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Controllable crRNA Self-Transcription Aided Dual-Amplified CRISPR-Cas12a Strategy for Highly Sensitive Biosensing of FEN1 Activity

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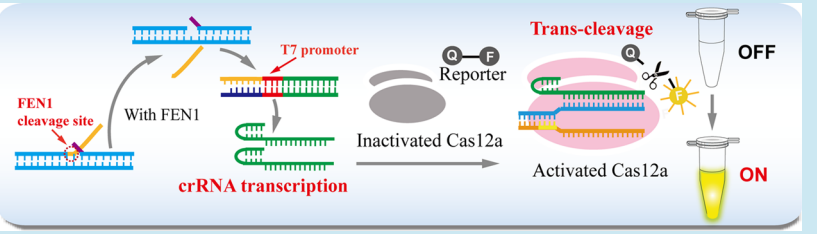

ABSTRACT: A controllable crRNA self-transcription aided dual-amplified CRISPR-Cas12a strategy (termed CST-Cas12a) was developed for highly sensitive and specific biosensing of flap endonuclease 1 (FEN1), a structure-selective nuclease in eukaryotic cells. In this strategy, a branched DNA probe with a 5′ overhanging flap was designed to serve as a hydrolysis substrate of FEN1. The flap cut by FEN1 was annealed with a template probe and functioned as a primer for an extension reaction to produce a double-stranded DNA (dsDNA) containing a T7 promoter and crRNA transcription template. Assisting the T7 RNA polymerase, abundant crRNA was generated and assembled with Cas12a to form a Cas12a/crRNA complex, which can be activated by a dsDNA trigger and unlock the indiscriminate fluorophore−quencher reporter cleavage. The highly efficient dual signal amplification and near-zero background enabled CST-Cas12a with extraordinarily high sensitivity. Under optimized conditions, this method allowed highly sensitive biosensing of FEN1 activity in the range of 1×10^{-5} U μL^{-1} to 5×10^{-2} U μL^{-1} with a detection limit of 5.2×10^{-6} U μL^{-1} and achieved excellent specificity for FEN1 in the presence of other interfering enzymes. The inhibitory capabilities of chemicals on FEN1 were also investigated. Further, the newly established CST-Cas12a strategy was successfully applied to FEN1 biosensing in complex biological samples, which might be a reliable biosensing platform for highly sensitive and specific detection of FEN1 activity in clinical applications.

KEYWORDS: *CRISPR/Cas12a, trans-cleavage, crRNA, FEN1, biosensor*

Flap endonuclease 1 (FEN1), which plays essential roles in almost all cellular processes, is ubiquitously upregulated in highly proliferative tissues because of its involvement in the DNA metabolic pathway *via* DNA repair and replication.[1−3] FEN1 can catalyze the removal of the 5′ overhang from branched double-stranded DNA (dsDNA) structures, and the FEN1 cleavable overhang is termed the 5′ flap.[4] The expression level of FEN1 is generally elevated in cancer cells and correlated to tumor aggressiveness and progression.[5,6] Thus, FEN1 has been identified as a promising diagnostic and monitoring biomarker for various types of cancers, such as hepatocellular carcinoma and breast tumors.[7] Since inhibition of FEN1 can suppress tumor invasion and metastasis, FEN1 is also considered to be a potential therapeutic target of anticancer drugs.[8] On the basis of the medical significance of FEN1 activity, accurate FEN1 biosensing is of great value in clinical fields.

Unfortunately, there has been limited progress in developing sensing methods to monitor FEN1 activity. Conventional prevalent approaches for FEN1 detection, such as immunochemistry analysis, western blotting, and enzyme-linked immunoassays (ELISAs), mainly rely on the antigen−antibody interaction and suffer from the intrinsic limitation of detection sensitivity and stability due to the fragile recognition entities.[9−11] The sophisticated testing equipment, professional technicians, and relatively high costs of these methods also impeded their widespread applications. Recent studies have developed novel biosensing strategies to circumvent the aforementioned drawbacks of conventional technologies. For example, Zhang et al. conjugated fluorophore-labeled DNA probes to graphene oxide (GO) nanosheets as carriers to construct a variety of nanodevices for in situ monitoring of FEN1 activity.[12] Wang et al. proposed a platform consisting of gold nanoparticles, functional DNA, and organic dyes to quantify FEN1 in clinical samples.[13] Despite remarkable

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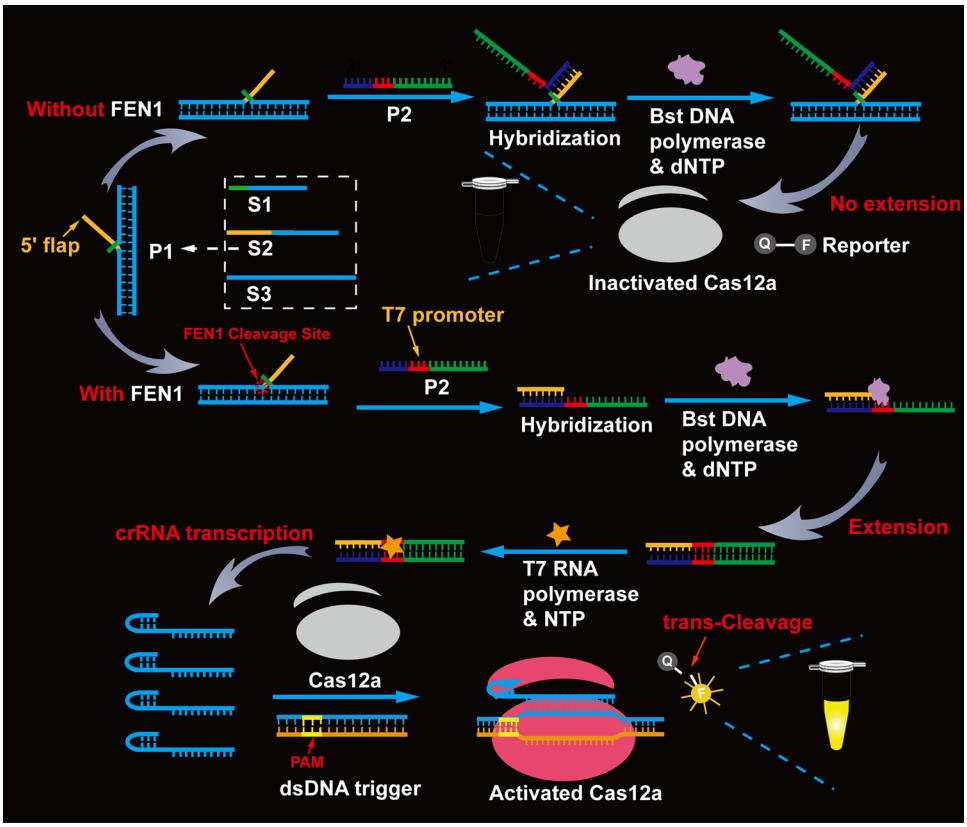
Scheme 1. Schematic Illustration of CST-Cas12a Based on Target-Responsive crRNA Self-Transcription and the Trans-Cleavage Activity of CRISPR-Cas12a for FEN1 Biosensing



progress in these biosensors, the majority of them still face troublesome multiple washing steps, harsh buffer/temperature requirements, and complex nanomaterial synthesis and modification. Therefore, developing a reliable biosensing platform with high sensitivity and specificity, convenience, and affordable costs is still a challenge.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins are originally derived from the adaptive immune system in prokaryotes, which have become a revolutionary tool for genome editing and have also shown great potential for the development of next-generation biosensing platforms.[14−16] The CRISPR-mediated diagnostics mainly relies on the collateral cleavage activities of some Cas proteins (e.g., Cas12a, Cas13a, and Cas14), and the collateral cleavage activity is referred to as trans-cleavage.[17−19] This unique feature makes the Cas proteins an excellent actuator for exploiting biosensors, not only converting biometric events into detectable signals but also easily amplifying the output signals. Among the CRISPR family, CRISPR-Cas12a, a class 2 type V-A CRISPR system, can be complexed with a specific guide CRISPR RNA (crRNA) and triggered to indiscriminately trans-cleave any nearby single-stranded DNA (ssDNA) substrates by dsDNA containing the protospacer adjacent motif (PAM) sequence or crRNA-complementary ssDNA.[20,21] The trans-cleavage effect of the Cas12a protein can therefore be monitored with a fluorophore−quencher dual-modified ssDNA, which will release a fluorescence signal after cleavage by crRNA-target-binding-activated Cas12a nuclease. CRISPR-Cas12a has recently been exploited to establish faster, more portable, and cost-effective biosensing methods for the determination of various bioanalytes, including nucleic acid sequences, small molecules, protein biomarkers, and enzyme activities.[22−32] However, the drawback of most existing CRISPR-based biosensors is related to the insufficient sensitivity of the Cas protein.[23] In addition, the trans-cleavage capability of CRISPR-Cas12a may be suppressed or non-specifically triggered by target-independent factors.

Herein, we proposed a dual-amplified biosensing strategy by combining target-responsive crRNA self-transcription and CRISPR-Cas12a trans-cleavage (termed CST-Cas12a) for highly sensitive detection of FEN1 activity. Briefly, FEN1-initiated primer extension and T7 transcription were employed to generate numerous crRNA and assembled with the Cas12a protein to form a Cas12a/crRNA complex, which can be activated by a dsDNA trigger with a PAM sequence and unlock the nonspecific trans-cleavage capability for developing a powerful signal amplification system. Owing to the integration of the high efficiency of crRNA self-transcription amplification and the CRISPR-Cas12a trans-cleavage capability, CST-Cas12a achieves an extremely low background and a high signal-to-background ratio, thus realizing the highly sensitive detection of FEN1 activity down to 5.2×10^{-6} U μL^{-1}, which is much lower than that of most existing methods. Furthermore, FEN1 enzyme inhibitor screening and FEN1 activity detection in diluted serum samples and cell lysates were conducted to investigate the practicability of the biosensing method.

RESULTS AND DISCUSSION

Working Principle of CST-Cas12a. The working principle of CST-Cas12a for FEN1 biosensing has been

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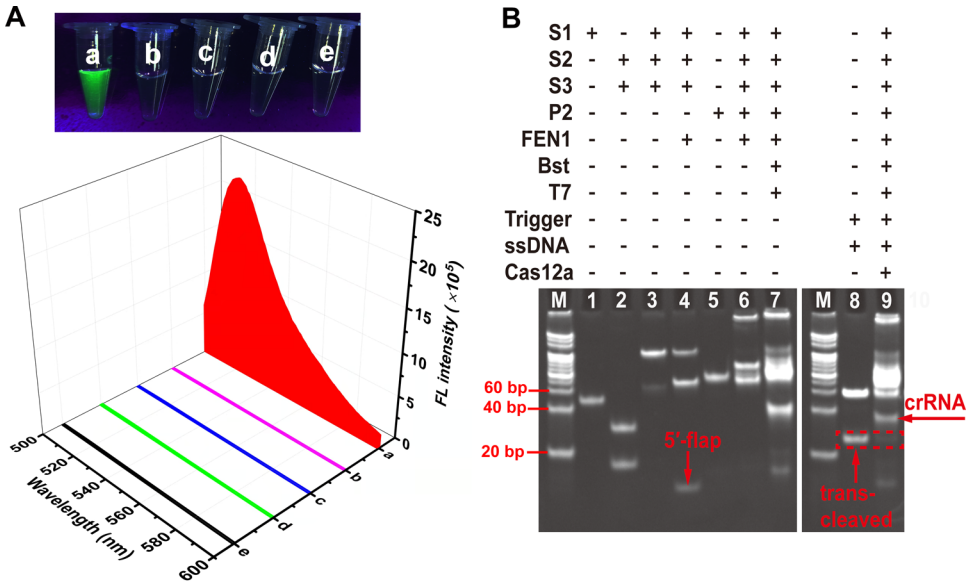

Figure 1. (A) Fluorescence visualization and corresponding spectra of CST-Cas12a obtained from different samples. a, Without Cas12a; b, without T7 RNA polymerase; c, without Bst DNA polymerase; d, without FEN1; and e, with FEN1. (B) 12% PAGE analysis: lane 1, S1; lane 2, S2 + S3; lane 3, S1 + S2 + S3; lane 4, FEN1 + P1; lane 5, P2; lane 6, FEN1 + P1 + P2; lane 7, FEN1 + P1 + P2 + Bst + T7; lane 8, dsDNA trigger + ssDNA; and lane 9, FEN1 + P1 + P2 + Bst + T7 + Cas12a + Trigger + ssDNA. [FEN1] = 1×10^{-2} U μL^{-1}, [S1] = 1 μM, [S2] = 1 μM, [S3] = 1 μM, [P2] = 1 μM, [Bst] = 5×10^{-2} U μL^{-1}, [T7] = 0.5 U μL^{-1}, [ssDNA] = 1 μM, [Cas12a] = 50 nM, and [dsDNA] = 50 nM.

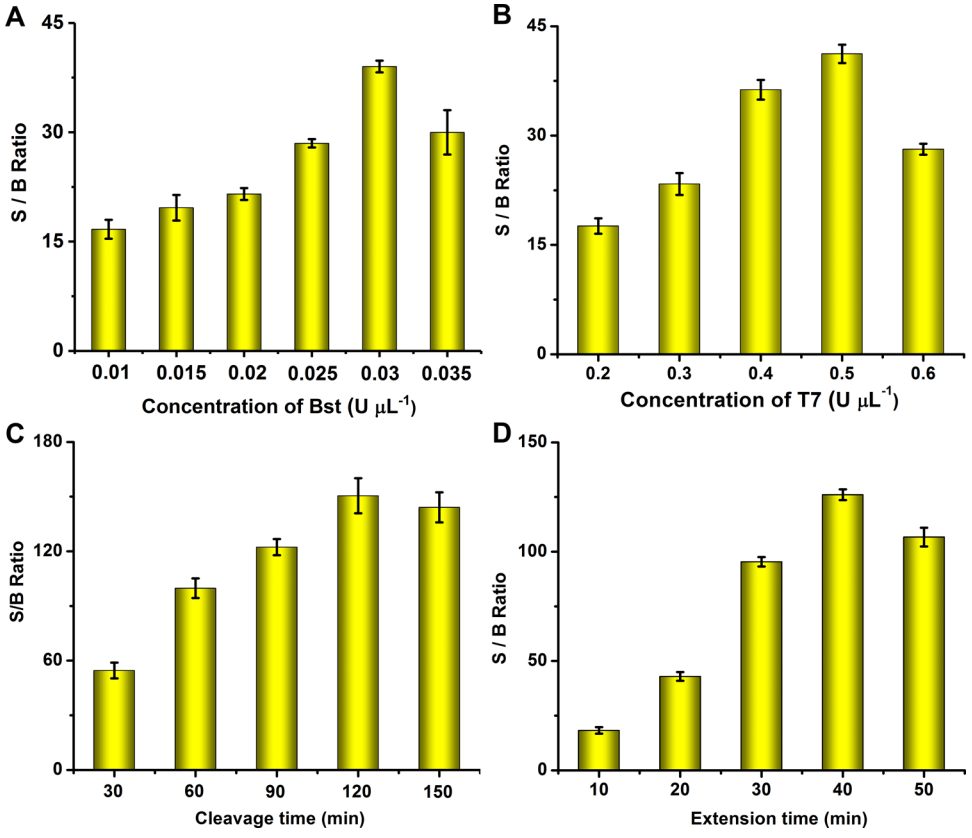

Figure 2. Effect of (A) the concentration of Bst DNA polymerase, (B) the concentration of T7 RNA polymerase, (C) the FEN1 cleavage time, and (D) the primer extension time. Error bars, SD, n = 3. λ_{ex}: 490 nm, λ_{em}: 520 nm. [FEN1] = 1×10^{-3} U μL^{-1}, [ssDNA-FQ] = 500 nM, [Cas12a] = 50 nM, and [dsDNA] = 50 nM.

illustrated in Scheme 1. A branched dsDNA probe (P1) with a 5′ overhanging flap for FEN1 cleavage was obtained by annealing S1, S2, and S3. In the presence of FEN1, the 5′ flap of P1 can be recognized and cleaved; then, the released 5′ flap with 3′-OH ends hybridized with a template probe (P2) and functioned as a primer to trigger an extension reaction. Here, P2 consists of three domains: a 5′ flap binding domain (blue), a T7 promoter complementary domain (red), and a tran-

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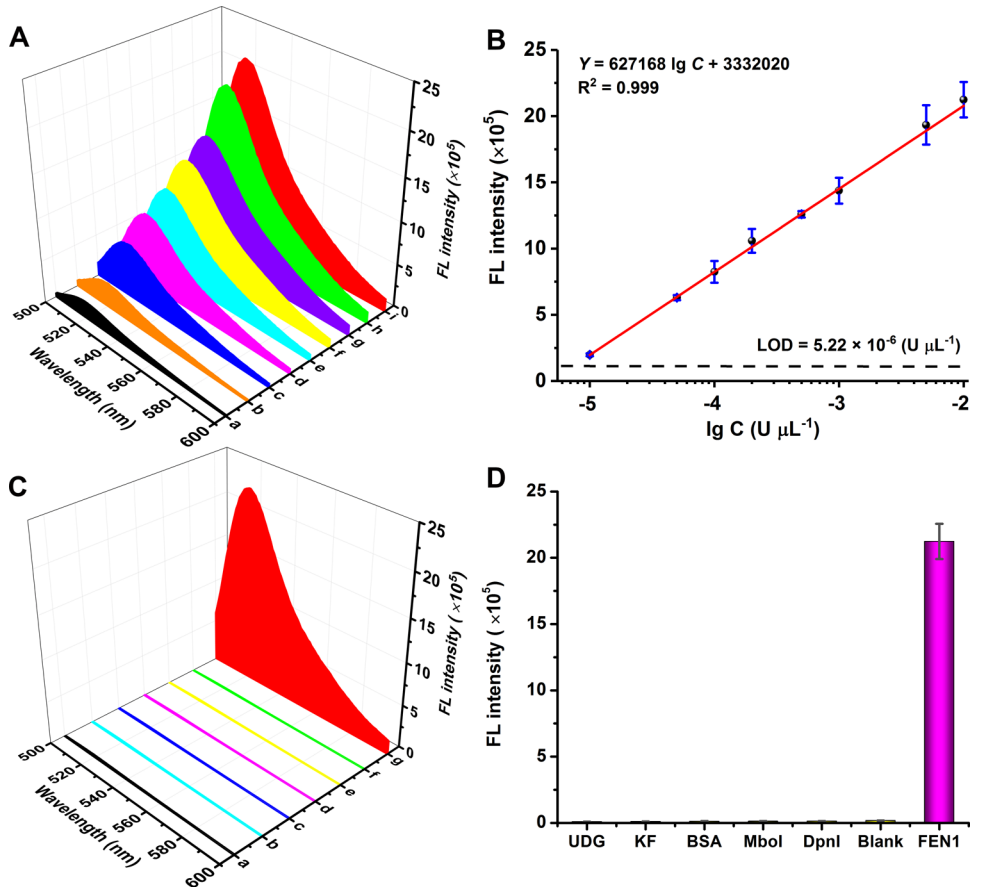

Figure 3. (A) Fluorescence spectra responding to 1×10^{-5}, 5×10^{-5}, 1×10^{-4}, 5×10^{-4}, 1×10^{-3}, 5×10^{-3}, 1×10^{-2}, and 5×10^{-2} U μL^{-1} (from a to h) of FEN1. (B) Linear relationship between the fluorescence intensity and the logarithm concentration of FEN1 ranging from 1×10^{-5} to 5×10^{-2} U μL^{-1}. Panels (C) and (D) Show the fluorescence spectra and corresponding fluorescence intensity of the biosensor toward 1 U μL^{-1} UDG, 1 U μL^{-1} KF, 1 U μL^{-1} *Mbo*I, 1 U μL^{-1} *Dpn*I, 1 mg mL^{-1} BSA, blank, and 1.0×10^{-2} U μL^{-1} FEN1 (from a to g). Error bars, SD, $n = 3$. λ_{ex}: 490 nm, λ_{em}: 520 nm. [Bst] = 3.0×10^{-2} U μL^{-1}, [T7] = 0.5 U μL^{-1}, [ssDNA-FQ] = 500 nM, [Cas12a] = 50 nM, and [dsDNA] = 50 nM.

scription template of crRNA (green). Thus, amounts of dsDNA containing T7 promoter were produced, which can be transcribed by the T7 RNA polymerase to generate numerous crRNAs. Subsequently, the Cas12a protein assembled with crRNA to form a Cas12a/crRNA complex, which can be activated by a dsDNA trigger with a PAM sequence and unlock the trans-cleavage activity. As a result, the activated Cas12a indiscriminately cleaved nearby ssDNA reporters labeled with FAM and BHQ1 (ssDNA-FQ) at both ends, resulting in FAM far away from BHQ1 and thereby producing a significantly amplified fluorescence response. Conversely, without FEN1, the primer extension and crRNA transcription reaction will not be initiated, and thus the nonspecific cleavage capability of Cas12a will not be triggered even if numerous dsDNA triggers are present. As a result, the nonspecific background fluorescence can be substantially eliminated since Cas12a itself does not have any cleavage activity. Benefiting from the high efficiency of the FEN1-initiated crRNA self-transcription and the multiple turnover trans-cleavage of Cas12a/crRNA, a novel biosensing method with an extremely low background and a high signal-to-background ratio was constructed for highly sensitive and specific monitoring of FEN1 activity.

Feasibility of CST-Cas12a. To test the possibility of the proposed CST-Cas12a biosensing strategy, fluorescence experiments were first performed, and ssDNA-FQ was introduced as the substrate for Cas12a trans-cleavage. As depicted in Figure 1A, when the target FEN1 was present, the fluorescence response of the biosensor was significantly enhanced (curve a), indicating that the ssDNA-FQ reporters were efficiently cut off and fluorescence signals were recovered. In contrast, in the absence of FEN1 (blank group), the fluorescence response was nearly annihilated (curve b). In the absence of the Cas12a protein, T7 RNA polymerase, or Bst DNA polymerase, the fluorescence signal was negligible and comparable to that of the blank group (curves c, d, and e). The above-mentioned results suggested that the CST-Cas12a strategy was successfully constructed and that the Cas12a protein, T7 RNA polymerase, and Bst DNA polymerase play an essential role in the CST-Cas12a system. To further confirm the results from fluorescence experiments, 12% PAGE was performed. As shown in Figure 1B, S1 (lane 1) hybridized with S2 and S3 (lane 2) to form a dsDNA (lane 3). In the presence of FEN1, the single-stranded 5′ flap was cut from the dsDNA (lane 4). The P2 containing 5′ flap binding region (lane 5) combined with the cleaved 5′ flap to form a partial double-stranded DNA structure (lane 6). Then, the extension and transcription were initiated by Bst DNA polymerase and T7 RNA polymerase and a ~41 nt band (lane 7) was generated, which suggested that the crRNA was successfully produced. Finally, the added ssDNA (25 nt) was trans-cleaved by Cas12a with the trigger of the dsDNA (lane 9). All of these results confirmed the feasibility of CST-Cas12a for FEN1 detection.

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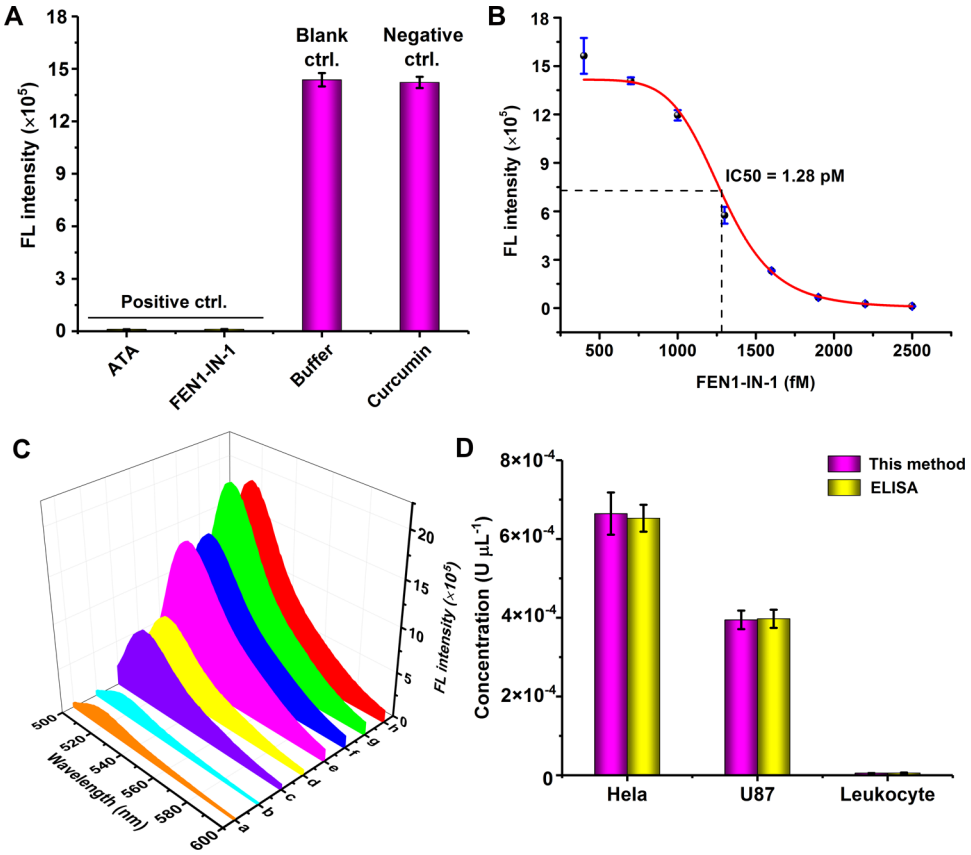

Figure 4. (A) Investigation of the inhibitory effect of ATA, FEN1-IN-1, and curcumin on FEN1 activity. (B) Relationship between the concentration of FEN-IN-1 and the fluorescence intensity. (C) Fluorescence spectra of different concentrations of FEN1 added into a buffer solution and 10% diluted healthy human serum. a, 1×10^{-5} U μL^{-1} FEN1 in diluted serum; b, 1×10^{-5} U μL^{-1} FEN1 in water; c, 1×10^{-4} U μL^{-1} FEN1 in diluted serum; d, 1×10^{-4} U μL^{-1} FEN1 in water; e, 1×10^{-3} U μL^{-1} FEN1 in diluted serum; f, 1×10^{-3} U μL^{-1} FEN1 in water; g, 1×10^{-2} U μL^{-1} FEN1 in diluted serum; and h, 1×10^{-2} U μL^{-1} FEN1 in water. (D) FEN1 quantification in HeLa (1×10^4 cells), U87 (1×10^4 cells), and leukocyte (1×10^4 cells) using the developed biosensor and commercial ELISA kits. Error bars, SD, $n = 3$. λ_{ex}: 490 nm, λ_{em}: 520 nm. [Bst] = 3.0×10^{-2} U μL^{-1}, [T7] = 0.5 U μL^{-1}, [ssDNA-FQ] = 500 nM, [Cas12a] = 50 nM, and [dsDNA] = 50 nM.

Optimization of Experimental Parameters. To obtain a better detection performance of CST-Cas12a, some critical experimental parameters were optimized. Figure 2A,B shows the effect of the concentration of Bst DNA polymerase and T7 RNA polymerase on the experimental results. When the concentration of Bst DNA polymerase was 0.03 U μL^{-1} and T7 RNA polymerase was 0.5 U μL^{-1}, a better signal-to-background (S/B) ratio can be obtained. As a result, the optimal concentrations of Bst DNA polymerase and T7 RNA polymerase were set as 0.03 and 0.5 U μL^{-1}, respectively. It is well known that the incubation time is a very important parameter for enzymatic reactions, as proteases will gradually deactivate over time and the background signal may increase accordingly. Figure 2C shows that the S/B ratio increased significantly with the augment of the FEN1 cleavage time and had a maximum value when the FEN1 cleavage time reached 120 min, and then its value instead decreased. Therefore, the optimized FEN1 cleavage time was 120 min. As illustrated in Figure 2D, the S/B ratio increased rapidly when the primer extension time was increased from 10 min to 40 min, but it exhibited a decrease when the primer extension time increased unceasingly. So, we selected 40 min as the optimal primer extension time. Furthermore, with the increase of the crRNA transcription time, the S/B ratio gradually increased and reached a maximum value when the crRNA transcription time was 2.5 h. Thus, 2.5 h was selected as the optimal transcription time in the following experiments (Figure S1).

Analytical Performance. Under optimized experimental parameters, various concentrations of FEN1 were detected to investigate the analytical performance of the established biosensing method. As depicted in Figure 3A, the fluorescence signal increased proportionately over a FEN1 range of 1.0×10^{-5} U μL^{-1} to 5.0×10^{-2} U μL^{-1}, as expected. The fluorescence intensity at 520 nm showed a good linear relationship with the logarithm of the FEN1 concentration over the investigated concentration range (Figure 3B). The corresponding regression equation was $Y = 627168 \lg C + 3332020$ ($R^2 = 0.9991$), where Y represents the fluorescence intensity and C represents the concentration of FEN1. The limit of detection (LOD) was calculated to be 5.2×10^{-6} U μL^{-1} based on the mean of the blank plus three times the standard deviation, which is competitive with previously proposed approaches (Table S2). Such a low detection limit of the biosensor essentially benefits from three factors: (i) highly efficient target-responsive transcription assisted by T7 RNA polymerase; (ii) signal amplification induced by the Cas12a-catalyzed multi-turnover cleavage capability; and (iii) significant elimination of the background signal with the transcription-unleashed crRNA self-supply.

To investigate the specificity of our method, four interfering proteases were used. As shown in Figure 3C,D and Table S3,

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ACS Synth. Biol. XXXX, XXX, XXX−XXX

the fluorescence intensity of UDG, KF, *Mbo*I, and *Dpn*I was remarkably lower than that of FEN1 and almost the same as those of the unrelated protein (BSA) and blank group, although their amount was 100 times more than that of FEN1, indicating that the proposed biosensor exhibited excellent specificity. In addition, five parallel measurements of 0.01 U μL^{-1} FEN1 were performed to evaluate the reproducibility of the biosensor. A superior reproducibility has been observed with a low relative standard deviation (RSD < 5%).

Inhibition Assay. Since FEN1 has been considered a potential drug target, it is of great importance to screen pharmacological inhibitors of FEN1 activity for tumor chemotherapy.[7,33] Based on previous reports, we selected ATA and FEN1-IN-1 as model inhibitors, curcumin as a negative control, and buffer as a blank control.[12,13] As depicted in Figure 4A and Table S4, the fluorescence signal was significantly inhibited by ATA and FEN1-IN-1, indicating that the inhibitors strongly suppressed the activity of FEN1 and blocked the cleavage induced extension, thus interrupting the entire experimental process. In contrast, intense fluorescence was observed from blank and negative controls. Figure 4B shows the fluorescence intensity when different doses of FEN1-IN-1 were added. The half-maximal inhibitory concentration (IC50) for FEN1 was estimated to be 1.28 pM. The above-mentioned results indicated that CST-Cas12a can be exploited to screen FEN1 inhibitors and investigate the interaction between FEN1 and its inhibitors.

Applicability of CST-Cas12a. To investigate the practicability of CST-Cas12a in complex biological samples, FEN1 with various concentrations was spiked into a 10-fold diluted human serum sample according to the standard addition method. It was found that the serum had almost no effect on the performance of the biosensor (Figures 4C and S2). The recoveries of the samples were in the range of 95.14% to 102.07%, and the RSD ranged between 3.44 and 9.99%, which illustrated that CST-Cas12a has good performance in a complex environment (Table S5). To further investigate the application potential of this biosensor, fluorescence measurements were carried out in several different cell lines. Results in Figure 4D revealed that the persistent upregulation of FEN1 was observed in HeLa and glioma U87 cell lysates, but almost no expression was observed in leukocytes, which is consistent with previous literature studies[16,34] and the results quantified by ELISA using a commercially available kit. These results suggested that the method has great potential for reliable biosensing of FEN1 in real samples.

CONCLUSIONS

In summary, a dual-amplified fluorometric biosensor based on target-responsive crRNA self-transcription and the trans-cleavage capability of CRISPR-Cas12a has been developed (which we term CST-Cas12a), and it was successfully used for highly sensitive detection of FEN1 activity. Under the strict identification of target molecules, this strategy enables the production of large amounts of crRNA, which can assemble with Cas12a to form a Cas12a/crRNA complex and activate its trans-cleavage by a dsDNA trigger. More importantly, since crRNA is produced by the detection system itself through transcription, the nonspecific background signal is largely eliminated. The LOD achieved is as low as 5.2×10^{-6} U μL^{-1} with linearity between 1.0×10^{-5} U μL^{-1} and 5.0×10^{-2} U μL^{-1} (more than three orders of magnitude). In addition, the practical application of CST-Cas12a was confirmed for inhibitor screening and FEN1 detection in complex biological samples. However, enzyme consumerism and mutual interference in biological mixtures may compromise the analytical performance of this method; thus, immobilization of the CRISPR-Cas12a system at nanomaterial interfaces for the design of biosensing strategies and devices would be the right direction in the future.[35] Overall, CST-Cas12a provides a heuristic avenue for advancing CRISPR-Cas12a-based biosensors and has broad application prospects in biomedical research and clinical practice.

EXPERIMENTAL SECTION

Procedure of the CST-Cas12a Reaction. Initially, P1 was obtained by annealing three complementary sequences S1, S2, and S3 in 1 × NEBuffer 3.0 (0.1 M NaCl, 0.05 M Tris-HCl, 0.01 M $MgCl_2$, 0.001 M dithiothreitol, pH 7.9) at 90 °C for 5 min, followed by incubation at 37 °C for 2 h. Incubation of 1μL of 1 μM P1 and specific concentration of FEN1 in 1 × Thermo Pol reaction buffer (0.01 M KCl, 0.02 M Tris-HCl, 0.01 M $(NH_4)_2SO_4$, 0.002 M $MgSO_4$, 0.1% Triton X-100, pH 8.9) was carried out at 65 °C for 2 h in the total volume of 20 μL. Subsequently, 1 μL of 1 μM P2, 1 μL of 0.03 U μL^{-1} Bst 3.0 DNA polymerase, 1 μL of 25 mM dNTPs, and 4.4 μL 10 × NEBuffer 3.0 were added into a final volume of 50 μL, and the mixture was incubated at 65 °C for 40 min. Afterward, the Cas12a-induced cleavage reaction was performed by mixing a reaction solution containing 50 nM Cas12a, 50 nM dsDNA complex, 40 U RNase inhibitor, 0.5 U μL^{-1} T7 RNA polymerase, and 500 nM ssDNA-FQ in 1 × NEBuffer 3.0 in the total volume of 100 μL at 37 °C for 2.5 h. The fluorescence spectrum was measured by an FS5 fluorescence spectrophotometer using an excitation wavelength of 492 nm and an emission intensity of 520 nm.

FEN1 Biosensing in Cell Lysates. The HELA and glioma U87 cell lines were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and 1% streptomycin−penicillin. The cells were incubated at 37 °C in a humidified 5% CO_2-containing atmosphere. Proteins were extracted from these two cell cultures using RIPA lysis buffer. The collected lysates were then used to detect FEN1 activity immediately or stored at −80 °C until utilization. The FEN1 activity analysis in cell lysates was performed according to the above-mentioned procedures.

Native Polyacrylamide Gel (PAGE) Electrophoresis. The 12% PAGE analysis was carried out to confirm the FEN1 cleavage, primer extension, crRNA transcription, and trans-cleavage capability of Cas12a. Electrophoresis was carried out in 1 × TBE buffer (2 mM EDTA, 90 mM Tris-borate, pH 8.4) at 110 V for 45 min at room temperature. Afterward, the gel was immersed in a freshly prepared stain-all solution (containing 4 μL of GoldView in 80 mL of 1 × TBE) for 35 min. Subsequently, the PAGE results were scanned by a gel image analysis platform.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.2c00420.

Materials and apparatus; sequences of oligonucleotides used in this work; comparison between the CST-Cas12a strategy and various analytical methods; investigation of the specificity of CST-Cas12a; investigation of the

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ACS Synth. Biol. XXXX, XXX, XXX−XXX

inhibitory effect of chemicals; recovery experiments; effect of the crRNA transcription time; and fluorescence intensities of different concentrations of FEN1 added into the buffer solution and 10% diluted healthy human serum (PDF)

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Author Contributions

#Y.S. and K.G. contributed equally to this work. Y.S.: Conceptualization, methodology, software, investigation, data curation, and writing−original draft; K.G.: methodology, software, formal analysis, and investigation; X.C.: methodology, formal analysis, and software; W.C.: supervision and methodology; S.D.: supervision and data curation; D.Z.: conceptualization, writing−review & editing, and resources; and S.D.: supervision, methodology, data curation, funding acquisition, and project administration.

Notes

The authors declare no competing financial interest.

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