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Trans-cleavage activity of Cas12a effectors can be unleashed by both double-stranded DNA and single-stranded RNA targeting in absence of PAM

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Abstract

CRISPR-Cas12a is a powerful tool in nucleic acid detection, but the relationship between its trans-cleavage activity and protospacer adjacent motif (PAM) sequences remains incompletely understood. In this study, we synthesized diverse PAM-sequence substrates and conducted systematic cis-cleavage and trans-cleavage experiments with three Cas12a orthologs. We found that double-stranded DNA (dsDNA) can activate Cas12a's trans-cleavage activity even without PAM and this activation occurring independently of cis-cleavage. Notably, our results also revealed that single-stranded RNA (ssRNA) can directly initiate the trans-cleavage activity of Cas12a.We also experimentally validated the feasibility of CRISPR-Cas12a in detecting target dsDNA lacking PAM sequences, including identifying mutated sites in clinical samples. Structural prediction using AlphaFold 3 revealed the potential mechanism of Cas12a's PAM-independent trans-cleavage. Our research expands the understanding of Cas12a's trans-cleavage mechanism and demonstrates its potential for nucleic acid detection beyond PAM-dependent targets. This discovery broadens the application scope of Cas12a, providing new opportunities for developing highly sensitive and versatile diagnostic platforms.

Key words: CRISPR-Cas12a, Cis-cleavage, Trans-cleavage, Protospacer Adjacent Motif

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Introduction

The CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated proteins) system has emerged as a revolutionary technology in the fields of genetics, synthetic biology, and gene editing[1–4]. It functions as a natural adaptive immune mechanism in prokaryotes, empowering them to safeguard against invading nucleic acids, such as viruses and plasmids[5–7]. Among the diverse CRISPR-Cas systems, Class 2 systems, which include Cas9 and Cas12a (also referred to as Cpf1), have garnered significant attention due to their simplicity and versatility in genetic manipulations[8–10]. A noteworthy feature of Cas12a is its trans-cleavage activity. Once activated by target recognition, Cas12a can non-specifically degrade ssDNA[11, 12]. This property has been exploited in the development of highly sensitive nucleic acid detection platforms, such as the DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter) platform and the HOLMES (an one-HOur Low-cost Multipurpose highly Efficient System) platform[11, 12]. Cas12a recognizes a specific PAM adjacent to the target sequence. This PAM serves as a vital identifier, enabling Cas12a to distinguish between self and non-self DNA.[9, 13, 14]. The PAM sequence is crucial for Cas12a binding and activation, as it initiates the cleavage of the target DNA[9, 15–17].

Significant attention has focused on the PAM requirement for the cis-cleavage of targets. LbCas12a exhibits the utmost efficiency in recognizing the canonical TTTV PAM. The T-rich sequence forms a narrow minor groove that precisely aligns with the WED, REC1, and P1 domains, thereby triggering an allosteric shift towards a

catalytically active configuration[13, 15]. In contrast, non-canonical PAMs, such as TCTA, TCCA, or CCCA, can activate Cas12a. However, they result in less efficient cis-cleavage due to structural perturbations in both the enzyme and target duplex that diminish binding stability[15]. While the effects of canonical and non-canonical PAMs in the substrate DNA sequence on the cis-cleavage activity of Cas12a and their underlying mechanisms are now well understood, the influence of non-canonical PAMs on the trans-cleaving activity of Cas12a remains largely unexplored.

In this study, we aimed to comprehensively investigate the role of non-PAM elements in the trans-cleavage activity of Cas12a. Strikingly, we found that Cas12a effectors can be activated and exhibit robust trans-cleavage activity even in the absence of a canonical PAM sequence. Moreover, we demonstrated that direct RNA targeting can activate Cas12a's trans-cleavage function, thus expanding its potential applications. These research results not only broaden the scope of Cas12a's DNA detection capabilities but also introduce promising new avenues for RNA detection. Looking forward, the synergistic integration of Cas12a and Cas13a proteins holds great potential for revolutionizing molecular diagnostics. It may enable the simultaneous detection of multiple targets within a single reaction.

Materials and methods

Materials

LbaCas12a nuclease, which is derived from *Lachnospiraceae bacterium* (Cat. No.: E373-YH01) was purchased from Novoprotein Scientific Inc. (Shanghai, China). FnCas12a (Cat. No.: 32106), derived from *Francisella tularensis*, and AsCas12a (Cat.

No.: 32104), derived from *Acidaminococcus sp. BV3L6* were respectively purchased from Beyotime Biotech Co., Ltd. (Shanghai, China) and Tolo Biotech Co., Ltd. (Shanghai, China). Nuclease-free water (Cat. No.: BL1040A) was purchased from Biosharp Life Science Co., Ltd. (Beijing, China). The PCR kits (2 × Taq Master Mix, Cat. No.: P111), Ultra GelRed (Cat. No.: GR501) used for nucleic acid gel staining, T7 High Yield RNA Transcription Kit (Cat. No.: TR101), VAHTS RNA Clean Beads (Cat. No.: N412), VAHTS DNA Clean Beads (Cat. No.: N411) were all purchased from Vazyme Biotech, Inc. (Nanjing, China). RNase A (Cat. No.: 2158) and Proteinase K (Cat. No.: 9034) were purchased from Takara Biomedical Technology (Beijing) Co., Ltd. (Beijing, China). Bacterial DNA Extraction Kit (Cat. No.: 60300K-50T) was purchased from Shanghai Jinnuo Biotechnology Co., LTD. (Shanghai, China). Sputum samples were collected from confirmed TB patients at Shenzhen Third People's Hospital as well as from healthy individuals. The DNA extracted from Mtb clinical strains resistant to RIF and INH had been previously stored in our laboratory. All RNA and DNA oligonucleotide sequences used in this study were synthesized by Sangon Biotech Inc. (Shanghai, China) and the detailed sequences are listed in Supplementary Table 1.

Preparation of dsDNA substrates for cleavage assays

In this study, DNA oligonucleotide duplexes were formed by combining two complementary strands in a 1:1 molar ratio within a Nuclease-Free buffer environment. Subsequently, the duplexes underwent a heat treatment protocol, involving incubation at 95°C for 5 minutes followed by gradual cooling to 12°C. The

prepared duplexes were then stored at -20°C prior to their utilization. The IS6110 sequence, sourced from Mycobacterium tuberculosis, served as the target DNA substrate for cleavage assays. This gene sequence was commercially synthesized and then cloned into the pUC57 vector. To obtain amplified IS6110 fragments, PCR was performed using either specific primer pairs designed for the pUC57 vector or those derived from the DNA of clinical sample. The composition of the PCR reaction mixture, which had a total volume of 50 μL, consisted of 10 pg of template DNA, 25 μL of 2× Taq Master Mix, and 0.4 μM concentrations of both forward and reverse primers. The PCR protocol commenced with an initial denaturation step at 94°C for 1 minute, followed by 35 cycles of denaturation (94°C for 5 seconds), annealing (65°C for 5 seconds), and extension (72°C for 10 seconds). This was concluded with a final extension step at 72°C for 3 minutes, followed by cooling to 4°C. The resulting PCR products were analyzed using 1% agarose gel electrophoresis and subsequently purified using VAHTS DNA Clean Beads. The concentration of the purified PCR products was accurately determined using a Nanodrop 2000 spectrophotometer. Finally, these purified products were stored at -20°C for future use in the subsequent experiments of this study.

Preparation of IS6110 RNA substrates for cleavage assays

To prepare the IS6110 RNA substrates for the cleavage assays, we first integrated the T7 transcription promoter sequence into the downstream primer, IS6110-RNA-R. This primer was specifically designed for the amplification of the IS6110 fragment. Subsequently, using the primer pair consisting of IS6110-RNA-R and IS6110-RNA-F,

we amplified the transcription template DNA of IS6110. The template for this amplification was the pUC57 vector that harbored the IS6110 sequence. After the PCR amplification, we purified the resulting PCR product. Next, 100 ng of the purified DNA is taken as the transcription template, and in vitro transcription is carried out using the T7 High Yield RNA Transcription Kit, following the instructions provided in the kit. Upon completion of transcription, 1 μL of DNase I is added to digest the template DNA. The transcribed RNA is then purified using VAHTS RNA Clean Beads, quantified with Nanodrop 2000, and stored at -80°C for downstream experiments.

Trans-cleavage assays

The Cas12a trans-cleavage assay was generally carried out in a final volume of 20 μL, which included 2 μL of 10 × Cas12a buffer, 1 μL of Cas12a (1 μM), 1 μL of crRNA (1 μM), 1 μL of FQ probe (10 μM), 1 μL synthesized substrates (1 μM) or 100 ng purified IS6110 fragment and Nuclease-free water added to reach a final volume of 20 μL. Fluorescence signal was measured at 2-minute intervals over a 2 hours duration at 37°C using an Applied Biosystems™ 7500 Real-time PCR system (Thermo Fisher Scientific, MA, USA). To visualize the results in a more intuitive manner, the reaction mixture was observed by the naked eye under blue light illumination. This was achieved using a Blue Light Gel Imager (Sangon Biotech. Inc., Shanghai, China). The visual observations were then recorded using a smartphone, allowing for easy documentation and further analysis of the experimental outcomes.

Cis-cleavage assays

The Cas12a cis-cleavage assay was typically carried out in a final reaction volume of 20 μL, consisting of 2 μL of 10× Cas12a buffer, 1 μL of Cas12a (10 μM), 1 μL of crRNA(10 μM), and either 5 μL of synthetic substrates (1 μM) or 300 ng of purified IS6110 fragment. Nuclease-free water was then added to achieve the desired 20 μL volume. Following incubation at 37 °C for 30 minutes, 1 μL of RNase A (10 μg/μL) was introduced into the reaction tubes and allowed to incubate at room temperature for an additional 10 minutes. Subsequently, 1 μL of Proteinase K was added, and the mixture was further incubated at 42 °C for 10 minutes. For synthetic substrates, the reaction products were analyzed via 15% denaturing PAGE, whereas for IS6110 substrates, 2% agarose gel electrophoresis was employed. The resulting gels were visualized using a Tanon MINI Space 100 (Tanon, Shanghai, China). For the cis-cleavage assay with FAM-labeled targets, gel images were recorded using the Bio-Rad ChemiDoc MP Imaging system. The DNA cut cleavage ratio (%) was defined as the dsDNA target cleavage percentage calculated from gels quantitative analysis using Image-J software.

Application of CRISPR-Cas12a in nucleic acid detection in absence of PAM

The sputum specimens underwent decontamination utilizing the NALC-NaOH method, with a concluding NaOH concentration of 1%. Following decontamination, the concentrated sediment was resuspended in 1.5 milliliters of sterile phosphate buffer (pH 6.8) and subsequently employed for DNA extraction, adhering strictly to the manufacturer's instructions of the Bacterial DNA Extraction Kit (Catalog No.: 60300K-50T). The purity and concentration of the extracted DNA were quantified

using a Qubit 4.0 instrument. The target DNA was then amplified via PCR in a final reaction volume of 50 μL, comprising either 50 ng of sputum-derived DNA or 100 pg of Mtb genomic DNA, 25 μL of 2 × Taq Master Mix, and 0.4 μM concentrations of both forward and reverse primers. The PCR protocol initiated with a denaturation step at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 10 seconds, annealing at 65°C for 10 seconds, and extension at 72°C for 20 seconds, concluding with a final extension at 72°C for 5 minutes, and cooling to 4°C. For the trans-cleavage assay, 2 μL of unpurified PCR products were incorporated into CRISPR reaction mixtures. Fluorescence signals were monitored at 37°C, with measurements taken at 2-minute intervals over a period of 2 hours, utilizing the Applied Biosystems™ 7500 Real-time PCR system (Thermo Fisher Scientific, MA, USA).

Structural simulation of protein-nucleic acid complexes

The online tool AlphaFold 3 (https://golgi.sandbox.google.com/) was used to construct the complex model of proteins and nucleic acids[18]. The structure of Fncas12a (PDB: 5NG6) was utilized as the initial structural model for the structural simulation process. In the course of modeling, the number of runs was configured as 3, and the top 5 ranked models were generated. Ultimately, the models were chosen as experimental models in accordance with the scoring systems of iPTM and pTM[18, 19]. Both pTM and ipTM scores are derived from a metric named the Template Modeling (TM) score. A pTM score exceeding 0.5 implies that the overall predicted folding of the complex is likely to bear resemblance to the actual structure.

The ipTM serves to evaluate the predicted accuracy of the relative positions of subunits within the complex. A value greater than 0.8 denotes a high-quality prediction with substantial confidence, whereas a value less than 0.6 indicates a probable failed prediction.

Statistical Analysis

The t-test (unpaired t-test) was performed to assess significant differences between groups. A *P*-value less than 0.05 was considered statistically significant. *P*-values less than 0.0001, 0.001, 0.01, and 0.05 were denoted as ****, ***, **, and *, respectively. The bar plots show the means ± SD. Statistical analyses were conducted using GraphPad Prism 10.0.

Results

PAM-free dsDNA activates Cas12a's trans - cleavage

It is widely recognized that Cas12a can be activated by ssDNA to execute both specific and non-specific cleavage, independent of PAMs. Generally, Cas12a orthologs recognize a T-rich PAM located at nucleotides -2, -3, and -4 upstream of the target dsDNA sequence. Our investigation was aimed at clarifying the role of PAMs in modulating the dsDNA-triggered, non-specific ssDNA cleavage activity of Cas12a. To this end, we made use of three Cas12a orthologs—LbCas12a from *Lachnospiraceae bacterium*, FnCas12a from *Francisella tularensis*, and AsCas12a from *Acidaminococcus* sp. BV3L6. Each of these orthologs is known for recognizing distinct PAMs: TTTN, TTN, and TTTV (V=A/C/G), respectively[9]. As shown in Supplementary Figure 1, we designed four different crRNAs, namely crRNA-a,

crRNA-b, crRNA-c, and crRNA-d. For each crRNA, we synthesized eight complementary dsDNA substrates. These substrates incorporated various PAMs at positions -1 to -4 relative to the first nucleotide of the crRNA-targeted sequence. Our results, As presented in Figure 1A, our results revealed a substantial increase in the fluorescence signal for nearly all dsDNA substrates, with the exception of one containing an AAAC PAM. This indicates that most dsDNAs effectively activate the trans-cleavage activity of all three Cas12a orthologs. Notably, once activated, the trans-cleavage function of Cas12a progresses rapidly, reaching fluorescence signal saturation within approximately 30 minutes (Supplementary Figure 2). Significantly, dsDNAs bearing canonical PAMs (TTTV or TTN) exhibited comparable trans-cleavage efficiency comparable to those without PAMs, suggesting that there is no PAM preference for non-specific ssDNA cleavage dsDNA activation.

To conduct a more realistic study on the impact of PAM on the trans-cleavage activity of Cas12a, we shifted to using PCR-amplified IS6110 fragments derived from *M. tuberculosis* genomic DNA as the dsDNA substrates for the trans-cleavage assay. We designed ten crRNAs targeting different regions of IS6110, with target10 uniquely featuring a TTTT canonical PAM (Figure 1B). Upon incubation with CRISPR mixtures, all crRNAs, except for crRNA1, crRNA2, and crRNA3 (which target sequences with GGAG, CGGG, and GACA PAMs, respectively), induced strong fluorescence signals (Figures 1 C and D). Interestingly, although the CGGG PAM dsDNA showed high trans-cleavage efficiency with synthetic substrates (Figure 1A), its PCR-amplified counterpart exhibited reduced activity. This discrepancy suggests a

at the combined influence of PAM and target sequence on Cas12a trans-cleavage efficiency. Notably, the TTTT PAM in target10 accelerated the trans-cleavage rate, as evidenced by a more rapid stabilization of the fluorescence signal (Figure 1D). Collectively, our findings emphasize the role of PAM in enhancing Cas12a's trans-cleavage efficiency while highlighting that it is not essential for activating this activity.

CRISPR-Cas12a with PCR equally detects PAM and PAM-free DNA

Cas12a can be activated to exhibit its trans-cleavage activity by dsDNA independently of the PAM, a feature that significantly broadens the potential applications of nucleic acid detection using Cas12a. However, the sensitivity of nucleic acid detection is crucial for the development and implementation of detection methods and technological platforms. Therefore, we designed a CRISPR trans-cleavage experiment to determine whether there is a difference in sensitivity when Cas12a detects nucleic acids with or without the PAM. Specifically, we intended to use crRNAs targeting IS6110 to evaluate the sensitivity of CRISPR in detecting various loci of IS6110. Due to their suboptimal trans-cleavage efficiency, crRNA1, crRNA2, and crRNA3 were excluded from the limit of detection (LOD) assessments, while crRNA5 through crRNA10 were selected for these evaluations. To precisely quantify the analytical sensitivity of the CRISPR system, we systematically diluted the pUC57 vector harboring the IS6110 fragment from 3 million copies per μl down to 3 copies per μl, thereby facilitating an accurate assessment of LOD. Recognizing the limited sensitivity of the CRISPR-Cas12a system for nucleic acid detection, we integrated

nucleic acid amplification technology with CRISPR to enhance detection capabilities. For each PCR reaction, we added 1 μl of each diluted template, along with an equal volume of TE buffer as a negative control. Subsequently, 2 μl of unpurified PCR products were introduced into individual CRISPR reaction mixtures. The fluorescence intensities of the CRISPR reaction tubes were recorded at 30, 60, and 120 minutes intervals (Figure 2). By comparing the fluorescence signal values of the CRISPR reactions, we observed that after 30 minutes, when the substrate concentration was 30 copies, the fluorescence signal value in the experimental group was significantly higher than that of the negative control group (Figure 2A). After 60 and 120 minutes of the CRISPR detection reaction, all tested reactions exhibited a significant enhancement in fluorescence signal values (Figures 2B and C). However, the detection limit remained constant at 30 copies per reaction. Additionally, we found that the trans-cleavage efficiencies of crRNA6, crRNA7, and crRNA9 were lower than those of the other crRNAs, especially when the IS6110 substrate concentration was below 300 copies. Our results suggested that the sensitivity of CRISPR-Cas12a in detecting dsDNA substrates without a PAM is comparable to that of detecting substrates with a PAM when combined with nucleic acid amplification techniques.

Activation of Cas12a cis-cleavage is not essential for its trans-cleavage activity

It has become a consensus that PAM sequences play a pivotal role in enabling CRISPR systems to precisely cleave their intended targets. Our results, illustrated in Figure 1, demonstrated that the PAM motif is not essential for activating the trans-cleavage activity of Cas12a orthologs. This observation prompted us to inquire

whether the activation of Cas12a's trans-cleavage activity is contingent upon its cis-cleavage activity. To investigate the interplay between cis- and trans-cleavage activation, we evaluated Cas12a's cis-cleavage activity using the same dsDNA substrates employed in trans-cleavage assays. Cas12a/crRNA complexes were incubated with respective substrates at 37 °C for 30 minutes, followed by PAGE analysis to quantify cleavage efficiency (Supplementary Figure 3). The cleavage ratios for each substrate were systematically calculated and visualized in Figure 3. As shown in the PAGE gel image in Supplementary Figure 3A and the cleavage efficiency plots in Figures 3A-D, , we found that all Cas12a orthologs exhibited the strongest cleavage activity when the DNA substrates contained a "T" base at the -2 and -3 positions of the PAM, which is consistent with the canonical Cas12a TTTN PAM motif. More notably, we observed that many dsDNA substrates lacking the PAM could also be cleaved, at least partially, by Cas12a orthologs. LbCas12a demonstrated minimal cleavage of substrates from the target-a and target-b series, while AsCas12a and FnCas12a were able to cleave most of these substrates, though some exhibited weaker activity. Overall, the cleavage activity of LbCas12a towards nearly all substrates was significantly weaker compared to that of the AsCas12a and FnCas12a orthologs.

To more accurately reflect the cis-cleavage efficiency of Cas12a independent of the PAM, we employed PCR-amplified IS6110 as the substrate for the cis-cleavage assay, consistent with previous experiments. The IS6110 gene fragments were obtained through PCR amplification and purified to prepare the required reaction

substrates, following the procedures described in the Materials and Methods section.

As shown in the PAGE image in Supplementary Figure 3B, after incubation of various Cas12a/crRNA complexes with IS6110, the majority of the bands remained intact, indicating that Cas12a/crRNA was unable to cleave these substrates (Figure 3E). A noteworthy observation emerged: all Cas12a orthologs, when charged with crRNA10, which is complementary to target 10 featuring a TTTN PAM motif, almost completely cleaved the IS6110 substrates. Intriguingly, we discerned clear cleavage specifically in IS6110 substrates when combined with AsCas12a/crRNA4 or FnCas12a/crRNA4, but not with LbCas12a/crRNA4. This particular crRNA4 was complementary to an IS6110 region characterized by a non-canonical ATCA PAM sequence. Similarly, albeit to a lesser extent, we detected faint cleavage in substrates mixed with AsCas12a/crRNA6 or FnCas12a/crRNA6, excluding LbCas12a/crRNA6, where crRNA6 targets an IS6110 region marked by a non-canonical GTGC PAM. Furthermore, minimal cleavage was observed in substrates involving AsCas12a/crRNA5 or AsCas12a/crRNA8, whose respective crRNAs match IS6110 regions characterized by non-canonical PAMs TTGG and CCGC. This observation suggested that the AsCas12a exhibited a greater tolerance for PAM variations compared to the LbCas12a and FnCas12a. Collectively, considering the data presented in Figure 1, we concluded that the activated cis-cleavage activity of Cas12a enhances their trans-cleavage activity but not essential for activation of their trans-cleavage activity.

Structural simulation uncovers PAM-independent mechanism of Cas12a

trans-cleavage

To elucidate the mechanism underlying PAM-independent activation of Cas12a's trans-cleavage activity, we employed the AlphaFold v3 for structural simulation of the Cas12a-crRNA-dsDNA ternary complex. Among three Cas12a orthologs analyzed, FnCas12a was prioritized for its structural clarity and functional relevance. We established an experimental framework pairing crRNAa with eight distinct dsDNA substrates (a1-a8) to probe sequence-agnostic activation mechanisms. As shown in Figure 4A, the RuvC catalytic domain (mediating dual cis/trans-cleavage functions)[11, 20, 21] remains occluded in the apo FnCas12a/crRNA complex due to steric constraints imposed by REC2-Nuc domain interactions. This locked conformation renders the enzyme catalytically inert. Strikingly, introduction of dsDNA substrates (a1-a8) induces immediate disruption of REC2-Nuc interdomain contacts. This allosteric rearrangement triggers global conformational reorganization of FnCas12a, fully exposing the RuvC active sites. The resultant catalytically competent FnCas12a/crRNA/dsDNA ternary complex demonstrates robust cleavage activity independent of PAM sequence recognition, as evidenced by uniform activation across all eight substrates.

To systematically evaluate PAM sequence tolerance, we performed structural alignment of all eight FnCas12a/crRNAa/dsDNA complexes (a1-a8) through superposition analysis (Figure 4B). Notably, these complexes exhibited minimal conformational divergence (root mean square deviation <1.5 Å across all pairwise comparisons), demonstrating remarkable structural conservation regardless of PAM

presence. This structural parity confirms that both TTTN-containing and TTTN-deficient dsDNAs can effectively activate Cas12a's trans-cleavage function, with activation capacity showing no strict correlation with canonical PAM motifs.

RNA targeting directly activates Cas12a's tran-Cleavage

We have demonstrated that dsDNA without a PAM can trigger the trans-cleavage activity of Cas12a, a discovery that has not been well documented in the existing literature. Inspired by this finding, we initiated an investigation to determine whether RNA could directly stimulate the trans-cleavage activity of Cas12a, despite the absence of prior reports on such direct activation. To this end, we employed the previously utilized crRNA-b, crRNA-c and crRNA-d as crRNAs for the assay and synthesized their corresponding RNA substrates. We utilized LwCas13a, an RNA-targeting nuclease, as a positive control in this assay[22]. Figure 5A presented an intriguing outcome: when RNA target b served as the CRISPR substrate, it effectively activated the non-specific ssDNA cleavage activity of both LbCas12a and AsCas12a, but notably failed to elicit a response from FnCas12a. Surprisingly, LwCas13a, our positive control, exhibited minimal trans-cleavage activity under these conditions. When RNA target c and RNA target d were introduced as CRISPR substrates, they successfully activated the trans-cleavage activity of LwCas13a, LbCas12a, and AsCas12a, with LwCas13a displaying the most robust activity among them. Nevertheless, neither RNA target c nor RNA target d could stimulate the trans-cleavage activity of FnCas12a. To evaluate the activation efficiency of RNA relative to DNA, we compared the trans-cleavage activities of Cas12a induced by

different activators. As illustrated in supplementary Figure 4, the fluorescence signals in reactions activated by dsDNA or ssDNA reached saturation within a mere 30 minutes. In contrast, RNA-activated reaction showed no signal saturation even after 2 hours of monitoring. These findings conclusively indicate that although RNA is capable of directly activating Cas12a's trans-cleavage activity, its efficiency is markedly lower than that of DNA-based activators.

We hypothesized that the inability of RNA to activate FnCas12a's trans-cleavage activity might be influenced by its cis-cleavage activity. To test this hypothesis, we labeled RNA substrates with FAM and observed the cleavage results via gel electrophoresis (Figure 5B). Prior to gel electrophoresis, all reaction mixtures were treated with Proteinase K to digest Cas12a proteins. Surprisingly, in the FnCas12a reaction system, the binding between crRNAs and RNA targets was significantly weaker compared to that observed in the LbCas12a and AsCas12a mixtures. This observation may provide an explanation for why RNA target b, c, and d are unable to directly activate Cas12a's trans-cleavage activity. However, the underlying reason for the failure of crRNA-b, c, and d to bind to their respective RNA substrates in the FnCas12a reaction system remains elusive and necessitates further investigation.

To rigorously validate RNA’s ability to directly activate Cas12a trans-cleavage, we performed experiments using in vitro transcribed IS6110 RNA as the substrate. Prior to assays, RNA purity was ensured through DNase I treatment to eliminate residual DNA templates, followed by purification. We tested all previously designed crRNAs (crRNA1–crRNA10) against the IS6110 RNA substrate across three Cas12a

orthologs (LbCas12a, FnCas12a, AsCas12a).As shown in Figure 5C, the fluorescence intensity in reactions containing LbCas12a/crRNA complexes increased significantly over time when bound to their corresponding RNA substrates, whereas the negative control showed negligible signal changes. Fluorescence values measured at 120 minutes were statistically compared to the negative controls (Figure 5D), revealing a significant enhancement in signal intensity for LbCas12a/crRNA-substrate complexes. When using FnCas12a, similar results were observed, with all FnCas12a/crRNA complexes significantly increasing fluorescence intensity, although the activated fluorescence intensity may be lower than that of LbCas12a/crRNA. In contrast, in the AsCas12a reaction system, only the crRNA3, crRNA4, and crRNA10 systems showed an increase in fluorescence intensity over time (Figure 5C). After 120 minutes, we also found that only the crRNA3, crRNA4, and crRNA10 reaction systems exhibited significantly higher fluorescence intensities compared to the negative controls (Figure 5C). Notably, IS6110 RNA10 emerged as the most potent activator across all Cas12a orthologs, suggesting potential PAM-like sequence effects in RNA-mediated activation. In summary, all these results indicate that RNA can directly activate the trans-cleavage activity of Cas12a, and this activity is influenced by the type of Cas12a and the RNA sequence.

CRISPR-Cas12a nucleic acid detection without PAM using clinical samples

In an effort to explore the application of LbCas12a in detecting DNA lacking a PAM sequence, we sought to assess the practicality of utilizing LbCas12a for identifying target nucleic acids devoid of PAM in clinical samples. We collected sputum samples

from tuberculosis patients and healthy individuals. IS6110 gene was selected as the target DNA for detection. For Cas12a, we chose crRNA5, crRNA6, crRNA7, crRNA8, and crRNA9, which can recognize IS6110 DNA regions without PAM, as the guiding crRNAs. Additionally, crRNA10, which targets an IS6110 DNA region containing a TTTT PAM, was included as a control. As shown in Figure 6A, all PAM-free targets in TB-positive sputum samples were successfully detected by their respective LbCas12a/crRNA complexes, and the outcomes were consistent with those from LbCas12a/crRNA10 complex. This finding indicated that PAM - free targets could be viable candidates for nucleic acid detection. To provide more robust experimental evidence for the concept that the activation of Cas12a's trans-cleavage activity is independent of the specific PAM motif of dsDNA, we designed an additional set of nine crRNAs targeting the IS6110 gene fragment. Notably, the regions of IS6110 complementary to these crRNAs lack the specific TTTN PAM motif in their adjacent areas. As illustrated in Figure 6B, the presence of the target IS6110 from Mtb positive sputum activated the trans-cleavage function of all LbCas12a/crRNA complexes, generating intense fluorescence signals. Conversely, in the absence of the target, no such fluorescence was observed. These results further corroborated that PAM-free targets were suitable for nucleic acid detection. Simultaneously, after transcribing IS6110 into its corresponding RNA, which was then used as a substrate, incubating these substrates with the LbCas12a/crRNA complex successfully activated the trans - cleavage activity of LbCas12a. This strongly suggested that RNA had the ability to directly activate the trans-cleavage activity of Cas12a.

Drug resistance in *M. tuberculosis* often arises from changes in amino acid sequences due to single-base mutations in specific genes. These mutated sites seldom contain suitable PAM sequences, which has previously limited their use as targets for CRISPR-Cas12a. Here, we aimed to investigate whether CRISPR-Cas12a could detect drug-resistance mutations in absence of PAM sequences. We selected the 1349th base of the *RpoB* gene associated with rifampicin resistance, the 944th base of the katG gene related to isoniazid resistance, and the 15th base of the inhA gene promoter also related to isoniazid resistance for CRISPR detection. As shown in Figure 6C, we designed crRNAs specific to wild-type gene sites and performed CRISPR detection. Notably, we observed that all target regions lacking PAM sequences activated the trans-cleavage activity of LbCas12a (Figure 6D). Both LbCas12a/crRNA and FnCas12a/crRNA could effectively distinguished between wild-type and mutant alleles at the 1349th position of the RpoB gene and the -15th position of the inhA gene promoter, while AsCas12a was unable to differentiate between the wild-type and mutant allele of these two genes. Surprisingly, however, the crRNA designed to complement the wild-type katG 944 site induced stronger fluorescence from the mutant substrate than from the wild-type, suggesting that fluorescence intensity may be influenced by the preference of the crRNAs for their substrate sequence. In summary, and most significantly, all these drug resistance-related genes lacking PAM were effectively detected by Cas12a, underscoring the potential of CRISPR-Cas12a for detecting base mutations at various genomic locations.

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Discussions

CRISPR technology is being widely employed in various forms of nucleic acid detection, including viruses, bacteria, and tumor mutations, due to its superior performance in sensitivity and specificity, making it a promising next-generation molecular diagnostic tool[23-25].It is well established that the cleavage of targets by CRISPR-Cas12a relies on the PAM sequence present in the substrate[9,11]. Nevertheless, there have been reports of non-canonical PAM sequences activating the cis-cleavage activity of Cas12a [13,15]. Previously, it was believed that substrates lacking PAM sequences could not activate the cis-cleavage, and consequently, the trans-cleavage activity of Cas12a, despite a lack of definitive evidence.This limited the applicability of CRISPR-based nucleic acid detection methods, particularly in targeting specific loci. However, it has been demonstrated that ssDNA can activate both the cis- and trans-cleavage activities of Cas12a in the absence of PAM motifs[11,12].Consequently, researchers have explored alternative approaches to overcome Cas12a's PAM dependence in dsDNA detection by converting dsDNA detection into ssDNA detection, employing asymmetric PCR or enzymatic digestion of non-target DNA strands to convert dsDNA PCR products into ssDNA, which are then subjected to CRISPR detection[26,27]. Others have reported fusing PAM sequences recognized by Cas12a onto primers to overcome PAM limitations in CRISPR detection[20,28]. However, these methods complicate the CRISPR detection process, hindering its widespread adoption.

Our findings underscore a crucial discovery: Cas12a effectors can exhibit robust

trans-cleavage activity even in the absence of canonical PAM sequences. This observation challenges the long-held belief that PAM is indispensable for Cas12a activation, revealing a fundamental distinction between its cis- and trans-cleavage modes of action. Our conclusion aligns with recently published work by Eric A. Nalefski et al [29]. Importantly, we demonstrate that the trans-cleavage activity of Cas12a can be activated solely by binding to the target substrate, independent of cis-cleavage activation, thereby shedding new light on the mechanisms of this versatile enzyme. This may explain why PAM motifs are not essential for activating Cas12a's trans-cleavage activity. Moreover, while PAM sequences are not strictly necessary, they enhance the trans-cleavage rate of Cas12a effectors following dsDNA activation, which contradicts Eric's report that canonical TTTN does not affect Cas12a's trans-cleavage activity [29]. This finding is intriguing as it suggests that PAM sequences modulate the kinetics of Cas12a's non-specific ssDNA cleavage activity. However, our detection analysis limits reveal that PAM sequences do not significantly improve the overall sensitivity of Cas12a in nucleic acid detection when combined with nucleic acid amplification techniques, indicating that Cas12a can be utilized for PAM-free target nucleic acid detection without compromising sensitivity, as validated by subsequent clinical sample testing.

Traditionally, Cas12a's trans-cleavage activity has been considered to be activated exclusively by DNA substrates[11, 12].Therefore, when using Cas12a for RNA detection, it was necessary to reverse transcribe RNA into DNA first[30-32]. Several studies have also reported that strategies involving the splitting of

CRISPR-Cas12a's crRNA or activator can be employed for the detection of miRNA[33-36]. In this study, we discovered that direct RNA targeting can activate Cas12a's trans-cleavage function, thereby expanding its potential applications in RNA detection. However, the efficiency of RNA in activating Cas12a's trans-cleavage activity is lower than that of a DNA activator. Further investigations are needed to enhance its sensitivity for practical RNA detection. Recent publications support our findings, indicating that RNA can directly activate Cas12a's trans-cleavage activity[37,38]. Our data demonstrated that different Cas12a orthologs exhibit varying degrees of RNA-activated trans-cleavage activity, with LbCas12a displaying the most potent response. This contrasts with Zhang's report, which suggests that AsCas12a exhibits stronger RNA-activated trans-cleavage activity than LbCas12a[37]. These discrepancies underscore the need for further research to elucidate the underlying mechanisms. Additionally, we found that Cas12a's RNA-activated trans-cleavage activity may preferentially target substrates containing AAAA motifs, similar to Oleg Dmytrenko's report on Cas12a2, an RNA-activated nuclease capable of cleaving ssRNA, ssDNA, and dsDNA, which shows a preference for GAAAG motifs[39,40].

The practical implications of our findings are profound. Traditionally, CRISPR-Cas12a-based nucleic acid detection platforms have relied on the presence of a PAM sequence for target recognition and cleavage. However, our results demonstrate that Cas12a can be effectively used for detection in the absence of a PAM. This approach not only overcomes the limitation of PAM-dependency but also enhances the versatility of Cas12a in detecting targets with mutated PAM sites or

those lacking a PAM altogether. Notably, our study highlights the potential of CRISPR-Cas12a in detecting drug-resistance mutations without PAM sequences. This is particularly significant in the context of tuberculosis diagnostics, where drug resistance often arises from mutations in genes that do not harbor suitable PAM motifs.

In conclusion, our findings provide new insights into the mechanistic underpinnings of Cas12a's trans-cleavage activity and demonstrate its potential in nucleic acid detection applications beyond PAM-dependent targets. The discovery that Cas12a can be activated by dsDNA and RNA in the absence of a PAM sequence significantly broadens its application horizon, offering new opportunities for the development of highly sensitive and versatile diagnostic platforms. Further studies are warranted to fully explore the therapeutic and diagnostic potential of Cas12a's non-PAM-dependent trans-cleavage activity.

CRediT authorship contribution statement

Guohui Xiao: Writing - original draft, Conceptualization, Methodology, Validation, Investigation, Project administration. **Hongyu Shi:** Writing - original draft, Methodology, Data curation, Formal analysis, Visualization. **Meixia Liu:** Writing - original draft, Methodology, Data curation, Formal analysis. **Min huang:** Resources, Data curation, Formal analysis. **Siqi Li:** Resources, Data curation, Formal analysis. **Xuefeng Zhou:** Resources, Data curation, Formal analysis. **Hengfei Li:** Resources, Data curation, Formal analysis. **Guoliang Zhang:** Writing - review & editing, Conceptualization, Methodology, Project administration, Funding acquisition,

Supervision.

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Declaration of competing interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Activation of Cas12a's trans-cleavage activity by dsDNA in the absence of PAM sequences. (A) Assessment of the trans-cleavage activity of Cas12a effectors using synthesized dsDNA substrates that incorporate a variety of PAM sequences. **(B)** The sequence of IS6110, where the target regions complementary to crRNA1-10 are marked in red and PAM sequences are marked in yellow. **(C)** Assessment of the trans-cleavage activity of Cas12a effectors by employing PCR-amplified IS6110 substrates containing various PAM sequences. **(D)** Time-dependent fluorescence signals obtained from trans-cleavage assays of Cas12a effectors with PCR-amplified

IS6110. Data are presented as the mean ± standard deviation (n = 3 technical replicates); RFU: Raw Fluorescence Units. NTC: Non-target control.

Figure 2. Assessment of LOD for CRISPR-Cas12a detection of IS6110 target. IS6110 substrate was diluted across a series of concentration gradients, followed by PCR amplification. Subsequently, the PCR products were incubated with the LbCas12a mixture. The LOD values for each crRNA were calculated at 30 minutes **(A)**, 60 minutes **(B)**, and 120 minutes **(C)** post-incubation. The x-axis represents the copy number of IS6110 fragments in each reaction. RFU: Raw Fluorescence Units. The data are reported as the mean ± standard deviation (n = 3 technical replicates); statistical significance is indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, ns indicating no significance.

Figure 3. The effects of PAMs on cis-cleavage activity of Cas12a effectors.

(A-D) In the cis-cleavage assay, a variety of synthesized dsDNA substrates were utilized. These substrates were incubated with individual CRISPR mixtures for 30 minutes, followed by analysis using PAGE. **(E)** PCR-amplified IS6110 fragments were utilized to evaluate the cis-cleavage activity of Cas12a effectors. The PCR-amplified fragments were also incubated with each CRISPR mixture for 30 minutes and then analyzed by agarose electrophoresis. The extent of cleavage was quantified by measuring the electropherograms of the cleavage products.

Figure 4: Structural representations of FnCas12a/crRNA complexes with dsDNAs containing various PAM sequences.

(A) Depiction of the control FnCas12a/crRNA complex on the left, and

FnCas12a/crRNA/dsDNA complexes with various PAM sequences (labeled below each structure, such as TTT A, TAAA etc.) on the right. The key domains including REC1, REC2, RuvC, and Nuc are labeled in red for easy identification. Different colors are used to distinguish various structural domains of the complexes. **(B)** A superpose view of the FnCas12a/crRNA/dsDNA complexes, highlighting the overall interaction. The dsDNA and PAM regions are labeled in red, while the important domains (REC1, REC2, RuvC, Nuc) are also indicated in red, showing their relative positions within the complex.

Figure 5. RNA targeting directly activates Cas12a's tran-Cleavage. (A) Illustration of the activation of Cas12a effector's trans-cleavage activity through RNA targeting. Synthesized RNA targets b, c, and d were employed as CRISPR substrates in trans-cleavage assays, and the fluorescence signals of each CRISPR reaction were recorded at the 60-minute time point. **(B)** Cis-cleavage assay of Cas12a effectors, where FAM-labeled synthesized RNA targets b, c, and d served as CRISPR substrates. **(C)** Time-course analysis of fluorescence signals from trans-cleavage assays of Cas12a effectors using IS6110 RNA. **(D)** Fluorescence signals of each CRISPR reaction at the 120-minute time point. Data are presented as the mean ± standard deviation (n = 3 technical replicates). Statistical significance is indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, and “ns” indicates no significance. RFU stands for Raw Fluorescence Units.

Figure 6. CRISPR-Cas12a nucleic acid detection without PAM using clinical samples. (A) Detection of the IS6110 gene in clinical sputum samples using

LbCas12a in combination with crRNA5 - crRNA10 . Samples 1 - 10 have been verified as Mtb-positive, while samples 12 - 15 have been confirmed as Mtb-negative. **(B)** An additional set of nine crRNAs targeting the IS6110 gene fragment were used. The regions in IS6110 complementary to these crRNAs lack the specific TTTN PAM sequences in their vicinity. Green fluorescence indicates the outcomes of reactions with LbCas12a/crRNA complexes, where either IS6110 dsDNA or transcribed RNA was used as substrates. A non-target sample served as a negative control. Visual results were captured by smartphone under blue light illumination. **(C)** Design of customized crRNAs for the detection of genetic mutations. **(D)** Identification of genetic mutations associated with Mtb drug resistance. WT: wild type; MT: mutation gene type; NTC: Non-target control; RFU: Raw Fluorescence Units. The data are reported as the mean ± standard deviation (n = 3 technical replicates); statistical significance is indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, “ns” indicating no significance.

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