Current updates on the structural and functional aspects of the CRISPR/Cas13 system for RNA targeting and editing: A next-generation tool for cancer management (Review)

KHALED S. ALLEMAILEM[1], ARSHAD HUSAIN RAHMANI[1], NAHLAH MAKKI ALMANSOUR[2], FAHAD M. ALDAKHEEL[3], GHADAH MOHAMMAD ALBALAWI[4], GHADEER MOHAMMED ALBALAWI[5] and AMJAD ALI KHAN[6]

[1]Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University, Buraydah 51452, Saudi Arabia; [2]Department of Biology, College of Science, University of Hafr Al Batin, Hafr Al Batin 31991, Saudi Arabia; [3]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud University, Riyadh 11433, Saudi Arabia; [4]Department of Laboratory and Blood Bank, King Fahd Specialist Hospital, Tabuk 47717, Saudi Arabia; [5]Department of Laboratory and Blood Bank, Maternity and Children Hospital, Tabuk 47717, Saudi Arabia; [6]Department of Basic Health Sciences, College of Applied Medical Sciences, Qassim University, Buraydah 51452, Saudi Arabia

Received January 22, 2025; Accepted April 2, 2025

DOI: 10.3892/ijo.2025.5748

Abstract. For centuries, a competitive evolutionary race between prokaryotes and related phages or other mobile genetic elements has led to the diversification of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated sequence (Cas) genome-editing systems. Among the different CRISPR/Cas systems, the CRISPR/Cas9 system has been widely studied for its precise DNA manipulation; however, due to certain limitations of direct DNA targeting, off-target effects and delivery challenges, researchers are looking to perform transient knockdown of gene expression by targeting RNA. In this context, the more recently discovered type VI CRISPR/Cas13 system, a programmable single-subunit RNA-guided endonuclease system that has the capacity to target and edit any RNA sequence of interest, has emerged as a powerful platform to modulate gene expression outcomes. All the Cas13 effectors known so far possess two distinct ribonuclease activities. Pre-CRISPR RNA processing is performed by one RNase activity, whereas the two higher eukaryotes and prokaryotes nucleotide-binding domains provide the other RNase activity required for target RNA degradation. Recent innovative applications of the type VI CRISPR/Cas13 system in nucleic acid detection, viral interference, transcriptome engineering and RNA imaging hold great promise for disease management. This genome editing system can also be employed by the Specific High Sensitivity Enzymatic Reporter Unlocking platform to identify any tumor DNA. The discovery of this system has added a new dimension to targeting, tracking and editing circulating microRNA/RNA/DNA/cancer proteins for the management of cancer. However, there is still a lack of thorough understanding of the mechanisms underlying some of their functions. The present review summarizes the recent updates on the type VI CRISPR/Cas system in terms of its structural and mechanistic properties and some novel applications of this genome-editing tool in cancer management. However, some issues, such as collateral degradation of bystander RNA, impose major limitations on its *in vivo* application. Furthermore, additional challenges and future prospects for this genome editing system are described in the present review.

Correspondence to: Dr Amjad Ali Khan, Department of Basic Health Sciences, College of Applied Medical Sciences, Qassim University, P.O. Box 6666, Buraydah 51452, Saudi Arabia
E-mail: akhan@qu.edu.sa

Key words: CRISPR/Cas13, RNA targeting, gene expression, genome editing, cancer management, tumor-targeting

Contents

1. Introduction

Most bacteria and archaea have an adaptive defense system termed the Clustered Regularly Interspaced Short Palindromic

Repeats (CRISPR) and CRISPR-associated sequence (Cas) genome editing systems, which protect them from attack by phages, viruses, plasmids and other foreign genetic materials (1,2). With the discovery of the CRISPR/Cas system, recent advances in genome editing technology hold great promise for improving the environment, agriculture and human health (3-5). The hallmark feature of CRISPR/Cas systems is the memory acquisition of previous infections from mobile genetic elements (MGEs) in the form of short sequences (spacers) incorporated into the CRISPR array (6,7). The CRISPR array of adjacent regions also consists of genes encoding Cas proteins. Cas proteins have a notable role in CRISPR/Cas-driven immunity, including adaptation, CRISPR RNA (crRNA) expression and interference (8,9).

More recently, the type VI CRISPR/Cas system, an exclusively single-stranded RNA (ssRNA)-targeting platform, has been developed (10,11). All the Cas13 subtype effectors are functional crRNA-guided RNases with two definite and independent catalytic centers. The pre-crRNA is processed by one catalytic center and contains two R-X_4-H motifs, termed higher eukaryote and prokaryote nucleotide-binding (HEPN) domains, responsible for the cleavage of ssRNA (12). The novel performance of Cas13 effectors has been used as a tool for targeting and manipulating different RNAs, although practical applications of this technique are still in their infancy (13-15).

Until recently, approaches for targeted cancer therapy using small-molecule drugs and monoclonal antibodies have become significant. However, certain complications of this approach, such as off-target effects and the limited availability of cancer-driven proteins, limit their broader application (16,17). Furthermore, molecular analysis of cancer using diagnostic methods is very expensive and is performed using sophisticated equipment (18,19). To overcome these limitations of cancer management, researchers are seeking a precise, efficient and versatile platform for targeted cancer treatment (20,21).

CRISPR-mediated genetic manipulation has expanded rapidly in recent years. RNA manipulation using the type VI CRISPR/Cas13 system is a promising tool for cancer research, diagnosis and therapy (22,23) (Fig. 1). Additionally, Cas13-based RNA detection and targeting allows for the early monitoring of tumor markers from different bodily fluids without the need for sophisticated instrumentation. Furthermore, Cas13-based RNA editing offers significant prospects for understanding drug resistance biology in addition to the discovery of novel therapeutic targets (24). Cas13 can also be mutated to dead Cas13 (dCas13), an RNA-binding effector that can be easily programmed for novel tasks. dCas13 is formed from native Cas13 by the mutation of its HEPN domain residues, which are involved in RNA cleavage (25). dCas13 has significant applications when bound to other proteins for cell imaging in its living state, RNA interaction mapping, mRNA splicing, RNA base editing, RNA methylation/demethylation and blocking other RNA-binding proteins (26,27) (Fig. 1).

The present review summarizes the recent updates on the CRISPR/Cas13 system in view of its structural, mechanistic and novel applications in cancer management. In addition, the present review will be helpful in understanding CRISPR/Cas13-related novel techniques that can be used as innovative tools for cancer diagnostics and targeted therapeutics. Furthermore, some limitations and future prospects must be addressed before this RNA-editing tool can be used more efficiently.

2. Classification of the CRISPR/Cas system

The CRISPR/Cas system in bacteria and archaea uses three stages to defend against viruses or other MGEs: Protospacer acquisition, expression and target recognition (4,28). In the first stage, a protospacer is incorporated in the host CRISPR locus as spacers between crRNA repeats (29). In the next stage, the expression of Cas proteins occurs and the spacers are transcribed as pre-crRNA repeats (1,29,30). In the third stage, the interaction between the target site of some invading viruses or other MGEs (protospacers) occurs with the help of crRNA (spacers), leading to genome cleavage (29,31). Most CRISPR/Cas systems use a sequence-specific protospacer adjacent motif adjacent to the crRNA-specific site within the target genome to distinguish self from foreign genetic elements (32).

In recent years, the number and diversity of CRISPR/Cas systems have significantly increased. The most updated classification system for this genome editing tool consists of two classes (class 1 and class 2), six types (type I to type VI) and 33 subtypes (33). The CRISPR/Cas system classification is primarily based on the composition of the Cas protein and the divergence of the sequence between the effector modules (34,35). In the class 1 system, several effector proteins are required for RNA-guided target cleavage. However, only one RNA-guided endonuclease is required in class 2 systems to cleave foreign DNA/RNA sequences (34,36,37). The class 1 system of this genome editing tool is divided into three types: Types I, III and IV, while the class 2 system is classified as types II, V and VI (34,38,39) (Fig. 2). In the type I system, the Cas3 signature gene is located at the CRISPR/Cas locus; it encodes a large protein possessing helicase activity to unwind RNA-DNA and DNA-DNA duplexes (40,41). The type II system is one of the most well-known and widely used CRISPR/Cas systems, as only a single multidomain protein targets and cleaves double-stranded DNA (dsDNA) in this system (42,43). In the type III CRISPR/Cas system, Cas10 acts as a signature gene that encodes a multidomain protein for the target detection and cleavage of single-stranded DNA (ssDNA) (44,45). The type IV CRISPR/Cas system has no Cas nucleases and integrases as typically seen in other CRISPR systems, but it does have CRISPR-associated splicing factor 1, and deeper details about its function have yet to be explored (34,46). In the type V CRISPR/Cas system, Cas12 acts as a signature gene, known as CRISPR from Prevotella and Francisella 1 (Cpf1), C2c1 (class 2, candidate 1) or C2c3 (class 2, candidate 3) proteins, that encodes the RuvC domain, which cleaves both ssDNA and dsDNA (47,48). The type VI CRISPR/Cas system contains Cas13 (C2c2), which encodes the nucleotide binding domain (HEPN) of higher eukaryotes and prokaryotes and can cleave ssRNA (49,50) (Fig. 2).

3. Type VI CRISPR/Cas system

Cas13 is a single effector protein found in the type VI CRISPR/Cas system with the ability to process crRNA, recognize invaders and trigger the immune system (25,51) (Figs. 1 and 2).

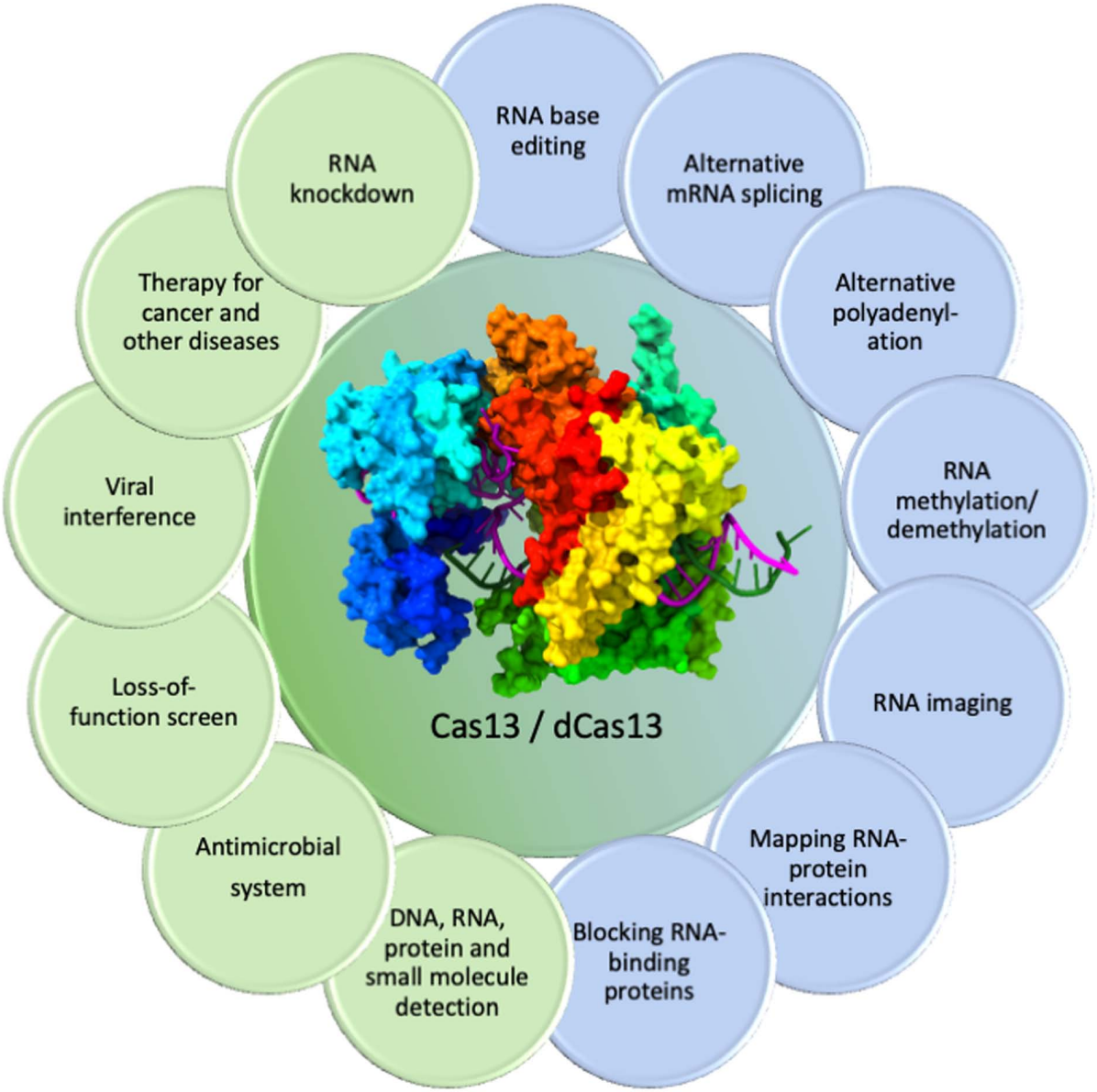

Figure 1. Some novel applications of Cas13 and dCas13 in RNA targeting and editing. Activated Cas13 is employed for different applications including disease therapeutics, RNA knockdown, viral intervention, detection of biological agents, loss-of-function screening and development of antimicrobials. The RNA sequence can also be targeted by dCas13, which is catalytically inactive and can be coupled to other proteins for the execution of live cell RNA tracking and imaging, target mRNA splicing/polyadenylation, single base editing, methylation/demethylation of target RNA, RNA-binding protein blocking and exploration of RNA-protein interactions. As an example, for Cas representation, this figure shows the ternary complex of LbuCas13a, downloaded from the PDB website https://www.rcsb.org/ (PDB ID: 5XWP) and was edited using UCSF ChimeraX, version 1.8 (https://www.rbvi.ucsf.edu/chimerax). Cas13, Clustered Regularly Interspaced Short Palindromic Repeats-associated sequence 13; dCas13, dead Cas13; PDB, protein data bank.

To date, six distinct type VI subtypes have been identified: VI-A to VI-D, Cas13X and Cas13Y (39,52) (Fig. 3). The functional characterization of the VI-A, VI-B and VI-D subtypes is now understood in more detail in relation to their respective effectors compared with the other subtypes (53). However, there are other subtypes of this system, such as Cas13e and Cas13i (54).

A single feature common to Cas13 proteins from different subtypes is the shared inclusion of two HEPN domains (39,55). This effector protein has a bilobed shape, wherein RNA nuclease activity is attributed to one lobe and RNA target recognition to the other. Cas13 can detect a target RNA in a population of non-target RNAs with femtomolar sensitivity (56). Binding of the target RNA to Cas13 induces conformational changes in the nuclease lobe, which contains two HEPN domains. This conformational change results in a stable composite RNase pocket that mediates the cleavage of the target RNA and bystander RNA (57-59), resulting in the inhibition of invading DNA viruses (60,61). The different subtypes of Cas effectors in the type VI CRISPR/Cas system are elaborated below.

CRISPR/Cas13a. Cas13a, an effector of the VI-A CRISPR/Cas system, was previously termed class 2, candidate 2 (C2c2) and was first discovered in 2015 using a computational pipeline by Shmakov *et al* (62). In contrast to the other CRISPR/

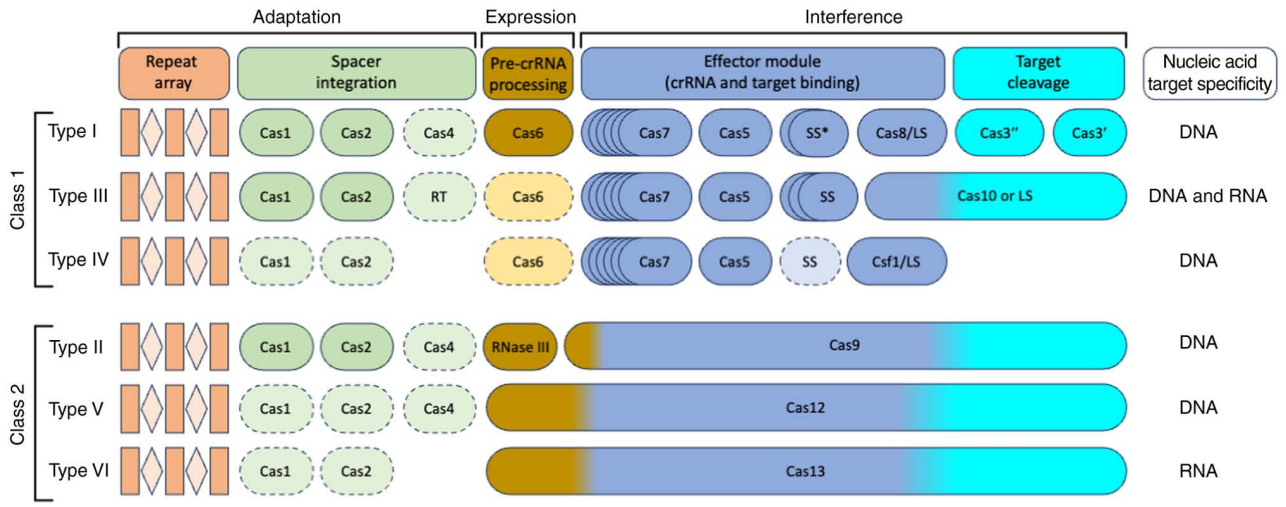

Figure 2. CRISPR/Cas system organization showing two classes and six types with genetic and functional organization. In some subtypes and variants, dashed outline areas indicate dispensable and/or missing proteins (such as Cas1 and Cas2 in the type VI CRISPR/Cas system). The gradient of three colors in Cas13 reflects that this effector contributes to different response stages of the type VI CRISPR/Cas system. * indicates SS that may bind with large subunit. SS, small subunit; LS, large subunit; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas13, CRISPR-associated sequence 13.

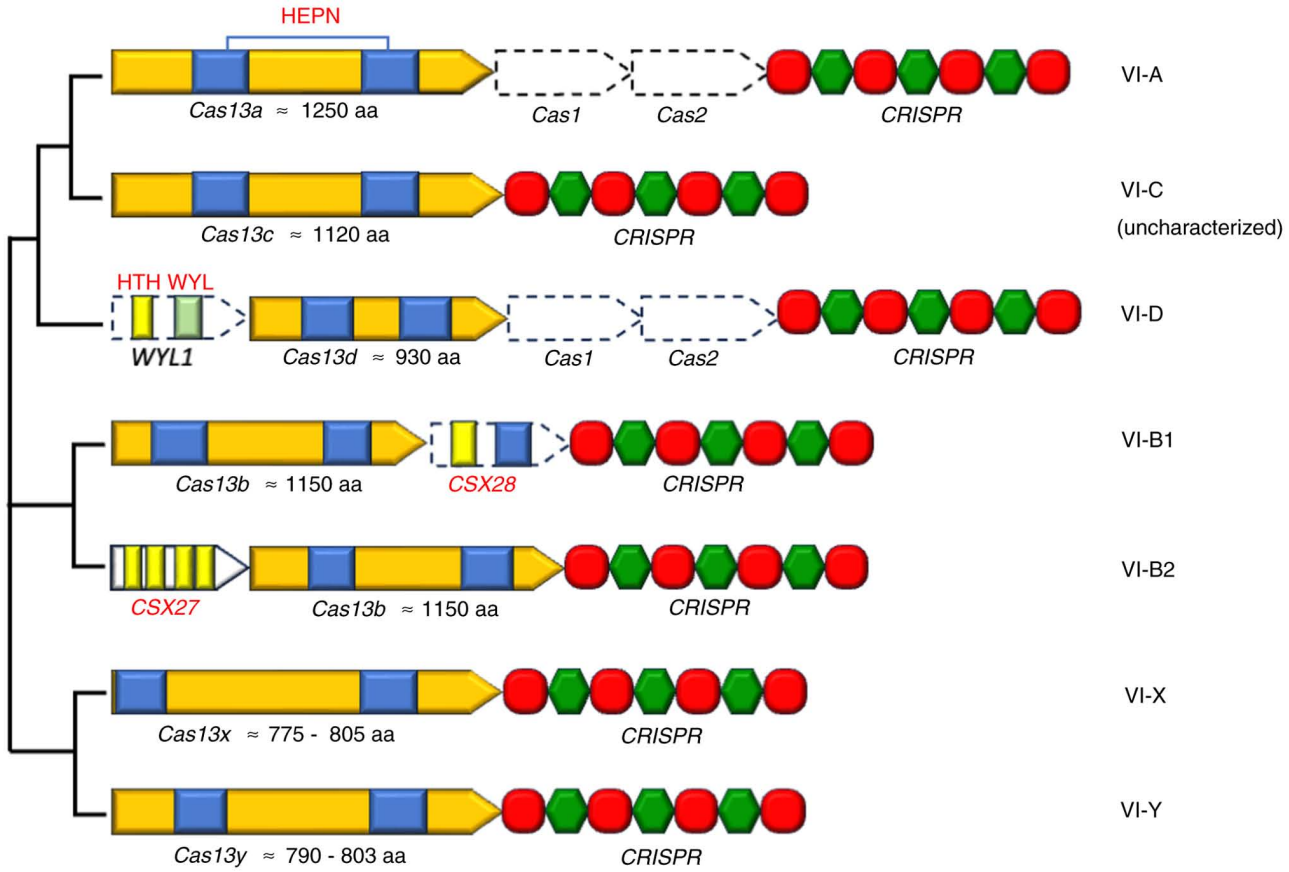

Figure 3. Genomic architecture of the type VI CRISPR/Cas locus showing different subtypes and their phylogenetic relationship. A single Cas effector protein and CRISPR locus is present in each subtype of this system. In this phylogenetic tree, different functional domains are represented by different colors. The dark blue areas in each effector protein represents the HEPN domains. The approximate number of aa in each domain for the effector proteins and its conserved domain are indicated in the gene box. aa, amino acids; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas, CRISPR-associated sequence; HEPN, higher eukaryote and prokaryote nucleotide-binding.

Cas loci, the Cas1 and Cas2 genes are absent in the type VI-A loci, but it does contain a CRISPR array along with a Cas13 gene (Fig. 3). This CRISPR array is notably unstructured and heterogeneous, with 35-39 bp direct repeats (35,62). Cas13a is currently a well-understood type VI effector protein with >1,000 amino acids, and its primary structure lacks noticeable similarity with other known Cas effectors (15,62,63). Based on cleavage preference and non-cognate crRNAs, the

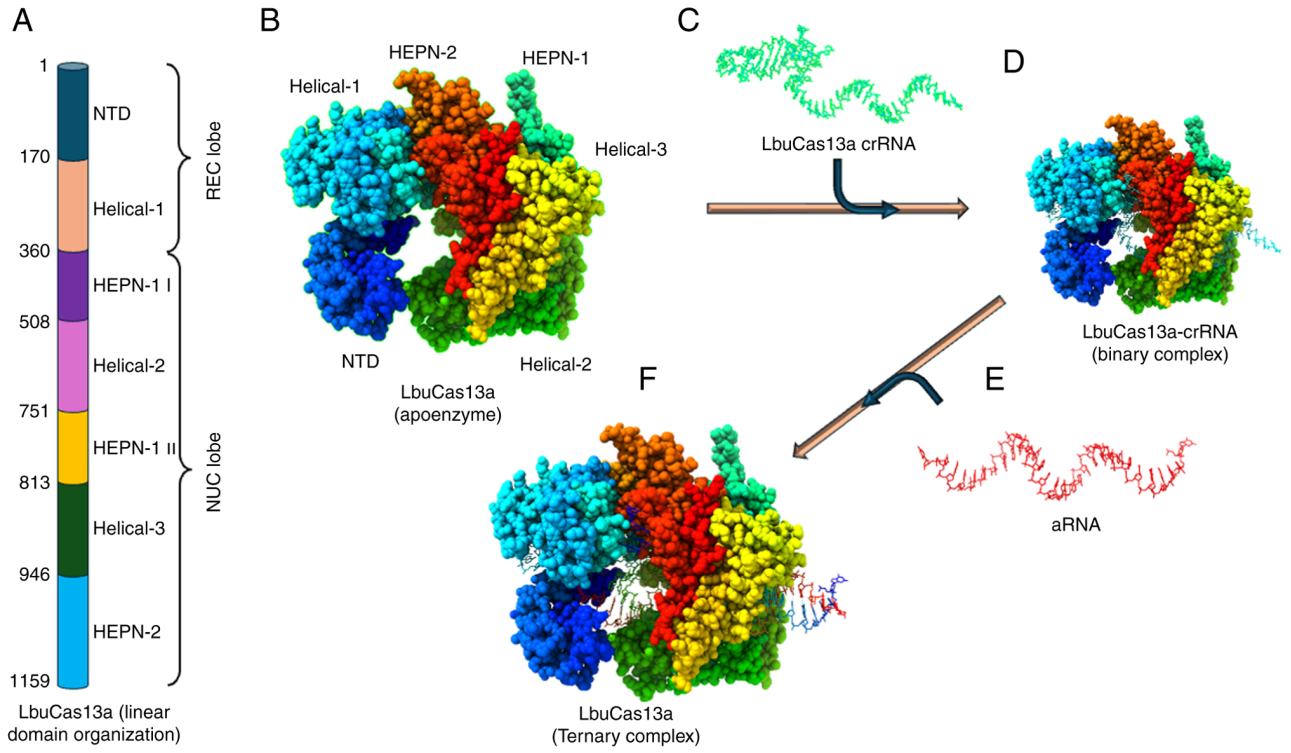

Figure 4. Basic structural features of type VI-A CRISPR/Cas effectors. (A) Linear domain organization of LbuCas13a, presenting different domains (REC and NUC lobes) annotated with the corresponding amino acid number. (B) Cartoon representation of the LbuCas13a apoenzyme. (C) Secondary structure of LbuCas13a crRNA and its nucleotide sequence. (D) Binary structure of the LbuCas13a-crRNA complex. (E) Secondary structure of aRNA. (F) LbuCas13a-crRNA-aRNA ternary complex. The crystal structure of LbuCas13a-crRNA-target RNA ternary complex was downloaded from the PDB website https://www.rcsb.org/ (PDB ID: 5XWP) and was edited using UCSF ChimeraX, version 1.8 (https://www.rbvi.ucsf.edu/chimerax). LbuCas13a, *Leptotrichia buccalis* Cas13a; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas, CRISPR-associated sequence; HEPN, higher eukaryote and prokaryote nucleotide-binding; REC, recognition; NUC, nuclease; crRNA, CRISPR RNA; aRNA, activator RNA; PDB, protein data bank; NTD, N-terminal domain.

currently recognized Cas13a orthologs are distributed as either A-cleaving or U-cleaving (64,65). pre-crRNA maturation is not essential for RNA interference; however, it can enhance activity by releasing crRNA from the CRISPR array (64).

The domain organization of several Cas13a and crRNAs in complex with activator RNA (aRNA) have been reported. Some well-known examples include Lachnospiraceae bacterium Cas13a (LbaCas13a), *Leptotrichia shahii* Cas13a (66) and *Leptotrichia buccalis* Cas13a (LbuCas13a) (51,65) (Fig. 4). Cas13a is predominantly α-helical, possessing a recognition (REC) lobe and nuclease (NUC) lobe (Fig. 4A). However, the 3D structure notably differs at the levels of architectural and domain organization (51,65,66). The REC lobe consists of a strongly basic cleft, which accommodates the repeat region of crRNA between the N-terminal domain (NTD) and Helical-1 domain (Fig. 4C and D). The NUC lobe comprises the HEPN-1, Helical-2, HEPN-2 and Helical-3 domains (Fig. 4A and B). The Helical-2 domain divides HEPN-1 further into two distinct subdomains, HEPN-1I and HEPN-1II (51,65). Cas13a undergoes a significant conformational rearrangement upon crRNA binding and forms a compact structure. This complex facilitates aRNA binding by closing the crRNA-binding channel (51) (Fig. 4E and F). In both U-cleaving and A-cleaving Cas13a effectors, recognition of the repeat region of the crRNA occurs in a sequence- and structure-specific manner. It is well known that Cas13a is a single effector protein performing the crRNA processing and interferences steps without the help from other accessory proteins. For pre-crRNA processing, critical catalytic residues have been identified for all three Cas13a orthologs, but the A-cleaving LbaCas13a presents a more detailed acid-base mechanism (51,65,66).

Cas13a uses spacers with different sequence identities and lengths (20-28 nucleotides) to successfully utilize crRNAs (64,65). The 5' region of the spacer is hidden in the NUC lobe cavity in binary complex structures (Fig. 4D) that are currently known, and it adopts a deformed conformation stabilized by significant interactions with the sugar-phosphate backbone (51,65,66). The central portion of the spacer surfaces out from the NUC lobe and passes through a groove created between the NTD and Helical-2 domains (51,65,66). In Cas13a-crRNA binary complexes, the center and 3' region maintain an almost A-form helical conformation; however, this is not apparent in all orthologs (51,53).

The Cas13a functional aspects, including aRNA binding, HEPN nuclease site activation and RNA interference, have been well-studied. Sequence interrogation and propagation of the crRNA spacer-aRNA duplex begins when a possible aRNA binds to the central seed region of the crRNA (57,65). To further stabilize the duplex, base pairing between aRNA and the remaining spacer is necessary (57). Sequence interrogation and propagation of the crRNA spacer-aRNA duplex eventually leads to synergistic conformational changes in both crRNA and Cas13a, thus forming a ternary complex

with a fully active HEPN nuclease position that is prepared to interfere with RNA (Fig. 4F). Exposure of the hidden R-X_4-H domain of the HEPN-1 region towards the exterior shifts it closer to the R-X_4-H domain in the HEPN-2 region, which activates the HEPN nuclease active site, permitting interactions within the NUC duplex lobe and widening the binding channel to accommodate the crRNA-aRNA duplex (51,57,65,66). The LbuCas13a binary complex and its ternary complex provide the most pertinent insights into these conformational changes (51).

The aRNA-bound spacer of crRNA in the ternary complex of LbuCas13a approached a normal A-form helix (51,67). The duplex base pairs were mainly in contact with the HEPN-1, Helical-2 and Helical-3 domains, while they were bound inside the positively charged central channel of the NUC lobe. LbuCas13a primarily interacts with nucleotides 7-15 (which often correspond to the seed area and portions of the HEPN region), some nucleotides of spacer crRNAs and aRNA nucleotides (51,53). A multitude of residues for spacer interaction in LbuCas13a are important for ssRNA cleavage (51). The reason that Cas13a effectors can employ spacers of >20 nucleotides with comparable efficacy is because the duplex beyond the 24th base pair is situated outside of the protein (51,68). Cas13a effectors cleave ssRNA sequences in either cis or trans mode during RNA interference (65,69,70). A groove on the protein surface houses the HEPN active site, which is located away from the central channel where the crRNA can bind with aRNA to form a crRNA-aRNA complex. However, the catalytic centers of type II and V Cas proteins are embedded within the proteins (51,65,66). For cleavage in trans, any RNA in solution can readily approach the catalytic center; however, for cleavage in cis, the effector-bound aRNA must be sufficiently long.

The structure of the LbuCas13a ternary complex demonstrated how RNA interacts with HEPN nuclease residues during interference (Fig. 4F and G). This interaction is performed by the nucleotide at the 5' terminal of aRNA swinging away from the crRNA-activator RNA duplex and being inserted into the HEPN active center of the adjacent LbuCas13a (65,71). An HEPN-1 β-hairpin stretches into the main groove of the crRNA-aRNA duplex and contacts it through some weak interactions and catches RNA in the immediate region of the catalytic site (51,72). Its importance in capturing ssRNA is demonstrated by the reduction in both trans and cis RNA intrusion that result from interaction with the 5' nucleotide of aRNA (51).

CRISPR/Cas13b. In 2016, new computational procedures such as position-specific iterative basic local alignment search tool and hidden Markov models prediction, in addition to the ideas from previous views and no firm requirement for cas1 and cas2 identification in this type, led to the discovery of Cas13b. Cas13b consists of 1,100-1,200 amino acid effectors of type VI-B systems (62,73). This subtype was found in bacteria supported by other CRISPR/Cas systems, getting support from cas1 and cas2 genes. This suggests that the VI-B platform acquire spacers in trans (73).

Type VI-B systems have several unique features: i) The orientation of the processed mature crRNA is different from that of the Cas13a and Cas13d crRNAs (73); ii) the size (36 nucleotides) of crRNA direct repeat sequences (DRs) are all conserved; iii) the 5' non-C and the 3' NNA or NAN protospacer flanking sequences (PFSs) limit the interference caused by Cas13b; iv) a small accessory protein (~200 amino acids), either Csx27 or Csx28, controls the interference of Cas13b; and v) ssRNA cleaves at the pyrimidine base, preferring to cleave uracil. Csx28 boosts Cas13b-mediated cleavage, which is routinely observed in subtype VI-B2 systems, whereas Csx27 represses Cas13b-mediated cleavage. Csx27 is infrequently present in subtype VI-B1 systems. Both accessory proteins possess the potential to regulate orthogonal type VI-B systems, thereby increasing their potential applications (73,74).

The general domain organization and mechanistic action of Cas13b have recently been identified by determining the structures of subtype VI-B1 *Bergeyella zoohelcum* (Bz) (Fig. 5) and subtype VI-B2 *Prevotella buccae* (Pbu) Cas13b-crRNA binary complexes (75-77). Cas13b adopts a distinct pyramidal shape in both configurations with a positively charged central cavity that holds the crRNA. The binary complex of Cas13b-crRNA lacks a bilobed architecture, and its domain organization is notably different from that of other type VI effectors (75,76). The two termini of the protein consist of two domains, HEPN-1 and HEPN-2, placing the two R-X_4-H motifs relatively close to each other (Fig. 5B and C) (75,76). A sizable positively charged channel connecting the solvent and the internal cavity was found at the base of the pyramidal structures of both PbuCas13b and BzCas13b (75,76). The Helical-1 and Helical-2 regions surround this channel in BzCas13b and extend to resemble pincers (Fig. 5B and C) (75). However, the purpose of these pincers, which are specific to PbuCas13b, is not fully understood. Cas13b recognizes its specific crRNA, resembling an L-shaped architecture, with the 3' segment of the spacer region and the bulk of the repeat region protected inside the protein (75). The twisted hairpin loop (36 nucleotide repeat region) is almost perpendicular to the spacer direction. The repeat region has four distinct subregions: Stem-1, stem-2, internal loop and U-rich loop. Watson-Crick base pairing and multiple H-bonds are responsible for maintaining intramolecular contacts (75,76) Additionally, crRNAs have several flipped nucleotides in their repeat regions. Except for HEPN-2, crRNA interacts extensively with all Cas13b domains; the majority of these interactions occur with the repeat region interacting domain-2, which includes the lid and Helical-1 III (78).

The interactions between the repeat region of crRNA and Cas13b stabilize the phosphate backbone of this RNA type, and this interaction is based more on the crRNA structure than its nucleotide sequence; however, this collaboration is ortholog-specific (75,76). The spacer region of the crRNA in BzCas13b is surrounded by different regions, such as the HEPN-1, Helical-1 and repeat region domains on one side, and the Helical-2 domain on the other (75). Due to the extreme spacer flexibility, the central region (nucleotides 9-15) is invisible (75). The nucleotide sequence between 16-22 is in the surface groove near the surface of the protein. The spacer orientation was modified in both PbuCas13b and BzCas13b in relation to RR by preventing the initial spacer nucleotide from moving, although this was achieved through different sets of contacts (75). This shows that for both Cas13b orthologs, effective activator RNA binding and spacer trajectory determination depend on the proper placement of the first spacer nucleotide. The lid protein domain processes the pre-crRNA

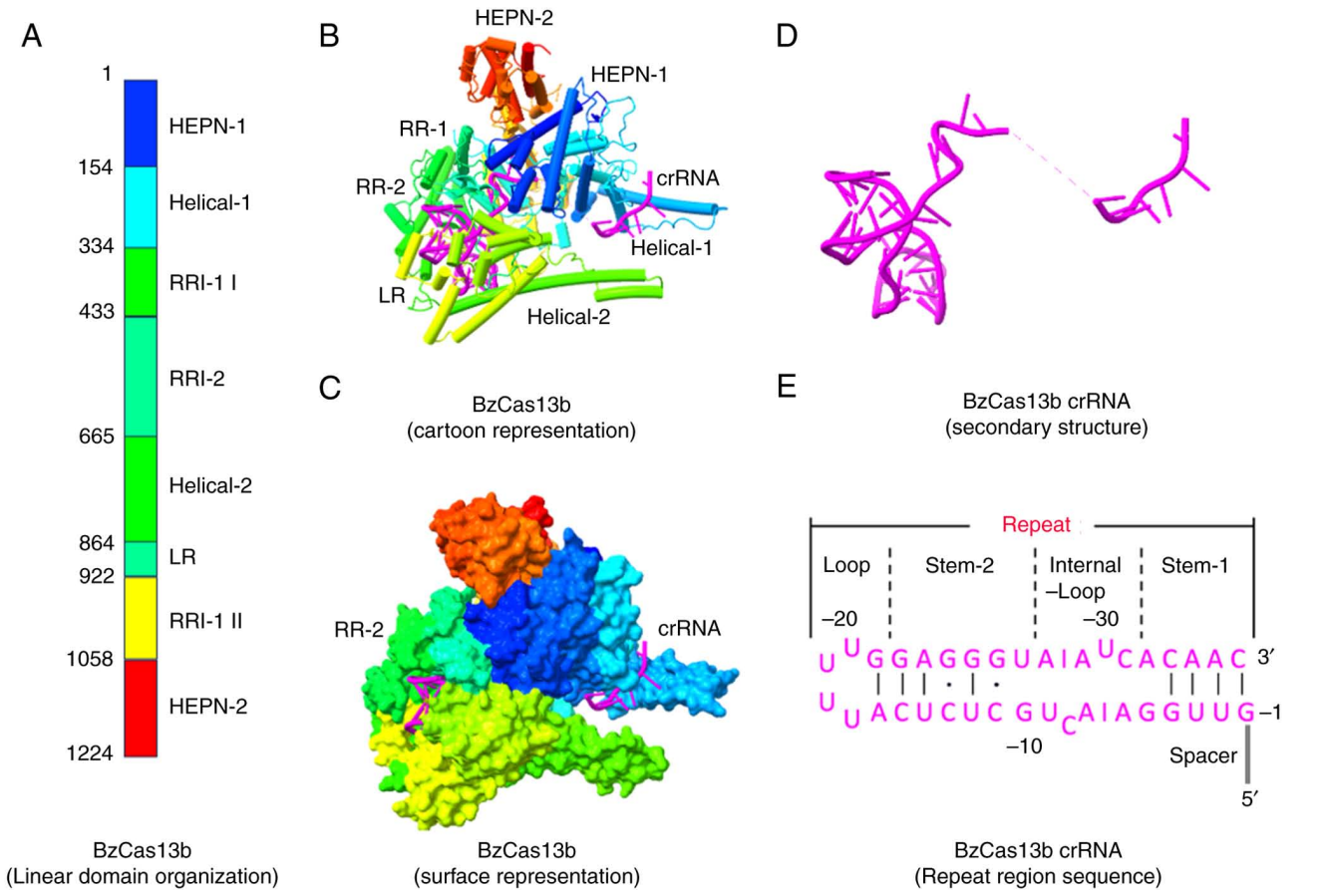

Figure 5. Diagrammatic representation of type VI-B CRISPR/Cas effectors employing the BzCas13b-crRNA binary complex. (A) Linear domain organization of BzCas13b. (B) Cartoon representation of BzCas13b. (C) Surface representation of BzCas13b. (D) secondary structure of BzCas13b crRNA. (E) BzCas13b crRNA repeat region nucleotide sequence. The crystal structure of the BzCas13b binary complex was downloaded from the PDB website https://www.rcsb.org/ (PDB ID: 6AAY) and was edited using UCSF ChimeraX, version 1.8 (https://www.rbvi.ucsf.edu/chimerax). BzCas13b, *Bergeyella zoohelcum* Cas13b; crRNA, CRISPR RNA; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas, CRISPR-associated sequence; HEPN, higher eukaryote and prokaryote nucleotide-binding; RRI, repeat region interacting domain; RR, repeat region.

towards the downstream using tightly conserved arginine and lysine residues (75,76). Nucleotide A(-37) was not recognized by catalytic residues in a base-specific way (75).

A Cas13b-based computational model was analyzed by studying the crystal structure of PbuCas13b complexed with 36-nucleotide direct repeat sequence and a 5-nucleotide spacer at 1.6 Å and illustrated aRNA binding. According to this model, Cas13b binds to crRNA and probes ssRNA at the spacer repeat region-proximal (3'-end) (76). If complementarity occurs, the HEPN-1 and Helical-2 domains open in response to the initial binding of the potential aRNA to the crRNA spacer, allowing the RNA to enter the positively charged core cavity. Prior to full conformational activation of the bipartite HEPN site, the remaining RNA sequence is scanned to complete complementarity using the spacer (76). Nevertheless, the channel that has been suggested as a pathway for aRNA to enter the central cavity is absent from the structure of the BzCas13b-crRNA binary complex (75,76). Furthermore, the narrower PbuCas13b channel might not be open enough to maintain aRNA since the interdomain linker connects the HEPN-1 and Helical-1 domains, which probably prevents the HEPN-1 domain from moving as much. Additionally, in both PbuCas13b and BzCas13b, the spacer 3' region is buried within the polypeptide chain. However, in the binary molecule BzCas13b-crRNA, the 9-15 nucleotides traverse wide solvent-reachable channel-like type VI-A systems (75). Accordingly, while tandem mismatches are intolerant across the spacer, only one mismatch is sufficient to eliminate PbuCas13a HEPN enzymatic activity (76). As a result, the spacer middle region most likely probes aRNA first, with aRNA binding then moving towards the spacer 5' and 3' ends.

CRISPR/Cas13c. The Cas13c subtype has not been well studied, mostly due to its lower efficiency in RNA targeting and interference compared with the other Cas13 subtypes (53). The Cas13c *Fusobacterium perfoetens* ortholog is mostly used in the limited investigations that have been conducted (79,80). Cas13c was initially discovered in *Fusobacterium* and *Clostridium* using bioinformatics; however, compared with the other Cas13 types, its functional characterization is far less comprehensive. With ~1,120 amino acids, this protein is identical to Cas13a in terms of locus and crRNA structure. However, like Cas13b, it lacks an adaptation module (Cas1 and Cas2). On the 5' end of the crRNA, this subtype displays a DR with a spacer size of 28-30 nucleotides (80).

CRISPR/Cas13d. To date, Cas13d is the smallest type VI effector with ~930 amino acids (10,11,81). Extending the search to smaller effectors and refining the search tactics

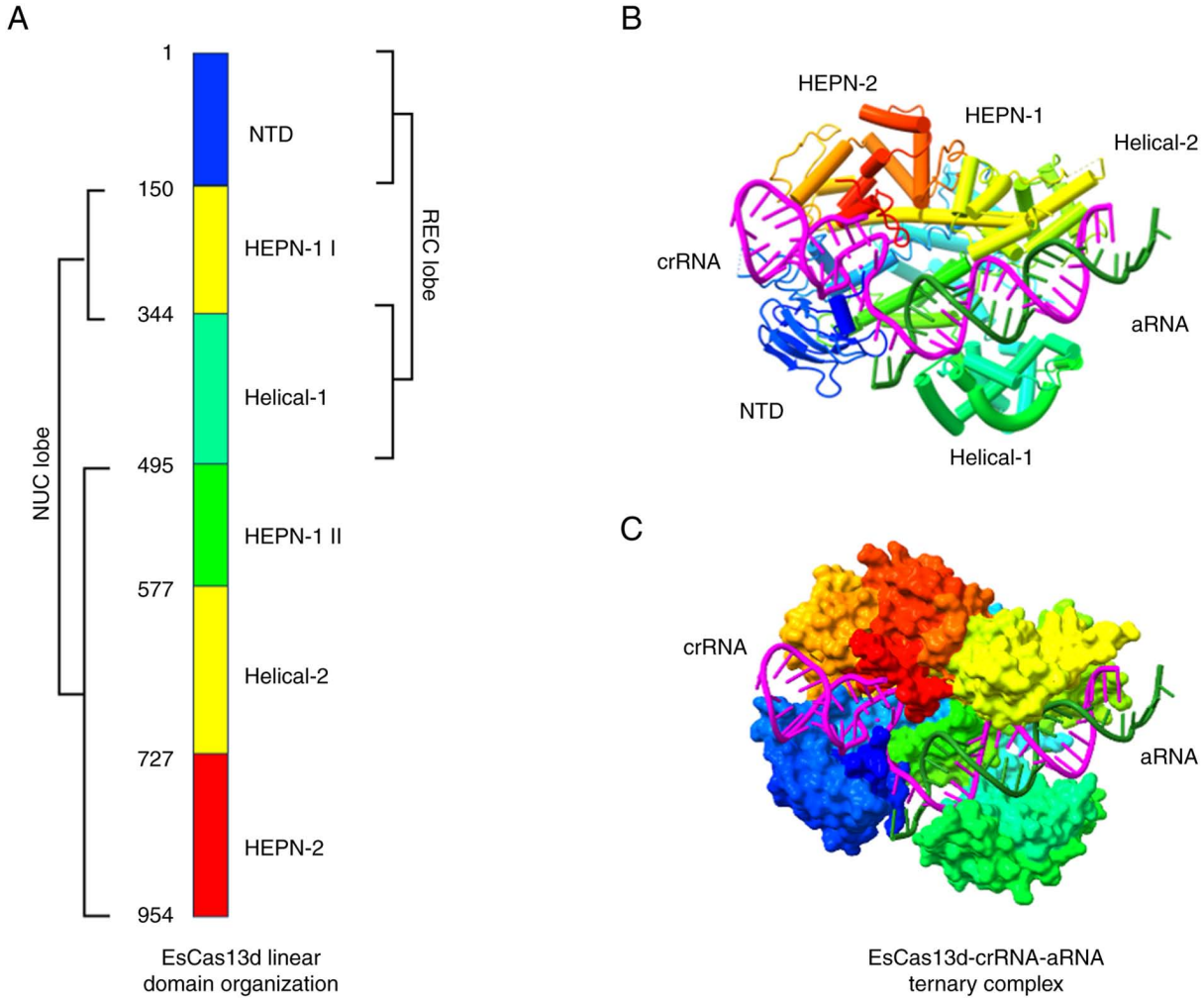

Figure 6. Diagrammatic representation of type VI-D CRISPR/Cas effectors employing the EsCas13d-crRNA-aRNA ternary complex. (A) Linear organization of EsCas13d showing the REC and NUC lobes. (B) Cartoon representation of EsCas13d presenting the position of crRNA (magenta) and aRNA (mountain green). (C) Surface representation of EsCas13d. The crystal structure of EsCas13d ternary complex was downloaded from the PDB website https://www.rcsb.org/ (PDB ID: 6E9F) and edited using UCSF ChimeraX, version 1.8 (https://www.rbvi.ucsf.edu/chimerax). EsCas13d, *Escherichia coli* Cas13d; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas, CRISPR-associated sequence; HEPN, higher eukaryote and prokaryote nucleotide-binding; REC, recognition; NUC, nuclease; crRNA, CRISPR RNA; aRNA, activator RNA; PDB, protein data bank; NTD, N-terminal domain.

of earlier computational pipelines led to the discovery of Cas13d (10,11,82). The core structure of Cas13d has only a distant relationship with Cas13a or other type-VI effectors outside the two HEPN domains (Fig. 3) (10,11,13). However, its ternary structure is similar to that of Cas13a effectors (Fig. 6).

Ruminococcus and *Eubacterium* are two benign gram-positive gut bacteria that are the source of the majority of type VI-D CRISPR/Cas loci (10,11,83,84). Type VI-D loci are characterized by notable divergence in locus organization and, in most cases, by the absence of the Cas1 or Cas2 adaptation system in their immediate proximity (Fig. 3) (10,11,85). For this effector, the repeat section in the crRNA is highly conserved, with a stem containing an A/U-rich loop (10,86). It has been observed that Cas13d targets phage DNA rather than phage RNA, as assumed earlier, which is consistent with other type VI systems (11,58).

The CRISPR array is processed by Cas13d and forms mature crRNAs with a 5'-30 nucleotide repeat region (10,13). pre-crRNA processing tests conducted *in vitro* have suggested that Cas13d does not completely process the 3' direct-repeat region. This results in truncation, producing mature crRNAs with varying spacer lengths (11). Overall, it is hypothesized that Cas13d uses a base-catalyzed method to process pre-crRNA in which the highly conserved basic residues of the HEPN-2 domain are essential (13). Metal ions are not necessary; however, their presence improves processing by increasing Cas13d binding affinity for crRNA at lower Cas13d-crRNA ratios (13,75). Similar to other effectors of Cas13, the Cas13d RNase potential is dependent on the availability of Mg^{2+} ions and spacer-matching aRNA (10,11). With little secondary structure, preference for uracil bases and no restrictions imposed by PFS, Cas13d cleaves ssRNA sequences (10,11,77). Although strong collateral non-specific RNase activity of Cas13d has been described *in vitro*, no similar activity has been observed in mammalian cells (10).

The cryo-electron microscopy structure of EsCas13d in an apo-to-ternary state exhibits a bilobed presentation (Fig. 6) (13,87,88). The Cas13d protein is composed of five domains that are separated into two lobes: The NUC lobe is composed of HEPN-1, Helical-2, Helical-1 and NTD domains,

whereas the REC lobe is composed of Helical-1 and NTD domains. The HEPN-1 domain functions as a hinge and structural scaffold that joins the two lobes (13,75). Except for the Helical-1 domain of Cas13a, all Cas13a domains have equivalents in the basic sequence Cas13d (11). However, all five protein segments are necessary for the enzymatic activity of Cas13d due to their compact size (75). Binding of crRNA leads to the stabilization of the REC segment and portions of the HEPN-2 domain. This phenomenon also causes the development of a positively charged area connecting the REC and NUC domains (75). The spacer is enclosed in an area composed of all protein segments other than the N-terminus, and the repeat region of the crRNA is fastened within the HEPN-1, HEPN-2 and NTD domains of this channel (13,75). Due to the small size of Cas13d, a portion of the repeat region stem-loop protrudes into the solvent, enabling truncation of the superfluous crRNA region in this area (13). Cas13d forms extensive contact with the crRNA repeat region, sugar-phosphate backbone and nucleobases (13,75). Conserved base-specific contacts are crucial for preserving the appropriate crRNA binding and placement (13,75).

For ssRNA cleavage, the repeat region nucleotide sequence of crRNA and its structure are important (13). The sugar-phosphate backbone plays a major role in the interaction between Cas13d and the spacer region, stabilizing its configuration and setting it up for aRNA attachment (13,75). The spacer is reordered to create an A-form RNA helix with aRNA upon engagement, eliminating most of its prior interactions with Cas13d (75). Simultaneously, new contacts are created between the crRNA spacer, aRNA and different domains of Cas13d. Cas13d undergoes multiple conformational changes from binary to ternary states, repositioning HEPN for ssRNA breakdown and placing the target RNA in the channel (75). Strong associations exist between aRNA binding and HEPN active-site activation. Partial conformational rearrangement of Cas13d requires a minimum of 18 nucleotides with strict complementarity, half-maximum cleavage activity requires 18-20 nucleotides and optimal cleavage activity requires >21 nucleotides with complementarity (34,75). It was first considered that the Cas13d crRNA spacer lacks a distinct seed region (75). Based on the available data, it appears that a mismatch tolerance exists that is likely ortholog-dependent, and a very mismatch-intolerant area inside the central 3' spacer nucleotides exists.

CRISPR/Cas13x and CRISPR/Cas13y. Cas13x (also known as Cas13bt3) and Cas13y have been identified as subtypes of Cas13b. Using metagenomic data, a recent study suggested additional categories of Cas13b exist, based on clades of the derived phylogenetic tree (54,89). Undoubtedly, the effectiveness of each subtype differs, depending on several factors. Being the smallest Cas proteins, Cas13x and Cas13y have lengths between 775 and 800 amino acids, whereas the other Cas proteins have lengths between 1,000 and 1,200 amino acids (52,85) (Fig. 7A). Furthermore, the corresponding single guide RNAs (sgRNAs) differ fundamentally from one another in terms of sequence and structure (for example, the sgRNA mounted by Cas13b has a spacer at its 5' end, whereas the sgRNA mounted by Cas13a has a spacer at its 3' end) (55). Such CRISPR/Cas13 systems have been used for a number of purposes other than their intended use in relatively recent times (71,90).

The VI-X and VI-Y families of bacteria produce all known Cas13 orthologs. These orthologs originate from hypersaline habitats, which lowers the possibility of preexisting immunity, as observed in Cas9 and Cas12 orthologs that were found in pathogenic bacterial samples that are closely related to humans (91). Additionally, to circumvent the difficulty of delivering several large Cas13-based base editors *in vivo*, Cas13x.1 has been truncated from amino acids 775 to 445 via structure-guided engineering. This allowed for the creation of a minimum RNA base editor for C-to-U or A-to-I editing of a variety of RNA loci in mammalian cells (52). Furthermore, it is anticipated that *in vivo* RNA-editing-based research and therapeutic applications will benefit from the use of these RNA editors with a compact Cas13x.1 system (92-94). The summarized differences between the various subtypes of the type VI CRISPR/Cas system are presented in Table I.

4. Innovative applications of CRISPR/Cas13 platforms for the detection of tumor-related nucleic acids and proteins

With great efficiency and precision, the CRISPR/Cas13 system can be employed for targeted cancer therapy by destroying and modifying the transcripts associated with malignancy. Furthermore, CRISPR/Cas13-based RNA editing is a valuable choice for studying the biology of drug resistance mechanisms, cancer research and the identification of new therapeutic targets (22,23,95). Recently, the identification of cancer-based RNAs, proteins and circulating tumor DNA (ctDNA) from liquid biopsy tests has been successfully performed using Cas13-powered biosensors (96). *In vitro* and *in vivo* Cas13 has also been utilized to efficiently degrade a variety of cancer-associated RNAs. Furthermore, a plethora of RNA-manipulation platforms based on the CRISPR/Cas13 system have been engineered, providing a vital toolkit for modeling, researching drug-resistance biology and identifying new therapeutic targets in cancer (97-99). The adaptability of the Cas13 systems and their applications in tumor research, therapy and diagnosis are discussed below. In addition, the benefits and drawbacks of recent research and the promise of Cas13-based techniques in the emerging fields of cancer treatment and diagnosis are discussed.

Identification of different circulating RNAs (cRNAs). Compared with single RNA marker assessment, the identification of numerous cRNAs in different biological samples provides improved specificity and sensitivity for detecting the initial stages of cancer (100-102). In this context, early-stage non-small cell lung cancer (NSCLC) detection has been performed by identifying the expression levels of two mRNAs, epidermal growth factor receptor (EGFR) and thyroid transcription factor-1, and four micro (mi)RNAs (miR-17, miR-19b, miR-155 and miR-210), based on an electrochemical biosensor provided by the CRISPR/Cas13 system (23,103).

In one biosensor, a catalytic hairpin assay was initiated by the collateral cleavage activity of Cas13, which ultimately produced a current signal. The biosensor achieved high sensitivity with a limit of detection (LOD) of 50 aM for RNA detection, thus increasing the overall signal owing to

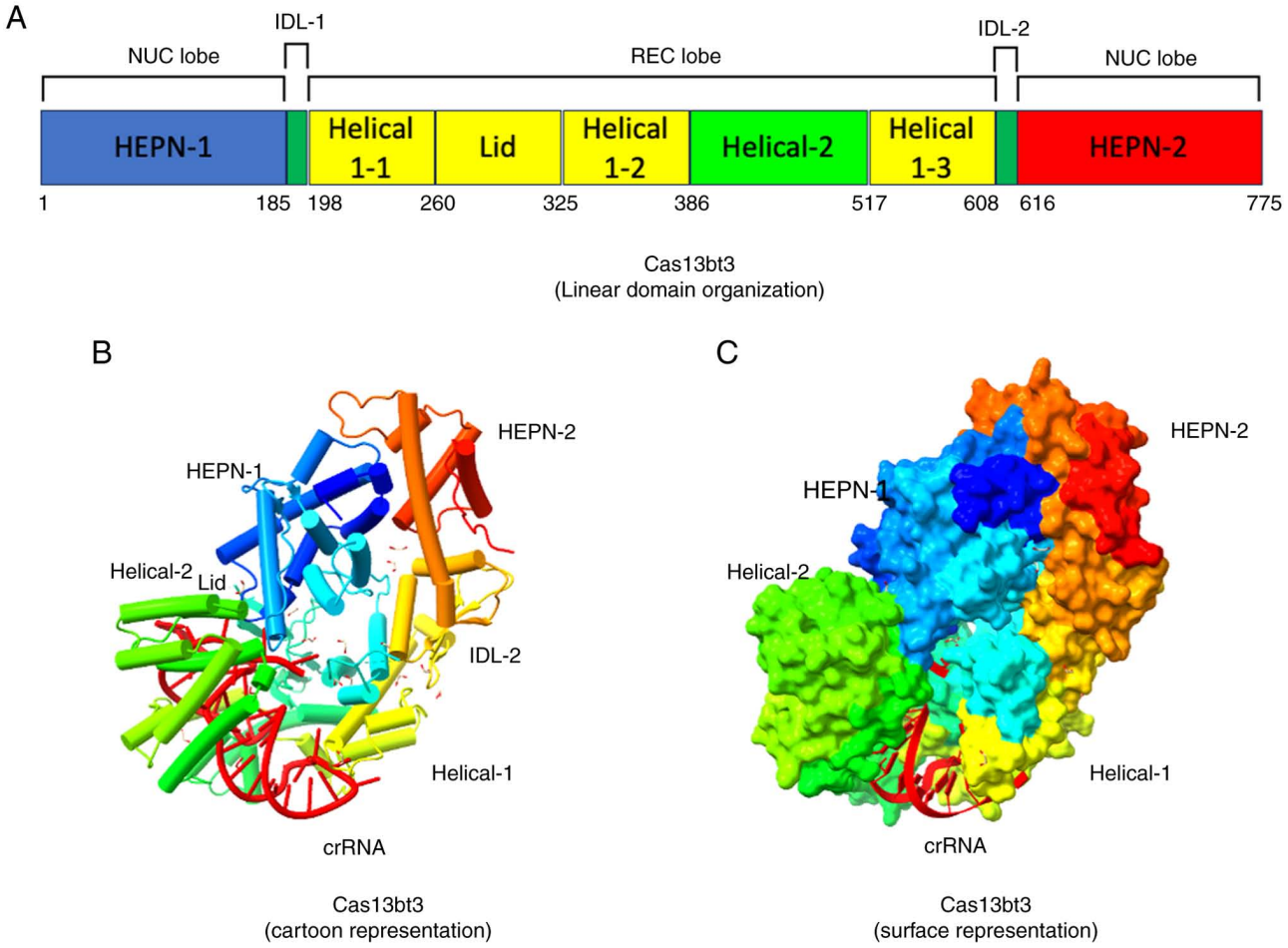

Figure 7. Structural features of the VI-X CRISPR/Cas effector binary complex. (A) Linear domain organization of Cas13bt3, presenting different domains (REC and NUC lobes) annotated with corresponding amino acid numbers. (B) Cartoon representation presenting the position of crRNA (red). (C) Surface representation of Cas13bt3. The protein structure was downloaded from the PDB website https://www.rcsb.org/ (PDB ID: 7VTI) and the binary complex was edited using UCSF ChimeraX, version 1.8 (https://www.rbvi.ucsf.edu/chimerax). Cas13bt3, *Planctomycetota bacterium* Cas13x; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas, CRISPR-associated sequence; HEPN, higher eukaryote and prokaryote nucleotide-binding; REC, recognition; NUC, nuclease; crRNA, CRISPR RNA; PDB, protein data bank.

the combined effect of both the catalytic hairpin assay and collateral RNA breakdown. The turnaround time was 36 min, and it was possible to use a single biosensor chip 37 times continuously without any sensitivity loss. Serum samples were obtained from four cohorts of patients with NSCLC: 30 healthy individuals, 12 patients with benign lung disease, 20 patients in the early stage (stages I-II) of the disease and 55 patients in the late stage (stages III-IV) of the disease. In total, six different RNA types from serum samples were selected for disease evaluation. Of the 117 samples, 114 were classified using this biosensor. Additionally, this platform demonstrated 97.3% sensitivity and 95.2% specificity in differentiating patients with NSCLC (stages I-IV) from non-cancer populations, with 90% sensitivity and 95.2% specificity in differentiating patients with initial-phase NSCLC (stages I and II). Therefore, compared with reverse transcription-quantitative PCR (RT-qPCR), the proposed biosensor offers more notable serum sample discrimination. In this study, the detection cost was estimated as $1.62 for each of the six RNAs; thus, multiple marker analysis with this biosensor is reasonably priced (22). However, this method requires two steps, which makes it less user friendly. Moreover, although the biosensor can be reused, it cannot identify multiple RNAs simultaneously. This lengthens the duration of multiple RNA analysis. However, by improving the Cas13 platform for RNA detection within the chip, a single-step technique could be developed. This platform can also be coupled with a multichannel biosensor. This research demonstrates that various RNAs in biopsy samples can be analyzed by employing Cas13-based electrochemical sensors to help diagnose cancer at the early stages. These precise and sensitive biosensors can also be utilized to track clonal evolution, therapeutic responses and tumor heterogeneity (22).

Identification of ctDNA. A promising marker for managing and diagnosing cancer is the detection of ctDNA (104,105). The concept of CRISPR-based diagnostics employing Cas13 was first presented by Zhang *et al* (106). This approach can be used in several applications, such as genotyping, the identification of cancer-based alterations in DNA samples from cellular extracts and the identification of harmful bacteria and viruses (56).

For the rapid identification of RNA or DNA sequences, the Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) platform was engineered using a recombinase

Table I. Comparison between different subtypes of type VI CRISPR/Cas systems.

Characteristics	VI-A	VI-B	VI-C	VI-D	VI-X	VI-Y
Cas effector	Cas13a	Cas13b	Cas13c	Cas13d	Cas13x	Cas13y
Size (no. of amino acids)	~1,250	~1,150	~1,121	~930	~775	~790
Cas architecture	REC and NUC lobe	Pyramidal (binary complex)	ND	REC and NUC lobe	REC and NUC lobe	ND
Location of pre-crRNA processing	Helical-1 and HEPN-2 domains	RRI-2 domain	ND	HEPN-2 domain	Not process	ND
Site of RNase activity	Surface-exposed	Surface-exposed	ND	Surface-exposed	ND	ND
Preference for ssRNA cleavage	A- and U-cleaving effectors	Pyrimidine base (mostly U)	ND	U	ND	ND
Specificity	High	High	Unclear	High	High	High
Efficiency (for KRAS), %	57.5	62.9	Low	>90.0	>90.0	>90.0
Represented by	LwaCas13a	PspCas13b	FpCas13c	CasRx	Cas13x.1	Cas13y.1
PFS	5' non-G	5' non-C, 3'NAN or NNA	ND	No restrictions	No constraints	ND
Requirement of small accessory proteins	None	Csx27 and Csx28	ND	WYL domain-containing proteins	ND	ND
crRNA orientation	5'-3'	3'-5'	ND	5'-3'	3'-5'	ND
crRNA repeat length, nucleotides	Varies, 27-32	36 (short), 88 (long)	ND	30	36	ND
Repeat architecture	Stem-loop	Distorted stem-loop	ND	Stem-loop	ND	ND
Applications	RNA knockdown and nucleic acid detection	RNA knockdown, imaging and epigenetic engineering	Unclear	RNA knockdown, imaging, epigenetic engineering and circular RNA screening	RNA knockdown and epigenetic engineering	RNA knockdown

Cas, CRISPR associated sequence; HEPN, higher eukaryotes and prokaryotes nucleotide-binding domain; KRAS, Kirsten rat sarcoma virus; LwaCas, Leptotrichia wadei Cas; PspCas, Streptococcus pyogens; RRI-2, repeat region interacting domain-2; ND, no data available; REC, recognition; NUC, nuclease; pre-crRNA, pre-CRISPR RNA; ssRNA, single-stranded RNA; U, uracil.

polymerase amplification (RPA) system and collateral RNA breakdown potential provided by Cas13 (107). SHERLOCK possesses a high sensitivity and specificity for RNA and DNA identification (Fig. 8). Cancer-related BRAFV600E and EGFR-L858R mutations have been identified using the SHERLOCK approach, and as few as 0.1% of mutant alleles were identified in mock cell-free DNA samples (Table II) (56). The sensitivity of SHERLOCK is comparable to those of qPCR and droplet digital PCR. In another study on patients with NSCLC, liquid biopsy was used to identify EGFR exon 19, EGFR-T790M and EGFR-L858R deletion changes in DNA samples using fluorescence and readout techniques (108). The SHERLOCK approach was able to significantly identify the mutant allele, even though in one patient with the EGFR-T790M

Table II. Summary of the different markers present in various cancer types and their detection by the CRISPR/Cas13-based approach.

First author/s, year	Cancer type	Sample used	Marker type and target used	Method of preamplification	Readout test, sensitivity and assay time	(Refs.)
Tian *et al*, 2021	Breast cancer	Cancer cell extract and patient serum	miRNA (miR-17 and miR-21)	Free of amplification	Fluorescence, 100 aM and 60 min	(156)
Shan *et al*, 2019	Breast cancer	Mock serum, cancer cell extract and tumor tissue	miRNA (miR-17)	Amplification-free	Fluorescence, 450 fM and 30 min	(14)
Zhou *et al*, 2021	Breast cancer	Cancer cell extract and mock serum	miRNA (miR-10b)	Rolling circle amplification	Colorimetric, 1 fM and 120 min	(157)
Sha *et al*, 2021	Breast cancer	Cancer cell extract, mock serum and patient serum	miRNA (miR-17)	Amplification free	Fluorescence, 1.33 fM and 125 min	(158)
Zhou *et al*, 2020	Breast cancer	Cancer cell extract	miRNA (miR-17)	Isothermal exponential amplification	Electrochemilumi nescence, 1 fM and 90 min	(112)
Cui *et al*, 2021	Breast cancer	Mock serum	miRNA (miR-21)	Catalytic hairpin assembly	Electrochemical, 2.6 fM and 150 min	(159)
Huang *et al*, 2020	Breast cancer	Cancer cell extract and mock serum	miRNA (miR-17)	Hyper-branching rolling circle amplification	Fluorescence, 200 aM and 170 min	(160)
Abudayyeh *et al*, 2016	NSCLC	Mock serum	ctDNA (EGFR, L858R and BRAF-V600E)	RPA and T7 transcription	Fluorescence, 0.1% of mutant allele and 150 min	(25)
He *et al*, 2020	NSCLC	Patient serum	Protein (serum exosomal PD-L1)	T7 and RPA transcription	Fluorescence, 10 particles/ml and 60 min	(120)
Gootenberg *et al*, 2018	NSCLC	Patient serum	ctDNA (EGFR-T790M, EGFR L858R and EGFR exon 19 deletion)	T7 and RPA transcription	Fluorescence lateral-flow, 0.6% of mutant allele and 60-90 min	(108)
Sheng *et al*, 2021	NSCLC	Patient serum	miRNA and mRNA (miR-17, miR-19b, miR-155, miR-210 and TTF-1 and EGFR mRNAs)	Catalytic hairpin assembly	Electrochemical, 50 aM and 36 min	(103)
Bruch *et al*, 2021	Medullo-blastoma	-	miRNA (miR-19b and miR-20a)	Amplification-free	Electrochemical 10 nM (on-chip), 210 min	(114)
Bruch *et al* 2019	Medullo-blastoma	Patient serum	miRNA (miR-19b and miR-20a)	Amplification-free	Electrochemical, 10 pM (off-chip), 2.2 nM (on-chip) and 210 min	(113)

BRAF, B-Raf proto-oncogene; ctDNA, circulating tumor DNA; EGFR, epidermal growth factor receptor; miRNA, microRNA; NSCLC, non-small cell lung cancer; PD-L1, programmed death-ligand 1; RPA, recombinase polymerase amplification; TTF-1, thyroid transcription factor-1.

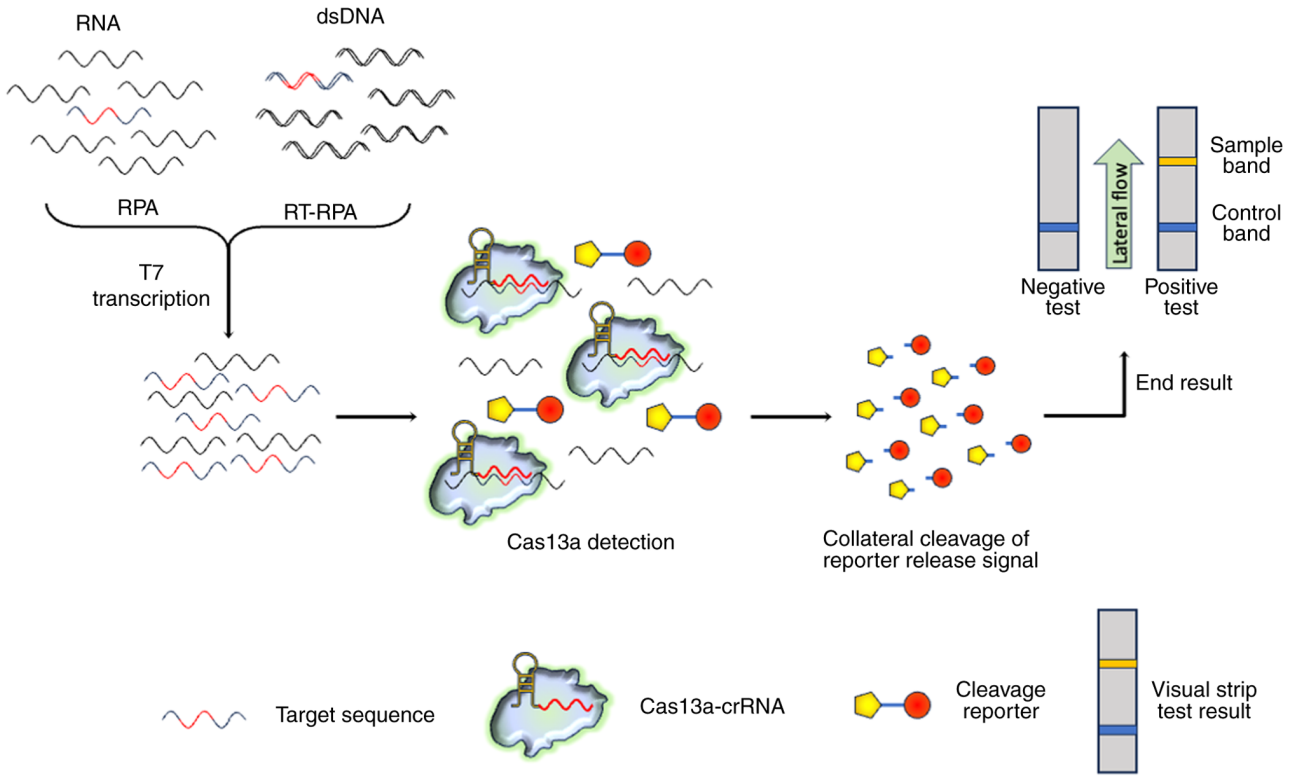

Figure 8. SHERLOCK detection assay. Assay steps in SHERLOCK are initiated by the pre-amplification of either the RNA or DNA target input. T7 transcription converts the amplified targets to RNA and the Cas13-crRNA complex detects the target RNA sequences. Activated Cas13-crRNA cleaves the fluorescent RNA reporters. SHERLOCK, Specific High-Sensitivity Enzymatic Reporter UnLOCKing; RPA, recombinase polymerase amplification; dsDNA, double-stranded DNA; RT-RPA, reverse transcriptase-recombinase polymerase amplification; crRNA, CRISPR RNA; Cas13, CRISPR-associated sequence 13.

mutation, a 0.6% ratio of mutant alleles in cell-free DNA was observed (108). SHERLOCK provides attomolar sensitivity and single-base specificity for the detection of DNA and RNA in clinical samples (97,109). This approach works well with quick and easy techniques for the isolation of nucleic acids (56). Additionally, each SHERLOCK reagent costs less than $0.60 per test and can be utilized in one-step reactions after freeze-drying (56). Moreover, orthogonal Cas13 enzymes can identify several tumor-related nucleotide changes in a single tube (108). A thorough procedure that encompasses the LwCas13a enzyme purification step has been previously suggested for the SHERLOCK assay (97). In conclusion, extremely sensitive and specific ctDNA identification can be achieved at cheaper rates without the need for complex instrumentation with Cas13-mediated detection.

Identification of circulating miRNA. The identification of tumors at early stages by the detection of circulating miRNAs represents an innovative strategy for cancer diagnosis (110,111). The development of fast, affordable, sensitive and targeted miRNA detection based on CRISPR systems may help make cancer screening more common and enable early detection. Compared with other CRISPR-associated enzymes utilized in nucleic acid detection, Cas13 targets ssRNA directly and, when activated, it demonstrates collateral ssRNA cleavage (15). Cas13 can be employed for RNA detection without the need for reverse transcription or nucleic acid amplification, as shown in Fig. 8. In this context, a one-step Cas13a-based miRNA detection technique was designed to allow for amplification-free measurement of miRNAs with a sensitivity of 450 fM and single-base specificity. In this procedure, miR-17 was measured in the total RNA from mock serum samples, cancer cell lines and tumor tissues from patients with mammary cancer to highlight the potential of this fluorescent-mediated technique (14). The Cas13-based approach showed a potential increase in miR-17 levels in 18 out of 20 cancer tests, and the results were comparable to those of RT-qPCR. According to this study, tissue and liquid biopsy samples can be used to test for cancer using the Cas13-based miRNA detection approach. To improve the sensitivity of the original technique, Zhou *et al* (112) developed several novel biosensors based on this innovative approach (Table II).

A Cas13-mediated biosensor has been designed to identify miR-19b and miR-20, which are elevated in patients with medulloblastoma (113). In this assay, the binding of miRNA-activates the Cas13 endonuclease. The FAM-tagged reporter ssRNA is cleaved by active Cas13, leading to the unbound washing out of glucose oxidase labeled with an anti-FAM antibody. Glucose oxidase generates H_2O_2 in electrochemical cells when the target miRNA is absent from the sample. Consequently, the presence of the sample led to a decrease in the signal. The instrument allowed the testing of 10 pM miR-19b and miR-20a when the Cas13-based miRNA detection phase was carried out outside the chip. Patients with medulloblastoma in both a full remission state and a progressive state could be identified by the biosensor. Furthermore, for the additional optimization of this technique to achieve multiway detection, a new biosensor was designed to simultaneously detect up to eight miRNAs (114).

This led to the successful detection of miR-20a and miR-19b of up to a10 nM range. These studies demonstrated the role of Cas13-based electrochemical miRNA sensors as potential tools for cancer diagnosis.

Detection of liquid biopsy proteins. Extracellular vesicles known as Exosomes are released by various cell types including cancer cells (115). These vesicles are present in bodily fluids such as saliva, blood and urine, and are composed of lipids, proteins and nucleic acids. Exosomes are intriguing candidates for liquid biopsy investigations in cancer diagnosis (116). In particular, several exosomal proteins have been identified for the identification of cancer in its early stages, tracking the disease course and estimating the prognosis (117,118).

Traditional protein analysis techniques, such as mass spectrometry, ELISA and immunoblotting, require considerable processing time, labor and expensive equipment. These techniques also require several purification steps. New protein analysis techniques have been developed to identify exosomal proteins, including nanoplasmonic exosome sensors and flow cytometry. However, these techniques are costly and require specialized equipment (119). To overcome these complications, RPA- and Cas13-mediated signaling has been performed using DNA aptamer-based protein identification to engineer an exosomal protein detection biosensor (120). This biosensor was devised to identify exosomal programmed death ligand 1 (PD-L1), an important biomarker for monitoring immune-based therapies. Exosomes were extracted from the blood of patients using a mixture of antibodies. The exosomes that were bound were mixed with DNA-based aptamers that exclusively attached to PD-L1. After the unbound aptamers were removed by washing, aptamers bound to PD-L1 were removed, and their amplification was performed using RPA and T7 to produce RNAs. This triggered Cas13a to break the reporter ssRNAs based on fluorescence detection. This approach resulted in an LOD of 10 particles/ml, and the detection range was notably more precise than that of ELISA for identifying minute quantities of exosomal PD-L1. The researchers followed a treatment plan with anti-PD-1 therapy for non-progressive and progressive NSCLC. The PD-L1 levels were examined in two groups of patients, and the biosensor verified that only the progressive disease patient group showed a statistically significant increase in exosomal PD-L1 expression.

In a different study, Chen *et al* (121) designed an immunosorbent assay driven by Cas13 that could detect proteins at a femtomolar level and was at least 100 times more sensitive than regular ELISA testing. This technique is based on the idea that when a target antigen is present in the sample, it attaches to a dsDNA sample and adheres to the well via a sensing antibody. The dsDNA template is transcriptionally transcribed by T7 to produce RNA copies, which trigger Cas13 to break the ssRNA, a fluorescence-based reporter. This procedure is called the CRISPR/Cas13a signal amplification linked immunosorbent assay (CLISA). The detection range of CLISA is very high, and the detection range for VEGF and IL-6 was shown to be 0.81 and 2.29 fM, respectively (121) (Table II). According to these experiments, tumor marker protein detection techniques with Cas13-enhanced protein detection can be carried out quickly, quantitatively, sensibly and specifically, without the requirement for sophisticated equipment. By facilitating biopsy analysis for tumor detection and therapeutic strategies, these techniques can improve treatment outcomes and enable the initial stage of tumor diagnosis.

Although, the CRISPR/Cas13 system shows great promise for cancer diagnostics, a fully integrated Cas13-based biosensor has not yet been engineered. A multiplex hypothetical biosensor for the diagnosis of NSCLC-based nucleic acid detection has been proposed for future cancer detection (22) (Fig. 9). This biosensor can extract nucleic acids from whole blood, which are then divided into multiple channels for detection by Cas13 present in the detection panel of the biosensor. This biosensor model was proposed to facilitate the initial detection of cancer and to provide an overview of the response to treatment therapy (22).

5. Delivery and expressional approaches of CRISPR/Cas13 in cancer cells

The targeting of Cas13 expression in cancer cells holds great promise; however, careful consideration of certain factors such as off-target effects, immune response, tumor heterogeneity and delivery challenges is crucial to maximize the therapeutic efficacy. Therefore, Cas13-based cancer targeting can be made safer by using delivery techniques that are specific to tumor tissue or expression that is particular to cancer cells. Due to the high specificity of Cas13, tumor-specific delivery techniques and cancer cell-specific expression, studies employing CRISPR/Cas13 for cancer cell targeting have been conducted without causing harm to normal cells *in vitro* (122-124) or to normal tissues *in vivo* (125,126). The safety of CRISPR-Cas13-based RNA-targeting may be further enhanced using newly identified anti-CRISPR inhibitors of Cas13a (127,128) for the optogenetic regulation of Cas13 activity (129) or the cancer tissue-specific activation of Cas13a enzymes (130). For the selective Cas13a-mediated degradation of oncogenic mRNAs in cancer cells, Fan *et al* (131) integrated tumor-specific delivery, tumor-specific expression and laser-controlled release techniques in a well-designed study. They coupled vascular endothelial growth factor receptor 2 (VEGFR2) monoclonal antibodies with the lipid component of nanoparticles for tumor-specific delivery since invasive bladder cancer has upregulated VEGFR2 expression (132). Cas13a was expressed from human telomerase reverse transcriptase (hTERT), a synthetic promoter unique to bladder cancer (133). Using a photothermal agent, liposomes were released under laser control. By combining these strategies, intelligent liposomes were installed in the bladder and orthotopic bladder cancer tumors were effectively targeted (131).

Using promoters unique to cancer cells is another approach of cancer cell targeting. Gao *et al* (99) employed a decoy minimum promoter, which is unique to nuclear factor κB (NF-κB), to express Cas13a exclusively in cancer cells. It has been demonstrated that this promoter only permits effective transcription when NF-κB, which is upregulated in a number of malignancies, is present in high concentrations (134-136). Gao *et al* (99) demonstrated that only the tumor tissue expressed the Cas13a enzyme and that the target transcripts were exclusively broken down in the tumor tissue, even though adeno-associated viruses (AAVs) carrying

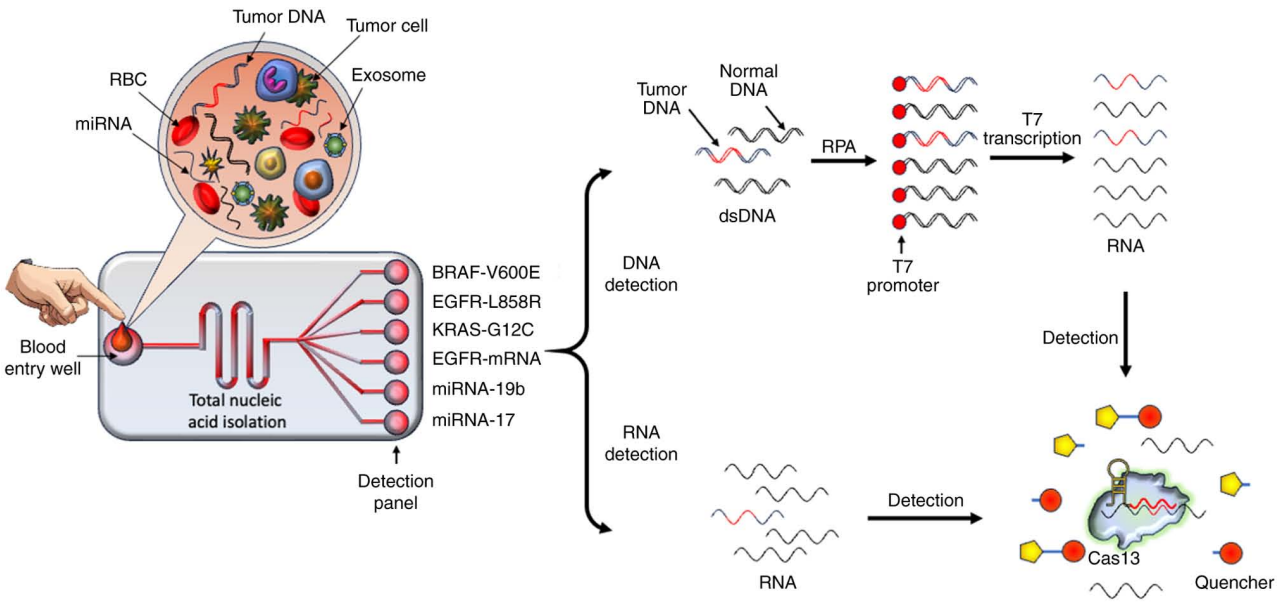

Figure 9. A proposed all-in-one Cas13-based biosensor devise for future cancer diagnosis. As a microfluidic sensor, the sample preparation and Cas13-based RNA and DNA detection steps are integrated for initial-stage NSCLC diagnosis. The patient's blood possesses cancer-derived cells, miRNAs, circulating mRNAs, tumor DNAs and exosomes. In this proposed device, the patient's blood sample is introduced into the entry well directly. Following the quick nucleic acid isolation step, the RNAs and DNAs flow within the channel and proceed to the detection panel. In this panel, each well for detection contains reagents needed to check for the NSCLC-based DNA mutation or RNA quantification. Specific RNAs are targeted directly by Cas13, which is activated in the presence of the CRISPR RNA spacer sequence. The fluorescent reporter as well as target RNA are cleaved by the activated Cas13. Additionally, cancer-associated DNAs are amplified by RPA. After the addition of T7 promoter, the transcription of RPA amplicons is performed. The mutation-based transcripts after binding with Cas13 subsequently activate it, which concomitantly cleaves the fluorescent reporter RNAs, thus providing a detectable fluorescent signal. Cas13, CRISPR-associated sequence 13; NSCLC, non-small cell lung cancer; miRNA, microRNA; RPA, recombinase polymerase amplification; RBC, red blood cell; dsDNA, double-stranded DNA.

Cas13a and crRNAs were administered intravenously to numerous tissues (99).

Zhang *et al* (124) employed chemically-locked nanoparticles containing plasmids encoding PD-L1-targeting crRNA and Cas13a. Due to the low pH and high H_2O_2 concentration found in the tumor microenvironment, these nanoparticles were engineered to only release the plasmids in tumor tissues, and the Cas13a and crRNA-encoding plasmids were primarily absorbed by cancer cells. Similarly, Jiang *et al* (125) ensured targeted delivery of Cas13d/crRNA expression cassettes to the pancreas of orthotopic tumor-bearing mice using a capsid-optimized AAV8. A study that used the CRISPR-Cas13d technology to treat age-related macular degeneration also found no *in vivo* harm (93). Together, these findings demonstrate that the RNA-targeting CRISPR/Cas13 system is highly precise and that both tumor tissue-specific viral and non-viral delivery strategies, as well as expression unique to cancer cells, can guarantee safety.

6. Pre-clinical and clinical applications of CRISPR/Cas13 for the management of cancer

In addition to the detection of different tumor related markers such as cRNAs, ctDNAs, circulating miRNAs and proteins, the CRISPR/Cas13a system has also been used in real-world clinical application for cancer therapy (22). By precisely identifying and lowering the mRNA expression of oncogenes in cancer cells, the CRISPR/Cas13a system has been used to stop tumor growth. This has resulted in regulated incidental breakage and the induction of programmed cell death in cancer cells (95). However, more research is necessary to determine whether CRISPR/Cas13a, a potent RNA knockdown technique, can be used in all types of cancer cells. The pre-clinical and clinical applications of CRISPR/Cas13 system for the management of different cancers is elaborated on below.

Pancreatic cancer. Due to its quick onset, low incidence of early diagnosis, high surgical mortality rate and low cure rate, pancreatic cancer has surpassed breast cancer; it is a very challenging task to identify and treat pancreatic cancer and this disease is expected to be the third leading cause of cancer-related death in the United States (137,138). Due to the high mortality rate of pancreatic cancer, effective novel therapeutic approaches are desperately needed (122). A mutant oncogene, known as Kirsten rat sarcoma viral oncogene homolog (KRAS), is the main cause of pancreatic cancer (139). One successful antitumor method is to suppress the expression of the KRAS mutant at the mRNA level. It is reported that the expression of mutant KRAS mRNA is markedly reduced by the bacterial Cas13a protein and crRNA (25), demonstrating that the CRISPR/Cas13a system can achieve up to 94% expressional knockdown. In several KRAS-driven pancreatic cancer models, the Cas13a-crRNA complex efficiently inhibits the KRAS-G12D mutation signaling pathway, resulting in apoptosis and tumor growth inhibition both *in vitro* and *in vivo* (140). This suggests that the CRISPR/Cas13a system can be employed as a targeted therapy for patients with pancreatic cancer harboring mutant KRAS (141). More notably, CRISPR/Cas13a knockdown of KRAS-G12D can stop the growth of pancreatic cancer cells, and CRISPR/

Cas13a-mediated mRNA downregulation can cause tumor cells to undergo apoptosis and cause marked tumor shrinkage *in vitro*. Therefore, it can be concluded that the CRISPR/Cas13a system is the most effective method for influencing the efficient and focused suppression of carcinogenic mRNAs since it is a flexible targeted therapeutic tool (22,142).

In a recent study, researchers employed an innovative approach known as Cas13a Allele-Specific PCR Enzyme Recognition (CASPER), an allele-specific PCR preamplification technique, to increase the sensitivity and specificity of detecting KRAS G12D with minimal DNA input (140). CASPER identified KRAS mutations in DNA samples obtained from patients with pancreatic cancer by ultrasound-guided fine-needle aspiration fluid; thus, implementing this approach may be a versatile and reliable method for the detection of point mutations in pancreatic cancer DNA samples.

Bladder cancer. Globally, bladder cancer is one of the most prevalent cancer types, with a high incidence and recurrence rate (143). This type of cancer had a 5-year frequency of >1.7 million cases in 2020, making it the sixth most common cancer worldwide (144). To the best of our knowledge, the use of CRISPR/Cas13a-based therapeutic approaches for intravesical instillation in bladder cancer is still unexplored, although several experimental procedures have shown effectiveness in enabling execution of CRISPR/Cas13a-based cellular functions. In one study, the researchers used a CRISPR/Cas13a nanoplatform that significantly reduced the expression of programmed death ligand 1 (PD-L1) after its intravesical instillation. The transmembrane peptide, trans-activator of transcription and CRISPR/Cas13 were genetically fused to generate the fusion protein, calpastatin (CAST). Fluorinated chitosan (FCS), a transepithelial delivery vehicle was assembled with CAST, which acted as a powerful transmembrane RNA editor. The CAST-crRNAa/FCS nanoparticles had notable transepithelial properties and markedly reduced PD-L1 expression in tumor tissues when administered intravesically into the bladder (145). The researchers used a fenbendazole (FBZ) intravesical system to increase immune activation in the tumor microenvironment. This system was constructed by bovine serum albumin encapsulation and FCS, which establishes FBZ as a potent chemo-immunological agent. This nanoformulation method reorganized the immunological microenvironment, synergistically decreased PD-L1 expression and showed marked tumor cell death in a bladder cancer model. The study suggested a possible synergistic treatment approach and revealed a unique RNA editor nano-agent formulation. This strategy greatly increased therapeutic efficacy and showed potential for the clinical application of cancer perfusion therapies based on CRISPR/Cas13 (145).

Cervical cancer. Cervical cancer is a common cancer that is a major threat to women's health and is a major global public health issue. Globally, there were 348,189 cervical cancer-related deaths and 661,021 new cases in 2022 (146). Multiple types of human papillomavirus (HPV) have been linked to cervical cancer; however, the primary causes are the high-risk HPVs, HPV16 and HPV18 (147). Cervical cancer is largely caused by the E6 and E7 oncoproteins, which are encoded by the HPV genome and lower the expression levels of the tumor suppressor proteins, p53 and retinoblastoma. This results in increased cell proliferation and decreased apoptosis, which furthers the development of cervical cancer (148). Therefore, it is anticipated that antiviral drugs that suppress the production of E6/E7 oncoproteins will be used to treat cervical cancer. According to previous research, the CRISPR/Cas13a system can efficiently and precisely knockdown HPV16/18 E6/E7 mRNA (123). The HPV16 knockdown by the CRISPR/Cas13a system markedly decreased tumor weight and volume in a subcutaneous xenograft tumor growth model. According to the aforementioned study, the ability of the CRISPR/Cas13a system to target HPV E6/E7 mRNA may make it a viable treatment option for cervical cancer linked to HPV.

Glioma. The most prevalent type of malignant brain cancer is glioblastoma multiform (GBM), with an incidence rate of 3.23 per 100,000 population globally (149). Since there are several forms of GBM and its prognosis is typically poor, research on its therapeutic approach is especially crucial (150). The CRISPR/Cas13a system can kill glioma cells that have upregulated EGFR VIII expression, a distinct mutant subtype of EGFR observed in glioma. The CRISPR/Cas13a system has been characterized by non-specific RNA cleavage, according to a single-cell RNA sequencing investigation conducted on U87-Cas13a-EGFR VIII cells (151). In addition, CRISPR/Cas13a has been shown to inhibit glioma formation in intracranial tumors. In a mouse tumor model, U87-F3-T3 cells expressing Cas13a prevented glioma cells from proliferating and growing, demonstrating CRISPR/Cas13a-based suppression (151).

7. Challenges and prospects

Understanding the scope and limitations of RNA editing by the Cas13 system and avoiding potential errors are crucial, although this system has numerous uses in clinical diagnostics, scientific research and possible therapeutic applications. For targeted RNA knockdown, high-fidelity Cas13 variants reduce the risk of unintentional collateral RNA degradation. However, recent research has revealed unexpected effects of expressing either Cas13 or gRNA alone. These unexpected effects include off-target effects, intrinsic targeting of host RNA and cellular toxicity (70). The creation of an innovative Cas13 platform for a range of uses will be made easier with an improved comprehension of these findings and the underlying mechanisms.

The prospects to enhance the specificity and minimize unintended RNA cleavage by the CRISPR/Cas13 system in the near future may be challenging, which include different novel approaches, such as i) optimizing the gRNA design: The new designs of the gRNAs can significantly improve the targeting specificity within cancer cells. This approach includes the chemical modification of nucleotides and other structural alterations to enhance the gRNA stability and reduce off-target effects. The chemical modifications into gRNAs can enhance their binding affinity and specificity for target RNA sequence (78); ii) use of high-fidelity Cas13 variants: The off-target effects of Cas13 can be further reduced by using

high-fidelity Cas13 variants. These variants are engineered to have high specificity for their target RNA sequence (152); and iii) employment of computational tools: The prediction and minimization of Cas13 off-target effects for potential gRNA-target interactions can be advanced by computational tools and algorithms (153).

Additional challenges regarding the use of the Cas13 system within the cells of interest include its delivery options. The Cas13 system may have difficulty in penetrating certain tissues or cell types (such as HeLa and 293T cells), which limits its effectiveness. Off-target effects or unintended consequences can raise ethical concerns, particularly in clinical applications. Specifically, germline editing using this RNA editing system raises ethical questions regarding the modification of future generations. Despite these limitations, ongoing research is addressing these challenges and exploring innovative approaches to improve the specificity of the Cas13 RNA-editing strategy, enhance its specificity and improve delivery approaches for potential therapeutic applications.

Optimizing the delivery of CRISPR/Cas13 to cancer cells is challenging but highly significant for enhancing its therapeutic efficacy. Some novel strategies to improve the delivery of CRISPR/Cas13 in target cells include: i) Viral vectors: CRISPR/Cas13 components can be easily delivered by AAVs and lentiviruses into target cells. These vectors can be modified to target cancer cells specifically, ensuring efficient delivery and expression of the CRISPR system (153); ii) nanoparticle-based delivery: CRISPR/Cas13 components can also be delivered efficiently in target cancer cells by utilizing nanoparticles, such as lipid nanoparticles or polymer-based nanoparticles. These nanoparticles can be engineered to target specific tumor cells, enhancing specificity and reducing off-target effects (154); iii) cell-penetrating peptides (CPPs): CPPs can facilitate the delivery of CRISPR/Cas13 components into cancer cells by enhancing cellular uptake. These peptides can be conjugated with CRISPR components to improve their intracellular delivery (152); iv) electroporation: This method involves applying an electric field to cells to increase the permeability of the cell membrane, allowing CRISPR/Cas13 components to enter the cells more effectively. It is particularly useful for *ex vivo* applications, such as modifying immune cells before reintroducing them into the patient (155); and v) targeted delivery systems: Developing targeted delivery systems that use ligands or antibodies to recognize and bind to specific receptors on cancer cells can enhance the precision of CRISPR/Cas13 delivery. This approach minimizes off-target effects and maximizes therapeutic impact (154). All these strategies collectively contribute to a more effective and precise delivery of CRISPR/Cas13 in cancer therapy, potentially leading to improved treatment outcomes.

Furthermore, the prospects of Cas13 RNA-targeting and editing approaches in the near future include the correction of genetic defects, such as in cystic fibrosis, Huntington's disease and sickle cell anemia, for proper disease management. In addition, this system could be employed to target and destroy viral RNA to combat viral infections, such as HIV and influenza, and emerging viruses, such as SARS-CoV-2. Cas13-based diagnostic tools can be developed for rapid and sensitive detection of genetic mutations, pathogens and other biomarkers.

8. Conclusions

Since its discovery in 2016, the CRISPR/Cas13 platform has revolutionized the art of RNA-based diagnostics and therapeutics, in addition to the fundamental study of RNA biology. Cas13 is a potent gene targeting tool due to its ability to precisely and efficiently target nearly any nuclear or cytoplasmic RNA sequence. The potential of CRISPR/Cas13 as a therapeutic platform is further enhanced by its small size and inherent flexibility. With its single-nucleotide accuracy and specificity, the constantly growing CRISPR/Cas13 toolset enables a wide variety of RNA modifications. Safer treatments for a variety of illnesses are made possible by the capacity to accurately modify RNAs, including substitutions or nucleotide additions, without changing DNA. However, the structural and biochemical aspects of the gene editing strategies of the CRISPR/Cas13 system are still insufficient to comprehend its thorough use in the clinical field. The prospects to enhance the specificity and minimize unintended RNA cleavage by the CRISPR/Cas13 system is highly significant but challenging. These challenges can be circumvented by using new gRNA designs, high-fidelity Cas13 variants and the employment of new computational tools. Furthermore, optimizing the delivery approaches of the CRISPR/Cas13 system into cancer cells may be utilized by engineered nanoparticles, viral vectors, CPPs, electroporation and other targeted delivery systems. We anticipate that Cas13 technologies will continue to advance, opening fascinating possibilities for RNA-based diagnostics, RNA-targeting treatments and biological research.

Acknowledgements

The researchers would like to thank the Deanship of Graduate Studies and Scientific Research at Qassim University for financial support (QU-APC-2025).

Funding

This study was supported by the Deanship of Graduate Studies and Scientific Research at Qassim University (QU-APC-2025).

Availability of data and material

Not applicable.

Authors' contributions

Conceptualization was conducted by KSA and AAK; writing the original draft was conducted by KSA, AHR and AAK; reviewing and editing the manuscript was conducted by KSA, AHR, NMA, FMA, GhadahMA, GhadeerMA and AAK; data curation was conducted by KSA, AHR, NMA, FMA, GhadahMA, GhadeerMA and AAK. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patent consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Marraffini LA: CRISPR-Cas immunity in prokaryotes. Nature 526: 55-61, 2015.
2. Allemailem KS, Alsahli MA, Almatroudi A, Alrumaihi F, Alkhaleefah FK, Rahmani AH and Khan AA: Current updates of CRISPR/Cas9-mediated genome editing and targeting within tumor cells: An innovative strategy of cancer management. Cancer Commun (Lond) 42: 1257-1287, 2022.
3. Piergentili R, Del Rio A, Signore F, Umani Ronchi F, Marinelli E and Zaami S: CRISPR-Cas and its wide-ranging applications: From human genome editing to environmental implications, technical limitations, hazards and bioethical issues. Cells 10: 969, 2021.
4. Allemailem KS, Almatroudi A, Rahmani AH, Alrumaihi F, Alradhi AE, Alsubaiyel AM, Algahtani M, Almousa RM, Mahzari A, Sindi AAA, *et al*: Recent updates of the CRISPR/Cas9 genome editing system: Novel approaches to regulate its spatiotemporal control by genetic and physicochemical strategies. Int J Nanomedicine 31: 5335-5363, 2024.
5. Meng X, Wu TG, Lou QY, Niu KY, Jiang L, Xiao QZ, Xu T and Zhang L: Optimization of CRISPR-Cas system for clinical cancer therapy. Bioeng Transl Med 8: e10474, 2022.
6. Heler R, Marraffini LA and Bikard D: Adapting to new threats: The generation of memory by CRISPR-Cas immune systems. Mol Microbial 93: 1-9, 2014.
7. Faure G, Shmakov SA, Yan WX, Cheng DR, Scott DA, Peters JE, Makarova KS and Koonin EV: CRISPR-Cas in mobile genetic elements: Counter-defence and beyond. Nat Rev Microbiol 17: 513-525, 2019.
8. Hille F, Richter H, Wong SP, Bratovič M, Ressel S and Charpentier E: The biology of CRISPR-Cas: Backward and forward. Cell 172: 1239-1259, 2018.
9. Van Der Oost J, Westra ER, Jackson RN and Wiedenheft B: Unravelling the structural and mechanistic basis of CRISPR-Cas systems. Nat Rev Microbiol 12: 479-492, 2014.
10. Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN and Hsu PD: Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors. Cell 173: 665-676.e14, 2018.
11. Yan WX, Chong S, Zhang H, Makarova KS, Koonin EV, Cheng DR and Scott DA: Cas13d is a compact RNA-targeting type VI CRISPR effector positively modulated by a WYL-domain-containing accessory protein. Mol Cell 70: 327-339.e5, 2018.
12. Zhao F, Zhang T, Sun X, Zhang X, Chen L, Wang H, Li J, Fan P, Lai L, Sui T and Li Z: A strategy for Cas13 miniaturization based on the structure and AlphaFold. Nat Commun 14: 5545, 2023.
13. Zhang B, Ye Y, Ye W, Perčulija V, Jiang H, Chen Y, Li Y, Chen J, Lin J, Wang S, *et al*: Two HEPN domains dictate CRISPR RNA maturation and target cleavage in Cas13d. Nat Commun 10: 2544, 2019.
14. Shan Y, Zhou X, Huang R and Xing D: High-fidelity and rapid quantification of miRNA combining crRNA programmability and CRISPR/Cas13a trans-cleavage activity. Anal Chem 91: 5278-5285, 2019.
15. Yang H and Patel DJ: Structures, mechanisms and applications of RNA-centric CRISPR-Cas13. Nat Chem Biol 20: 673-688, 2024.
16. Zhong L, Li Y, Xiong L, Wang W, Wu M, Yuan T, Yang W, Tian C, Miao Z, Wang T and Yang S: Small molecules in targeted cancer therapy: Advances, challenges, and future perspectives. Signal Transduct Target Ther 6: 201, 2021.
17. Allemailem KS, Almatroodi SA, Almatroudi A, Alrumaihi F, Al Abdulmonem W, Al-Megrin WAI, Aljamaan AN, Rahmani AH and Khan AA: Recent advances in genome-editing technology with CRISPR/Cas9 variants and stimuli-responsive targeting approaches within tumor cells: A future perspective of cancer management. Int J Mol Sci 24: 7052, 2023.
18. Pulumati A, Pulumati A, Dwarakanath BS, Verma A and Papineni RVL: Technological advancements in cancer diagnostics: Improvements and limitations. Cancer Rep (Hoboken) 6: e1764, 2023.
19. Wang L, Dai C, Jiang L, Tong G, Xiong Y, Khan K, Tang Z, Chen X and Zeng H: Advanced devices for tumor diagnosis and therapy. Small 17: e2100003, 2021.
20. Allemailem KS, Alsahli MA, Almatroudi A, Alrumaihi F, Al Abdulmonem W, Moawad AA, Alwanian WM, Almansour NM, Rahmani AH and Khan AA: Innovative strategies of reprogramming immune system cells by targeting CRISPR/Cas9-based genome-editing tools: A new era of cancer management. Int J Nanomedicine 18: 5531-5559, 2023.
21. Allemailem KS, Almatroudi A, Alrumaihi F, Alradhi AE, Theyab A, Algahtani M, Alhawas MO, Dobie G, Moawad AA, Rahmani AH and Khan AA: Current updates of CRISPR/Cas system and anti-CRISPR proteins: Innovative applications to improve the genome editing strategies. Int J Nanomedicine 19: 10185-10212, 2024.
22. Palaz F, Kalkan AK, Can Ö, Demir AN, Tozluyurt A, Özcan A and Ozsoz M: CRISPR-Cas13 system as a promising and versatile tool for cancer diagnosis, therapy, and research. ACS Synth Biol 10: 1245-1267, 2021.
23. Durán-Vinet B, Araya-Castro K, Calderón J, Vergara L, Weber H, Retamales J, Araya-Castro P and Leal-Rojas P: CRISPR/Cas13-based platforms for a potential next-generation diagnosis of colorectal cancer through exosomes micro-RNA detection: A review. Cancers (Basel) 13: 4640, 2021.
24. Xu D, Cai Y, Tang L, Han X, Gao F, Cao H, Qi F and Kapranov P: A CRISPR/Cas13-based approach demonstrates biological relevance of vlinc class of long non-coding RNAs in anticancer drug response. Sci Rep 10: 1794, 2020.
25. Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DBT, Shmakov S, Makarova KS, Semenova E, Minakhin L, *et al*: C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science 353: aaf5573, 2016.
26. Núñez-Álvarez Y, Espie-Caullet T, Buhagiar G, Rubio-Zulaika A, Alonso-Marañón J, Luna-Pérez E, Blazquez L and Luco RF: A CRISPR-dCas13 RNA-editing tool to study alternative splicing. Nucleic Acids Res 52: 11926-11939, 2024.
27. Apostolopoulos A, Kawamoto N, Chow SYA, Tsuiji H, Ikeuchi Y, Shichino Y and Iwasaki S: dCas13-mediated translational repression for accurate gene silencing in mammalian cells. Nat Commun 15: 2205, 2024.
28. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J and Charpentier E: CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471: 602-607, 2011.
29. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA and Horvath P: CRISPR provides acquired resistance against viruses in prokaryotes. Science 315: 1709-1712, 2007.
30. Rananaware SR, Vesco EK, Shoemaker GM, Anekar SS, Sandoval LSW, Meister KS, Macaluso NC, Nguyen LT and Jain PK: Programmable RNA detection with CRISPR-Cas12a. Nat Commun 14: 5409, 2023.
31. Allemailem KS: Recent advances in understanding the molecular mechanisms of multidrug resistance and novel approaches of CRISPR/Cas9-based genome-editing to combat this health emergency. Int J Nanomedicine 19: 1125-1143, 2024.
32. McGinn J and Marraffini LA: Molecular mechanisms of CRISPR-Cas spacer acquisition. Nat Rev Microbiol 17: 7-12, 2019.
33. Makarova KS, Wolf YI and Koonin EV: Evolutionary classification of CRISPR-Cas systems. Crispr: Biology and Applications, pp13-38, 2022.
34. Koonin EV, Makarova KS and Zhang F: Diversity, classification and evolution of CRISPR-Cas systems. Curr Opin Microbiol 37: 67-78, 2017.
35. Yan F, Wang W and Zhang J: CRISPR-Cas12 and Cas13: The lesser known siblings of CRISPR-Cas9. Cell Biol Toxicol 35: 489-492, 2019.
36. Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, Moineau S, Mojica FJ, Wolf YI, Yakunin AF, *et al*: Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol 9: 467-477, 2011.
37. Steens JA, Bravo JPK, Salazar CR, Yildiz C, Amieiro AM, Köstlbacher S, Prinsen SHP, Andres AS, Patinios C, Bardis A, *et al*: Type III-B CRISPR-Cas cascade of proteolytic cleavages. Science 383: 512-519, 2024.

38. Shabalina SA and Koonin EV: Origins and evolution of eukaryotic RNA interference. Trends Ecol Evol 23: 578-587, 2008.
39. Makarova KS, Wolf YI, Iranzo J, Shmakov SA, Alkhnbashi OS, Brouns SJJ, Charpentier E, Cheng D, Haft DH, Horvath P, *et al*: Evolutionary classification of CRISPR-Cas systems: A burst of class 2 and derived variants. Nat Rev Microbiol 18: 67-83, 2020.
40. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV and Van Der Oost J: Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 321: 960-964, 2008.
41. Chen XD, Chen Z, Wythes G, Zhang Y, Orr BC, Sun G, Chao YK, Navarro Torres A, Thao K, Vallurupalli M, *et al*: Helicase-assisted continuous editing for programmable mutagenesis of endogenous genomes. Science 386: eadn5876, 2024.
42. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA and Charpentier E: A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337: 816-821, 2012.
43. Chylinski K, Makarova KS, Charpentier E and Koonin EV: Classification and evolution of type II CRISPR-Cas systems. Nucleic Acids Res 42: 6091-6105, 2014.
44. Marraffini LA and Sontheimer EJ: CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science 322: 1843-1845, 2008.
45. Stella G and Marraffini L: Type III CRISPR-Cas: Beyond the Cas10 effector complex. Trends Biochem Sci 49: 28-37, 2024.
46. Zhou Y, Bravo JPK, Taylor HN, Steens JA, Jackson RN, Staals RHJ and Taylor DW: Structure of a type IV CRISPR-Cas ribonucleoprotein complex. iScience 24: 102201, 2021.
47. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, Van Der Oost J, Regev A, *et al*: Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 163: 759-771, 2015.
48. Gao P, Yang H, Rajashankar KR, Huang Z and Patel DJ: Type V CRISPR-Cas Cpf1 endonuclease employs a unique mechanism for crRNA-mediated target DNA recognition. Cell Res 26: 901-913, 2016.
49. Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, Cox DB, Kellner MJ, Regev A, *et al*: RNA targeting with CRISPR-Cas13. Nature 550: 280-284, 2017.
50. van Beljouw SPB, Sanders J, Rodríguez-Molina A and Brouns SJJ: RNA-targeting CRISPR-Cas systems. Nat Rev Microbiol 21: 21-34, 2023.
51. Liu L, Li X, Ma J, Li Z, You L, Wang J, Wang M, Zhang X and Wang Y: The molecular architecture for RNA-guided RNA cleavage by Cas13a. Cell 170: 714-726.e10, 2017.
52. Xu C, Zhou Y, Xiao Q, He B, Geng G, Wang Z, Cao B, Dong X, Bai W, Wang Y, *et al*: Programmable RNA editing with compact CRISPR-Cas13 systems from uncultivated microbes. Nat Methods 18: 499-506, 2021.
53. Perčulija V, Lin J, Zhang B and Ouyang S: Functional features and current applications of the RNA-targeting type VI CRISPR-Cas systems. Adv Sci (Weinh) 8: 2004685, 2021.
54. Hu Y, Chen Y, Xu J, Wang X, Luo S, Mao B, Zhou Q and Li W: Metagenomic discovery of novel CRISPR-Cas13 systems. Cell Discov 8: 107, 2022.
55. O'Connell MR: Molecular mechanisms of RNA targeting by Cas13-containing type VI CRISPR-Cas systems. J Mol Biol 431: 66-87, 2019.
56. Gootenberg JS, Abudayyeh OO, Lee JW, Essletzbichler P, Dy AJ, Joung J, Verdine V, Donghia N, Daringer NM, Freije CA, *et al*: Nucleic acid detection with CRISPR-Cas13a/C2c2. Science 356: 438-442, 2017.
57. Tambe A, East-Seletsky A, Knott GJ, Doudna JA and O'Connell MR: RNA binding and HEPN-nuclease activation are decoupled in CRISPR-Cas13a. Cell Rep 24: 1025-1036, 2018.
58. Meeske AJ, Nakandakari-Higa S and Marraffini LA: Cas13-induced cellular dormancy prevents the rise of CRISPR-resistant bacteriophage. Nature 570: 241-245, 2019.
59. Tong H, Huang J, Xiao Q, He B, Dong X, Liu Y, Yang X, Han D, Wang Z, Wang X, *et al*: High-fidelity Cas13 variants for targeted RNA degradation with minimal collateral effects. Nat Biotechnol 41: 108-119, 2023.
60. VanderWal AR, Park JU, Polevoda B, Kellogg EH and O'Connell MR: CRISPR-Csx28 forms a Cas13b-activated membrane pore required for robust CRISPR-Cas adaptive immunity. Science 380: 6643, 2023.
61. Guan J, Oromí-Bosch A, Mendoza SD, Karambelkar S, Berry J and Bondy-Denomy J: RNA targeting with CRISPR-Cas13a facilitates bacteriophage genome engineering. BioRxiv: doi: https://doi.org/10.1101/2022.02.14.480438.
62. Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, Minakhin L, Joung J, Konermann S, Severinov K, *et al*: Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. Mol Cell 60: 385-397, 2015.
63. Deng X, Osikpa E, Yang J, Oladeji SJ, Smith J, Gao X and Gao Y: Structural basis for the activation of a compact CRISPR-Cas13 nuclease. Nat Commun 14: 5845, 2023.
64. East-Seletsky A, O'Connell MR, Burstein D, Knott GJ and Doudna JA: RNA targeting by functionally orthogonal type VI-A CRISPR-Cas enzymes. Mol Cell 66: 373-383.e3, 2017.
65. Knott GJ, East-Seletsky A, Cofsky JC, Holton JM, Charles E, O'Connell MR and Doudna JA: Guide-bound structures of an RNA-targeting A-cleaving CRISPR-Cas13a enzyme. Nat Struct Mol Biol 24: 825-833, 2017.
66. Liu L, Li X, Wang J, Wang M, Chen P, Yin M, Li J, Sheng G and Wang Y: Two distant catalytic sites are responsible for C2c2 RNase activities. Cell 168: 121-134.e12, 2017.
67. Charles EJ, Kim SE, Knott GJ, Smock D, Doudna J and Savage DF: Engineering improved Cas13 effectors for targeted post-transcriptional regulation of gene expression. BioRxiv: doi: https://doi.org/10.1101/2021.05.26.445687.
68. Molina Vargas AM, Sinha S, Osborn R, Arantes PR, Patel A, Dewhurst S, Hardy DJ, Cameron A, Palermo G and O'Connell MR: New design strategies for ultra-specific CRISPR-Cas13a-based RNA detection with single-nucleotide mismatch sensitivity. Nucleic Acids Res 52: 921-939, 2024.
69. East-Seletsky A, O'Connell MR, Knight SC, Burstein D, Cate JHD, Tjian R and Doudna JA: Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. Nature 538: 270-273, 2016.
70. Ai Y, Liang D and Wilusz JE: CRISPR/Cas13 effectors have differing extents of off-target effects that limit their utility in eukaryotic cells. Nucleic Acids Res 50: e65, 2022.
71. Zhang T, Zhao Y, Ye J, Cao X, Xu C, Chen B, An H, Jiao Y, Zhang F, Yang X and Zhou G: Establishing CRISPR/Cas13a immune system conferring RNA virus resistance in both dicot and monocot plants. Plant Biotechnol J 17: 1185-1187, 2019.
72. Kick LM, Von Wrisberg MK, Runtsch LS and Schneider S: Structure and mechanism of the RNA dependent RNase Cas13a from Rhodobacter capsulatus. Commun Biol 5: 71, 2022.
73. Smargon AA, Cox DBT, Pyzocha NK, Zheng K, Slaymaker IM, Gootenberg JS, Abudayyeh OA, Essletzbichler P, Shmakov S, Makarova KS, *et al*: Cas13b is a type VI-B CRISPR-associated RNA-guided RNase differentially regulated by accessory proteins Csx27 and Csx28. Mol Cell 65: 618-630.e7, 2017.
74. VanderWal AR, Park JU, Polevoda B, Nicosia JK, Molina Vargas AM, Kellogg EH and O'Connell MR: Csx28 is a membrane pore that enhances CRISPR-Cas13b-dependent antiphage defense. Science 380: 410-415, 2023.
75. Zhang B, Ye W, Ye Y, Zhou H, Saeed AFUH, Chen J, Lin J, Perčulija V, Chen Q, Chen CJ, *et al*: Structural insights into Cas13b-guided CRISPR RNA maturation and recognition. Cell Res 28: 1198-1201, 2018.
76. Slaymaker IM, Mesa P, Kellner MJ, Kannan S, Brignole E, Koob J, Feliciano PR, Stella S, Abudayyeh OO, Gootenberg JS, *et al*: High-resolution structure of Cas13b and biochemical characterization of RNA targeting and cleavage. Cell Rep 26: 3741-3751.e5, 2019.
77. Bhoobalan-Chitty Y, Stouf M and De Paepe M: Genetic manipulation of Bacteriophage T4 utilizing the CRISPR-Cas13b system. bioRxiv: doi: https://doi.org/10.1101/2024.07.15.603621.
78. Liu L and Pei DS: Insights gained from RNA editing targeted by the CRISPR-Cas13 family. Int J Mol Sci 23: 11400, 2022.
79. Kavuri NR, Ramasamy M, Qi Y and Mandadi K: Applications of CRISPR/Cas13-based RNA editing in plants. Cells 11: 2665, 2022.
80. Umaña A and Slade DJ: CRISPR-Cas systems in *Fusobacterium*: An untapped genetic frontier. bioRxiv: doi: https://doi.org/10.1101/2022.01.21.477265.
81. Hussein M, Liu Y, Vink M, Kroon PZ, Das AT, Berkhout B and Herrera-Carrillo E: Evaluation of the effect of RNA secondary structure on Cas13d-mediated target RNA cleavage. Mol Ther Nucleic Acids 35: 102278, 2024.
82. Wessels HH, Stirn A, Méndez-Mancilla A, Kim EJ, Hart SK, Knowles DA and Sanjana NE: Prediction of on-target and off-target activity of CRISPR-Cas13d guide RNAs using deep learning. Nat Biotechnol 42: 628-637, 2024.
83. Gupta R, Ghosh A, Chakravarti R, Singh R, Ravichandiran V, Swarnakar S and Ghosh D: Cas13d: A new molecular scissor for transcriptome engineering. Front Cell Dev Biol 10: 866800, 2022.

84. Tang XZ, Tan SX, Hoon S and Yeo GW: Pre-existing adaptive immunity to the RNA-editing enzyme Cas13d in humans. Nat Med 28: 1372-1376, 2022.
85. Kordyś M, Sen R and Warkocki Z: Applications of the versatile CRISPR-Cas13 RNA targeting system. Wiley Interdiscip Rev RNA 13: e1694, 2022.
86. Liu Y, Jing P, Zhou Y, Zhang J, Shi J, Zhang M, Yang H and Fei J: The effects of length and sequence of gRNA on Cas13b and Cas13d activity in vitro and in vivo. Biotechnol J 18: e2300002, 2023.
87. Zhang C, Konermann S, Brideau NJ, Lotfy P, Wu X, Novick SJ, Strutzenberg T, Griffin PR, Hsu PD and Lyumkis D: Structural basis for the RNA-guided ribonuclease activity of CRISPR-Cas13d. Cell 175: 212-223.e17, 2018.
88. Zhang K, Zhang Z, Kang J, Chen J, Liu J, Gao N, Fan L, Zheng P, Wang Y and Sun J: CRISPR/Cas13d-mediated microbial RNA knockdown. Front Bioeng Biotechnol 8: 856, 2020.
89. Nakagawa R, Kannan S, Altae-Tran H, Takeda SN, Tomita A, Hirano H, Kusakizako T, Nishizawa T, Yamashita K, Zhang F, *et al*: Structure and engineering of the minimal type VI CRISPR-Cas13bt3. Mol Cell 82: 3178-3192.e5, 2022.
90. Mahas A, Wang Q, Marsic T and Mahfouz MM: A novel miniature CRISPR-Cas13 system for SARS-CoV-2 diagnostics. ACS Synth Biol 10: 2541-2551, 2021.
91. Mehta A and Merkel OM: Immunogenicity of Cas9 protein. J Pharm Sci 109: 62-67, 2020.
92. Kushawah G, Hernandez-Huertas L, Abugattas-Nuñez Del Prado J, Martinez-Morales JR, DeVore ML, Hassan H, Moreno-Sanchez I, Tomas-Gallardo L, Diaz-Moscoso A, Monges DE, *et al*: CRISPR-Cas13d induces efficient mRNA knockdown in animal embryos. Dev Cell 54: 805-817.e7, 2020.
93. Zhou C, Hu X, Tang C, Liu W, Wang S, Zhou Y, Zhao Q, Bo Q, Shi L, Sun X, *et al*: CasRx-mediated RNA targeting prevents choroidal neovascularization in a mouse model of age-related macular degeneration. Natl Sci Rev 7: 835-837, 2020.
94. Zhou H, Su J, Hu X, Zhou C, Li H, Chen Z, Xiao Q, Wang BO, Wu W, Sun Y, *et al*: Glia-to-neuron conversion by CRISPR-CasRx alleviates symptoms of neurological disease in mice. Cell 181: 590-603.e16, 2020.
95. Granados-Riveron JT and Aquino-Jarquin G: CRISPR-Cas13 precision transcriptome engineering in cancer. Cancer Res 78: 4107-4113, 2018.
96. Yazdi ZF, Roshannezhad S, Sharif S and Abbaszadegan MR: Recent progress in prompt molecular detection of liquid biopsy using Cas enzymes: Innovative approaches for cancer diagnosis and analysis. J Transl Med 22: 1173, 2024.
97. Kellner MJ, Koob JG, Gootenberg JS, Abudayyeh OO and Zhang F: SHERLOCK: Nucleic acid detection with CRISPR nucleases. Nat Protoc 14: 2986-3012, 2019.
98. Patchsung M, Jantarug K, Pattama A, Aphicho K, Suraritdechachai S, Meesawat P, Sappakhaw K, Leelahakorn N, Ruenkam T, Wongsatit T, *et al*: Clinical validation of a Cas13-based assay for the detection of SARS-CoV-2 RNA. Nat Biomed Eng 4: 1140-1149, 2020.
99. Gao J, Luo T, Lin N, Zhang S and Wang J: A new tool for CRISPR-Cas13a-based cancer gene therapy. Mol Ther Oncolytics 19: 79-92, 2020.
100. Usuba W, Urabe F, Yamamoto Y, Matsuzaki J, Sasaki H, Ichikawa M, Takizawa S, Aoki Y, Niida S, Kato K, *et al*: Circulating miRNA panels for specific and early detection in bladder cancer. Cancer Sci 110: 408-419, 2019.
101. Liu R, Chen X, Du Y, Yao W, Shen L, Wang C, Hu Z, Zhuang R, Ning G, Zhang C, *et al*: Serum microRNA expression profile as a biomarker in the diagnosis and prognosis of pancreatic cancer. Clin Chem 58: 610-618, 2012.
102. Shi J, Li X, Zhang F, Zhang C, Guan Q, Cao X, Zhu W, Zhang X, Cheng Y, Ou K, *et al*: Circulating lncRNAs associated with occurrence of colorectal cancer progression. Am J Cancer Res 5: 2258-2265, 2015.
103. Sheng Y, Zhang T, Zhang S, Johnston M, Zheng X, Shan Y, Liu T, Huang Z, Qian F, Xie Z, *et al*: A CRISPR/Cas13a-powered catalytic electrochemical biosensor for successive and highly sensitive RNA diagnostics. Biosens Bioelectron 178: 113027, 2021.
104. Cescon DW, Bratman SV, Chan SM and Siu LL: Circulating tumor DNA and liquid biopsy in oncology. Nat Cancer 1: 276-290, 2020.
105. Rothwell DG, Ayub M, Cook N, Thistlethwaite F, Carter L, Dean E, Smith N, Villa S, Dransfield J, Clipson A, *et al*: Utility of ctDNA to support patient selection for early phase clinical trials: The TARGET study. Nat Med 25: 738-743, 2019.
106. Zhang X, Tian Y, Xu L, Fan Z, Cao Y, Ma Y, Li H and Ren F: CRISPR/Cas13-assisted hepatitis B virus covalently closed circular DNA detection. Hepatol Int 16: 306-315, 2022.
107. Antropov DN and Stepanov GA: Molecular mechanisms underlying CRISPR/Cas-based assays for nucleic acid detection. Curr Issues Mol Biol 45: 649-662, 2023.
108. Gootenberg JS, Abudayyeh OO, Kellner MJ, Joung J, Collins JJ and Zhang F: Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. Science 360: 439-444, 2018.
109. Myhrvold C, Freije CA, Gootenberg JS, Abudayyeh OO, Metsky HC, Durbin AF, Kellner MJ, Tan AL, Paul LM, Parham LA, *et al*: Field-deployable viral diagnostics using CRISPR-Cas13. Science 360: 444-448, 2018.
110. Bianchi F, Nicassio F, Marzi M, Belloni E, Dall'Olio V, Bernard L, Pelosi G, Maisonneuve P, Veronesi G and Di Fiore PP: A serum circulating miRNA diagnostic test to identify asymptomatic high-risk individuals with early stage lung cancer. EMBO Mol Med 3: 495-503, 2011.
111. Zhang L, Xu Y, Jin X, Wang Z, Wu Y, Zhao D, Chen G, Li D, Wang X, Cao H, *et al*: A circulating miRNA signature as a diagnostic biomarker for non-invasive early detection of breast cancer. Breast Cancer Res Treat 154: 423-434, 2015.
112. Zhou T, Huang R, Huang M, Shen J, Shan Y and Xing D: CRISPR/Cas13a powered portable electrochemiluminescence chip for ultrasensitive and specific MiRNA detection. Adv Sci (Weinh) 7: 1903661, 2020.
113. Bruch R, Baaske J, Chatelle C, Meirich M, Madlener S, Weber W, Dincer C and Urban GA: CRISPR/Cas13a-powered electrochemical microfluidic biosensor for nucleic acid amplification-free miRNA diagnostics. Adv Mater 31: e1905311, 2019.
114. Bruch R, Johnston M, Kling A, Mattmüller T, Baaske J, Partel S, Madlener S, Weber W, Urban GA and Dincer C: CRISPR-powered electrochemical microfluidic multiplexed biosensor for target amplification-free miRNA diagnostics. Biosens Bioelectron 177: 112887, 2021.
115. Ruivo CF, Adem B, Silva M and Melo SA: The biology of cancer exosomes: Insights and new perspectives. Cancer Res 77: 6480-6488, 2017.
116. Contreras-Naranjo JC, Wu HJ and Ugaz VM: Microfluidics for exosome isolation and analysis: Enabling liquid biopsy for personalized medicine. Lab Chip 17: 3558-3577, 2017.
117. Li W, Li C, Zhou T, Liu X, Liu X, Li X and Chen D: Role of exosomal proteins in cancer diagnosis. Mol Cancer 16: 145, 2017.
118. Li A, Zhang T, Zheng M, Liu Y and Chen Z: Exosomal proteins as potential markers of tumor diagnosis. J Hematol Oncol 10: 175, 2017.
119. Shao H, Im H, Castro CM, Breakefield X, Weissleder R and Lee H: New technologies for analysis of extracellular vesicles. Chem Rev 118: 1917-1950, 2018.
120. He Y, Wu Y, Wang Y, Wang X, Xing S, Li H, Guo S, Yu X, Dai S, Zhang G, *et al*: Applying CRISPR/Cas13 to construct exosomal PD-L1 ultrasensitive biosensors for dynamic monitoring of tumor progression in immunotherapy. Adv Therap 3: 2000093, 2020.
121. Chen Q, Tian T, Xiong E, Wang P and Zhou X: CRISPR/Cas13a signal amplification linked immunosorbent assay for femtomolar protein detection. Anal Chem 92: 573-577, 2020.
122. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2018. CA Cancer J Clin 68: 7-30, 2018.
123. Chen Y, Jiang H, Wang T, He D, Tian R, Cui Z, Tian X, Gao Q, Ma X, Yang J, *et al*: In vitro and in vivo growth inhibition of human cervical cancer cells via human papillomavirus E6/E7 mRNAs' cleavage by CRISPR/Cas13a system. Antiviral Res 178: 104794, 2020.
124. Zhang Z, Wang Q, Liu Q, Zheng Y, Zheng C, Yi K, Zhao Y, Gu Y, Wang Y, Wang C, *et al*: Dual-locking nanoparticles disrupt the PD-1/PD-L1 pathway for efficient cancer immunotherapy. Adv Mater 31: e1905751, 2019.
125. Jiang W, Li H, Liu X, Zhang J, Li T, Liu L and Yu X: Precise and efficient silencing of mutant $Kras^{G12D}$ by CRISPR-CasRx controls pancreatic cancer progression. Theranostics 10: 11507, 2020.
126. Yue H, Huang R, Shan Y and Xing D: Delivery of Cas13a/crRNA by self-degradable black phosphorus nanosheets to specifically inhibit Mcl-1 for breast cancer therapy. J Mater Chem B 8: 11096-11106, 2020.
127. Lin P, Qin S, Pu Q, Wang Z, Wu Q, Gao P, Schettler J, Guo K, Li R, Li G, *et al*: CRISPR-Cas13 inhibitors block RNA editing in bacteria and mammalian cells. Mol Cell 78: 850-861, 2020.

128. Meeske AJ, Jia N, Cassel AK, Kozlova A, Liao J, Wiedmann M, Patel DJ and Marraffini LA: A phage-encoded anti-CRISPR enables complete evasion of type VI-A CRISPR-Cas immunity. Science 369: 54-59, 2020.
129. Bubeck F, Hoffmann MD, Harteveld Z, Aschenbrenner S, Bietz A, Waldhauer MC, Börner K, Fakhiri J, Schmelas C, Dietz L, *et al*: Engineered anti-CRISPR proteins for optogenetic control of CRISPR-Cas9. Nat Methods 15: 924-927, 2018.
130. Lee J, Mou H, Ibraheim R, Liang SQ, Liu P, Xue W and Sontheimer EJ: Tissue-restricted genome editing in vivo specified by microRNA-repressible anti-CRISPR proteins. RNA 25: 1421-1431, 2019.
131. Fan J, Liu Y, Liu L, Huang Y, Li X and Huang W: A multifunction lipid-based CRISPR-Cas13a genetic circuit delivery system for bladder cancer gene therapy. ACS Synth Biol 9: 343-355, 2020.
132. Kopparapu PK, Boorjian SA, Robinson BD, Downes M, Gudas LJ, Mongan NP and Persson JL: Expression of VEGF and its receptors VEGFR1/VEGFR2 is associated with invasiveness of bladder cancer. Anticancer Res 33: 2381-2390, 2013.
133. Liu L, Liu Y, Zhang T, Wu H, Lin M, Wang C, Zhan Y, Zhou Q, Qiao B, Sun X, *et al*: Synthetic Bax-Anti Bcl2 combination module actuated by super artificial hTERT promoter selectively inhibits malignant phenotypes of bladder cancer. J Exp Clin Cancer Res 35: 3, 2016.
134. Wang D, Tang H, Xu X, Dai W, Wu J and Wang J: Control the intracellular NF-κB activity by a sensor consisting of miRNA and decoy. Int J Biochem Cell Biol 95: 43-52, 2018.
135. Dai W, Wu J, Wang D and Wang J: Cancer gene therapy by NF-κB-activated cancer cell-specific expression of CRISPR/Cas9 targeting telomeres. Gene Ther 27: 266-280, 2020.
136. Wang D, Dai W and Wang J: A cell-specific nuclear factor-kappa B-activating gene expression strategy for delivering cancer immunotherapy. Hum Gene Ther 30: 471-484, 2019.
137. Rahib L, Wehner MR, Matrisian LM and Nead KT: Estimated projection of US cancer incidence and death to 2040. JAMA Netw Open 4: e214708, 2021.
138. Halbrook CJ, Lyssiotis CA, di Magliano MP and Maitra A: Pancreatic cancer: Advances and challenges. Cell 186: 1729-1754, 2023.
139. Linehan A, O'Reilly M, McDermott R and O'Kane GM: Targeting KRAS mutations in pancreatic cancer: Opportunities for future strategies. Front Med (Lausanne) 11: 1369136, 2024.
140. Amintas S, Cullot G, Boubaddi M, Rébillard J, Karembe L, Turcq B, Prouzet-Mauléon V, Bedel A, Moreau-Gaudry F, Cappellen D and Dabernat S: Integrating allele-specific PCR with CRISPR-Cas13a for sensitive KRAS mutation detection in pancreatic cancer. J Biol Eng 18: 53, 2024.
141. Zhao X, Liu L, Lang J, Cheng K, Wang Y, Li X, Shi J, Wang Y and Nie G: A CRISPR-Cas13a system for efficient and specific therapeutic targeting of mutant KRAS for pancreatic cancer treatment. Cancer Lett 431: 171-181, 2018.
142. Zhang Y, Li S, Li R, Qiu X, Fan T, Wang B, Zhang B and Zhang L: Advances in application of CRISPR-Cas13a system. Front Cell Infect Microbiol 14: 1291557, 2024.
143. Kluth LA, Black PC, Bochner BH, Catto J, Lerner SP, Stenzl A, Sylvester R, Vickers AJ, Xylinas E and Shariat SF: Prognostic and prediction tools in bladder cancer: A comprehensive review of the literature. Eur Urol 68: 238-253, 2015.
144. Wéber A, Vignat J, Shah R, Morgan E, Laversanne M, Nagy P, Kenessey I and Znaor A: Global burden of bladder cancer mortality in 2020 and 2040 according to GLOBOCAN estimates. World J Urol 42: 237, 2024.
145. Liang M, Wang Y, Liu L, Deng D, Yan Z, Feng L, Kong C, Li C, Li Y and Li G: Synergistic intravesical instillation for bladder cancer: CRISPR-Cas13a and fenbendazole combination therapy. J Exp Clin Cancer Res 43: 223, 2024.
146. Zhou L, Li Y, Wang H, Qin R, Han Z and Li R: Global cervical cancer elimination: Quantifying the status, progress, and gaps. BMC Med 23: 67, 2025.
147. Matsukura T and Sugase M: Pitfalls in the epidemiologic classification of human papillomavirus types associated with cervical cancer using polymerase chain reaction: Driver and passenger. Int J Gynecol Cancer 18: 1042-1050, 2008.
148. Goodwin EC and DiMaio D: Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. Proc Natl Acad Sci USA 97: 12513-12518, 2000.
149. Pellerino A, Caccese M, Padovan M, Cerretti G and Lombardi G: Epidemiology, risk factors, and prognostic factors of gliomas. Clin Transl Imaging 10: 467-475, 2022.
150. Verhaak RGW, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, *et al*: Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 17: 98-110, 2010.
151. Wang Q, Liu X, Zhou J, Yang C, Wang G, Tan Y, Wu Y, Zhang S, Yi K and Kang C: The CRISPR-Cas13a gene-editing system induces collateral cleavage of RNA in glioma cells. Adv Sci (Weinh) 6: 1901299, 2019.
152. Di Carlo E and Sorrentino C: State of the art CRISPR-based strategies for cancer diagnostics and treatment. Biomark Res 12: 156, 2024.
153. Azeez SS, Hamad RS, Hamad BK, Shekha MS and Bergsten P: Advances in CRISPR-Cas technology and its applications: Revolutionising precision medicine. Front Genome Ed 6: 1509924, 2024.
154. Chehelgerdi M, Chehelgerdi M, Khorramian-Ghahfarokhi M, Shafieizadeh M, Mahmoudi E, Eskandari F, Rashidi M, Arshi A and Mokhtari-Farsani A: Comprehensive review of CRISPR-based gene editing: mechanisms, challenges, and applications in cancer therapy. Mol Cancer 23: 9, 2024.
155. Mousavi Kahaki SA, Ebrahimzadeh N, Fahimi H and Moshiri A: Development of an optimized protocol for generating knockout cancer cell lines using the CRISPR/Cas9 system, with emphasis on transient transfection. PLoS One 19: e0310368, 2024.
156. Tian T, Shu B, Jiang Y, Ye M, Liu L, Guo Z, Han Z, Wang Z and Zhou X: An ultralocalized Cas13a assay enables universal and nucleic acid amplification-free single-molecule RNA diagnostics. ACS Nano 15: 1167-1178, 2021.
157. Zhou T, Huang M, Lin J, Huang R and Xing D: High-fidelity CRISPR/Cas13a trans-cleavage-triggered rolling circle amplified DNAzyme for visual profiling of microRNA. Anal Chem 93: 2038-2044, 2021.
158. Sha Y, Huang R, Huang M, Yue H, Shan Y, Hu J and Xing D: Cascade CRISPR/cas enables amplification-free microRNA sensing with fM-sensitivity and single-base-specificity. Chem Commun (Camb) 57: 247-250, 2021.
159. Cui Y, Fan S, Yuan Z, Song M, Hu J, Qian D, Zhen D, Li J and Zhu B: Ultrasensitive electrochemical assay for microRNA-21 based on CRISPR/Cas13a-assisted catalytic hairpin assembly. Talanta 224: 121878, 2021.
160. Huang M, Huang R, Yue H, Shan Y and Xing D: Ultrasensitive and high-specific microRNA detection using hyper-branching rolling circle amplified CRISPR/Cas13a biosensor. Sens Actuat B Chem 325: 128799, 2020.