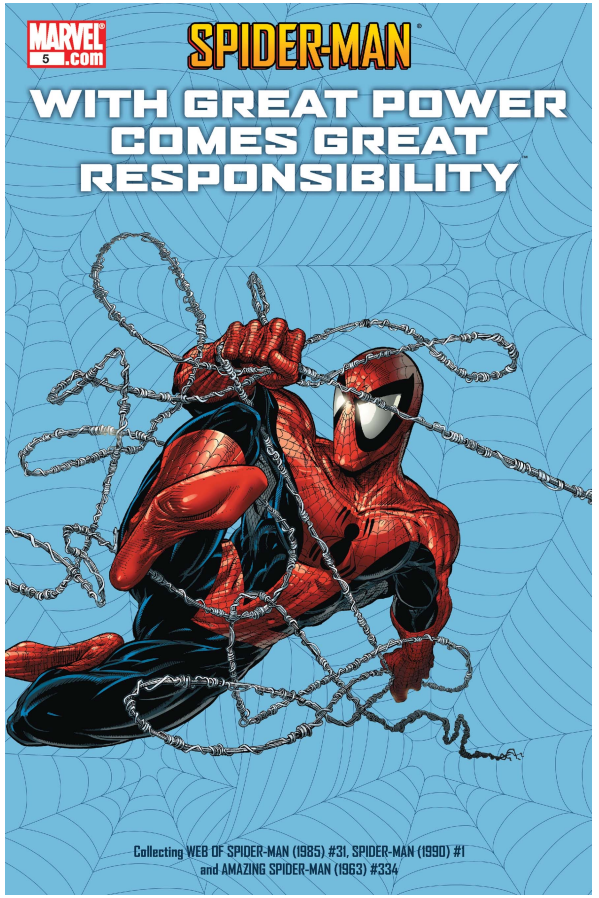
Source: "11 Amazing Feats the Gene-Editing Tool CRISPR Just Made Possible", https://www.nbcnews.com/mach/science/11-amazing-feats-gene-editing-tool-crispr-just-made-possible-ncna790911. Accessed Jul. 28, 2022.; "CRISPR-Cas9", https://www.whatisbiotechnology.org/index.php/science/summary/crispr. Accessed Jul. 28, 2022.; "Scientists Used CRISPR to Put a GIF Inside a Living Organism's DNA", https://www.technologyreview.com/2017/07/12/150497/scientists-used-crispr-to-put-a-gif-inside-living-dna/. Accessed Jul. 29, 2022.

Future: What is Possible?

Genetic mutations that change traits

- Steven Pete of Washington state carries a genetic mutation in the SCN9A gene, which causes insensitivity to pain. Unfortunately, Steven has broken more bones than he can count, and he once chewed off part of his tongue without realizing it.

- Scientists have experimented with ACTN3 gene mutations in mice and have found increased endurance.



Source: Marvel.

Source: "Captain Marvel's Powers: Could CRISPR Make Them a Reality?", https://www.synthego.com/blog/crispr-make-captain-marvel-powers. Accessed Jul. 28, 2022.; "Real-Life X-Men: How CRISPR Could Give You Superpowers in the Future", https://www.synthego.com/blog/could-crispr-make-x-men-a-realistic-possibility. Accessed Jul. 28, 2022.

Ethics, Issues, & Limitations

Ethics & Issues

Genetic manipulations can be passed on.

- **While we think we know what we're doing it's possible that we can miss something, or our technology might not catch the changes that were made.**

Germline cell and embryo genome editing bring up several ethical challenges.

- One of which is whether it would be permissible to use this technology to enhance normal human traits (such as height or intelligence).

Germline cell and embryo editing is banned.

- **Based on ethical and safety concerns, germline cell and embryo genome editing are currently illegal in the United States and many other countries.**

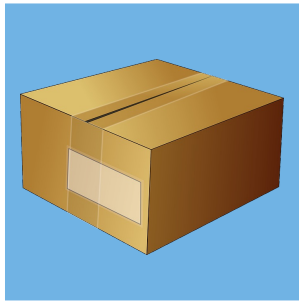
How to guide the field.

- Adopting a moratorium on heritable genome editing has become an idea to guide science.

Source: CompTIA.

Source: "What are genome editing and CRISPR-Cas9?", https://medlineplus.gov/genetics/understanding/genomicresearch/genomeediting/. Accessed Jul. 7, 2022.; "Adopt a moratorium on heritable genome editing", https://www.nature.com/articles/d41586-019-00726-5. Accessed Jul. 7, 2022.; "Is CRISPR Worth the Risk?", https://insights.som.yale.edu/insights/is-crispr-worth-the-risk. Accessed Jul. 25, 2022.

Limitations



Delivery

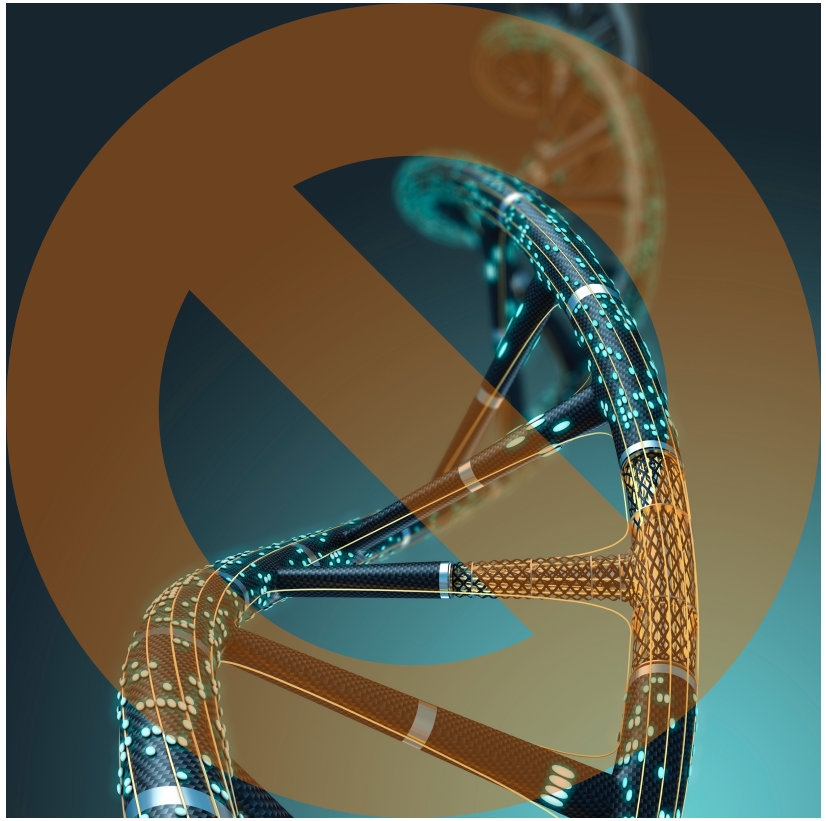
- **It's difficult to deliver the CRISPR/Cas material to mature cells in large numbers**, which remains a problem for many clinical applications.

Efficiency

- **CRISPR is not 100% efficient**, so even the cells that take in CRISPR/Cas may not experience genetic edits.

Accuracy

- **It's not 100% accurate**, and "off-target" edits, while rare, may have severe consequences, particularly in clinical applications.



Source: "WHAT IS CRISPR?", https://www.jax.org/personalized-medicine/precision-medicine-and-you/what-is-crispr. Accessed Jul. 7, 2022.

Notable Players

General Market Information

In 2021 the Gene Editing market surpassed USD 5.4 billion.

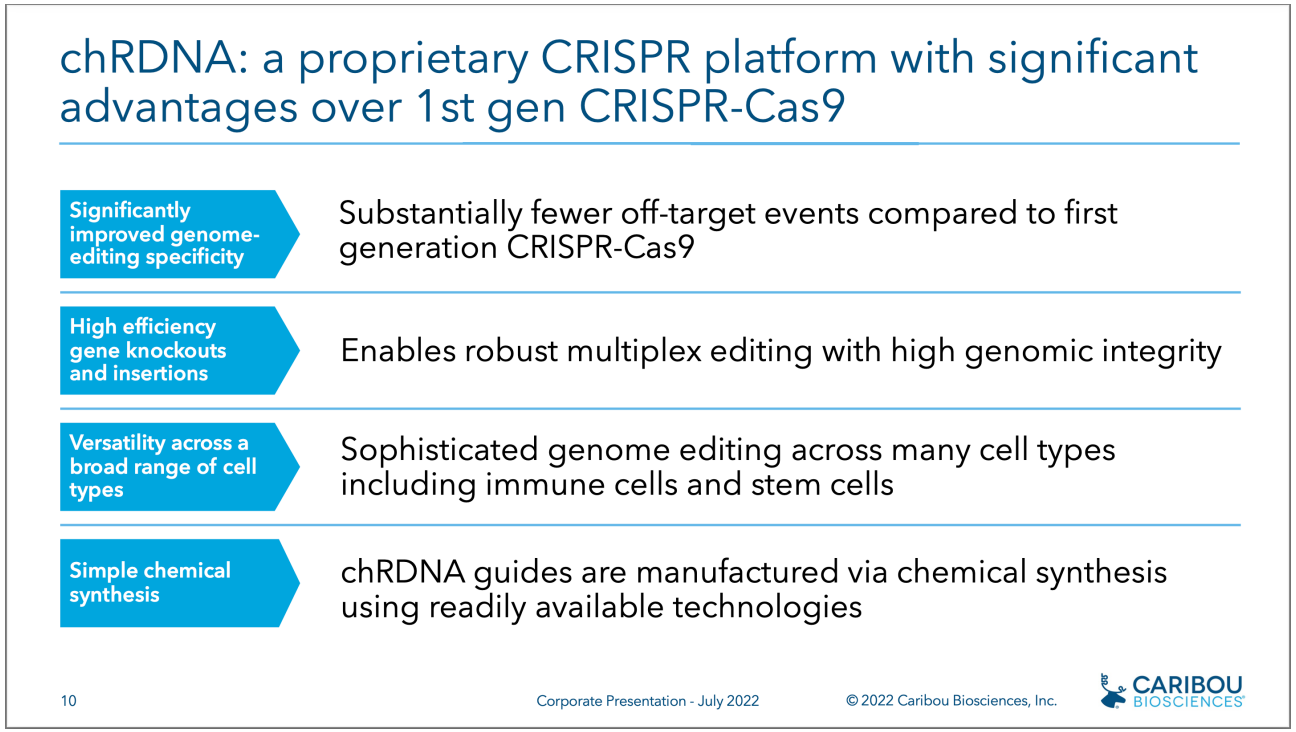
Gene Editing Market to hit USD 19.9 billion by 2030, says Global Market Insights Inc.

The 2022-2030 compounded annual growth rate is 15.5%.

Source: "Gene Editing Market Size by Application (Cell Line Engineering, Animal Genetic Engineering, Plant Genetic Engineering), Technology (CRISPR/Cas9, Zinc Finger Nucleases (ZFNs), TALENs) and End-user (Biotech and Pharma Companies, Contract Research Organizations (CROs), Research Institutes), Industry Analysis Report, Regional Outlook, Growth Potential, Competitive Market Share & Forecast, 2022 – 2030", https://www.gminsights.com/industry-analysis/gene-editing-market. Accessed Jul. 7, 2022.

Caribou Biosciences, Inc.(NasdaqGS: CRBU)

- **Mission**
  - Develop innovative, transformative therapies for patients with devastating diseases through novel genome editing.
- **chRDNA Platform**
  - chRDNA is a proprietary CRISPR platform with significant advantages over 1st gen CRISPR-Cas9.



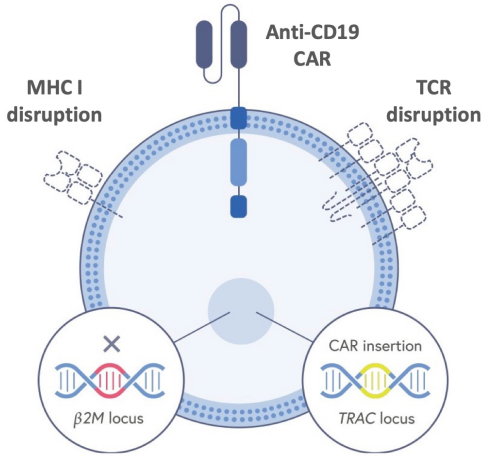
Source: "Corporate Presentation July 2022", https://investor.caribou bio.com/static-files/3168b9f6-48dd-4e44-8103-df5ff9013b6b. Accessed Jul. 25, 2022.

CRISPR Therapeutics AG (NasdaqGM: CRSP)

- **About**
  - CRISPR Therapeutics is a gene editing company focused on translating revolutionary CRISPR/Cas9 technology into transformative therapies.
- **Focus**
  - hemoglobinopathies, immuno-oncology, regenerative medicine, and in vivo approaches
- **CRISPR enables regenerative medicine**
  - CRISPR gene editing and pluripotent stem cell technology enable a new class of cell replacement therapies.

Multiplex CRISPR gene editing in one step designed to:

- **Improve persistence in the allo setting** via β2M knock-out to eliminate MHC I expression
- **Avoid need** for more toxic lymphodepletion regimens

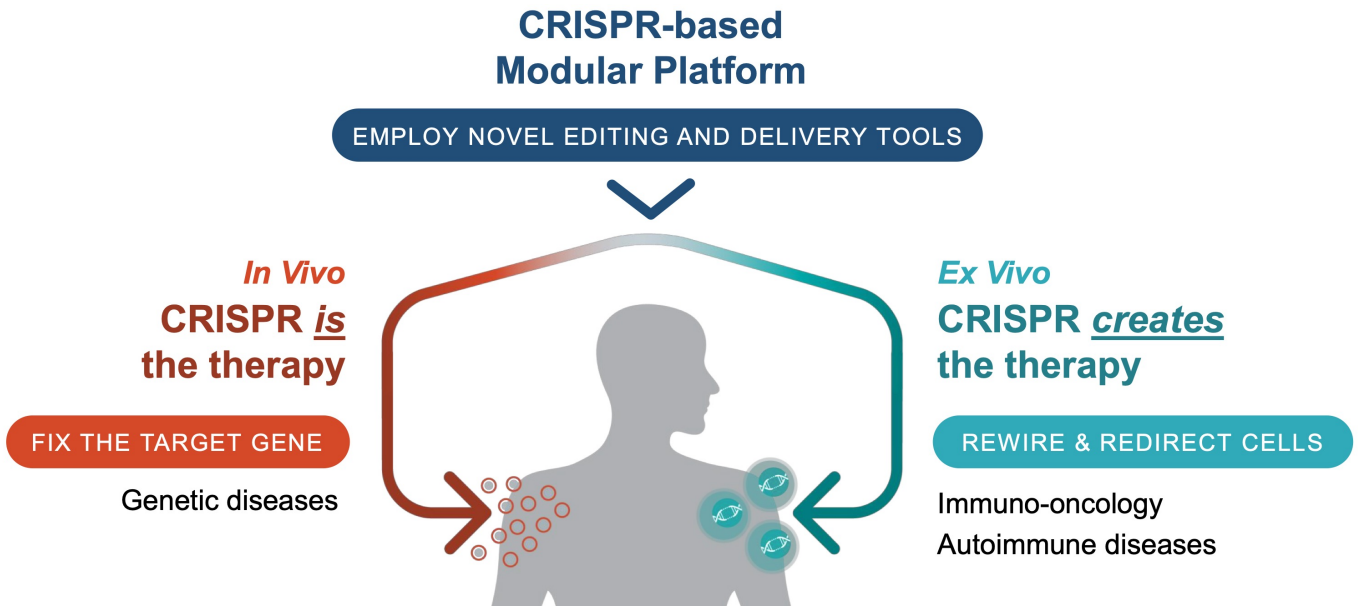


- **Prevent GvHD** via TCR disruption
- **Improve consistency and safety by precise insertion** of CAR construct into *TRAC* locus without using lentivirus or retrovirus

Source: "Creating transformative gene-based medicines for serious diseases", https://crisprtx.gcs-web.com/static-files/bfba41c6-5c28-4681-9002-bdfcef21c3f7. Accessed Jul. 25, 2022.

Intellia Therapeutics, Inc. (NasdaqGM: NTLA)

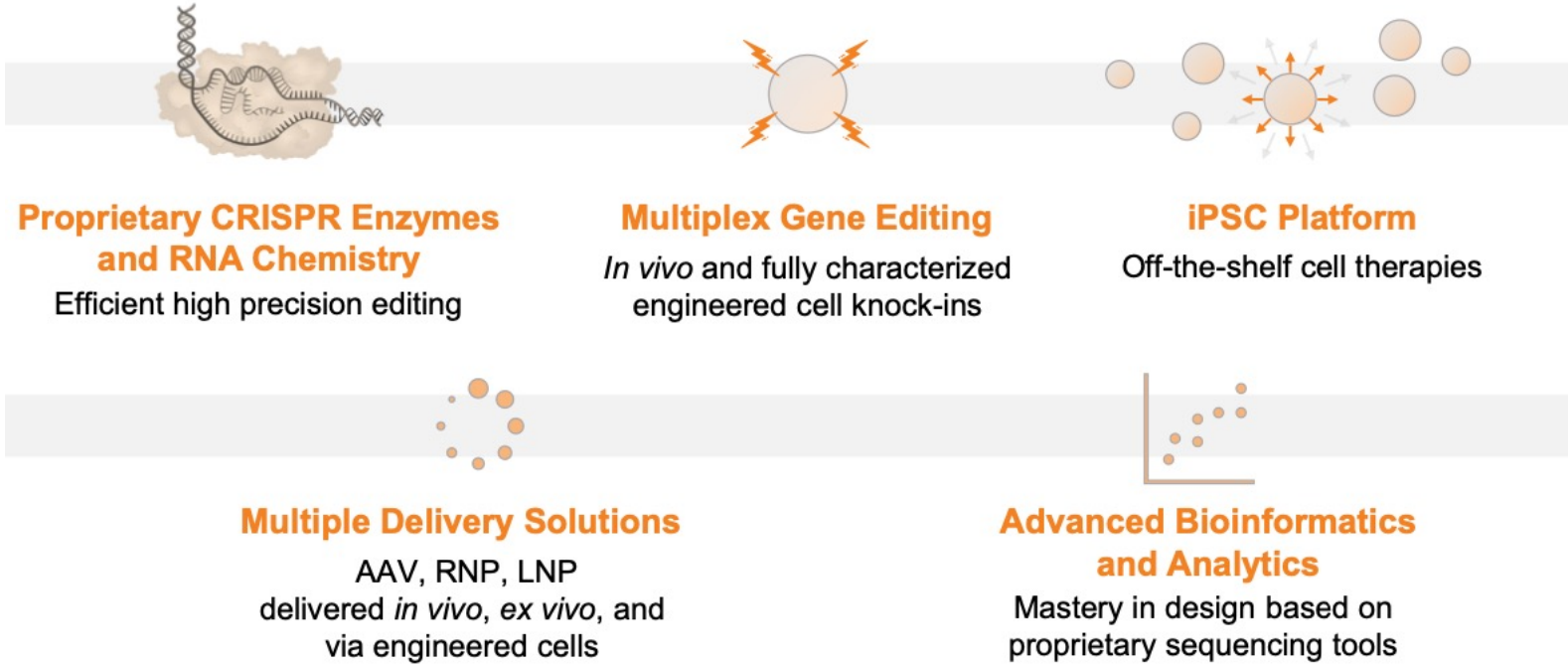
- **In vivo Crispr therapy**
  - Potentially curative in one dose, capable of delivering to multiple tissue types for various therapeutic applications, permanent gain of function with targeted gene insertion, and systemic non-viral delivery of CRISPR/Cas9 provides transient expression and potential safety advantages
- **Modular Delivery Platform Enables Rapid and Reproducible Path to Clinical Development**
- **Proprietary Engineering Platform to Power Next-Generation Engineered Cell Therapies**



Source:" Intellia is Leading the Genome Editing Revolution", https://ir.intelliatx.com/static-files/a57908a6-aaca-404d-9072-32471cd3d41e. Accessed Jul. 25, 2022.

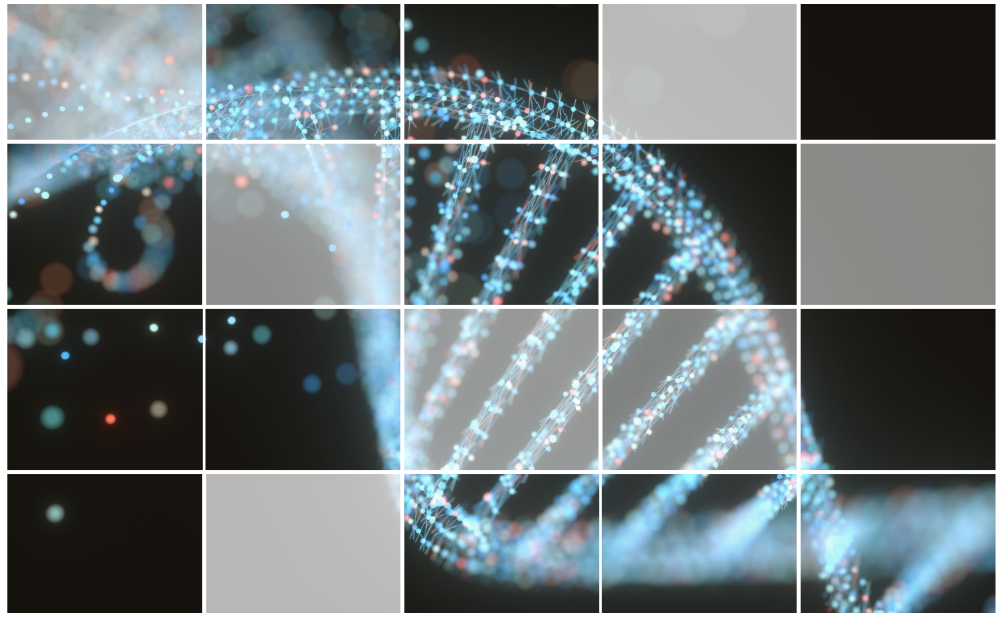
Editas Medicine, Inc. (NasdaqGM: EDIT)

- **Intellectual Property Portfolio in CRISPR Gene Editing**
  - Over 220 issued patents, over 800 applications pending
  - Multiple species and CRISPR forms to address widest range of diseases
  - Exclusive foundational IP for CRISPR/ Cas9 and Cas12a (Cpf1) editing in human therapeutic
  - Global coverage including US, Europe, Japan, Australia, Canada, China



Source:" Corporate Presentation May 2022", https://ir.editasmedicine.com/static-files/03350a40-33d7-413c-b2d6-c46f94ab273f. Accessed Jul. 15, 2022.

Thanks!



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