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Trans Single-Stranded DNA Cleavage Via CRISPR/Cas14a1 Activated by Target RNA without Destruction

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Abstract: As a CRISPR-Cas system (clustered regularly interspaced short palindromic repeats and CRISPR associated proteins), Cas14a1 can *cis*/*trans* cleave single-stranded DNA (ssDNA). Here, we discover an un-reported capacity of Cas14a1, RNA can trigger the *trans* ssDNA cleavage. This Cas14a1-based RNA-activated detection platform (Amplification, Transcription, Cas14a1-based RNA-activated *trans* ssDNA cleavage, ATCas-RNA) has an outstanding specificity for the detection of target RNAs with point mutation resolution, which is better than that of the Cas14a1-based ssDNA-activation. Using ATCas-RNA via a fluorophore quencher-labeled ssDNA reporter (FQ), we were able to detect 1 aM pathogenic nucleic acid within 1 h, and achieve 100% accuracy with 25 milk samples. This platform can serve as a new tool for high efficiency nucleic acid diagnostics. Importantly, this work can expand our understanding of Cas14a1 and inspire further mechanisms and applications of Class-2 Cas systems.

Introduction

CRISPR-Cas system in archaea and bacteria provides adaptive immunity against virus invasion by destroying their genetic elements.[1] Depending on the organization of Cas proteins, CRISPR-Cas systems are commonly classified into two distinct classes: class 1 systems include multiple sub-modules, while class 2 systems merge those functions into a single effector.[2] Due to their programmability, Class 2 systems have become a research hotspot.[3] After the pioneering discovery of specific bacterial gene cleavage by Cas9 endonuclease [4], a series of other effectors, such as Cas12[5]and Cas13[6], have been widely studied. Their applications span from nucleic acid diagnostic and multiplexed orthogonal genome editing to therapy of haplo-insufficiency using CRISPR activation.[7]

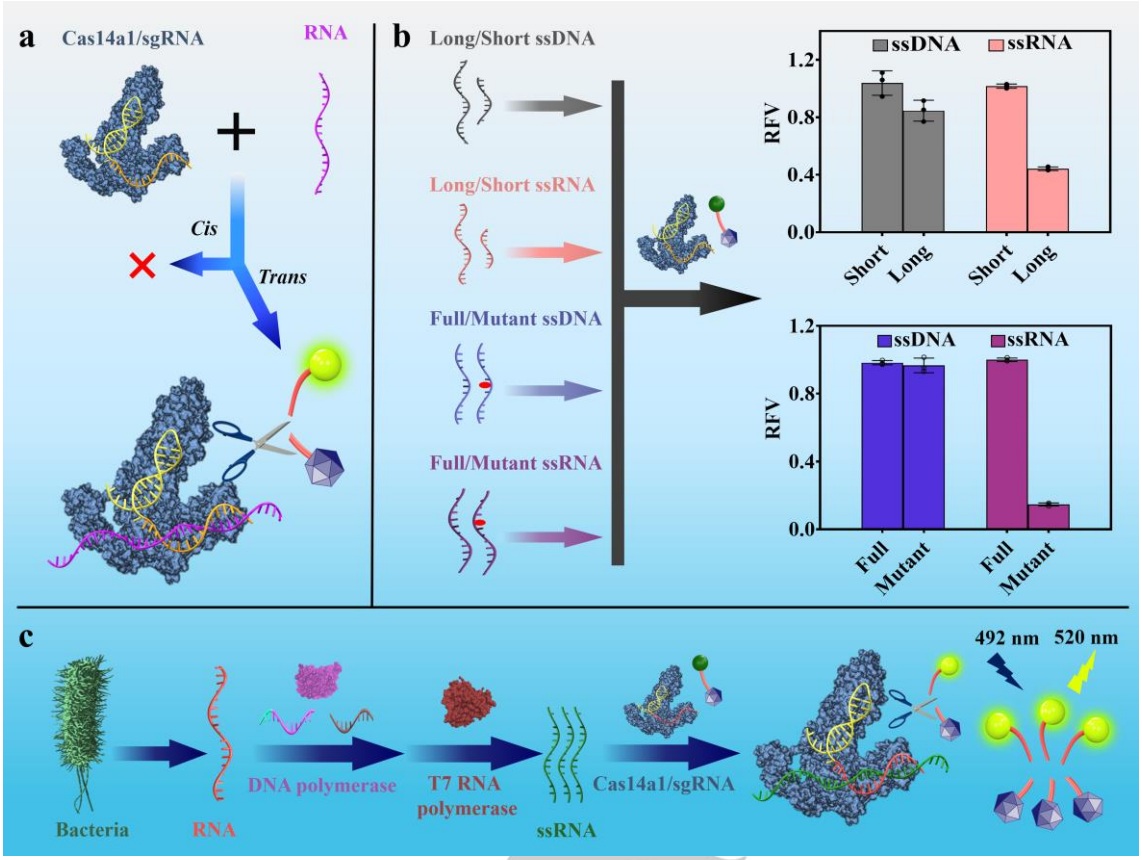
Cas14, a novel class 2 CRISPR-Cas system , was discovered by genome-resolved metagenomics from uncultivated archaea.[8] The bioinformatic analysis demonstrated that Cas14 proteins are putative type V variants genetically related to TnpB, a family of RuvC domain-containing proteins. Initially, Cas14, which is similar to Cas12a[9], was found to possess the ability of binding and cleaving single-stranded DNA (ssDNA) (both *cis*/*trans* cleavage) and PAM-double-stranded DNA (PAM-dsDNA) in *cis*-cleavage.[10] To investigate the mechanism of the Cas14a1-mediated dsDNA cleavage, the tertiary structure of Cas14a1 in biocomplex with the sgRNA and its target dsDNA with TTTG PAM site has been analyzed by cryoelectron microscopy (cryoEM). Unexpectedly, the observed structure indicated that two Cas14a1 molecules could bind with one sgRNA to form a ribonucleoprotein effector.[11] However，the self-assembly mechanism of the effector is still not completely understood. In addition, the Cas14a1 tertiary structure can provide totally different cleavage activities under different conditions (for example with varied NaCl concentration within the buffer) [11a], which inspired us to further explore the biochemical functionalities of Cas14a1.

In this work, we report, for the first time, the discovery of the capacity of Cas14a1 to provide *trans*-cleavage of ssDNA, specifically activated by a target RNA, without self-destruction (Scheme 1a, Figure1, and Figure S2a-b). To explore the influence of the active factor (target RNA or ssDNA) for the *trans*-cleavage activities of Cas14a1, we designed a series of target RNA and ssDNA with different lengths and mutation sites. We found that the self-assembled complex of Cas14a1/sgRNA-target RNA can only achieve *trans*-cleavage of the ssDNA FQ reporter (Scheme 1a). Furthermore, the substrate selectivity of this complex activated by target RNA can be higher than when activated by target DNA (Scheme 1b). Based on this discovery, we have developed a new ATCas-RNA platform that can detect pathogens via a FQ reporter with both high-selectivity and high-sensitivity (Scheme 1c), which is akin to that of Cas13-based RNA diagnostic system.[6a] Therefore, this new platform can expand the application of the CRISPR/Cas14a1 from cell biology research to precision medicine.[12]

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Scheme 1. The RNA target-activated *trans* ssDNA cleavage activity of the CRISPR/Cas14 and its application. a) Diagram of RNA target triggered *trans*-cleavage activity of the Cas14a1. b) The influence of the lengths (20 nt and 50 nt) and mutants of ssDNA or RNA target for triggering the *trans*-cleavage activity of the Cas14a1. c) The working principle of the ATCas-RNA platform. The 16S rRNA of bacteria was extracted from the artificially infected samples, then amplifications were followed by DNA polymerase and T7 RNA polymerase in series to generate short RNA target. Finally, the *trans*-cleavage activity of the Cas14a1/sgRNA complex was triggered by amplified RNA target to cleave FQ generating a fluorescent signal.

Results and Discussion

Cas14a1 is a ~62 kDa multi-functional Cas protein belonging to the Class 2 Type V CRISPR-Cas system (Figure S1). So far, studies have focused on DNA-related activities, including *cis*/*trans*-cleavage of target ssDNA[8] and *trans*-cleavage triggered by PAM-dsDNA with destruction[10], which is similar to the function of Cas12.[13] Recently, Cas12g derived from diverse functional type V systems, has been reported to display RNA-activated *cis*-cleavage of RNA and *trans*-cleavage of RNA and ssDNA.[14] We speculate that the *trans*-cleavage activity of Cas14a1 might be activated by RNA via pairing with the spacer sequence of sgRNA.

Inspired by the report about *cis*/*trans* RNA cleavage activity of the Cas12g system,[14] we tested our hypothesis and found that the Cas14a1/sgRNA complex can be activated by target RNA to trigger *trans*-cleavage of ssDNA FQ *in vitro* (Figure 1a), which was consistent with the target ssDNA as an activator (Figure 1d). To investigate whether the cas14a1/sgRNA complex can perform *cis* RNA cleavage, we used denaturing and non-denaturing acrylamide gels to test the *cis* -cleavage activity of Cas14a1, with *trans*-cleavage activity as well for comparison The acrylamide gel results displayed that the target RNA stayed in undamaged condition (Figure 1b and Figure S2a), which indicated that RNA cannot trigger the *cis*-cleavage activity of Cas14a1, while it could activate the *trans*-cleavage of ssDNA (Figure 1c and Figure S2b). However, both *trans*- and *cis*- cleavage activity of Cas14a1 could be successfully triggered by ssDNA (Figure 1e-f and Figure S2c-d), which was consistent with previous studies.[8] We thus conclude that RNA can trigger the *trans*-cleavage activity of Cas14a1，but not the *cis*-cleavage while ssDNA can do both.

To achieve better cleavage performance using this Cas14a1-based RNA activated platform, we optimized the reaction conditions including metal ions types (Fe^{2+}, Li^{+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, K^{+}, and Mg^{2+}), NaCl and Mg^{2+} concentration (5-120 mM), buffer types (HEPES, Tris-HCl, and PBS), temperature (30-55 ℃), and pH (7-9.5). As shown in Figure S3, under optimal reaction condition, 10 mM Mg^{2+} in 20 mM Tris, 20 mM NaCl buffer (pH 8.5) at 37 ℃, the real-time fluorescence response of the Cas14a1-based system for RNA sensing achieved the strongest intensity. The control assays for Cas14a1/sgRNA complexes either alone or in the presence of a non-complementary RNA produced a negligible signal related to a complementary RNA target. Therefore, the following experiments were performed under this optimal condition.

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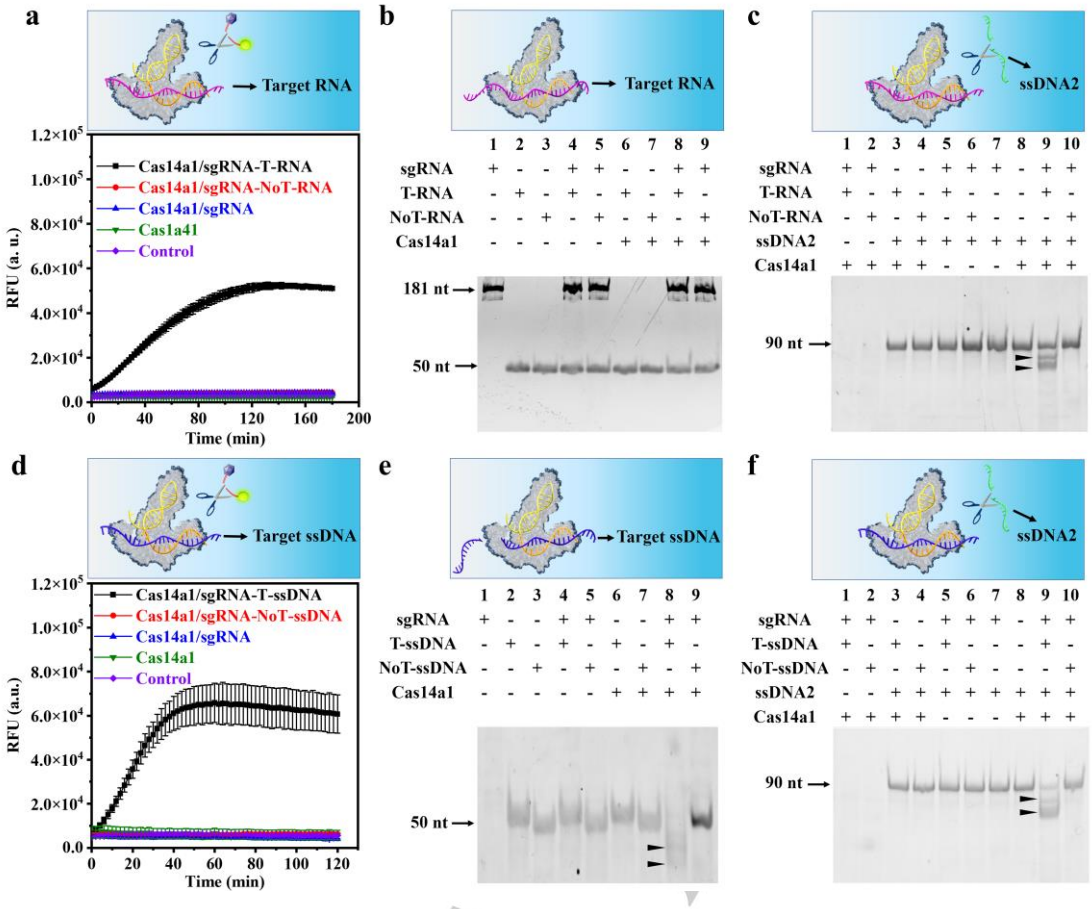


Figure 1. The comparison for *cis/trans* cleavage activity of Cas14a1/sgRNA complexes activated by targets (RNA and ssDNA). a and d) Trans nucleic acid cleavage kinetics of the Cas14a1/sgRNA complex activated by RNA (T-RNA (or T-ssDNA) represented target RNA (or ssDNA), NoT-RNA (or NoT-ssDNA) represented non-complementary target RNA (or ssDNA)). b) The *cis* RNA cleavage for the Cas14a1/sgRNA complex using denaturing acrylamide gels. c) The *trans* ssDNA cleavage of Cas14a1/sgRNA complex activated by the RNA using non-denaturing acrylamide gels. e) The *cis* ssDNA cleavage for Cas14a1/sgRNA complex using non-denaturing acrylamide gels. f) The *trans* ssDNA cleavage via Cas14a1/sgRNA activated by the ssDNA using non-denaturing acrylamide gels. (n=3 technical replicates, bars represent mean±s.d. the arrow represents the cleavage bands.)

To further understand the factors influencing the biochemical function of Cas14a1, various factors were investigated including the length for target sequences and sgRNA spacer, catalytic efficiency, and mismatches in the target.

The influence of target length in the *trans*-cleavage performance of Cas14a1 was studied by two approaches. (1) In approach 1, the length of targets are above 20nt, which used the middle 20 nucleic bases (dark orange color from sgRNA showed in Figure 2a) as the reference point, varied by extending to both sides, as shown in Figure 2a (detailed sequences information is in Table S2). (2) In approach 2, the lengths of target nucleic acid are below 20nt, varied by shortening from either side, as shown in Figure 2b.

In approach 1, different target sequences (20 nt, 30 nt, 50 nt, 70nt, and 90 nt) were tested at 100 nM using the fluorescent assay. As shown in Figure 2a and S4, the highest fluorescent signal was observed with 20 nt target sequence, and it was consecutively reduced as the target sequence increased from 20 nt to 90 nt. This might be attributed to the secondary structure of the target sequence, since longer target sequences have higher chances to form stable secondary structures that prevents the activation of Cas14a1. Simulation data from Figure S5 also validated this theory, showing much lower Gibbs free energy, more stable of the secondary structure. We also observed similar results for the *trans* cleavage with a PAM-containing dsDNA substrate (Figure S6), in accordance with previous report.

In approach 2, as in approach 1, target sequences (5' (or 3')-0 nt, 5' (or 3')-2 nt, 5' (or 3')-4 nt, 5' (or 3')-6 nt, and 5' (or 3')-8 nt) were also studied for the cleavage activation of Cas14a. As shown in Figure 2b, and as expected, we observed decreasing signal as the target sequences (ssDNA or RNA) varied from 5' (or 3')-0 nt to 5' (or 3')-8 nt. This could be explained by the fact that shorter target sequences become than the complementary fragment of sgRNA (20 nt) cannot totally trigger the cleavage activity of Cas14a1. Meanwhile, the data also suggested that the cleavage activity was more sensitive to modifications in its 3' end of target sequences (or 5' side of sgRNA).

We then designed different sgRNA to explore the optimal spacer length (10 nt, 15 nt, 20 nt and 25 nt; Table S3-S4 for more detail sequences information). The results showed that the most robust nonspecific DNase activity was exhibited when the target was 50 nt and the sgRNA spacer was 20 nt length (Figure 2c and Figure S7). These results were in accordance with the results of non-denaturing acrylamide gel analysis (Figure S2).

Next, we studied the kinetics of the Cas14a1-sgRNA complex activated by different nucleic acid substrates.[15] As shown in Figure S8, we found that Cas14a1/sgRNA bound to an RNA-activated

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trans ssDNA cleavage at a rate of ~0.2 turnovers per second and a catalytic efficiency (kcat/Km) of 4.57×10^6 s^{-1} M^{-1}. When bound to an ssDNA target, Cas14a1/sgRNA catalyzed at ~0.94 turnovers per second with a catalytic efficiency (kcat/Km) of 7.94×10^6 s^{-1} M^{-1}. When bound to a PAM-dsDNA target, Cas14a1/sgRNA catalyzed at ~0.45 turnovers per second with a catalytic efficiency (kcat/Km) of 3.03×10^6 s^{-1} M^{-1} (Table S1). The results suggested that the catalytic efficiency of the ssDNA activator was roughly two-fold higher than the PAM-dsDNA activator and RNA activator.

The mismatch tolerance of Cas14a1 was tested as well by tiling single or 2-nt across the targeted region in various 20 nt RNA and ssDNA substrates (Table S2). Surprisingly, single mismatches near the 3' terminal of the ssRNA target strongly inhibited the Cas14a1 activity, revealing a seed sequence of the 3' terminal for target RNA that was distinct from the seed region observed for ssDNA-targeting Cas14a1 activity. When using target ssDNA, the relative fluorescent signals were low if the single-mismatch locates in the middle of the target sequence. (Figure 2d, Figure S9). The results for the 2-nt mismatches of the targets were similar to the single mismatch (Figure S10) and consistent with previous work on Type V CRISPR-Cas enzymes targeting ssDNA.[8] Unlike Cas13a-based RNA detection[16], Cas14a1 had a high discrimination capacity against single mismatches in the target RNA.[17]

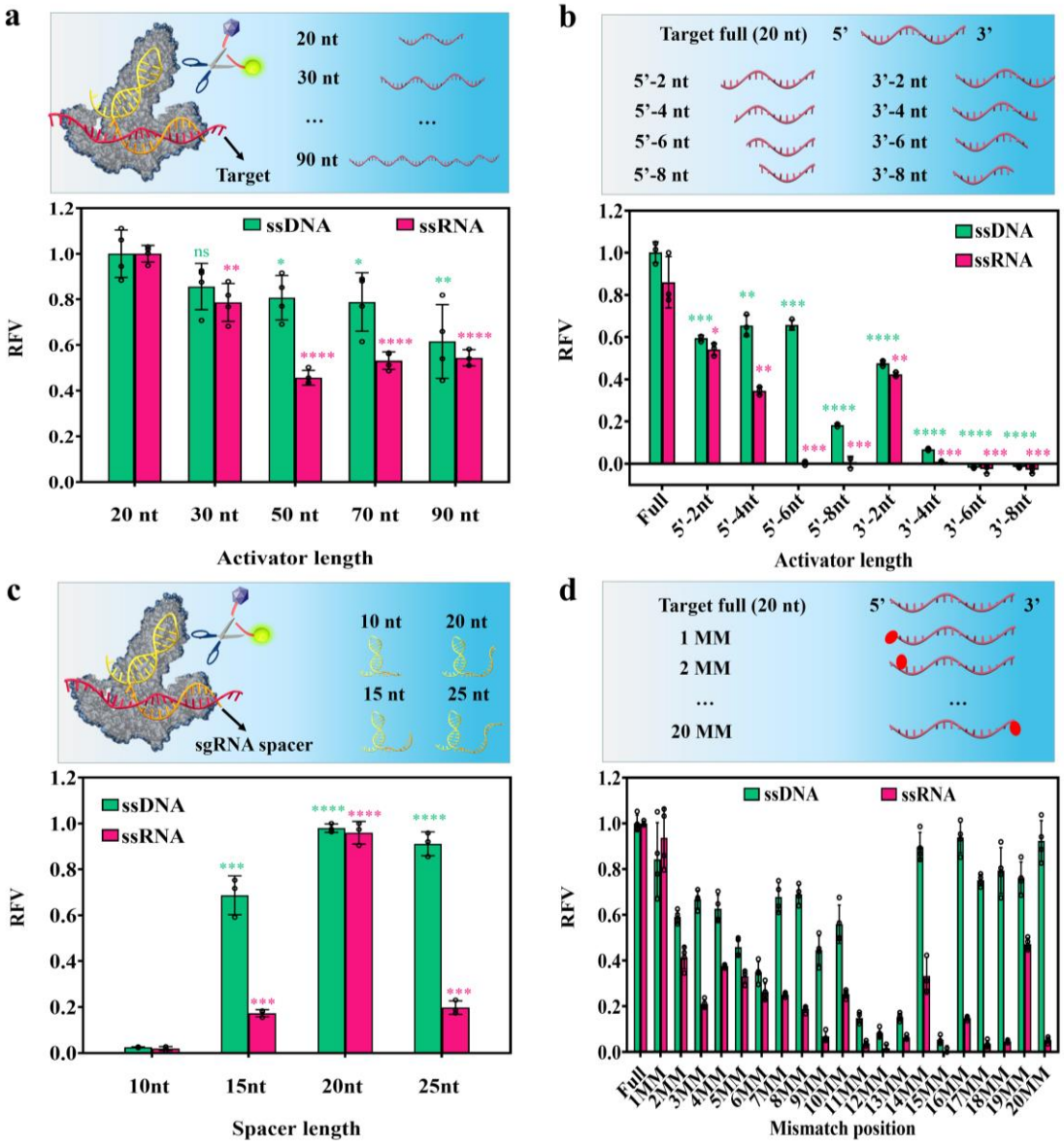


Figure 2. The impact of activators on *trans*-cleavage activation of the Cas14a1. a and b) The *trans*-cleavage activity of Cas14a1 triggered by different lengths of targeting substrates (a. Target lengths varied via extending its sequence length to both 3'and 5' end direction; b. Target lengths varied via missing fragment on 5' or 3' end from 20 nt full target (5' (or 3') - x nt target sequence meaning that 20 full-length 20 nt missing x nt from 5' (or 3') end.)). c). The *trans*-cleavage activity of Cas14a1 with varying the spacer length for sgRNA triggered by RNA (50 nt) and ssDNA (50 nt). d) *Trans*-cleavage activity of Cas14a1 triggered by target (RNA and ssDNA) with single-base mutations tiled across substrates. (3 technical replicates, points represent individual measurements, bars represent mean±s.d. *P<0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001)

CRISPR-Cas II effectors with *trans*-cleavage activity (more detail functional information of CRISPR-Cas II effectors shown Table S5), including Cas12[18], Cas13[19], and Cas14[20], have been used as biosensing platforms[21]. Numerous works have combined

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Cas effectors-based methods with isothermal amplification to obtain high detection sensitivity,[22] such as those obtained by recombinase polymerase amplification (RPA)[23] and strand displacement amplification.[24] To challenge the RNA-based Cas14a1 platform for pathogenic nucleic acid detection, we constructed a workflow consisting of three enzymatic reactions: an amplification by DNA polymerase, a transcription by T7 RNA polymerase, and a Cas14a1-based RNA-activated *trans*-cleavage (ATCas-RNA) reaction (Scheme 1c). A FQ reporter would be cleaved to produce a fluorescent response only in the presence of a specific target. We firstly optimized the ATCas-RNA system, including the transcription reaction time and the concentration of the T7 RNA polymerase (Figure S11). Subsequently, we tested the platform with sgRNA-*E.typhi* against a series of pathogens, including *K.peumonia*, *P.aeruginose*, *E.coli*, and *E.typhi* (Figure S12-S13). To further demonstrate the distinct capability of the ATCas-RNA platform for biosensing, slightly different short sequences from 16S rRNA were purposely selected as pairing substrates (the detail information of substrates sequences are shown in Table S6), as shown in Figure 3a and Figure S14. For comparison, we also obtained the ssDNA with same sequences by the asymmetric PCR. The results showed that the platform could distinguish target sequences both RNA (Figure 3b, Figure S15a, and Figure S16) and ssDNA (Figure 3c, Figure S15b, and Figure S17) with 1nt, 2nt, and 5 nt mismatches, respectively. And it also demonstrated a higher specificity for detecting RNA over ssDNA.

We then evaluated the sensitivity of the ATCas-RNA platform. The target RNA was obtained by amplification and transcription using a series of concentrated synthetic *E.typhi* DNA. Notably, a gradual increasement of fluorescence intensity was observed as the *E.typhi* target concentration varied from 1 aM to 10 pM (Figure S19). The logarithms of fluorescence intensity was linearly related to the logarithms of the target concentration (1×10^{-18} M to 1×10^{-15} M) with a correlation equation of $lgRFU=4.7+0.0667lgC_{E.typhi\ DNA}$ and calculated R^2 of 0.99. The limit of detection (LOD) was as low as 0.6 aM, displaying the high sensitivity of the ATCas-RNA platform (Figure S18-19). These results illustrated that this platform has a high specificity and great sensitivity. Similaraly to Cas13a with SHERLOCK for RNA detection,[6a] this platform is amenable to bacteria detection and nucleic acid analysis in practical applications.

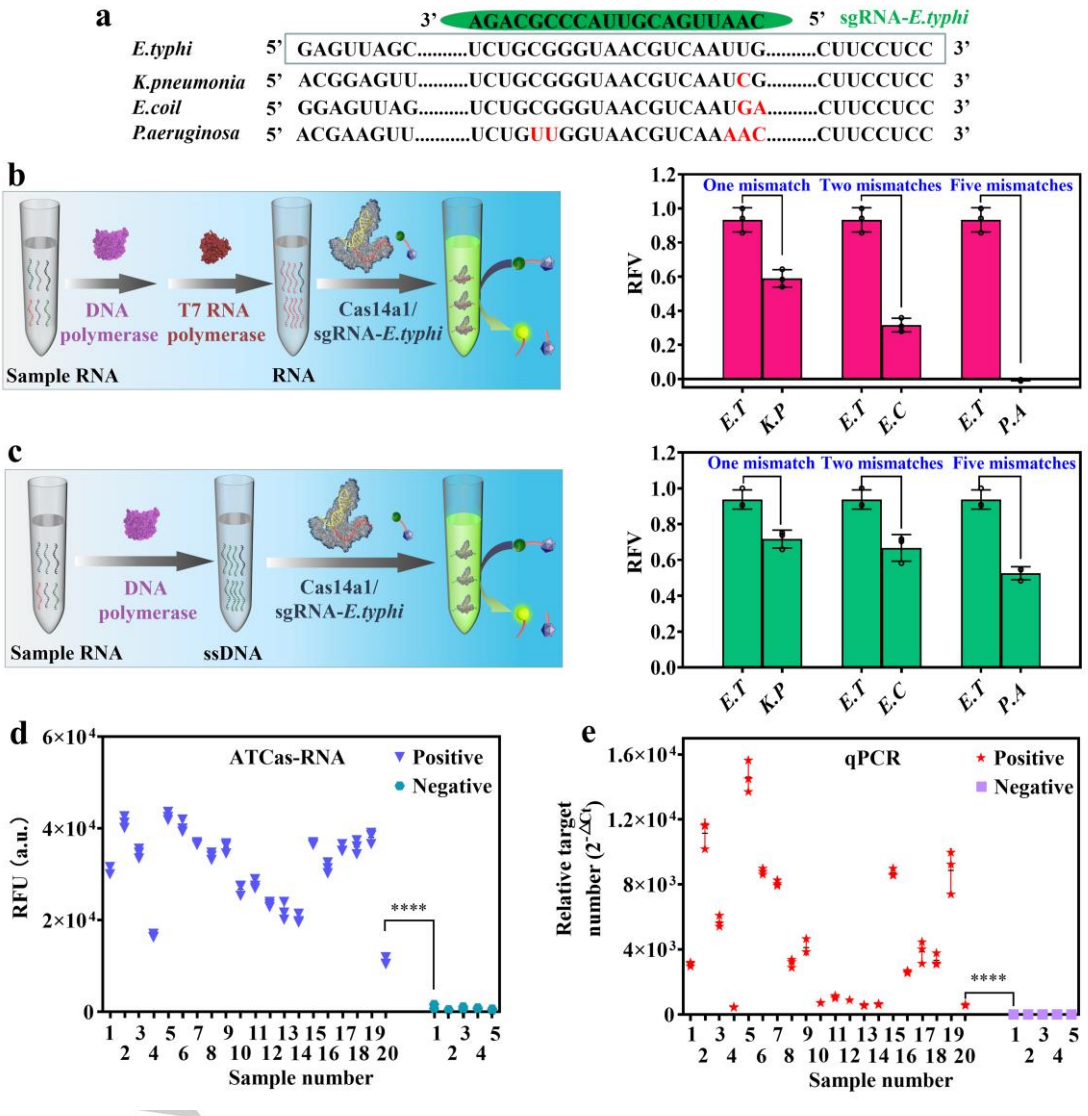


Figure 3. High fidelity RNA detection by ATCas-RNA platform with high specificity and sensitivity**.** a) Target sequences of 4 different bacterial genome types. sgRNA-*E.typhi* sequence highlighted in green color, mutant site of target sequences artificially infected in different bacteria highlighted in red. b) Diagram of ATCas-RNA platform (left) and

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different bacterial target RNA analysis using the platform with 1, 2 and 5 mutated bases, respectively(right). c) Diagram of Cas14a1-based system with RT-asymmetric PCR (left), and different bacterial target DNA analysis using this method with 1, 2 and 5 mutated bases, respectively(right). (*E.T* represent *E.typhi*, *K.P* represent *K.pneumonia*, *E.C* represent *E.coil*, *P.A* represent *P.aeruginosa*). Comparison of different method (d) ATCas-RNA and (e) RT-PCR platform for identification of *E.typhi* types samples (20 positives and 5 negatives) (n=3 technical replicates, points represent individual measurements, bars represent mean±s.d. *P<0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001).

Finally, the ATCas-RNA platform was employed to analyze deliberately infected milk samples with *E.typhi*. The results correctly confirmed all 20 positive samples (100%, 20 out of 20) and 5 negative samples (100%, 5 out of 5) (Figure 3d, S20). The gold standard qPCR method was also used as a comparison, and the same results were obtained, which were 20 positives samples with C_t values of 14-20 that were correlating with copying numbers of 10^4-10^6 copies/mL, and 5 undetectable ones (Figure 3e, S21). The results suggested the ATCas-RNA platform can detect RNA from real samples. Therefore, it has the potential to be used in clinic applications including RNA-based precision medicine and biological analysis.

Conclusion

In summary, we have discovered RNA-enabled *trans* cleavage activation of Cas14a1 and developed an ATCas-RNA platform for RNA biosensing. In this study, the platform was explored by varying the lengths of target and sgRNA spacer, by studying the tolerances of target mismatches, and by applying it to complex real samples. Our results show that the platform is capable of a low tolerance to single mismatches, which indicates a high specificity against target RNA, LOD of 1 aM with high sensitivity, and real sample detection with 100% accuracy. Moreover, the novel discovery of RNA-enabled *trans* cleavage activation of Cas14a1 without self-destruction will expand the applications of the CRISPR/Cas14a1 system in a variety of settings, including fundamental cell biological research, medical diagnostics, and microbial testing. The findings here described provide a new research direction for Cas14a1 and will inspire further exploration of the Class-2 Cas systems.

Author Contributions

Y.W. and Z.Y. contributed equally to this work. Y.W., Y.W. and J.L. conceived this study. C.Z., B.W., X.G., and X.T. performed the experiments and analyzed the data. X.L., Z.T., and P.W. designed the experiments. Y.W. and J.L. wrote the manuscript, which was read and approved by all authors.

Notes

The authors declare no competing financial interest.

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Keywords: CRISPR-Cas14a1 • RNA • *trans*-cleavage • pathogenic bacteria • RNA detection platform

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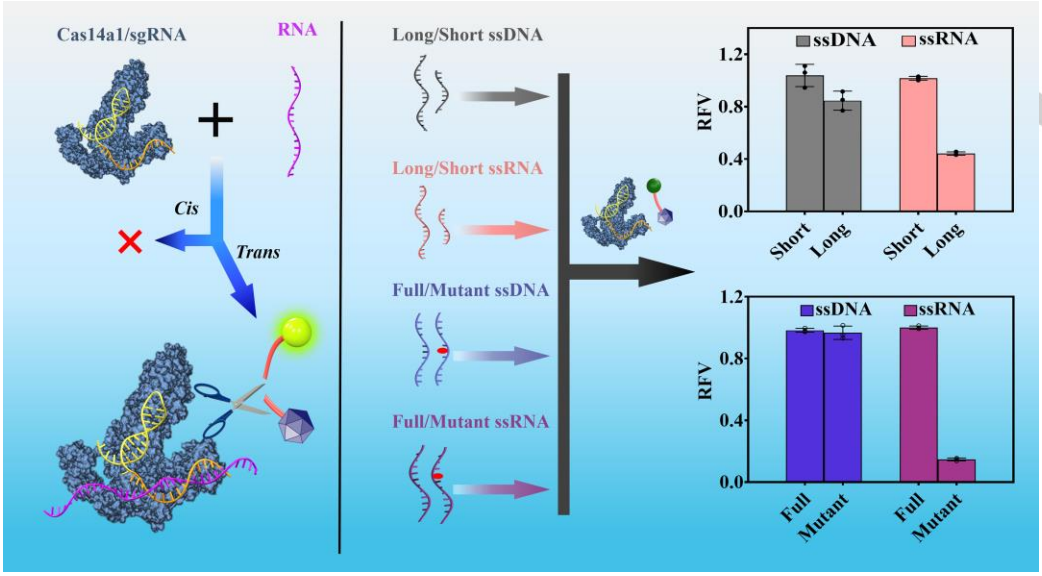
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Cas14a1-based RNA-activated detection platform (Amplification, Transcription, Cas14a1-based RNA-activated trans ssDNA cleavage, ATCas-RNA) has an outstanding specificity for the detection of target RNAs with point mutation resolution, which is better than that of the Cas14a1-based ssDNA-activation.

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