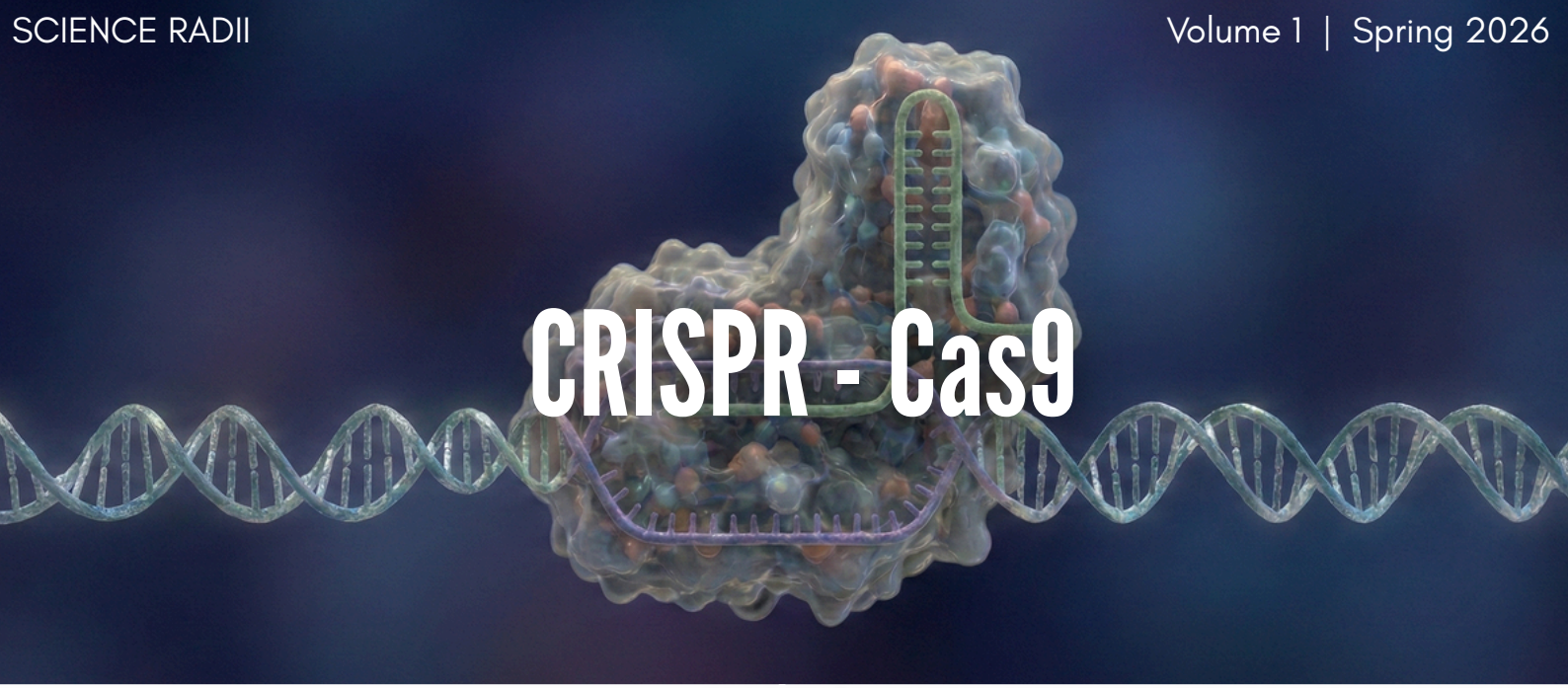

CRISPR - Cas9

EKIN SEZEN | BILKENT DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

Genome editing is an approach that allows making precise alterations in an organism's DNA, allowing researchers to add, remove, or change the genetic code for genetic studies, treatments, enhanced agricultural efficiency, and biotechnological studies. Targeted nucleases are powerful, commonly used tools for such genome editing processes. They are frequently used due to their precise binding ability to target sequences. Among all the tools, the CRISPR-Cas9 system stands out for several reasons. It offers a highly effective tool that is specific, simpler to design, and supports large-scale, simultaneous editing, compatible with many different cells [1].

What is CRISPR-Cas9? Its Origin and Mechanism

CRISPR (clustered regularly interspaced short palindromic repeats) is a specialized DNA sequence initially discovered in bacteria. Together with CRISPR-associated (Cas) proteins, this system provides defense against plasmid uptake and phage infection. The development of the CRISPR adaptation module was crucial for the evolution of prokaryotic adaptive immunity, allowing bacteria and archaea to store short fragments of foreign DNA from previous infections as spacers within their CRISPR arrays [2]. These short fragments of the invaders' DNA allowed the prokaryotic organisms to repair their genome by excising the foreign sequences, which are identified by the spacers. The CRISPR system is divided into two classes according to the differences in core Cas proteins, and each of the classes contains three subtypes with different proteins [3]. While some classes work on DNA, there are other classes that can work on RNA molecules. Among the second class, the type II system uses the Cas9 protein, an enzyme that works together with CRISPR to cut DNA at specific sites, thereby clearing integrated viral sequences. After the invasion of a phage, some of the genetic sequence of the invader gets integrated into the host's genome. After integration, the adaptation of the CRISPR system is initiated with the recognition and targeting of a foreign sequence, followed by the acquisition of this DNA and its integration as a new spacer into the CRISPR array [4]. This CRISPR array is made up of alternating repeats, originating from the host's genetic material, and spacer

sequences derived from the invader. Spacer sequences are non-coding pieces in between genes; in the system, they are placed between repeats and allow the system to identify specific sequences of foreign DNA. The mechanism used by scientists for genome editing is a repurposed version of the same mechanism found in bacteria. As Jiang and Doudna [5] note, the RNA-mediated targeting system can be manipulated using single-guide RNA libraries to pinpoint drug-target or disease-resistance genes in the genome, allowing quick assignment for drug targets.

Working Mechanism

The CRISPR system has three main stages in its working mechanism. The first step of the CRISPR-Cas9 working mechanism is recognition of the sequence to be cleaved. At the 5' end of the CRISPR RNA, there is the spacer sequence that complements the foreign genetic material, while at the 3' end, it contains a CRISPR repeat sequence. The repeat sequence does not directly target the DNA, but instead helps maintaining of the structure of precursor RNA. Using the spacer and its complementary target sequence, Cas nucleases can initiate cleavage of a specific sequence. Therefore, in the absence of single-guide RNA, the complex remains inactive.

The second step is cleavage. "The Cas9 nuclease makes double-stranded breaks (DSBs) at a site three base pairs upstream to PAM. The PAM sequence, standing for protospacer adjacent motif, is a short 2–5 base pair in length conserved DNA sequence, downstream of the cut site. The most commonly used nuclease in the genome-editing tool, Cas9 protein, recognizes PAM at 5′-NGG-3′ sequence where N can be any base. Once the Cas9 has found a target site with the appropriate PAM, it triggers local DNA melting followed by the formation of an RNA-DNA hybrid" [6]. After cleavage, the last step is the repair of the cut DNA. After the Cas9 complex disassociates from the strand, the DNA is induced to a double-strand break. The host cell's endogenous repair machinery detects this double-strand break and repairs it.

According to Xue et al., DNA damage can be resolved by multiple repair mechanisms, such as homologous recombination, classical non-homologous end joining, microhomology-mediated end joining, and single-strand annealing [7]. The first and most precise method is homologous recombination, which uses a homologous DNA template to lead the repair using either single-or double-stranded DNA molecules. The non-homologous end joining method recruits proteins that join the ends without any additional sequence. While it is fast and effective, it frequently leads to small, random mutations in the sequence. The Microhomology-mediated end joining method resects the broken ends of DNA until it finds small matching sequences, then ligates the ends from these sequences. The Single-strand annealing method also uses homologous repeats to ligate double-strand breaks. However, while the classical non-homologous end joining method requires only 1–4 nucleotide-long homology, microhomology-mediated end joining requires 1–16 nucleotide alignment, and single-strand annealing needs a wider alignment caused by directly repeated sequences. [8].

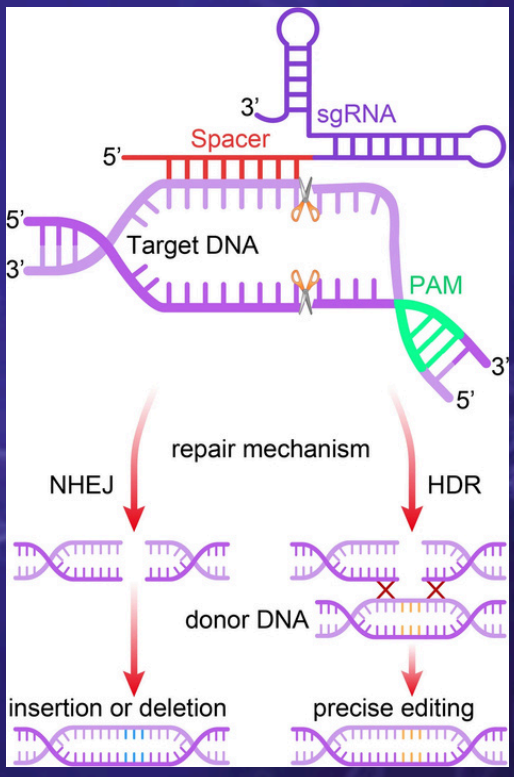

Figure 1. Recognition of target DNA is followed by the two repair mechanisms [9].

Upon recognition, and cleaveage steps, the double strand break is repaired using Non-Homologous End Joining via insertion or deletions, or by Homology Directed Repair that uses another DNA molecule as template [9].

Limitations of CRISPR-Cas9

While its cornerstone effect in genetic research and therapies cannot be underestimated, this method still has some limitations to overcome in order to achieve wide use [10]. The first being the off-target effects. When the tool is used for gene therapy, it has a high frequency of off-target effects [10]. Off-target effects on the topic are "deposition of unexpected, unwanted, or even adverse alterations to the genome" [11]. While these can be overlooked for some agricultural and educational studies, their effects on critical processes like treatment-purposed genome editing set a great limitation for approved usage.

The second limitation of the technique is that it requires a protospacer adjacent motif (PAM) alongside the target. Short canonical PAM recognition sites such as SpCas9 can be large and hard to pack into adeno-associated virus vectors, whereas SaCas9 (ortholog of SpCas9), which can be packaged more easily, can have longer PAM sequences. These problems narrow the therapeutic windows [10]. These create limits as desired sequences might be absent in the desired loci.

Another problem with this method is the immunogenic toxicity. The majority of humans are already immune to the standard CRISPR-Cas9 proteins. As discussed by Charlesworth et al [12], analysis of human serum reveals that a significant majority of the population carry a pre-existing adaptive immune response to Cas9 proteins, detecting both antibodies and specific T-cells against SaCas9 and SpCas9 in most donors. These findings can get in the way of common usage of the technique until technology advances toward the immune rejection problem in clinical applications.

Lastly, some ethical problems can arise during genome editing applications to germline or similar cell groups. Since changes in the genome can be carried on in generations, off-target effects can get even larger with accumulating mutations and crossing-overs. Furthermore, if these tools start to be used more frequently, children of families with access to higher technology treatments and the ability to cover their cost will have an unfair genetic advantage over other children.

Advantages of CRISPR-Cas9 Compared to Other Genome Editing Methods

One of the genome editing methods which can be used for similar applications is the Zinc Finger Nucleases (ZFNs). Zinc finger nucleases are programmable DNA-cutting enzymes that enable gene-targeting for targeted mutations or gene replacements [13]. Similarly, Transcription Activator-Like Effector Nucleases (TALENs) can be used. TALENs consist of two units as a non-spesific DNA-cleaving nuclease and a DNA binding unit to enable targeting [14]. The increasing potential for usage of genome editing tools, including CRISPR-Cas9 grows larger with advancements in the science field.

Some of the advantages Crispr-Cas9 mediated genome editing include a fast and flexible design. As we have discussed earlier, specific PAM sequences can create problems for the designing process; but new sequences are being discovered each year that have flexibility for sequencing (some have non-certain nucleotide sequences and can work with any of the four nucleotides).

As a second advantage of using CRISPR technology, its cost-effectiveness can be shown. CRISPR-Cas9 can offer significant financial efficiency compared to other gene manipulation tools, such as zinc finger nucleases and transcription activator-like effector nucleases (TALENs) that require protein engineering [15]. These older methods can be expensive preventing large-scale applications as they require custom proteins for each specific target, combined with a validation for the whole complex. In contrast, CRISPR system require only an RNA guide which can be fetched from a RNA library. This programmability reduces the time it takes to design and produce, costs, and trained specialist need to a certain extent.

Applications of CRISPR/Cas9

There are many potential usage areas for this relatively new genome editing tool. One of its most common uses is in gene therapy and the treatment of diseases and disorders that have a genetic foundation. CRISPR can correct mutations that cause diseases such as sickle cell anemia, cystic fibrosis, and cancer. Another use of it can be for infectious diseases. Rather than changing the patient's genome, the tool can directly target the virus itself.

When used in agriculture, the tool can also be used to enhance security, nutrition, and yield by modifying genes known for affecting these traits. The agricultural use can enable plantation with disease resistance and climate resilience that can enhance sustainability.

Some industrial applications of genome editing enable changed genomes for algae and yeast, and usage of them for producing lipids and alcohols, or plastic-digesting organisms.

CRISPR is not only an active tool for changing the genome but also for understanding it. Scientists can use it to study the functions of genes, model diseased organisms, and test newly developed drugs and therapies.

Did you know?

The 2020 Nobel Prize in Chemistry was awarded to Emmanuelle Charpentier and Jennifer A. Doudna for the discovery of the CRISPR/Cas9 genetic scissors.

Works Cited

1 - Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nature Protocols. 2013;8(11):2281-2308. doi:10.1038/nprot.2013.143

2 - Wang JY, Pausch P, Doudna JA. Structural biology of CRISPR–Cas immunity and genome editing enzymes. Nature Reviews Microbiology. 2022;20(11):641-656. doi:10.1038/s41579-022-00739-4

3 - Liu W, Li L, Jiang J, Wu M, Lin P. Applications and challenges of CRISPR-Cas gene-editing to disease treatment in clinics. Precision Clinical Medicine. 2021;4(3):179-191. doi:10.1093/pcmedi/pbab014

4 - Amitai G, Sorek R. CRISPR–Cas adaptation: insights into the mechanism of action. Nature Reviews Microbiology. 2016;14(2):67-76. doi:10.1038/nrmicro.2015.14

5 - Jiang F, Doudna JA. CRISPR–CAS9 structures and mechanisms. Annual Review of Biophysics. 2017;46(1):505-529. doi:10.1146/annurev-biophys-062215-010822

6 - Mengstie MA, Wondimu BZ. Mechanism and applications of CRISPR/CAS-9-Mediated Genome Editing. Biologics. 2021;Volume 15:353-361. doi:10.2147/btt.s326422

7 - Xue C, Greene EC. DNA repair pathway choices in CRISPR-CAS9-Mediated Genome Editing. Trends in Genetics. 2021;37(7):639-656. doi:10.1016/j.tig.2021.02.008

8 - Sfeir A, Symington LS. Microhomology-Mediated End Joining: a back-up survival mechanism or dedicated pathway? Trends in Biochemical Sciences. 2015;40(11):701-714. doi:10.1016/j.tibs.2015.08.006

9 - Zhang H, Qin C, An C, et al. Application of the CRISPR/Cas9-based gene editing technique in basic research, diagnosis, and therapy of cancer. Molecular Cancer. 2021;20(1):126. doi:10.1186/s12943-021-01431-6

10 - Uddin F, Rudin CM, Sen T. CRISPR gene therapy: applications, limitations, and implications for the future. Frontiers in Oncology. 2020;10:1387. doi:10.3389/fonc.2020.01387

11 - Guo C, Ma X, Gao F, Guo Y. Off-target effects in CRISPR/Cas9 gene editing. Frontiers in Bioengineering and Biotechnology. 2023;11:1143157. doi:10.3389/fbioe.2023.1143157

12 - Charlesworth CT, Deshpande PS, Dever DP, et al. Identification of preexisting adaptive immunity to Cas9 proteins in humans. Nature Medicine. 2019;25(2):249-254. doi:10.1038/s41591-018-0326-x

13 - Carroll D. Genome engineering with Zinc-Finger nucleases. Genetics. 2011;188(4):773-782. doi:10.1534/genetics.111.131433

14 - Joung JK, Sander JD. TALENs: a widely applicable technology for targeted genome editing. Nature Reviews Molecular Cell Biology. 2012;14(1):49-55. doi:10.1038/nrm3486

15 - Thomas C. What are the pros and cons of CRISPR-Cas9? | IDT. IDT. Published August 22, 2025. https://eu.idtdna.com/page/support-and-education/decoded-plus/crispr-cas9-what-are-the-pros-and-cons/