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A multi-functional synergistic platform of Cas12a split dsDNA activators†

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This work comprehensively explores the effects of various positions of Cas12a split activators. Based on this, a multi-functional synergistic platform was constructed. The construction of a structural dynamic network, the sensitive detection of APE1, and a time-controlled photo-activation have been achieved, demonstrating the potential for expanding Cas12a applications.

The CRISPR-Cas system (clustered regularly interspaced short palindromic repeats, CRISPR-associated system) is an immune system found in archaea and bacteria, which functions to eliminate the invasion of foreign nucleic acids.[1,2] Owing to its robust capabilities, including programmability, single-base recognition, and multi-turnover activity, it has subsequently been developed into a reliable tool for gene editing and molecular diagnostics.[3–6] Based on differences in structure and gene content, CRISPR-Cas can be categorized into two classes: class 1 (multi-protein complex with coordinated cascade)[7] and class 2 (single protein with multiple functional domains).[8] Among these, class 2 exhibits superior versatility and potential, with Cas12a, in particular, emerging as a representative effector protein of class 2 and achieving advancements in the fields of molecular biology and medicine.

Cas12a exhibits a unique indiscriminate non-specific cleavage activity on ssDNA following target-specific binding and cleavage, which is also known as trans-cleavage activity.[9] Initially, the specific binding targets were limited to ssDNA and dsDNA, necessitating reverse transcription or other conversion steps to obtain targets for Cas12a in RNA detection scenarios.[10–12] The concept of split activation has disrupted this paradigm, such as the splitting of the conserved repeat domain and the variable spacer domain of crRNA, using RNA targets as the spacer domain of crRNA to achieve detection purposes.[13–16] Additionally, the specific target can also be split, relying on the higher RNA tolerance at the distal end of the spacer region to expand target diversity. Previous studies have indicated that the ssDNA activators of Cas12a exhibit a synergistic effect, meaning that two split ssDNAs cannot activate Cas12a individually, but they can synergistically induce Cas12a to display trans-cleavage activity.[17,18] Our group has conducted detailed research on the synergistic effect of split ssDNA and proposed a modular synergistic activation platform for detection and logical operations.[19] Similarly, split dsDNA also exhibits a synergistic effect, and studies have shown that dsDNA is more stable than ssDNA and has a higher activation efficiency.[20] Therefore, exploring the activation of split dsDNA can enhance the effect of split activation and is beneficial for expanding the application scope of split activation modes. However, current research on split dsDNA activators of CRISPR-Cas12a remains insufficient.

Herein, we verified the synergistic activation effect of split dsDNA on Cas12a. By further transforming the various regions of the split dsDNA through deletions and extensions, it was found that the impact of the region proximal to the split gap is significantly greater than that of the region distal to the split gap. These findings endow the split dsDNA target substrates of Cas12a with functional domains, providing new methods for more precise activity regulation and molecular diagnostics. Based on these insights, we proposed strategies for direct detection of APE1, a light-responsive synergistic activation mode, and a self-regulatory network. These applications all demonstrate the regulatory diversity of split dsDNA and significantly advance the understanding of the synergistic activation of split dsDNA.

In the typical mode, the unwinding and binding of the distal split dsDNA depend on the facilitation of the proximal split activator.[21] To investigate this synergistic effect, we discussed the trans-cleavage activation under different combinations of the two parts of the split dsDNA. To ensure the stable binding

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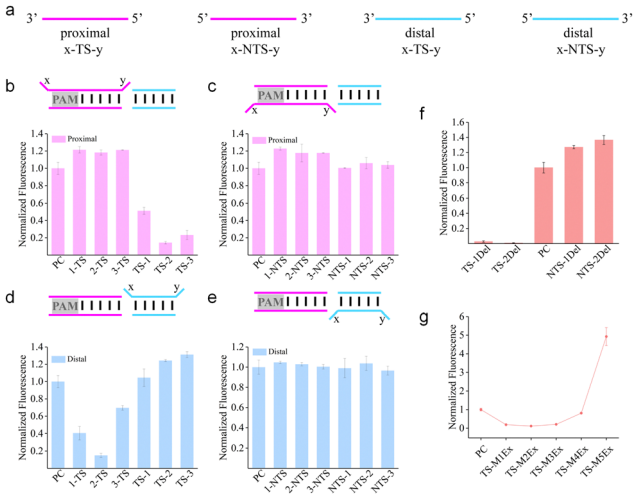


Fig. 1 (a) Schematic diagram of the various parts of the split dsDNA activator, where pink represents the proximal split activator, blue represents the distal activator, and *x* and *y* mean the number of bases. (b)–(e) Normalized fluorescence of different synergistic dsDNA activators for Cas12a. (f) Normalized fluorescence of base deletions in proximal TS/NTS. (g) Normalized fluorescence of matched extension in proximal TS. Error bar, SD, $n = 3$.

of the double strand, we designed a 24 nt spacer crRNA and the corresponding intermediate split dsDNA targets. The original split dsDNA targets are the proximal dsDNA containing PAM with 12 bp in the protospacer region (proximal ds12) and the distal dsDNA with 12 bp in the protospacer region (distal ds12). We compared the activation efficiency of the combination of dsDNA and ssDNA and found that the proximal dsDNA + distal dsDNA has the highest efficiency (Fig. S1, ESI†).

Then, we investigated the effect of extended bases on the activation of Cas12a. We designed proximal *x*-TS-*y*, proximal *x*-NTS-*y*, distal *x*-TS-*y*, and distal *x*-NTS-*y* in which *x* and *y* represent the extended base lengths compared to standard proximal ds12 or distal ds12 (Fig. 1a and Table S1, ESI†). We used the original split dsDNA targets as the positive control (PC). Interestingly, extensions to proximal *x*-TS and proximal *x*-NTS have a weaker impact on the activity of Cas12a, and the effects on the *y* side are mainly manifested on the TS strand, with only a 1 nt extension leading to significant activation differences (Fig. 1b and c). The above results also apply to distal ds12, but the influence of the seed region TS interior of distal ds12 (distal *x*-TS) is slightly lower than that of the seed region TS interior of proximal ds12 containing PAM (proximal TS-*y*), which also indirectly indicates the primary role of the proximal activator in synergistic activation, the distal region can only unwind and target binding with the assistance of the exon-unwinding and induced targeting effect of proximal region (Fig. 1d and e). For the proximal split activator, we also analyzed the impact of base deletions. The deletion of TS is intolerable, while the deletion of NTS promotes unwinding, leading to an increase in activity (Fig. 1f). For split activators, the extensions or deletions of proximal TS-*y* and distal *x*-TS can hinder the process of the formation of crRNA and TS, which is unfavorable for the unwinding of distal dsDNA and leads to differences in the activity of Cas12a. We also explored the competitive relationships of the extended structural regions, which means that the extended sequence is a complementary sequence to crRNA. The results showed that within a certain length range, there is an inhibitory effect, with the extended domain's inhibitory effect on the unwinding and binding of distal ds12 being dominant. However, as the length of the competitive strand increased by four bases in which the protospacer region of TS is 16 nt, Cas12a could be activated by the TS alone, showing a similar activation rate as the PC (Fig. 1g).

Based on the aforementioned findings, the multi-functional domains of split dsDNA activator also provide new possibilities for the construction of structural dynamic networks (CDNs)[22,23] to simulate natural processes such as adaptability, hierarchical adaptability, intercommunication, and feedback. The CDN network is composed of four components, A, A′, B, and B′, which interact with each other to facilitate transitions among AA′, AB′, BA′, and BB' states. Different triggers can be designed to manipulate the stability of each component within the CDN. When AA′ is stable, the CDN matches it to a new network, up-regulating AA′ and BB′ while down-regulating the corresponding AB′ and BA′. Conversely, when AB′ and BA′ are up-regulated, the corresponding AA′ and BB′ are down-regulated. Based on structural studies of the various positions of split dsDNA activators, we constructed a Cas12a-responsive self-regulatory dynamic network. We defined the perfectly matched TS as A, the perfectly matched NTS as A′, the seed region TS with an internal extended structure as B, and the seed region NTS with an internal extended structure as B′. Due to the complementary base pairing in the spacer region, they maintain the balance of AA′, AB′, BA′, and BB′ components in the [2 × 2] CDN.

Upon the introduction of Cas12a and the other half of the split dsDNA activator, the synergistic activation effect causes Cas12a to preferentially capture the dominant proximal split structure, *i.e.*, AA′. To mitigate the influence of the BA′ component on the self-upregulation of AA′, we increased the length of extended region. Under this condition, the synergistic effect between BA′ and the distal activator was partially suppressed, rendering the AA′ component as the optimal substrate choice in the network for the Cas12a RNP (Fig. S2, ESI†). The CDN network adapts to a new reaction equilibrium, up-regulating AA′ and BB′ components while down-regulating AB′ and BA′ components (Fig. 2a). We modified HEX and BHQ in A and A′, respectively. The quenching effect was more significant after adding RNP, indicating an increase in the concentration of AA′ component induced by RNP (Fig. 2b). In addition, the increase in the concentration of the better AA′ component has also been verified in terms of trans-cleavage activity. The self-regulation curve is the result of subtracting the fluorescence curve of the BA′ component from the fluorescence curve of the mixed solution. As AB′ and BB′ have almost no activity and can be ignored, the curve only represents the activity change of self-regulation (Fig. 2c). The successful validation of binding and trans-cleavage activity for self-regulation also suggests that

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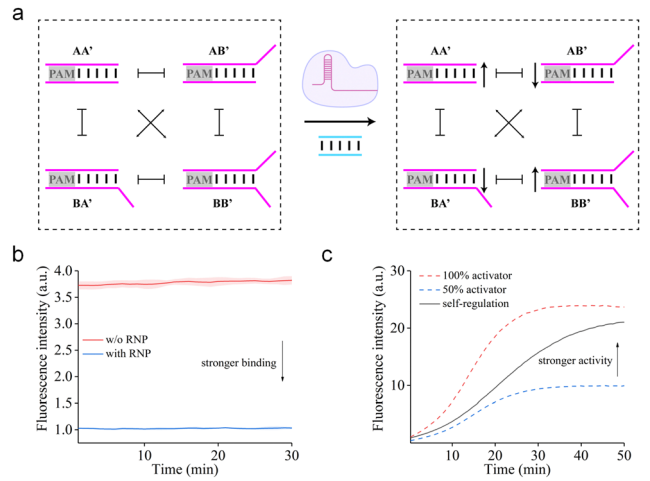


Fig. 2 (a) Mechanism diagram of CDNs based on synergistic activation. (b) and (c) Verification of synergistic activation-induced self-regulation network. Error bar, SD, n = 3.

future structural designs on another split dsDNA activator could achieve multi-network synergistic dynamic regulation. It is noteworthy that Cas12a serves not only as a trigger for manipulating the CDN but also as a key element for CDN signal output, which may facilitate the realization of more complex CDN designs.

Furthermore, we employed the proximal activator as the primary element for activity regulation. APE1, an indispensable component of the base excision repair (BER) pathway, has emerged as a crucial cancer biomarker due to its characteristic overexpression in cancer cells and presence in the blood, making it easy to operate in practical testing.[24] Through strategic modification of the AP site within the extended structure on the inside of the seed region TS, utilizing the AP site within the APE1 cleavage domain, and restoring it to the optimal split substrate structure, we achieved a switch of Cas12a from inactive (OFF) to active (ON) state, enabling APE1 detection (Fig. 3a).

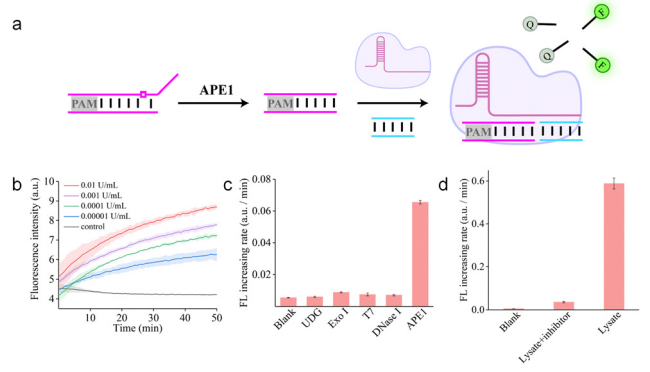
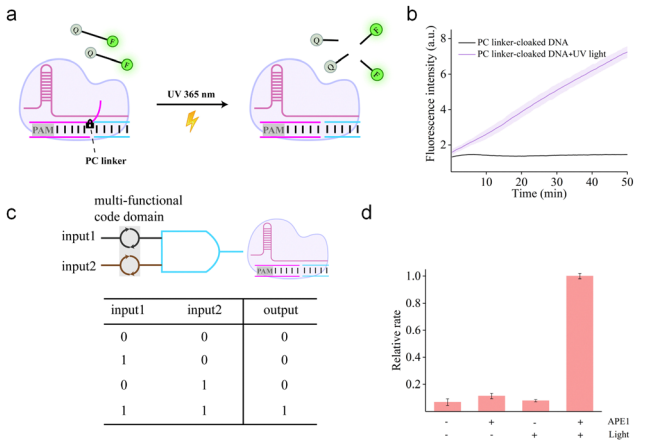


Fig. 3 (a) Mechanism diagram of synergistic platform for APE1 detection. (b) Fluorescence curves with different concentrations of APE1. (c) Selectivity of synergistic platform for APE1 detection. (d) Feasibility verification of synergistic platform for APE1 detection in cell lysate. Error bar, SD, n = 3.

We implemented the AP site on the TS-y-end of proximal ds12 containing PAM, with an additional 2 bp double-stranded structure, following our previous research on the substrate preferences of APE1.[25] The remaining 3 nt extra bases on the NTS after cleavage have also been proven not to affect the activity of Cas12a. Based on this, as low as 0.00001 U mL^{-1} APE1 can be effectively detected (Fig. 3b). The enhanced sensitivity compared to other methods (Table S2, ESI†) may be attributed to the better activation and regulatory efficiency of dsDNA on Cas12a. Additionally, in selectivity studies, other interfering nucleases failed to induce Cas12a activation, while exhibiting robust response exclusively to APE1 (Fig. 3c). Finally, we analyzed the APE1 in human colorectal cancer Lovo cell lysate, and the results indicated that detection can still be effectively carried out in complex systems (Fig. 3d).

Temporal precision control is of significant importance for reducing detrimental side effects. In this regard, light, due to its non-invasive nature and high spatiotemporal resolution, is considered an attractive trigger for altering nucleic acid structures.[26] Time-controlled light activation and the specific activation of nucleases can provide more effective synergistic advantages. We have engineered a photo-sensitive unit (PC linker) onto the extended structure inside the seed region TS of the proximal ds12 containing PAM. The photo-sensitive unit does not affect the inhibitory effect of the extended structure on the activity of Cas12a. After exposure to 365 nm ultraviolet light, the PC linker is cleaved and the activator restores to proximal ds12 to activate the Cas12a (Fig. 4a and b). Leveraging the multi-functional code domain of split activators, it is possible to achieve logical responses to inputs. We constructed an AND gate as a proof of concept. Similar to the design described previously, the proximal TS was modified with an AP site, while the distal TS was modified with a PC linker. A logical output of '1' is obtained only when both APE1 and light are present as inputs (Fig. 4c and d). This tandem of



input1	input2	output
0	0	0
1	0	0
0	1	0
1	1	1

Fig. 4 (a) Mechanism diagram of synergistic platform for time-controlled light activation. (b) Validation of synergistic activation of Cas12a *via* photo-induced deprotection effect. (c) Schematic diagram of synergistic AND gate. (d) Relative fluorescence rate of AND gate for APE1 and light. Error bar, SD, n = 3.

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endogenous and exogenous inputs also demonstrates the potential of this method for regulation *in vivo*.

In summary, we conducted a meticulous investigation of the split dsDNA synergistic activation effect of Cas12a. By modifying various parts of dsDNA to different extents, Cas12a′s activity can be precisely regulated. Based on the influence of each structural domain, we have achieved the construction of a self-regulatory structural dynamic network, the detection of APE1, and a light-responsive strategy. The structural domains of the split dsDNA activator allow for direct manipulation on the domains themselves, thereby eliminating the need for additional transformation processes and ensuring the accuracy of activity regulation. Moreover, compared to ssDNA split synergistic activation, the multi-position structural domain space endows the regulation function with diversity, which can contribute to the application of the Cas12a system in a broader range of fields.

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Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

There are no conflicts to declare.

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