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SIMPLIFYING COMPLEXITY: CRISPR-MEDIATED GENOME EDITING

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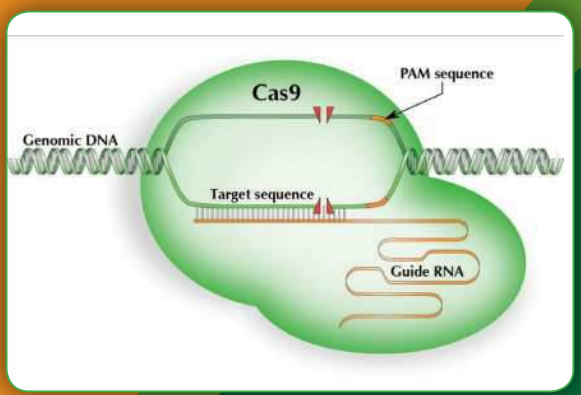

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- CRISPRa and CRISPRi kits

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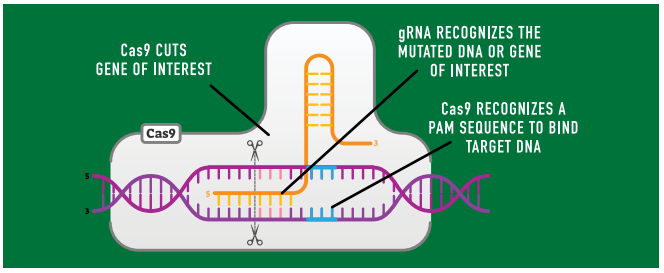
The Development of Genome Editing



The idea to correct "mistakes" in genomes has been around since the structure of DNA was discovered in the 1950s. However, the idea of genome editing really gained traction in the 1980s, when researchers recognized that most genetic errors associated with disease were passed on from parents to offspring as small changes in the genome. Scientists were excited by the notion of recognizing molecular mistakes, linking these mistakes back to a small part of the genome, and fixing the mistakes using gene therapy.

Early attempts at gene therapy focused on providing a functional copy of a mutated gene, either next to the "error" in the DNA, or outside of the genome, rather than removing or correcting the mistake. This worked for some diseases, but it was complicated and limited in scope. To make real changes, researchers had to work out how to break double-stranded DNA at a very precise location, and then find a way to repair it.

Early genome editing: Fingers and TALENS

Before CRISPR, two approaches were used to break double-stranded DNA:

ZFN fusion proteins are composed of specific DNA-binding domains and a subunit from the bacterial nuclease Fok1. To cut double-stranded DNA, researchers engineer two ZFN fusion proteins so that they will bind opposite DNA strands, allowing the Fok1 subunits to interact and cut the target DNA on both strands.

TALEN fusion proteins are comprised of DNA-binding domains derived from precise plant pathogen proteins, along with a Fok1 domain. They cut DNA in the same way as ZFN fusion proteins, introducing double-stranded breaks through dimerization of Fok1 nucleases. TALENS are easier to engineer than ZFNs, especially for longer DNA sequences.

CRISPR: Knocking out and knocking in

CRISPR, a microbial defense system, is currently the simplest, most versatile, and most precise method used for genetic manipulation. The CRISPR-Cas9 system comprises CRISPR associated protein 9 (Cas9), which acts as molecular scissors, and guide RNA (gRNA); gRNA usually consists of CRISPR RNA (crRNA) matching the gene of interest and tracer RNA (tracrRNA) which provides scaffolding for Cas9. Inside the cell, gRNA recognizes the gene of interest, and then Cas9 recognizes a short protospacer adjacent motif (PAM) sequence and cuts across the double-stranded DNA.

CRISPR knockout mutations are popular for researching gene function. Scientists knock out genes with CRISPR-Cas9 by creating a double-stranded cut and then letting the host cell's DNA repair mechanism fix the cut using non-homologous end joining (NHEJ), an imprecise method of repair.

Scientists use knock-in research models to study the effects of specific gene variants, add reporter genes such as green fluorescent protein, repair genes, and probe genome regulation. Knocking in requires Cas9 cuts to be repaired precisely with specific DNA bases. To do so, researchers must overcome a cell's NHEJ repair mechanism by pushing cells towards homology-directed repair (HDR), which is preferable compared to other repair pathways and happens only in the S and G2 phases of the cell cycle.

Comparison of common gene-editing techniques

	ZFNs	TALENs	CRISPR
Recognition complex	Protein-DNA	Protein-DNA	RNA-DNA
Ease of engineering	Difficult	Moderate	Easy
Constraints	Targeting G-rich sequences is difficult	Spacing and T positioning affect ability of Fok1 to bind	PAM must precede targeted sequence
Ease of packing into vectors	Easy due to small size	Difficult due to large size	Moderate due to size of Cas9, but smaller orthologs are available
Ability to perform multiple edits	Difficult	Difficult	Easy
Cost	High	High	Low

CRISPR-Cas9 Delivery Systems

CRISPR comes in many forms, and there are a variety of ways to deliver CRISPR-Cas9 into cells, giving researchers a multitude of options when it comes to designing a CRISPR experiment.

CRISPR Formats

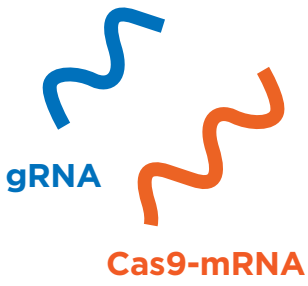
DNA

Enters the nucleus to be transcribed into Cas9 mRNA and gRNA. Cas9 is produced from the mRNA in the cytoplasm, then Cas9 and gRNA form a ribonucleoprotein (RNP) complex and travel to the nucleus to target genomic DNA.

CRISPR vectors

RNA

Cas9 mRNA is translated into Cas9 protein in the cytoplasm, gRNA and Cas9 form a RNP in the cytoplasm and target the nucleus.



- **Cas9 mRNA**
- **sgRNA**

Preformed RNP

No transcription or translation is required so RNPs can be delivered directly to the nucleus or cytoplasm.

- **Cas9 protein**
- **sgRNA**

CRISPR Formats	Delivery Methods
Plasmids (DNA)	• Transfection • Electroporation • Lentivirus • AAV
RNA (Cas9 mRNA, gRNA)	• Transfection • Electroporation • Microinjection
RNP (Cas9 protein, gRNA)	• Transfection • Electroporation • Microinjection

CRISPR in Research

Researchers use the CRISPR-Cas genome editing system in a multitude of research settings. Traditional CRISPR-Cas technology relies on the Cas9 endonuclease from *Streptococcus pyogenes*, but modified versions of Cas9 have expanded the toolkit. Researchers now use the technique to control transcription, modify epigenomes, image chromosomes, and screen genomes; uses for CRISPR in research are ever-expanding.

Genetic screening: CRISPRa and CRISPRi

Researchers first reported whole genome screening using the classic CRISPR-Cas9 toolkit in 2014[1,2]. Since then, development of Cas9 derivatives has expanded CRISPR technology. Researchers can now use CRISPR single-guide RNA (sgRNA) libraries with Cas9 derivatives to silence or upregulate gene expression for genetic screening.

CRISPR interference (CRISPRi) enables targeted yet reversible knockdowns of genes. Rather than knocking out genes completely, it silences them. The system uses a catalytically inactive version of Cas9 (dCas9), which targets the promoter region of a gene of interest, producing steric hindrance of transcriptional molecules[3].

CRISPR activation (CRISPRa) also makes use of dCas9. For this approach, researchers fuse transcriptional activators targeted to promoter or enhancer regions with dCas9 to activate and upregulate gene expression.

Next generation CRISPR-Cas systems

Initial successes with Cas9 alternatives and new gRNA constructs have inspired researchers to discover new ways to use and modify CRISPR-Cas genome editing technology. These new advances are improving researchers' understanding of the genome, and propelling genome editing toward medical and agricultural uses[4].

Cas9 alternatives: Although Cas9 was the original Cas protein used in CRISPR-Cas gene editing, several others are now available. Each one has advantages and disadvantages in the nascent field of gene editing.

High fidelity Cas9s: eSpCas9, SpCas9-HF1, and HypaCas9 are edited versions of Cas9, designed to lessen the chance of off-target genome editing.

Cas9 nickase: Produces single-strand breaks instead of double-strand breaks. Pairs of Cas9 nickases targeted to within 20 base pairs of each other create a double-stranded cut while minimizing off-target effects, because single-strand breaks are rapidly repaired, using the opposite DNA strand as a template with no ill effects.

xCas9: An engineered form of Cas9 that recognizes a broad range of PAM sequences, which increases genome target sites[5].

Cas12a: Produces a staggered cut in double-stranded DNA instead of the blunt cut produced by Cas9. It's also smaller and therefore easier to package into vectors, and only requires crRNA for successful targeting (does not requires tracrRNA)[6].

Cas12b: Smaller still than Cas9 and Cas12a, making it attractive for intracellular delivery systems, Cas12b was found in thermophilic bacteria, making it difficult to use at body temperature, and cuts the non-target strand of double-stranded DNA. However, recent modifications to the protein by Zhang et al. have made it more specific and enhanced its activity at body temperature[7].

Cas13: RNA-guided RNase (targets RNA instead of DNA). Has potential to be used as an antiviral therapy[8].

Cas13d: RNase that is around 20 percent smaller than Cas13, making it more convenient for delivering into cells[9].

Cas14a: A compact Cas nuclease from Archaea, which targets single-stranded DNA, does not require a PAM sequence for activation, and cuts other single-stranded DNA strands non-specifically after binding the target sequence[10]. Shows potential for use in diagnostics for recognizing single nucleotide polymorphisms.

CasX: Found in bacteria from groundwater and sediment, it can cut double-stranded DNA just like Cas9, but its smaller size is advantageous for vector delivery[11].

See references on page 8

Advancing the World: Present and Future Applications

From agriculture to genetic disease, CRISPR-Cas technology has revolutionized many areas of research, and it is likely to help produce world-altering advances in the near future. However, ethical questions have shrouded the technology since its introduction.

Food and livestock

Regulating gene-edited crops and foods has been controversial since the first prototypes were introduced. Historically, US regulators have been lenient with gene-edited products, stating that they do not need the stringent oversight of previous genetically modified organisms (GMOs) because they don't contain foreign DNA. However, the European Court of Justice ruled that gene-edited crops should receive the same regulation as other GMOs. Despite regulation, CRISPR has the potential to solve a range of food-related concerns.

Plant products: Scientists are using CRISPR to produce a multitude of edibles:

- Reduced-gluten wheat that can be tolerated by people with sensitivities[1]
- White button mushrooms that don't brown when cut[2]
- Soybeans with increased healthy fats[3]
- Groundcherries that are easier to grow domestically[4]
- Cacao beans that are resistant to crop-destroying viruses[5]

While no CRISPR-edited crops are currently on the market, oil from gene-edited soybeans, which inventors claim has no trans fats and a longer shelf life than other soybean oils, has been in use since 2018[3].

Livestock: Livestock breeders face long breeding cycles and limitations in genetic resources. CRISPR-Cas systems offer affordable solutions to these problems. For example, CD163 gene-edited pigs are resistant to porcine reproductive and respiratory syndrome (blue ear disease), and SP110 gene knock-in cows are less susceptible to tuberculosis[6]. CRISPR genome editing has been successfully applied to all species tested, including zebrafish, humans, mice, rats, monkeys, pigs, cattle, sheep, goats, and others.

Human gene editing

The potential exists for CRISPR-Cas9 to help treat or prevent many of the 6,000+ known genetic diseases. Scientists are currently exploring the possibility of applying the technology for several diseases:

- Cancer: Directly targeting tumors with CRISPR-Cas[7], or using CRISPR-Cas technology to improve T-cell therapy[8]
- Blood disorders: Replacing genes in sickle-cell disease and β-thalassaemia[9]
- HIV: Eliminating the virus from HIV-infected individuals[10]
- Muscular dystrophy: Correcting heart tissue defects caused by muscular dystrophy[11]
- Huntington's Disease: Ameliorating neurotoxicity[12]

CRISPR bioethics

CRISPR recently made news when Chinese Scientist He Jiankui reported using CRISPR to edit human embryos with the goal of protecting the resulting offspring against HIV. This controversial endeavor resulted in heated debates about the ethical framework and safety measures required for CRISPR[13], and prompted the World Health Organization to form a committee to set guidelines for human gene editing.

Key players in the CRISPR arena

Jennifer Doudna and Emmanuelle Charpentier—co-authors on the seminal 2012 CRISPR paper—and their respective research institutes have formed companies to commercialize the CRISPR-Cas system. Caribou Biosciences and Intellia Therapeutics are associated with Doudna, CRISPR Therapeutics, ERS Genomics, and Casebia Therapeutics are associated with Charpentier.

Feng Zhang and his team from the Broad Institute, who first demonstrated the applicability of CRISPR-Cas in eukaryotes, also has a spin-off company, Editas Medicine (which was originally co-founded with Doudna).

Other companies with stakes in CRISPR include Toolgen Inc., MilliporeSigma, Vilnius University, Cellectis, DowDuPont, and MPEG-LA[14].

See references on page 8

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