Review

Cas9 *Cis*/*Trans* Cleavage Mechanisms: Cross-System CRISPR Insights and Diagnostic Applications

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Abstract: CRISPR-Cas system is an adaptive immune system of prokaryotes against foreign invading viruses. Type II CRISPR-Cas9 system, as the earliest of the Class II systems to be discovered and applied to gene editing, has been recently found to possess *trans*-cleavage activity with other Class II systems not long ago. In this review, we summarize the molecular mechanism of *cis*- and *trans*-cleavage of target nucleic acids by the Class II type II CRISPR-Cas system, introduce the nucleic acid detection platforms developed based on its *trans*-cleavage activity, and compare them with those developed based on the type V, type VI, and type I CRISPR-Cas systems.

Keywords: CRISPR-Cas; Cas9; *cis*-cleavage; *trans*-cleavage; diagnostics

1. Introduction

In 1987, Ishino et al. detected repetitive sequences in the *E. coli* genome and found that these repetitive sequences were spaced apart by 32 base sequences [1]. In 2002, Jansen et al. analyzed this novel DNA family by bioinformatics methods and found that it exists only in bacteria and archaea, and named the direct repeats ranging in size from 21 bp to 37 bp as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), and the genes close to the CRISPR locus as cas (CRISPR-associated) [2]. In 2005, several studies by Mojica, Bolotin and Poursel et al. indicated that spacer sequences were derived from foreign invasive phages or plasmids and found an association between CRISPR and immunity [3–5]. In 2009, Mojica et al. found that proto-spacer adjacent motifs (PAM) sequences provide targets for CRISPR-Cas to play a role in recognition [6]. In 2011, a study by Deltcheva et al. revealed that tracrRNA (*trans*-activating CRISPR RNA) has a 24-nucleotide sequence complementary to the repeat region of pre-crRNA (precursor CRISPR RNA) and directs the maturation of crRNA through the involvement of RNase III and Csn1 [7]. In 2012, Jinek et al. discovered that CRISPR-Cas9 can be guided by RNA and used as a gene-editing tool [8]. Since then, there has been an outbreak of research on the CRISPR-Cas system. The discovery that the CRISPR-Cas system can be utilized as a gene-editing tool is groundbreaking and heralds a new era in genomic engineering. CRISPR-Cas9, the first CRISPR-Cas gene editing system to be developed, has been widely and intensively studied since its successful application to mammalian cells in 2013 [9–12]. As a revolutionary gene editing technology, the RNA-guided CRISPR-Cas9 system has become an indispensable tool in the field of gene editing and has been widely used in basic research, medical therapeutics, and agricultural biotechnology, among others, and its potential and impact are still expanding.

As an adaptive immune defense system used by bacteria and archaea to resist the invasion of mobile genetic elements (MGEs), the CRISPR-Cas system utilizes RNA-guided nucleic acid targeting and degradation consisting of three phases: adaptation, expression, and interference [13,14]. CRISPR systems can be categorized into two broad classes. Class I systems have effector complexes composed of multiple Cas protein subunits, including Type I, III, IV and VII. The effector complexes of class II systems are single multi-domain proteins, including type II, V, and VI types. Based on the structure of the CRISPR-Cas locus and the evolutionary relationships of Cas9, type II CRISPR systems are further classified into subtypes II-A, II-B, II-C and II-D [15–20]. Cas9 proteins range in size from 700 amino acids to greater than 1700 amino acids [21]. II-A is characterized by the inclusion of the csn2 gene, II-B is characterized by the long and most diverged Cas9 variants and the inclusion of the cas4 gene, II-C is characterized by the inclusion of only Cas9, Cas1 and Cas2, and II-D is characterized by the inclusion of the Zinc-finger motifs and high arginine content [15,16,20]. The CRISPR-Cas9 system exhibits notable diversity across

bacterial species, with distinct orthologues categorized into specific subtypes based on structural and functional characteristics. Among them, Cas9 orthologue from *Streptococcus pyogenes* (SpyCas9) and *Staphylococcus aureus* (SaCas9) are typical representatives of type II-A [22–25]. Type II-B systems are exemplified by the *Francisella novicida* Cas9 orthologue (FnCas9) [26–28], while type II-C systems feature *Neisseria meningitidis* Cas9 (NmeCas9) and *Campylobacter jejuni* Cas9 (CjCas9) as characteristic members [29,30]. A representative of the recently identified type II-D is the Cas9d-MG34-1, a compact CRISPR-Cas9 nuclease discovered from macrogenomic data [31]. Among these, SpyCas9 has emerged as the predominant model system for CRISPR-based genome engineering applications. As one of the earliest Cas9 proteins to be widely studied and adapted for CRISPR gene editing, SpyCas9-based variants have since fueled diverse research applications. Its molecular architecture requires a 20 nucleotides guide sequence paired with a canonical NGG PAM, features that confer both high targeting specificity and efficient DNA cleavage activity. Moreover, SpyCas9's ability to bind and cut DNA independently of each other has led to a wide range of applications in gene regulation and function research, and the widespread use of the SpyCas9 system has made it a standard tool in the field of gene editing. Of particular significance are its dual cleavage modalities—*cis*- and *trans*-cleavage—which govern target DNA processing and off-target interactions, respectively. Cis-cleavage refers to the enzymatic activity directed at the DNA strand physically bound by the Cas9-gRNA complex, while trans-cleavage describes the non-canonical cleavage of nucleic acids. Elucidating the molecular basis of these activities is critical for improving editing accuracy, reducing off-target effects, and developing novel Cas9 derivatives with programmable cleavage behavior. This review focuses on the molecular mechanism of SpyCas9 *cis*- and *trans*-cleavage activity of nucleic acids in detail, as well as summarizing the nucleic acid detection platforms developed based on the *trans*-cleavage activity of the CRISPR-Cas system. If not specified, Cas9 in the review refers to SpyCas9.

2. Mechanism of Nucleic Acid Cleavage by the CRISPR-Cas9 System

2.1. Mechanism of Cis-Cleavage of DNA by Cas9

Cas9 consists of two lobes, which can be divided into a REC (CRISPR RNA–TS (target strand) recognition) lobe with three regions (REC1-3) and a NUC (nuclease) lobe with RuvC, HNH and PI domain (PAM-Interacting domain containing Topoisomerase-homology and C-terminal domains) [22,32,33] (Figure 1A). The NUC and REC lobes are linked by an extended bridge helix that is rich in arginine residues [34] (Figure 1A). Cas9 is an endonuclease that is directed to target and cleave DNA by dual-RNAs, crRNA and tracrRNA (Figure 1A), which can also be chimerized to form a sgRNA (single-molecule guide RNA) to direct DNA targeting [8,35]. The binding of sgRNA induces significant rearrangement of the REC lobe in Cas9, wherein the CTD (C-terminal domain) of Cas9, which is involved in PAM recognition, transitions from a disordered to an ordered state [22,32,36,37]. This structural rearrangement is analogous to the conformation observed following DNA substrate binding, thereby stabilizing the Cas9-sgRNA pre-target conformation for PAM recognition [22]. Cas9-RNA searches for target DNA by three-dimensional collisions, and when searching for targets, it stays transiently on non-target DNA that lacks PAM, and interrogates and binds specifically on PAM-containing DNA [38]. During targeting, Cas9 proteins first recognize and bind to the PAM (Protospacer Adjacent Motif) sequence on the target DNA, which is critical for Cas9 to recognize and target DNA, and a prerequisite for the formation of the guide-RNA-target-DNA heteroduplex [39] (Table 1). The PAM duplex sits in the positively charged groove of the PI domain, where the GG dinucleotides (guanine nucleobases) in the non-target strand (NTS) are read out via major groove by base-specific hydrogen-bonding interactions with Arg (arginine residue) 1333 and Arg 1335 in the PI domain of Cas9, thus Cas9-mediated DNA cleavage requires recognition of the 5′-NGG PAM of the NTS [8,32,39] (Table 2). After recognizing the PAM, the PI domain of Cas9 further interacts with the minor groove of the PAM double strand, initiating strand separation and guiding base pairing of the target DNA strand with the guide RNA [32,38]. The DNA double helix bends at an angle of approximately 30° as it traverses Cas9, providing the structural distortion required for R-loop formation [40]. The guide RNA pairs with the TS and replaces the NTS, forming a rate-limiting R-loop [40,41]. During the formation of the R-loop, the Cas9-sgRNA complex undergoes at least one highly dynamic intermediate conformational state, and the stability of this intermediate state is closely related to the cleavage activity of the Cas9 protein [42,43]. At the initial stage of R-loop formation, the positively charged heteroduplex formed by the REC2 and REC3 domains binds to a portion of the PAM-distal duplex of the DNA substrate, and the interactions of REC2 and REC3 with the NTS and the TS, respectively, keep the structure stable [44]. At the middle and late stages of R-loop formation, guide RNA-TS hybridization forms a 10 bp heteroduplex, leading to the rearrangement of REC2 and REC3 domains; the PAM-distal DNA duplex is relocated into a positively charged central binding channel formed by REC3, RuvC, and HNH domains; the R-loop promotes the remodeling of REC2 and REC3 and the relocalization of the HNH nuclease structural domains to form an

inactivation checkpoint conformation [44]. To form a kinetically stable ternary complex with Cas9-sgRNA, the length of the target ssDNA must be at least 10 nucleotides [38]. RNA-DNA interactions in the R-loop hybridization strand and Cas9 protein interactions with the hybridization strand help the Cas9-sgRNA-DNA ternary complex form a steady state [40,44,45]. Cas9-sgRNA requires a 6–12 nucleotide seed sequence at the 3′ end of the sgRNA for target recognition [8,38]. This seed region is the first to form base pairing with the complementary DNA strand, anchoring the complex before R-loop propagation and cleavage [46,47]. In the targeting complex of the Class I CRISPR-Cascade system, the entire guide region of the crRNA is preordered, whereas in the targeting complex of Cas9, only the seed segment is preordered [37,48]. This proofreading mechanism is similar between Class I and Class II CRISPR-Cas systems [37,48]. Recognition of the target DNA induces a conformational change in the HNH nuclease domain, which oscillates violently from a position far away from the DNA, leading to disorganization [36,44], which in turn activates the nuclease activity of the RuvC domain [49]. Accompanying the structural reorientation of HNH is the structural rearrangement of the hinge region. Following target DNA binding, conformational changes manifest in the hinge regions, specifically L1 (residues 765–780) and L2 (residues 906–918), which structurally bridge the HNH and RuvC catalytic domains [40]. L1 collapses from a completely disordered state into an ordered loop and a short α-helix that lies between the unchained TS and NTS, while L2 unfolds from the α-helix structural state into an extended loop [40]. N863 of the HNH domain coordinates with the magnesium ion and shifts from conformational equilibrium to a catalytic state, forming a catalytic triad with D839 and H840 to cleave the phosphodiester bond of TS [41,50,51]. The RuvC domain contains a ribonuclease H (RNase H) fold composed of D10, E762, H983 and D986, which assists in cleaving the phosphodiester bonds of the NTS with the help of two catalytic magnesium ions [41,51,52]. The coordinated cleavage by the HNH and RuvC nuclease domains ensures the concerted cleavage of the target DNA double strands [48]. In the ternary complex structure, REC2 and most REC3 are ordered. REC2 interacts orderly with nucleic acids, while the non-catalytic domain REC3 binds to the RNA-DNA hybrid, helping to stabilize the complex and regulate the HNH nuclease to modulate overall catalytic activity [36,44]. In this stable ternary complex state, the target double-stranded DNA will undergo *cis*-cleavage, and the HNH and RuvC domains will target the TS and NTS to cleave the phosphodiester bond between the third and fourth nucleotides upstream of the -NGG PAM, resulting in a blunt-ended double-strand break (DSB) [8,39,48] (Figure 1A). In addition, by neighboring DNA metabolism or chromatin interference, the cleavage site of RuvC on the NTS may sometimes change, resulting in the formation of 5′ sticky ends [32,53–55].

Table 1. The sequential steps and structural changes involved in Cas9-mediated *cis*- and *trans*-cleavage.

Procedure	*Cis*	*Trans*
Target recognition	1. SgRNA binding induces REC lobe rearrangement to enable PAM recognition. 2. Cas9-RNA locates target DNA through 3D diffusion, forming transient contacts with non-PAM DNA and stabilizing binding exclusively at PAM-containing sites via sequence interrogation.	After recognizing and binding to the target DNA, Cas9 forms a channel that can accommodate non-specific ssDNA or ssRNA.
Structural changes	1.The DNA double helix bends at an angle of approximately 30°. 2. Formation of guide-RNA-target-DNA heteroduplex. 3. Remodeling of REC2 and REC3; Rearrangement of HNH. 4. RuvC domain is activated.	1. The same structural rearrangement as in cis-cleavage. 2. The REC1 domain shifts ~2.4 Å toward the HNH domain. 3. The sgRNA repeat: anti-repeat region displaces ~3.0 Å.
Cleavage results	HNH cleaves the TS; RuvC cleaves the NTS; Generating blunt-end double-strand breaks; occasional 5′ sticky ends due to DNA metabolism or under chromatin interference.	RuvC non-specifically cleaves ssDNA or ssRNA; Generating single-strand break.

Table 2. Comparison of the characteristics of different types of CRISPR-Cas systems with the same trans-cleaving activity.

Type	Type II	Type V	Type VI	Type I
Effector	Cas9	Cas12a	Cas13a	Cascade-Cas3
Nuclease domain	RuvC, HNH	RuvC	HEPN	HD
Guide RNA	crRNA, tracrRNA	crRNA	crRNA	crRNA
PAM/PFS	5′-NGG	5′-(T)TTV	3′-H (PFS)	variable, 5′-YCN (*Pfu*)
Cis target	dsDNA	dsDNA, ssDNA	ssRNA	dsDNA
Trans target	ssDNA ssRNA	ssDNA	ssRNA	ssDNA
Blunt/Staggered end	blunt	staggered	\	blunt
The length of spacer	18–21 nt	20–24 nt	20–28 nt	30–40 nt
Reference	[21,56]	[56,57]	[58,59]	[60,61]

N indicates A, T, G or C; V indicates A, C or G; H indicates A, U or C; Y indicates C or T. The PAM recognized by the type I CRISPR system are subtype-specific, and only the PAM recognized by *Pfu* Cascade-Cas3 are cited here as examples. PFS (Protospacer Flanking Sequence), a short nucleotide sequence adjacent to the target RNA sequence that helps guide the Cas13 to its target.

2.2. Mechanism of Trans-Cleavage of DNA by Cas9

Recently, it has been found that Cas9, a member of the class II CRISPR-Cas system, shares the *trans*-cleavage activity with type V Cas12 and type VI Cas13 [62]. In the Cas9-crRNA-tracrRNA or Cas9-sgRNA state, binding of target ssDNA, dsDNA, or ssRNA activates the activity of Cas9 for *trans*-cutting ssDNA and ssRNA substrates (Figure 1A) (Table 2). Upon formation of Cas9-sgRNA-DNA, channels are formed between the two catalytic structural domains that can accommodate and bind non-specific DNA (Table 1). The *trans*-cleavage activity of Cas9 mainly depend on RuvC domain. Moreover, structural repositioning of the HNH structural domain is required to activate the *trans*-cutting activity of Cas9. The *trans*-cleavage activity of Cas9 guided by crRNA-tracrRNA is higher than that guided by sgRNA. It has been shown that when the checkpoint and catalytic state Cas9-RNA-DNA complexes are superimposed, in addition to the massive rearrangement of the HNH, REC2 and REC3 domains, a slight conformational change in the REC1 domain, which is shifted by about 2.4 Å towards the HNH domain, is observed. The authors posit that interactions within the repeat may regulate the conformational changes observed in the REC1 domain: anti-repeat region of the sgRNA, as this region undergoes a displacement of approximately 3.0 Å during the transition from the checkpoint to the catalytic state. The repeat and anti-repeat regions of sgRNA adopt a stem-loop conformation connected by a loop structure, whereas the repeat and anti-repeat regions of crRNA and tracrRNA simply form an RNA duplex, and there is no linker between crRNA and tracrRNA. The authors of the article suggest that it may be this difference in repeat: anti-repeat regions that regulates REC1 domain dynamics and therefore, influences Cas9 activation. The activation efficiency of Cas9's *trans*-cleavage activity by ssDNA is higher than that by dsDNA and ssRNA. When dsDNA is used as the activator, the HNH domain active site is rotated toward the TS strand to expose the RuvC active site [44]. When ssDNA acts as an activator, it induces significant conformational changes in the REC2, REC3, and HNH domains. As a result, the guide-target duplex-binding channel, which is composed of these three domains, adopts a more compact conformation. When ssRNA is used as an activator, only the REC3 domain undergoes a marked conformational change, and its localization affects the PAM-distal end of the HNH domain conformation differently from the PAM-distal end of the HNH domain conformation when ssDNA is used as an activator [43]. The localization of the HNH domain is affected, which affects the activity of RuvC. Hence the HNH domain also affects the *trans*-cutting activity of Cas9. When the guide RNA is in the form of sgRNA, Cas9 prefers to cleave poly(T) and poly(C) ssDNA substrates, but barely cleaves poly(A) and poly(G), and similarly to Cas12b cleaves poly(T) ssDNA substrates with the highest efficiency. And when the guide RNA is in the form of crRNA- and tracrRNA, Cas9 produces weak ssDNase activity on poly(A), in addition to the same preference for cleaving ssDNA substrates of poly(T) and poly(C). Notably, biochemical characterization showed that Cas9 was most efficient at cleaving ssDNA substrates in a fully single-stranded conformation with no secondary structure.

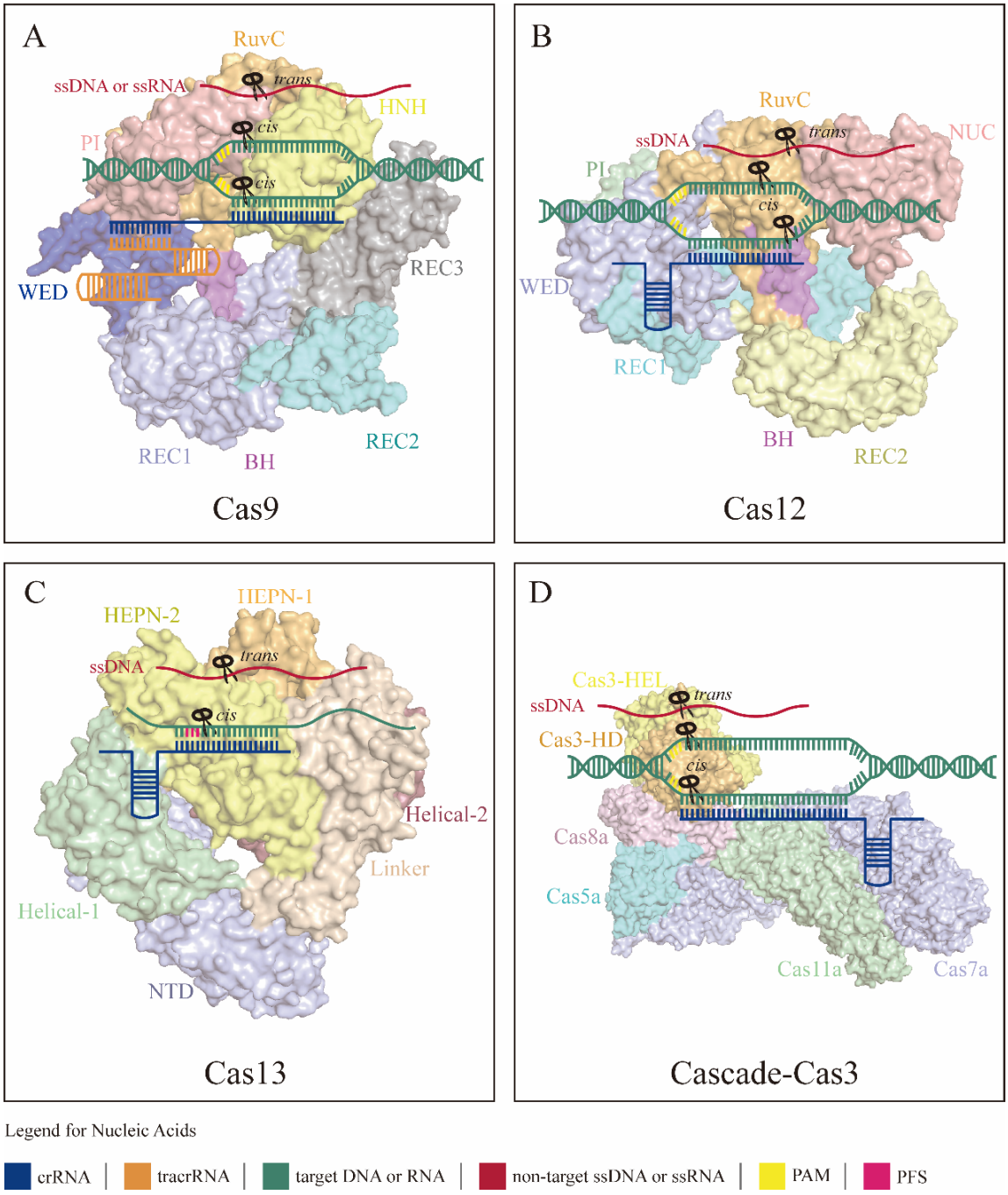

Figure 1. Different types of CRISPR-Cas systems for *cis*- and *trans*-cleavage. (**A**) Type II *cis*- and *trans*-cleavage of the CRISPR-Cas system. Cas9 structure is based on PDB ID: 8KAJ [62]. (**B**) Type V *cis*- and *trans*-cleavage of the CRISPR-Cas system. Cas12a structure is based on PDB ID: 8SFO [63]. (**C**) Type VI *cis*- and *trans*-cleavage of the CRISPR-Cas system. Cas13a structure is based on PDB ID: 5WTK [64]. (**D**) Type I *cis*- and *trans*-cleavage of the CRISPR-Cas system. Cascade-Cas3 structure is based on PDB ID: 7TR8 [60].

3. Structural Mechanisms Underlying *Trans*-Cleavage in Alternative CRISPR-Cas Systems

3.1. Structural Mechanisms Underlying Trans-Cleavage in Type V

In recent years, with the researchers' in-depth excavation and study of type V CRISPR-Cas12 proteins, the subtypes of Cas12 proteins have gradually become abundant. According to the differences of Cas12 protein's cleavage or binding mode to dsDNA, the transposition mechanism guided by RNA, or the biogenesis pathway and components of ribonucleoprotein complex (RNP), etc., it can be subdivided into 15 subtypes (V-A to V-O) [21,65–68]. Among them, Cas12a, Cas12b, Cas12c and Cas12f have stronger *trans*-cleavage activity than the other subtypes of effector proteins [69]. Cas12a utilizes the REC1 (recognition) domain to search for PAM, and upon recognition of the target PAM, the REC2 domain undergoes a rearrangement that exposes the catalytic active site of RuvC for

cis cleavage of the NTS, and the flexible REC2 domain repositions itself so that the NUC (nuclease) domain and the distal DNA are rearranged, exposing the TS to the RuvC domain for cis cleavage. This flexible oscillation of the REC2 domain leads to repeated exposure of the RuvC active site for trans cleavage [63] (Figure 1B). As part of the OMEGA (Obligate Mobile Element Guided Activity) system, the ancestor protein of Cas12, TnpB, and the ancestor protein of Cas9, IscB, both have a bilobed structure, as well as both containing a RuvC-like domain [70,71]. Similarly, the Cas12 protein has a bilobed structure like the Cas9 protein, consisting of Rec lobe and Nuc lobe [21]. However, unlike Cas9, Cas12 does not contain the HNH domain and uses only the RuvC domain to cleave the TS and NTS (Figure 1B), generating a staggered cut [72] (Table 2). Most Cas12 targets DNA, with only a few targeting RNA (Cas12g) or binding RNA (Cas12a2) [68,69,73,74]. Upon binding target nucleic acids, Cas12 also exhibits non-specific cleavage [75,76]. Apart from Cas12c2 (C2c3), Cas12k (C2c5), and Cas12m (C2c4) lacking *trans*-cleavage activity, and Cas12a2 being able to nonspecifically cleave ssRNA, ssDNA, and dsDNA, as well as Cas12o1 being able to nonspecifically cleave ssRNA and ssDNA, all other identified subtypes of type V can only nonspecifically cleave ssDNA [67–70,77,78].

3.2. Structural Mechanisms Underlying Trans-Cleavage in Type VI

Based on the differences in locus organization, Cas13 phylogeny and the positions HEPN domains of Cas13, type VI can be divided into four subtypes, VI-A, VI-B (which can be further subdivided into VI-B1, VI-B2 and distinctly branched VI-BT, of which VI-BT can be further categorized into two subtypes of Cas13X and Cas13Y with different effector proteins), VI-C (which also contains distinctly branched of VI-CT), and VI-D [79]. As an effector protein of Class II type VI CRISPR-Cas system, despite having a bilobed structure similar to that of other Class II effector proteins, i.e., REC lobe and NUC lobe. Cas13, unlike Cas9 and Cas12, lacks an identifiable DNase catalytic site and is an RNA-directed effector protein targeting RNA [80]. In general, REC lobe consists of N-terminal (NTD) and helical-1 domains, which is primarily responsible for the recognition of crRNA [79] (Figure 1C). NUC lobe consists of helical-2, HEPN-1 and HEPN-2 domains, which mainly contribute to accommodate and cut target RNA [79] (Figure 1C) (Table 2). The two HEPN domains of Cas13 contain well-conserved R-X_{4-6}-H (R, arginine; X_{4-6}, 4 to 6 random amino acids; H, histidine), which is used for RNA cleavage [64,80,81]. Upon binding to the target RNA, the two HEPN domains undergo symmetric conformational changes to form a catalytic site that mediates both sequence-specific *cis*-cleavage of the target RNA and non-specific trans-cleavage of bystander RNA [58,64] (Figure 1C).

3.3. Structural Mechanisms Underlying Trans-Cleavage in Type I

About 90% of natural CRISPR-Cas systems belong to Class I systems, which can be categorized into seven subtypes (I-A to I-G), and class I type I systems are the most diverse and widely distributed CRISPR-Cas systems, accounting for about 50% of all discovered CRISPR-Cas systems [82–84]. At present, the exact mechanism of Cas3-mediated cleavage and degradation of target DNA is still not fully elucidated [85]. Moreover, whether type I can be interpreted as having *trans*-cleavage activity is also an open question. Type I-A Cascade (CRISPR-associated complex for antiviral defense) -Cas3 is believed to possess *trans*-cleavage activity (Figure 1D) and its exact molecular mechanism has been revealed [60]. Moreover, EcoCas3/EcoCascade of type I-E, one of the most studied isoforms of the type I system, has also been shown to have *trans*-cleavage activity [85,86]. The current mechanism of type I cleavage of nucleic acids through Cas3-mediated cleavage is mainly considered in the following two ways: (1) Type I-E represented by EcoCascade, Cascade utilizes crRNA to recognize complementary DNA targets flanking PAM, and after the formation of a full R-loop, the structure of the R-loop induces the Cascade complex undergoes a conformational change, then recruits Cas3 helicase-nuclease, which repeatedly cleaves the NTS in *cis* and then the TS in *trans* [85,87,88]. (2) Type I-A system, on the other hand, Cas3 and Cascade first form an intrinsic complex, and the nuclease activity of Cas3 is in a state of autoinhibition before it recognizes and binds to the target DNA, and then activates the nuclease activity of Cas3 through allosteric regulation after it binds to the target DNA and forms a full R-loop [60] (Figure 1D). Type I-B, I-C, I-E, I-F, and I-G systems of these subtypes recruit and activate Cas3 activity consistent with type I-E systems [18,89–92]. In contrast, the type I-D system activates Cas3 in a manner similar to the type I-A system [18]. Moreover, regardless of the above ways of activating Cas3 nuclease activity, Cas3 does not exert nuclease activity to cleave the target DNA when forming a partial R-loop, i.e., when the Cascade is not fully bound to the target DNA, but catalyzes non-specific ssDNA cleavage through the HD (histidine-aspartate) nuclease domain [60,85] (Table 2).

4. Nucleic Acid Platform Based on *Trans*-Cleavage Activity

4.1. Studies Laying the Groundwork for the Use of Trans-Cleavage for Diagnostics

Prior to the discovery of trans-cleavage activity in Cas9, two Class II CRISPR-Cas effector proteins—Cas13 (2017) and Cas12 (2018)—were widely utilized in nucleic acid detection platform development following their identification as trans-cleaving nucleases [75,93] (Table 3). These systems garnered significant research attention during their early characterization [75,93]. Moreover, the recent discovery of multiple Cas12 subtypes exhibiting trans-cleavage activity has further advanced the development of CRISPR-based platforms for nucleic acid detection. The basic principle of these diagnostic platforms is to stimulate the *trans*-cleavage activity of Cas proteins through the specific binding of programmable crRNAs to target genes and non-specific cleavage of fluorescent reporter molecules in the reaction system, thereby enabling the detection of target nucleic acids. In 2017, Gootenberg et al. [93] developed a molecular diagnostic platform for nucleic acid detection against Zika and dengue viruses based on the RNA-guided, RNA-targeted Cas13 *trans*-cleavage activity on non-specific RNA, called SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) (Figure 2D), which is the first nucleic acid detection platform developed utilizing the *trans*-cleavage activity of the CRISPR-Cas protein. SHERLOCK technology first amplifies the sample using the isothermal amplification technique RPA (recombinase polymerase amplification), then *trans*cribes the amplification product into RNA, which binds to Cas13a using crRNAs containing sequences complementary to the target RNA. Upon detection of the target RNA, the *trans*-cleavage activity of Cas13 is triggered, which cleaves and fluoresces the ssRNA-FQ (fluorescent reporter) probe. Based on the development of this diagnostic platform, a series of nucleic acid detection platforms based on the CRISPR-Cas system have been developed. In 2018, Chen et al. [75] first discovered that Cas12 has *trans*-cutting activity and developed a novel nucleic acid detection platform for human papillomavirus (HPV) called DETECTR (DNA endonuclease-targeted CRISPR *trans* reporter) (Figure 2C), which allows for the direct detection of DNA samples. Also, by coupling with the isothermal amplification technology RPA, the ssDNA-FQ reporter probe can be cleaved and fluoresced using the *trans*-cutting activity of Cas12a, enabling the direct detection of DNA samples, omitting the in vitro transcription step compared to the SHERLOCK technology. Following closely, Li et al. [94] also developed a nucleic acid detection platform called HOLMES (one-HOur Low-cost Multipurpose highly Efficient System) for the *trans*-cleavage activity of Cas12a. The HOLMES system enables template amplification via a PCR step and demonstrates that that both ssDNA and dsDNA targets activate the *trans*-cutting activity of Cas12a. In the same year, Harrington et al. [95] identified the Cas14 (Cas12f) protein, which also has *trans*-cutting activity. Cas14 encodes a Cas protein of only 40–70 kDa in size, about half the size of other Cas proteins, and it is able to cleave targeted ssDNA without restriction sequence requirements. Based on DETECTR, they developed the Cas14-DETECTR nucleic acid detection platform by replacing Cas12a with Cas14a. The PAM-free and more compact features of the Cas14-DETECTR system enable high-fidelity detection of DNA single-nucleotide polymorphisms (SNPs) without PAM limitations. Subsequently, in the same year, Gootenberg et al. [96] modified the SHERLOCK technology and developed the SHERLOCK version 2 (SHERLOCKv2) system. The SHERLOCKv2 system, in combination with Cas12a and Csm6 proteins, is able to detect multiple target molecules in a single reaction with a 3.5-fold increase in signal strength, detects viral DNA sequences at concentrations as low as two copies per microliter of sample, and enables results to be obtained within one hour. In 2019, Teng et al. [97] developed a nucleic acid detection platform with higher sensitivity at the single base level based on the *trans*-cutting activity of Cas12b, called CDetection (Cas12b-mediated DNA detection). In the same year, Li et al. [98] improved the HOLMES system and developed the HOLMESv2 nucleic acid detection platform by combining the loop-mediated isothermal amplification (LAMP) technology. The HOLMESv2 system, besides improving the sensitivity of detection at the single-base level, facilitates the quantification of the target nucleic acids and avoids cross-contamination. Later that year, Wang et al. [99] developed a rapid CRISPR-Cas12-based detection method named Cas12aVDet (Cas12a-based Visual Detection). This method combined the two-step reaction of RPA amplification and Cas12a cleavage of fluorescence quenching groups into a single reaction. This approach eliminates the two-step process of first amplifying and then detecting, thereby circumventing the problem of uncapping contamination during the procedure. Moreover, to avoid low amplification efficiency caused by Cas12a cleaving substrate templates during amplification, Cas12a was pre-attached to the upper wall of the tube. After 15 min of RPA amplification, Cas12a was added to the reaction system via centrifugation. Additionally, it enables the completion of the detection process within 30 min.

Table 3. Characteristics of representative nucleic acid detection platforms for different CRISPR-Cas systems.

Platform	DACD/RACD	DETECTR	SHERLOCK	HASTE
Effector	Cas9	Cas12a	Cas13a	Cascade-Cas3
Activator	dsDNA; ssDNA; ssRNA	dsDNA; ssDNA;	ssRNA	dsDNA
Detection Limit	Attomolar sensitivity with RAA	Attomolar sensitivity with RPA	Attomolar sensitivity with RPA	Attomolar sensitivity with LAMP
Application scenario	Virus detection; SNP detection	Virus detection; SNP detection	Virus detection; genotyping	Virus detection

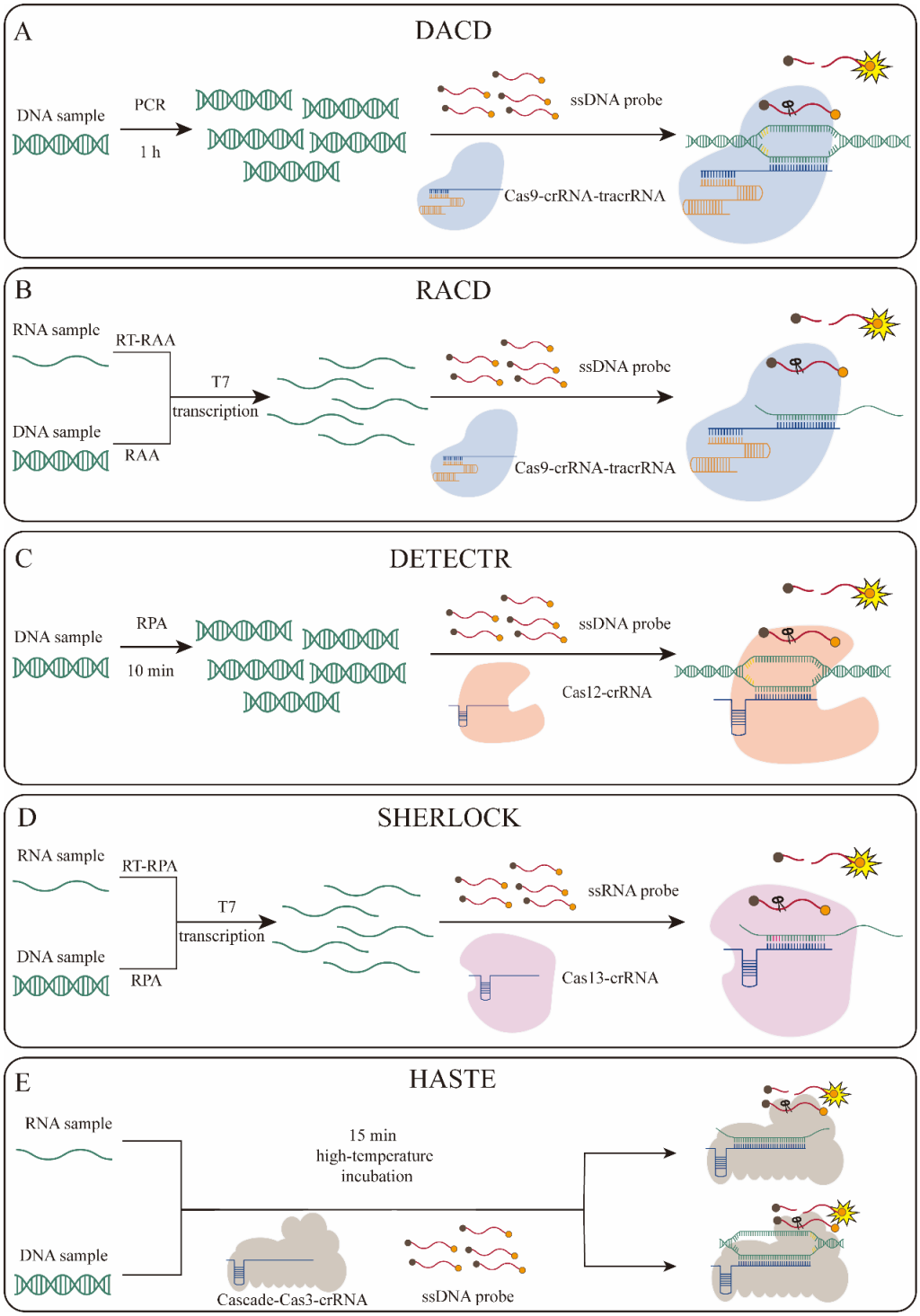

Figure 2. Representative of nucleic acid detection platforms based on the CRISPR-Cas system. (**A**) CRISPR-Cas9 based nucleic acid detection platform DACD. (**B**) CRISPR-Cas9 based nucleic acid detection platform RACD. (**C**) CRISPR-Cas12 based nucleic acid detection platform DETECTR. (**D**) CRISPR-Cas13 based nucleic acid detection platform SHERLOCK. (**E**) Cascade-Cas3 based nucleic acid detection platform HASTE.

4.2. Pioneering Research on the Trans-Cleavage Activity of Cas9 for Diagnostics

In 2024, Chen et al. [62] discovered for the first time that type II CRISPR-Cas9 proteins also have *trans*-cleavage activity, and developed RACD (RNA-activated Cas9 detection) (Figure. 2B) and DACD (DNA-activated Cas9 detection) (Figure. 2A) nucleic acid detection platform that can target both RNA and DNA. The *trans*-cutting activity of Cas9 can be activated by the target ssDNA, dsDNA or ssRNA (Table 3), and Cas9 can *trans*-cleave ssRNA-FQ or ssDNA-FQ. For the same guide RNA, the Cas9-based assay platform can detect both DNA and RNA samples without the need for additional *in vitro* transcription, and can be used with either RNA or DNA probes on demand. For the monkeypox (mpox) virus extracellular envelope protein B6R, the limit of detection for the DACD platform was 24 copies per μL, and the limit of detection for the RACD platform reached 2.4 copies per μL. Furthermore, based on the single base mismatch between Cas9 target DNA and the 17th position of crRNA, they established the SNP detection method.

4.3. Special Type I System for Diagnostics

Although in contrast to class II CRISPR-Cas systems, class I type I CRISPR-Cas systems require the assistance of multiple Cas protein subunits to exert their *trans*-cleavage activity. However, the mechanism of gradual activation of Cascade-Cas3 enzyme activity of class I type I systems is considered to have a lower off-target effect than that of Class 2 systems, which has its particular research value [60]. In 2022, Yoshimi K et al. [86] developed a Cas3-based (type I-E) novel in vitro nucleic acid detection platform by exploiting the *trans*-cleavage activity of EcoCas3/EcoCascade, an instrument-free detection method that can detect SARS-CoV-2 within 30–40 min, called CONAN (Cas3-Operated Nucleic Acid detection). CONAN shows high specificity for single-base-pair discrimination within the PAM site. In the same year, Hu et al. [60] developed an advantageous nucleic acid detection platform based on *Pfu* (*Pyrococcus furiosus*) Cascade-Cas3 (type I-A) that can simplify sample inactivation and nucleic acid detection into a single 15-minute high-temperature incubation step, called HASTE (heat-activated streamlined nucleic acid detection platform) (Figure 2E) (Table 3). The deconjugating enzyme (helicase) activity of Cas3 of this I-A system is strictly temperature-dependent, with heat-activated properties and very strong signal amplification in the temperature range of 45–85 °C, making possible the combination of cell lysis, pathogen inactivation, and nucleic acid detection in a single high-temperature incubation step. In 2024, Hu et al. [100] developed another new platform for molecular diagnostics against HPV called HAVE (Hyper-Active-Verification Establishment) using the *Tsi* (*Thermococcus siculi*) type I-A CRISPR-Cas3 system. Cas3 alone exhibits potent ssDNA cleavage activity, but it becomes autoinhibited and only exhibits trans-cleavage activity upon target binding when combined with Cascade. Therefore, the development of molecular diagnostic platforms using Cascade-Cas3 often suffers from high background noise and high false positives. And this TsiCas3 was found to remain inactive before binding to Cascade and target DNA. Therefore, by utilizing this property of TsiCas3, the authors they developed the HAVE platform that can complete the assay within 35 min with an accuracy close to the PCR-RDB method, which is the gold standard for identifying HPV genomic DNA, and the assay device is compact and portable.

4.4. Optimization and New Development of Diagnostic Platform Based on CRISPR-Cas System

Based on the fact that Cas12 and Cas13 have the advantage of *trans*-cutting action through a single nuclease, which has a broad prospect for nucleic acid detection applications, researchers have designed an improved nucleic acid detection platform through continuous development and optimization. Problems to be optimized mainly include: false positives caused by aerosol contamination in the two-step assay, low amplification efficiency caused by cutting of amplification substrate templates in the one-step assay, problems with assay throughput and efficiency, and problems faced by whether rapid diagnosis can be achieved, and so on.

The main limitation of the one-pot assay is the incompatibility between the amplification reaction and the Cas protein's cleavage activity. Current optimization directions are (1) physical isolation of Cas protein from the initial amplification reaction system, (2) modification of crRNA to achieve regulation of the equilibrium of the two reactions, and (3) modulation using photocontrolled CRISPR method to keep the two reactions separate. Specific examples include: (1) the Cas12aVDet platform described above, which physically isolates the Cas protein from the reaction system at the initial stage of amplification; and the opvCRISPR (one-pot visual reverse transcription [RT]-LAMP-CRISPR) platform, which avoids initial template cleavage by placing the Cas protein on the tube lid [101]. (2) Nguyen et al. [102] developed a new platform for nucleic acid detection based on the LbCas12a protein by modifying the crRNA by adding seven DNA nucleotides to the 3′ end of the crRNA. This heptamer changes its conformation upon *cis*-cleavage of LbCas12a, exposing the endonuclease structural domain of LbCas12a, which is more favorable for *trans*-cleavage, in a method called CRISPR-ENHANCE (ENHanced

Analysis of Nucleic acids with CrRNA Extensions). The assay can detect target DNA up to pM concentrations in combination with FRET-based reporter DNA molecules. In combination with an isothermal amplification step (LAMP/RT-LAMP), the assay can reach the detection limit of qPCR. The CasDOS (Cas12a-based single-stranded DNA (ssDNA)-modified crRNA (mD-crRNA)-mediated one-step diagnostic) platform modifies wild-type crRNA (wt-crRNA) by adding 9 AT-rich DNA bases to each end [103,104]. This modification results in lower cis-cleavage activity of the mD-crRNA when bound to Cas12a compared to wt-crRNA, thus shifting the reaction towards RPA and achieving a detection limit of 16.6 aM for pathogenic DNA [103,104]. (3) Hu et al. [105] temporarily blocked the activity of Cas12a by designing protective RNAs (p-RNAs) of R5-3PC (a sequence was supplemented with three PC (photocleavable) linkers for the spacer sequence and 5 additional bases complementary to the crRNA repeat region) to bind to the crRNA of Cas12a. After the RPA reaction was finished, the cutting activity of Cas12a was completely restored by 365-nm light irradiation, allowing the spatiotemporal isolation of the amplification reaction from the *cis*- and *trans*-cutting reactions of Cas12a. Considering that this photocontrol method prevents the crRNA from pre-binding to Cas proteins, which affects the stability of Cas12a-crRNA, the team then embedded 6-nitropiperonyloxymethyl-caged thymidine into the CRISPR-Cas12a crRNA [106]. This caged crRNA prevents Watson-Crick base pairing between the crRNA and the target DNA, achieving blockade of Cas12a activity without affecting its pre-binding to the crRNA. This photocontrol one-pot assay is 50 times more sensitive than the conventional two-step method.

Generally, CRISPR-Cas-based nucleic acid detection systems can detect only one pathogen target or several pathogen targets by multiplexing, making it difficult to realize high-throughput and high-efficiency detection. To address this problem, Ackerman et al. [107] developed a multiplexed nucleic acid assay based on Cas13 that can differentiate 169 human-associated viruses simultaneously, called CARMEN (Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids)-Cas13a. In this assay system, nanoliter droplets containing CRISPR nucleic acid detection reagents self-assemble into a matrix of microwells to pair with droplets of amplified samples, allowing for repetitive testing of crRNA against each sample, enabling efficient testing of over 4,500 crRNA-target pairs on a single array, which enables high-throughput, high-efficiency detection.

Whether the nucleic acid detection platform based on CRISPR-Cas system can realize Point-of-Care Testing (POCT) in clinical practice is also a key question [108]. Rapid diagnosis involves issues such as whether the samples for nucleic acid detection need to be pre-treated or not, whether pre-amplification is required, the detection limit of the detection system, the detection sensitivity and the time required for the detection, whether the signals of the test results can be read by the naked eye, and whether the test equipment is portable [108–113]. In recent years, researchers have developed and optimized novel diagnostic platforms to address these problems. In 2020, Iwasaki et al. [114] extended a Cas13-based molecular diagnostic method called SPRINT (SHERLOCK-based profiling of in vitro transcription). The SPRINT technology enables the detection of small molecules in a single experimental run and quantifies hundreds of reactions over several hours. In 2021, Sun et al. [115] developed OR-DETECTR, a single-tube platform for detecting SARS-CoV-2 that combines RT-RPA (Reverse Transcription and Recombinase Polymerase Isothermal Amplification) technology with DETECTR, producing results in about 50 min. In 2021, Sheng et al. [116] combined the CRISPR/Cas13a system and catalytic hairpin DNA circuit (CHDC) to develop a Cas-CHDC-powered electrochemical RNA-sensing technology (COMET) platform integrated on a reusable electrochemical biosensor, achieving a dual signal amplification and a detection limit of 50 aM. In 2021, Liu et al. [117] created a one-step assay called Fast Integrated Nuclease Detection In Tandem (FIND-IT) by combining Cas13 and Csm6 with chemically stable activators. This assay does not require high-temperature heating and is capable of detecting SARS-CoV-2 RNA at as low as 31 copies per microliter in as little as 20 min. In 2022, Tang et al. [118] developed a CRISPR-Cas12a-based diagnostic platform called CLIPON (CRISPR and Large DNA assembly Induced Pregnancy strips for signal-ON detection). The reaction system consists of four components: single-stranded DNA-hCG composite probe (NHP), CRISPR-Cas12a, crRNA, and cauliflower-like large-sized DNA assemblies (CLD), providing an innovative strategy for the development of universal point-of-care testing assays. RPA-CLIPON is capable of detecting HPV-16 from clinical samples and can detect low levels of SARS-CoV-2 RNA. In 2022, Broto et al. [119] combined a CRISPR-based diagnostic assay with a nano-enzyme-linked immunosorbent assay (NLISA) to develop a CrisprZyme platform that enabled the possibility of without preamplification. In 2022, Yang et al. [120] obtained variants with stronger *trans*-cutting activity by modifying *Leptotrichia wadei* (*Lwa*) Cas13a, which can achieve sensitive detection of the SARS-CoV-2 genome at the attomolar concentrations level without target preamplification. In 2024, Chen et al. [121] discovered for the first time that Cas12a can *trans*-cleave the spacer region of pre-crRNA through the RuvC structural domain under the activation of target nucleic acids. They utilized this property to develop a PDCD (Pre-crRNA-dependent Cleavage Detection) one-step nucleic acid detection platform by directly binding fluorescent and quenching moieties on both sides of the pre-crRNA spacer region cleavage site. In 2025, Dai et al. [122] developed the E-

CRISPR (CRISPR-Cas12a (cpf1) based electrochemical biosensor) platform by optimizing the *in vitro* transmembrane activity of Cas12a in conjunction with the development of a biosensor for electrochemically detecting CRISPR cleavage activity. The platform enables Point-of-Care Testing with detection limits up to the picomolar level.

5. Conclusions

This review analyzes the molecular mechanisms of *cis*- and *trans*-cutting in Cas9 and summarizes the diagnostic platforms developed based on the *trans*-cutting activity of the CRISPR-Cas system. Cas9 has been considered to lack *trans*-cutting activity since it was discovered to be used as a gene-editing tool in 2012, and it was not reported to have *trans*-cutting activity until 2024. The prolonged binding of Cas9-sgRNA to target DNA for several hours, without immediate dissociation after cleavage, may provide an opportunity for Cas9 to exhibit trans-cutting activity [123,124]. Cas9 has currently only been shown to have *trans*-cutting activity in vitro. Due to the complexity of the in vivo environment, it remains an unanswered question whether *trans*-cleavage also occurs when Cas9 is used for in vivo gene editing, so this reminds researchers that they should be mindful of the possible negative effects of its *trans*-cleavage activity when using Cas9 as a gene editing tool. Class II CRISPR-Cas system has the advantages of single nuclease with cleavage activity, simple reaction system and low cost, which is expected to develop into an ideal nucleic acid detection tool through continuous optimization. Despite these advantages, current CRISPR-Cas-based diagnostic platforms still require further refinement. For example, some current detection systems combine sample pre-processing with nucleic acid detection, which increases operational complexity and risk of contamination, and suffers from insufficient nucleic acid purity and low detection efficiency. To improve detection sensitivity, many nucleic acid detection platforms are often used in conjunction with amplification technologies such as RAA, RPA and LAMP, but this increases the cost, time and operational complexity of detection. In one-step assay, CRISPR-Cas may cut amplified template sequences, affecting amplification efficiency, but two-step assay increases the problem of operational steps and contamination risk. Off-target effects of CRISPR-Cas systems, such as dependence on PAM, limit the flexibility of detection targets and may lead to false-positive results. Moreover, with the increasing variety and number of target molecules in clinical testing, CRISPR-Cas nucleic acid detection systems need to further improve detection throughput and efficiency to cope with the demand for large-scale clinical validation. Based on these problems, future optimization efforts are needed in the following aspects. Firstly, optimizing sample pretreatment protocols enables direct extraction and detection of nucleic acids from complex matrices. A second strategic approach involves either the discovery of novel Cas proteins exhibiting *trans*-cleavage activity or the engineering of existing Cas enzymes to enable PAM-independent targeting while minimizing off-target activity, thereby addressing two critical limitations in current CRISPR-based applications. Concurrently, integrating these systems with auxiliary proteins may facilitate multiplexed detection platforms capable of identifying multiple pathogens simultaneously, thereby enhancing both detection efficiency and analytical throughput.

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