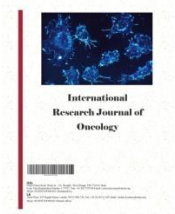

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A Review of the CRISPR/Cas System and Its Potential for Early Cancer Diagnosis

Ikalo David Oseghale [a*], Imade Omoregie [a], Courage Ushiobafoh Godday [b], Virtue Oniso Okhemukhokho [c] and Godwin Mmaduabuchi Ikokwu [d]

[a] Department of Biochemistry, University of Benin, Benin City, Nigeria.
[b] Department of Biomedical Sciences, Keele University, England, United Kingdom.
[c] University of Essex, United Kingdom.
[d] Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The range of CRISPR-based gene editing and modification technologies has quickly increased in recent years. In recent years, CRISPR/Cas has widened its application to diagnostics. CRISPR/Cas has the capacity to evade off targeting, which makes it both a powerful therapeutic tool and an excellent diagnostic tool for a variety of pathological diseases. The most common diagnostic tools currently in use are unable to properly target cancer, which is the number one cause of illness and mortality worldwide, because they can only detect it in advanced stages. As the next-generation molecular diagnostic technique, clustered regularly interspaced short palindromic repeats/CRISPR associated protein (CRISPR/Cas)-based in vitro diagnosis has considerable potential because it can streamline the detection processes and increase sensitivity and specificity. CRISPR/Cas systems have seen significant technical advancements that have demonstrated a high degree of effectiveness, specificity, and flexibility in targeting the targeted genomic location. A substantial amount of optimism is offered by recent advancements in CRISPR technology for the treatment of severe diseases like cancer. This article intends to give readers an overview of the CRISPR/Cas system's workings and to educate readers on the numerous Cas proteins that may be employed in the next generation of precise genome engineering.

**Corresponding author: E-mail: ikalo.oseghale@gmail.com;*

Keywords: CRISPR; Cas proteins; DNA; RNA; Protospacer Adjacent Motif (PAM).

1. INTRODUCTION

The DNA sequence family known as CRISPR, which stands for clustered regularly interspaced short palindromic repeats, is present in the genomes of prokaryotic organisms like bacteria and archaea. The DNA fragments of bacteriophages that previously infected the prokaryote are where these sequences were derived. During successive infections, they are used to locate and eradicate DNA from bacteriophages that are similar to them. As a result, these sequences are essential to prokaryotes' antiviral (or anti-phage) defensive system and offer a sort of acquired immunity. "About 50% of sequenced bacterial genomes and almost 90% of sequenced archaeal genomes contain CRISPR" [1-5]. "Adaptive immunity systems called CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated proteins) modules are encoded by the majority of archaea and many bacteria. They protect their host cells from foreign genetic material including viruses and plasmids. These defense mechanisms are encoded by operons with extremely varied architectural styles and rapid rates of evolution for both the Cas genes and the particular spacer content. Bacterial and archaeal genomes have distinctive arrays of short repeats with distinctive spacers for years. Computational methods have been used to thoroughly analyze the Cas proteins that are encoded by putative operons near CRISPR sequences, and it has been discovered that these proteins contain domains that are typical of a number of nucleases, a helicase, a polymerase, and various RNA-binding proteins" [6]. "Currently, there are two classes, six types, and numerous subtypes of CRISPR-Cas systems that can be classified based on evolutionary links. The ribonucleoprotein effector complex is what distinguishes the different classes of CRISPR-Cas systems: class 1 systems are characterized by a complex of many effector proteins, whereas class 2 systems include a single crRNA-binding protein" [7,8].

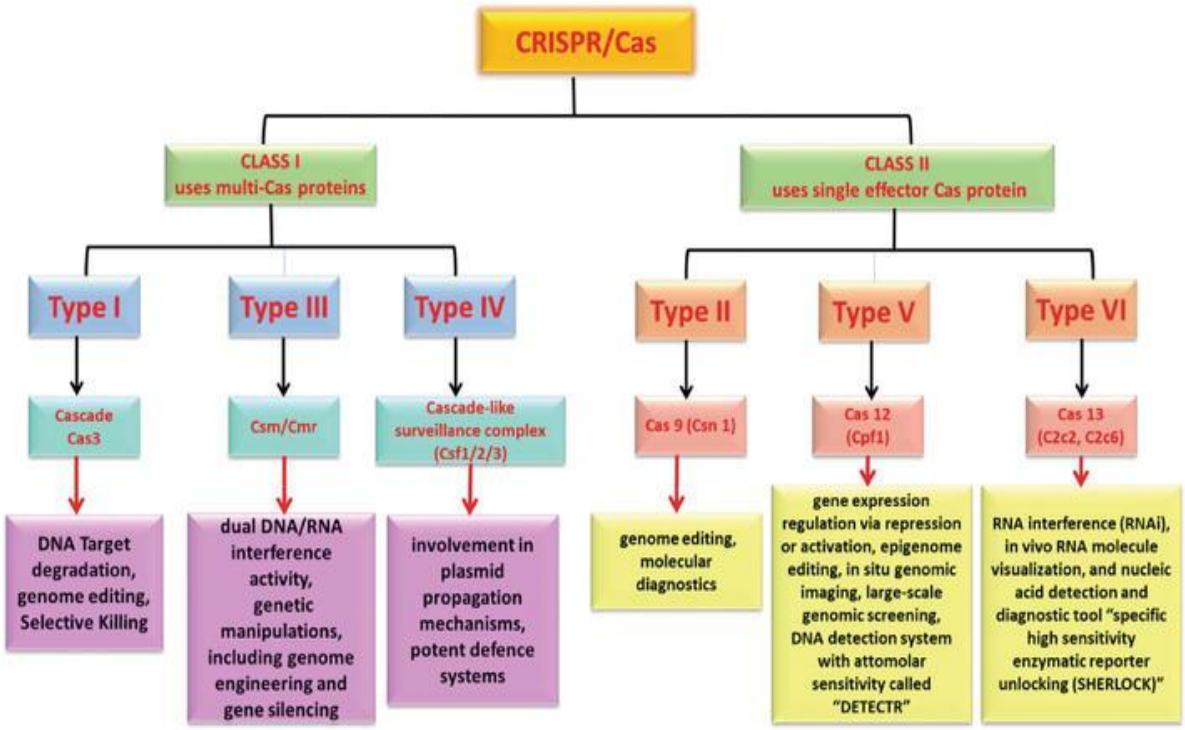

Fig. 1. Classification of CRISPR/Cas system with their emphasized application [9]

1.1 Classification of Crispr/Cas Systems

The CRISPR/Cas system is split into two categories as seen from Fig. 1 [9] based on the kind of Cas protein involved and the intricacy of the individual parts. In addition to being further divided into Class I and Class II, there are six types and 22 subtypes. Multiple Cas proteins are said to be involved in the class I classification, which includes type I, III, and type IV, whereas only one effector Cas protein is said to be associated with the CRISPR array of processed guide RNA, also known as crRNA, in the class II classification, which includes type II, V, and type VI. Type I, which is categorized under Class I, uses multiple Cas proteins and cascade Cas3 to carry out tasks such genome editing, selective death, and DNA target destruction. The Csm/Cmr is used in Type III (classified under Class I), which employs multi-Cas proteins, to carry out tasks such dual DNA/RNA interference and genetic manipulation. The cascade-like surveillance complex (Csf1/2/3) is used by Type IV (classified under Class I), which uses multiple Cas proteins, to carry out its involvement in the plasmid propagation mechanism and powerful defense system. Type II, which is categorized under Class II, uses a single Cas9 effector protein to carry out genome editing and molecular diagnostics. The forms of CRISPR/Cas involved in Class II Classification are said to be the simplest to use for successful genome editing and manipulation of nucleic acids without cells due to their inherent simplicity. The varieties of CRISPR/Cas systems generated from Class 2 classification, i.e., type II, V, and VI, are believed to be crucial in developing competent diagnostic platforms of illness detection in addition to being active in gene editing (Mubthasima et al. 2022).

2. THE CRISPR–CAS SYSTEMS THREE-STAGE DEFENSE PATHWAY PROCESS

1.) Short segments of DNA that are similar to viral or plasmid sequences, known as protospacers (a copy or fragment of invading nucleic acid), are produced and integrated into the CRISPR locus throughout the adaptation process. When a virus is challenged, a single virus-derived spacer that confers resistance is commonly inserted at the leader side of a CRISPR locus; internal insertions and the acquisition of numerous spacers from the same phage are less common. Each integration event duplicates a repeat, resulting in the formation of a new spacer-repeat unit. Recognition of proto-spacer-adjacent motifs appears to control the selection of spacer precursors (proto-spacers) from the invasive DNA (PAMs). The creation and/or integration of protospacers into CRISPR loci depend heavily on PAMs, which are short (3-6 nt) sequence elements found next to the protospacer in foreign nucleic acids. PAMs, which vary between CRISPR-Cas system types, are typically only a few nucleotides long. The adaptation mechanism most likely detects the PAM. Although Cas1 and Cas2, the most highly conserved Cas proteins, are the leading candidates for proteins with crucial roles in this process, there is currently no clear evidence for a mechanism of spacer acquisition [6,10,11].

2.) CRISPR RNA biosynthesis (crRNA) and expression are involved in stage 2 of CRISPR-Cas-mediated immunity. The long main transcript of a CRISPR locus (pre-crRNA) is produced and then processed into short crRNAs as part of biogenesis, which involves the synthesis of multiple distinct crRNAs from CRISPR locus transcripts. Endoribonucleases, which either function as a single enzyme or as a component of a larger complex (such as the CRISPR-associated complex for antiviral defense (Cascade) in Escherichia coli), catalyze the processing step (such as Cas6 in the archaeon Pyrococcus furiosus). In order to recognize foreign nucleic acids, CrRNAs join forces with Cas proteins to form ribonucleotide complexes. The lack of preference between coding and non-coding strands displayed by CrRNAs suggests the presence of an RNA-guided DNA-targeting machinery. [12,13,4]. Five Cas proteins must be coupled to one crRNA in order to form the type I-E complex, often known as Cascade. Promoters found at the leader ends are primarily responsible for transcription at CRISPR sites. The primary crRNA biosynthesis enzymes have been largely identified and characterized. CRISPR transcripts are cut by RAMP superfamily Cas proteins in a number of CRISPR-Cas systems. For instance, the core Cas protein Cas6 (from *P. furiosus*) and sub-type-specific Cas proteins Cse3

(from *E. coli*) and Csy4 (from *P. aeriginosa*) each recognize particular crRNA repeat sequences/structures and catalyze a single-cut within each repeat to release unit crRNAs (also referred to as 1 processing intermediates), which contain the 5′ and 3′ flanking repeat-derived sequences. Unknown mechanisms may be able to further reduce the repetition sequences. At the 5′ ends of CRISPR RNAs from a number of archaea and bacteria, an 8-nucleotide repeat-derived region is retained. This sequence probably plays a significant role as a crRNA "identification tag" that functions as a Cas protein binding site [6,10].

3.) The third step is interference. The PAM sequence is recognized on the complementary strand of the crRNA during the interference stage in type I systems and is necessary along with crRNA annealing. For instance, in Escherichia coli (*E. coli*), the cleavage is likely performed by the HD endonuclease domain of the Cas3 protein. In type I systems, proper base pairing between the crRNA and the protospacer signals a conformational shift in Cascade that recruits Cas3 for DNA degradation. Additionally, it appears that the PAMs are crucial to the interference process. Targeting either strand of the phage DNA gives immunity to the homologous phage in S. thermophilus and *E. coli*, a discovery that is most consistent with DNA being the target [6,10].

3. CRISPR/CAS APPLICATION IN CANCER BIOMARKER DETECTION

Detection of Circulating Tumor DNA (ctDNA). Cancer cells that have died or become necrotic largely release circulating tumor DNA (ctDNA), which then circulates in the bloodstream. It is a critical biomarker for the detection of cancer since it contains gene mutations found in both primary and metastasizing tumors. Epidermal growth factor receptor (EGFR) gene mutation identification can be utilized to assess the course of cancer and direct cancer treatment choices [14,15].

4. CRISPR/CAS9

“Cas9 protein has six domains (1) Recognition lobe (REC I), (2) REC II, (3) Arginine-rich bridge helix, (4) PAM Interacting, (5) HNH, and (6) RuvC. REC I is the major domain responsible for binding with the guide RNA (gRNA). As soon as it binds to specific sequences, the arginine-rich bridge helix starts the cleavage process. PAM specificity, which is responsible for binding with the target sequence, is conferred via the interaction with PAM. The target sequence is cut by the nuclease domains HNH and RuvC” [16]. “It has a four-part system that consists of two tiny molecules, trans-activating CRISPR RNA (tracrRNA) and crRNA, which together produce the gRNA (guide RNA)” [1]. “Streptococcus pyogenes Cas9 is a large (1,368 amino acid), multidomain, and multifunctional DNA endonuclease. It is also known as SpyCas9. It snips dsDNA 3 bp upstream of the PAM using two different nuclease domains: an HNH-like nuclease domain that cleaves the DNA strand opposite the complementary strand (target strand), and a RuvC-like nuclease domain that does the same (non-target strand). In addition to playing a crucial part in CRISPR interference, Cas9 also takes part in crRNA maturation and spacer acquisition” [17].

4.1 CRISPR/CAS9 Mechanism

Short pieces of foreign DNA are incorporated into the CRISPR repeat-spacer array in the host chromosome as new spacers during the immunization process after exposure to invading genetic elements from plasmids or phages. This process creates a genetic record of the prior infection that allows the host to fight off the same invader in the future. Short mature CRISPR RNAs are produced by further transcription of the CRISPR array and enzymatic processing of precursor-CRISPR transcripts through endonucleolytic cleavage (crRNAs). The lack of gRNA renders the Cas9 protein inactive. The modified gRNA takes the shape of a T with one tetra-loop and three stem-loops. The target sequence's complementary 5′ end is coded into the gRNA. The spacer, a short section of RNA that completes a sequence from a foreign genetic material, is located at the 5' end of the crRNA (gRNA), while the CRISPR repeat sequence is located at the 3' end. The dormant Cas9 protein is transformed into its active version when the planned gRNA attaches to the Cas9 and causes modifications in the protein [16]. “Upon a second infection, hybridization of the crRNA (gRNA) spacer with a complementary foreign target sequence (protospacer) causes Cas nucleases to specifically target and destroy the invasive DNA or RNA. Once activated, it looks for a sequence that matches the PAM

sequence (5′-NGG-3′) by attaching to it. Then Cas9 uses its HNH and RuvC domains to cleave dsDNA 3 bp upstream of the PAM. The 20-nucleotide sequence of the crRNA (target strand) is cut by the HNH domain, while the RuvC domain cuts the DNA strand in opposition to the complementary strand (non-target DNA strand)" [16]. "The assembly of mature crRNAs with Cas proteins form crRNA-effector complexes, which probe DNA targets and obliterate matched sequences in foreign nucleic acids, is a distinguishing characteristic of CRISPR-Cas systems. Notably, in most CRISPR-Cas systems, the PAM, a short-conserved sequence motif (2–5 bp) situated near to the crRNA-targeted sequence on the invading DNA, is crucial for target DNA selection and destruction" [17].

4.2 Targeting and Recognition by Cas9

To prevent an autoimmune reaction, CRISPR-Cas immune systems must distinguish between self and non-self. Potential targets in the host's CRISPR loci do not contain PAMs, thus RNA-guided interference complexes avoid them. In type I and type II systems, foreign DNA targets that contain contiguous PAM sequences are targeted for degradation. PAMs operate to both attract Cas9-guide RNA complexes (Cas9-gRNA) to probable target sites and to cause nuclease domain activation, according to single-molecule and bulk biochemical investigations. A 5'-NGG-3' PAM on the non-target (displaced) DNA strand is recognized by Cas9 from Streptococcus pyogenes, which raises the possibility that PAM identification could activate catalysis through allosteric control. Furthermore, Cas9's HNH nuclease domain is similar to other HNH domains that cleave RNA substrates and mediates target strand cleavage [18]. The complex is prepared to look for complementary target DNA locations once Cas9 binds its guide RNA. Both the presence of a conserved PAM sequence close to the target site and complementary base pairing between the 20-nt spacer sequence and a protospacer in the target DNA are necessary for target search and recognition. Typically utilized SpyCas9's original PAM sequence is 5-NGG-3, where N can be any of the four DNA nucleotides. Single-molecule investigations have shown that Cas9 searches for a suitable PAM sequence to start the target DNA search process before checking the surrounding DNA for potential guide RNA complementarity. Target recognition happens by three-dimensional collisions, in which Cas9 quickly separates from DNA that lacks the right PAM sequence. When the right PAM is present, dwell time is determined by the complementarity between the guide RNA and nearby DNA. "When Cas9 locates a target site with the proper PAM, it causes local DNA melting at the PAM-adjacent nucleation site, followed by invasion of an RNA strand to create an RNA-DNA hybrid and displacement of a DNA strand (known as an R-loop) from the PAM-proximal to PAM-distal ends" [17].

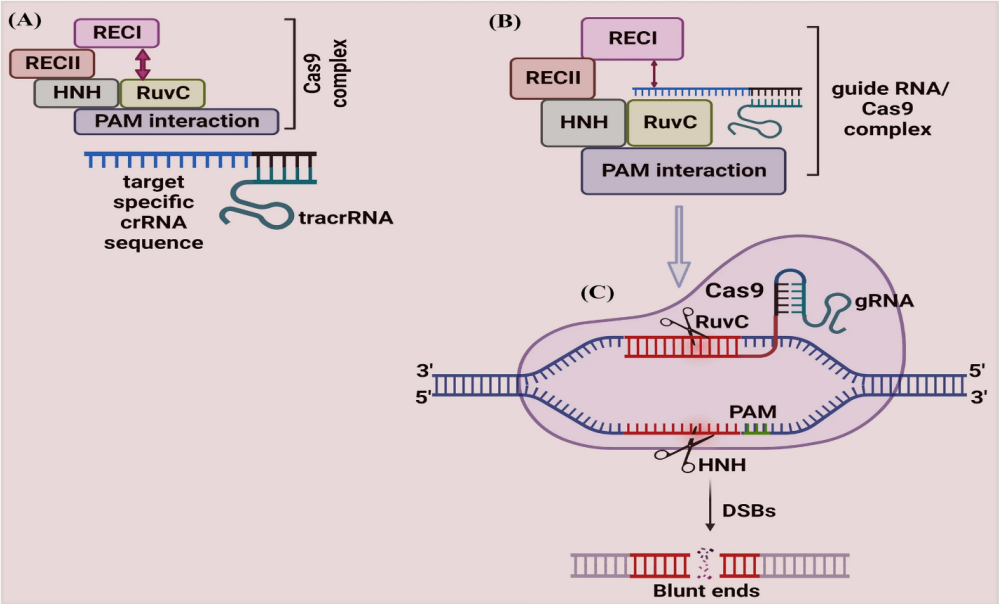

Fig. 2. Schematic illustration of CRISPR/Cas9 mechanism [16]

5. CAS12 PROTEIN

Cas12 is a flexible protein with more contemporary uses, such as epigenome editing. The type V CRISPR system includes the Cas12 protein. As an effector RNA-guided DNA endonuclease that replaces the Cas9 protein in genome editing, the Cas12 protein has just come to light. The Lachnospiraceae bacterium (LbCas12a) and Acidaminococcus species (AsCas12a), which defend against invading viruses, were the sources of the Cas12 protein. The Cas9 protein is more effective at detecting mismatches in the first 10 bp of the RNA-DNA helix closest to the PAM sequence. Additionally, Cas12 differs from the Cas9 protein in that it may process the precursor crRNA without the need of tracr-RNA or RNase III. Researchers may now use Cas12 protein for multiplex genome editing due to this method [16].

5.1 Mechanism of Cas12 Protein

"To efficiently cut ssDNA and dsDNA, the Cas12 protein just needs the crRNAs. For cleavage action, the Cas12 protein has the RuvC and nuclease lobe (NUC) domains. Similar to Cas9, Cas12 comes across a possible target site next to a PAM sequence. Once Cas12 encounters a target DNA strand, it starts the R-loop process, which creates base-pair hybridization between the target DNA strand and the crRNA. In this stage, Cas12 binds to the target sequence's 17th base pair, causing an R-loop to develop. Once an R-loop has been established, the Cas12 protein utilizes the PAM sequence along with its active RuvC domain to cleave the non-target strand. However, it is still unclear how the Cas12 protein's RuvC domain works to cleave the desired DNA strand" [16].

6. CAS13 PROTEIN

Cas13 is the most recent Cas protein to be identified. In order to protect archaea and bacteria against RNA invaders, the CRISPR/Cas13 system acts as an "adaptive" immune system. Previously, there were two subtypes of the Cas13 protein family: (1) Cas13a, from the Leptotrichia shahii bacterium, which is also known as C2c2 and belongs to type VI; and (2) Cas13b, from Prevotella sp., which is part of the type III CRISPR/Cas system [16]. Cas13a (formerly known as C2c2), Cas13b, Cas13c, and Cas13d are the four subtypes of the Cas13 protein families that have been identified in general, based on the phylogeny of their effector complexes. Cas13X and Cas13Y, two novel Cas13 proteins, have also been identified recently [19]. They all possess two HEPN domains, which are crucial for RNA breakdown in higher eukaryotes and prokaryotes. HEPN domain mutations would result in a catalytically inactive protein that retains the capacity to bind target RNA, which is a crucial component in RNA imaging, RNA tracking, and RNA modification. "Additionally, the CRISPR-Cas13 system may degrade single-strand RNA (ssRNA) non-specifically when activated by target RNA in vitro, a technique that is commonly employed in diagnostics, similarly like the CRISPR-Cas12 system, which has single-strand DNA collateral cleavage activity" [20].

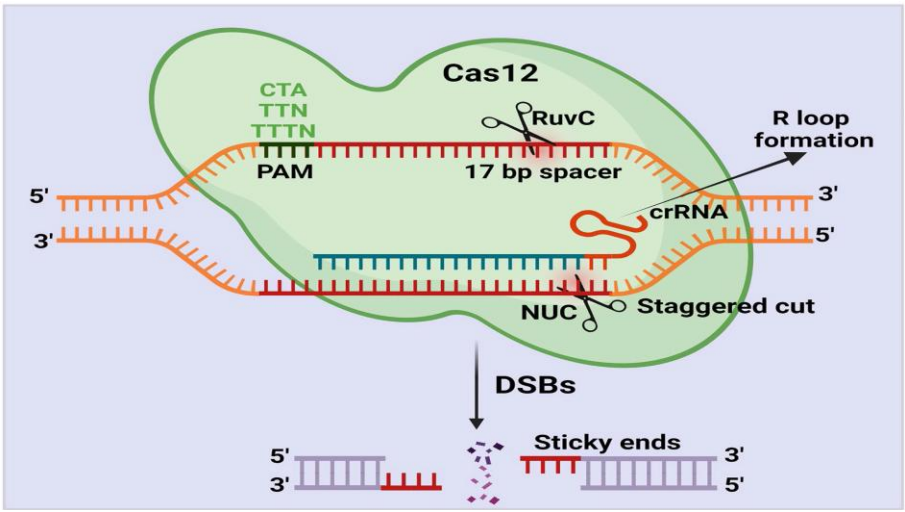

Fig. 3. Schematic illustration showing the CRISPR/Cas12 mechanism [16]

6.1 Mechanism of Cas13a Protein

The type VI-A CRISPR-Cas system includes a large protein called Cas13a. Both the crRNA recognition and nuclease lobes are present in Cas13a as shown in Fig. 4 [16]. The NUC lobe is made up of the helical-2 and helical-3 domains, two nucleotide-binding (HEPN) RNase domains for RNA targeting (HEPN-1 and HEPN-2). A cleft that accommodates the crRNA repeat region is located in the REC lobe, along with an N-terminal domain (NTD), a Helical-1 domain, and other structures. LshCas13a, LbuCas13a, and LwaCas13a are a few of the Cas13 proteins that have now been identified. These Cas13 proteins handle pre-crRNA, but their structural and domain organization makeup is also very diverse from one another. Upon identifying the target sequence (22–28 nt) complementary to the crRNA spacer, the LshCas13a cleaves ssRNA. A protospacer flanking site (PFS) with a bias to adenosine (A), uracil (U), and cytosine flanks the target sequence at its 3′ end (C). Without the help of the tracrRNA, LshCas13a and crRNA bind and cleave the target region of ssRNA. Recent research revealed that among 15 Cas13a orthologs lacking a major PFS motif, LwaCas13a was the most effective protein, offering a viable research platform for RNA targeting [19,16,20].

6.2 Mechanism of Cas13b Protein

Since a PFS flanks RNA targeting with an A, U, or G at the 5′ end and a PAM (NAN/NNA) at the 3′ end, the Cas13b protein is more strong and precise than the Cas13a protein. The mature crRNA is connected to the Cas13b protein. Inducing conformational changes at the target ssRNA through the CRISPR/Cas13b complex leads to nonspecific RNA cleavage. Scientists investigated the Cas13b protein's capacity for RNA editing, however the Cas13b protein's exact mechanism is yet unknown. Cas13b differs significantly structurally from other Cas13 proteins. The HEPN domains of Cas13b are located at the extreme N and C termini of the linear protein, in contrast to Cas13a, Cas13c, and Cas13d. Second, the 3′ end of the Cas13b crRNA contains the direct repeat. Thirdly, Cas13a and Cas13d require a shared solvent-exposed cleft that catches the target RNA, but PbuCas13b allows the target RNA to reach the opening central channel. Contrary to Cas13a, Cas13b contains 1150 amino acids, and in order to maximize its targeting capabilities, it requires a double-sided PFS, 50 PFS of D (A, U, or G), and 30 PFS of NAN or NNA [19,16,20].

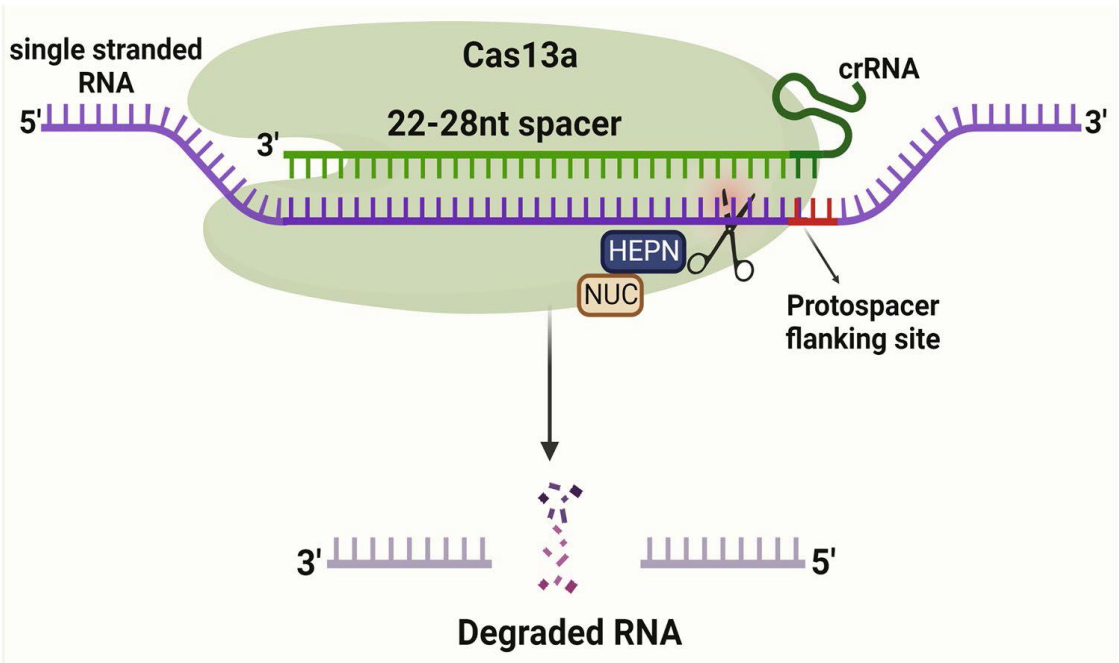

Fig. 4. Schematic illustration showing the CRISPR/Cas13a mechanism [16]

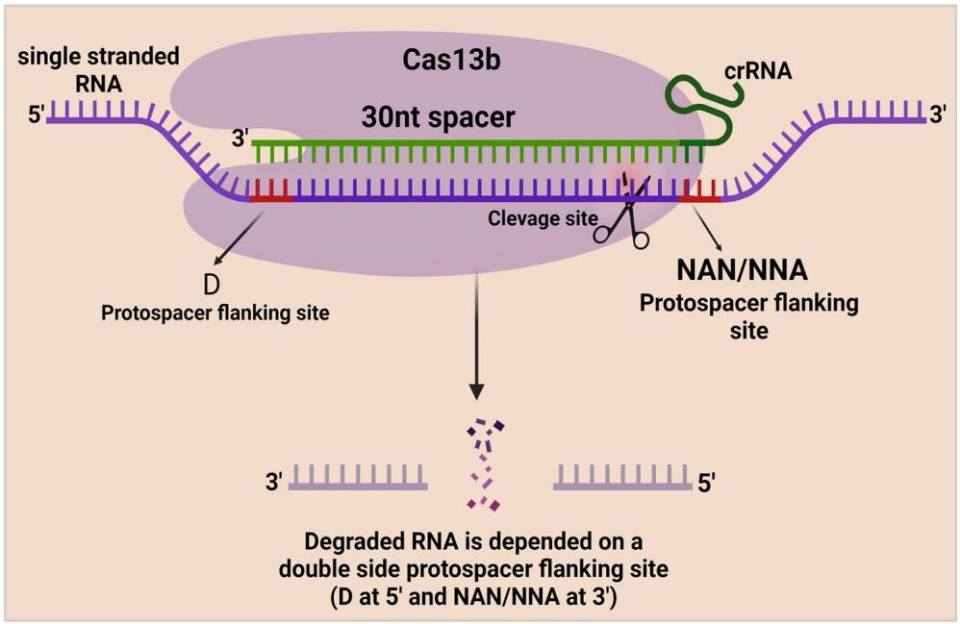

Fig. 5. Schematic illustration showing the CRISPR/Cas13b mechanism [16]

6.3 Mechanism of Cas13d Protein

Compared to Cas13a, which contains 1250 amino acids, Cas13d is a type VI CRISPR-Cas variation with a reduced size of approximately 930 amino acids. The 30 nt (5' direct repeat) crRNA of Cas13d is followed by a variable 3' spacer with a length that varies from 14 to 26 nt. Unlike other systems like RNAi, CRISPRi, and the other Cas13 members, the Cas13d-crRNA complex exerts strict sequence-specific RNA cleavage in vivo, which promises more distinctive RNA interference. Additionally, it has been demonstrated that CRISPR/Cas13d RNA target cleavage does not seem to depend on the PFS, which expands its target-sequence options [20]. Because Cas13d is more adaptable and has a smaller physical size than other Cas13 subtypes, it is used more frequently [21]. The absence of the Helical-1 domain in comparison to Cas13a is one major factor [21]. The target flanking sequences are not much constrained in Cas13d, however. Additionally, Cas13d has a unique method for digesting crRNA. Because of its very tiny size, Cas13d exhibits strong collateral RNase activity and target cleavage, making it appropriate for in vivo delivery [19].

6.4 Mechanism of Cas13X and Cas13Y Protein

Cas13X and Cas13Y are two novel Cas13 family members that were discovered in hypersaline samples. In comparison to Cas13Y, which can be separated into Cas13Y.1 to Cas13Y.5, Cas13X can be divided into Cas13X.1 and Cas13X.2. The smallest Cas13 protein currently exists, Cas13X.1, which has 775 fewer amino acids than the standard Cas13 protein. When Cas13X.1 is further trimmed from 775 to 445 aa, it can overcome the delivery challenges faced by different Cas13-based base editors in vivo, giving them a significant delivery advantage [22,23]. Cas13X.1 had the best knockdown efficiency and no PFS bias among the other CasX and CasY proteins [19,20].

7. CAS14 PROTEIN

By building a metagenomic database of the bacterial genome to look for uncharacterized Cas genes, the Cas system was investigated. The smaller Cas protein with MW 40–70 kd is encoded by the Cas14 protein, which was discovered. Compared to other Cas proteins that have been described, the Cas14 protein is significantly smaller (400–700 amino acids). Cas14 protein can target ssDNA without a PAM because of their tiny size [24,16].

7.1 Mechanism of Cas14 Protein

In addition to providing immunization against viruses with ssDNA genomes or mobile genetic components, the Cas14 protein also cleaves ssDNA (MGEs). The Cas14 protein cleaves ssDNA rather than dsDNA or ssRNA after recognizing the target ssDNA and mediating

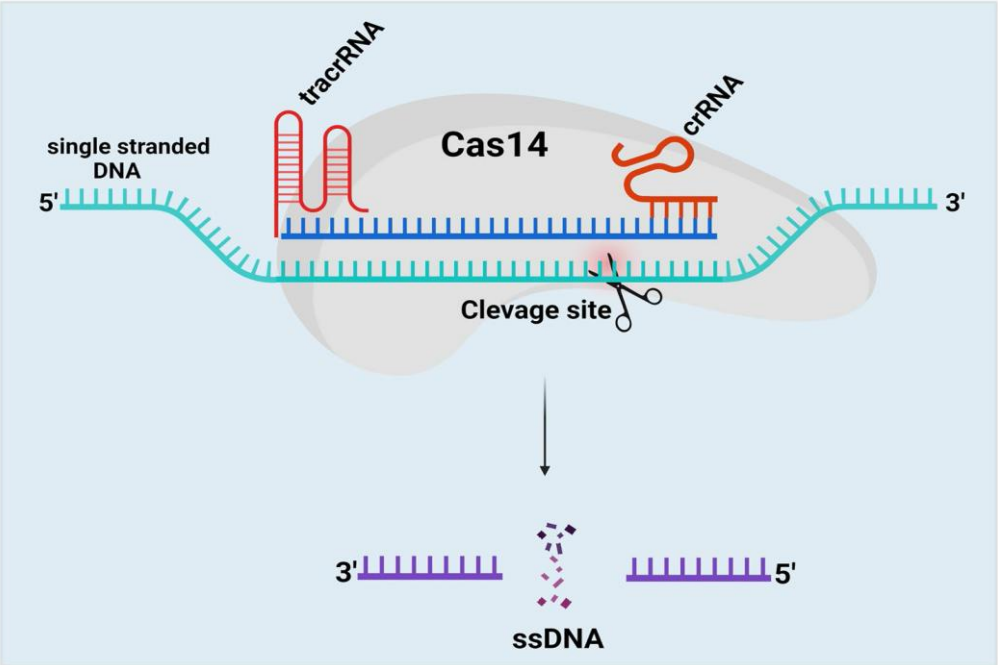

Fig. 6. Schematic illustration showing the CRISPR/Cas14 mechanism (Hillary and Ceasar 2022)

seed sequence contact with it as shown in Fig. 6 [16]. To target the ssDNA, the Cas14 protein also needs both tracrRNA and crRNA. When the PAM region is absent, the Cas14 protein cleaves more specifically than Cas9, Cas12, and Cas13 proteins [16].

8. CONCLUSION

The prevention of cancer-related fatalities has also been demonstrated through early cancer diagnosis. Scientists have utilized CRISPR gene editing to change immune cells such that they will recognize altered proteins particular to a person's tumor. These targets include DNA from cancer-causing viruses and RNA from cancer cells. Gain-of-function mutations, which activate oncogenes, promote carcinogenesis, and these oncogenes are only expressed by cancer cells. The CRISPR-Cas system's ability to knock out these genes makes it a desirable therapeutic target since it will stop the spread of cancer. The Cas system is an excellent tool for RNA imaging and the detection of RNA-protein interactions because of its precise targeting and customizable features. The search for novel Cas variations in nature is the subject of numerous investigations right now. The mechanism of Cas proteins is still not fully understood in many respects, though. Therefore, harnessing Cas proteins for precise genome engineering applications will be more likely if their molecular mechanism is understood, PAM-less Cas proteins are found, and appropriate targeting specificity is used to reduce off-target effects.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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