Catalytically inactive dKbCas12d guided by sgRNA and new insights into its binding through a single-molecule approach.

Aleksandr Alekseev[1], Valerii Klimko[1], Marina Abramova[1] ,Irina Franzusova[1], Victor Vinnik[1], Aleksandra Vasilieva[1,2], Polina Selkova[1,2], Mikhail Khodorkovskii[1] and Anatolii Arseniev[1,2]*

1. Peter the Great St. Petersburg Polytechnic University, Saint Petersburg, Russia
2. Complex of NBICS Technologies, National Research Center "Kurchatov Institute", 123182 Moscow, Russia

* To whom correspondence should be addressed. Email: arsenievanatoly@gmail.com

ABSTRACT

CRISPR-Cas12d is a distinct V-D type system discovered in metagenomes of Candida Phyla Radiation bacteria. It stands out from most closely related systems due to its 17-19 nucleotide short spacer region and specialized stabilizing scout RNAs. We have made significant improvements to this system by modifying its scout RNA to create sgRNA, which greatly simplifies its use. We found mutations in the RuvC domain of the effector protein KbCas12d that result in loss of nuclease activity. We obtained two catalytically inactive dKbCas12d variants D827A and E913A. Using the optical tweezers technique, we demonstrated high specificity of dKbCas12d in binding targets on individual DNA molecules. Engineered sgRNA and catalytically inactive dKbCas12d variants have promising applications in biotechnology for the precise regulation of gene expression and molecular diagnostics.

INTRODUCTION

CRISPR-Cas systems are defense mechanisms used by bacteria and archaea to provide adaptive immunity against various mobile genetic elements, such as transposons, bacteriophages, and plasmids [1,2] The operation of these systems is based on the action of RNA-protein complexes consisting of Cas effectors and guide RNAs. Complementary pairing of guide RNAs with the foreign genome enables Cas effectors to precisely recognize and cleave specific sites in the invader's nucleic acid, halting further infection. For genetic engineering purposes, Cas effectors can be directed towards desired sites in the genomes of prokaryotic and eukaryotic organisms by utilizing guide RNAs to selected sequences[3,4]. Characterization of novel CRISPR-Cas proteins allows us to expand the range of potential genome engineering tools and provides fundamental insights into the operation of bacterial defense systems.[5]

CRISPR-Cas12d (previously named CasY) systems were discovered by studying metagenomic data from the CPR (Candida Phyla Radiation) bacteria[6] The CPR bacteria are not culturable in the laboratory and represent more than 15% of all bacterial diversity. Presumably symbionts of other prokaryotes, these organisms are characterized by a compact cell size and small genomes. CRISPR CasY systems found in these bacteria are also characterized by a small size of effector genes [7].

The locus of the CRISPR-Cas12d system includes a CRISPR cassette, the gene Cas1, which is thought to be responsible for adaptation, and the Cas12d gene, which encodes a nuclease of about 1200 amino acids. The sequences between direct repeats of the CRISPR cassette are also reduced and represent short spacers of 17-19 nucleotides in length. It was previously shown that cells carrying the CRISPR-Cas12d locus are capable of interference defense against foreign mobile elements in vivo. [6,8]
Later research discovered [8] that CRISPR-Cas12d systems require additional scout RNA. In vitro experiments mainly focused on the CRISPR-CasY15 system, showing the presence of a TR – PAM sequence, the necessity of scout RNA for specific nuclease activity, collateral activity, and strong specificity of the system with spacer sequence substitutions. Similar to other type V systems, the RuvC domain that is responsible for DNA cleavage was determined.[7,9,10]

In this study, we aimed to identify mutations that would inactivate the active site of the Cas12d effector protein from Katanabacteria - KbCas12d. To achieve this goal, we produced recombinant wild-type and mutant proteins and compared their in vitro activities. Furthermore, in order to effectively utilize the system, we intended to fuse crRNA and scout RNA molecules to obtain single guide RNA. Since standard biochemical methods do not always provide a comprehensive assessment of DNA-protein interactions, we employed optical tweezers to gain a better understanding of the interaction between the catalytically inactive mutant and individual DNA molecules. This approach, along with the use of fluorescence imaging, allows for more direct visualization of specific, targeted DNA-protein interplay.

MATERIALS AND METHODS

Recombinant protein expression and purification

The wild-type and mutant forms of dCasY D827A and dCasY E913A genes were cloned into pET21a plasmids under the regulation of the T7 promoter. His and MBP tags were added to the C-terminus of the proteins at the cloning state for subsequent affinity purification. The resulting plasmids, pET21_CasY, pET21_dCasY D827A, and pET21_dCasY E913A, were transformed into competent Rosetta *E. coli* cells. A 10 ml overnight culture of transformed bacteria was diluted into 500 ml LB medium with ampicillin and cultured at 37°C until reaching an optical density of 0.6 OD. Expression of the effector proteins was induced by adding 1 mM IPTG, followed by incubation at 16°C for 16 hours. Cells were then lysed in 15 ml lysis buffer (50 mM Hepes-HCl pH=7.5, 500 mM NaCl, 10% glycerol, 1 mM beta-mercaptoethanol) with addition of 15 mg lysozyme.After sonication and centrifugation, the supernatant was filtered (0.22 µm pore size) and applied to a HisTrap HP 1 mL column (GE Healthcare) for affinity chromatography. After washing with lysis buffer plus 20 mM imidazole the protein was eluted with lysis buffer containing 300 mM imidazole. Fractions were concentrated using Amicon Ultra-4 centrifugal units (Merck Millipore) with a 100 kDa cutoff to a volume of 600 µl. At this stage, TEV protease was added to the samples, and incubation occurred overnight at 10°C. The resulting sample was applied to a Superdex 200 Increase 10/300 GL gel filtration column (GE Healthcare) equilibrated with 50 mM Hepes-HCl pH=7.5, 500 mM NaCl, 1 mM DTT, and 10% glycerol. Fractions containing monomeric protein fractions were collected, concentrated, and frozen in small volumes in liquid nitrogen with subsequent storage at -80C.

Detection of nuclease activity *in vitro.*

DNA cleavage reactions were performed using recombinant KbCas12d protein and linear dsDNA targets (818 b. p.) obtained by the PCR method from plasmid puc19 containing the native spacer of the KbCas12d system . The reaction was performed using 1× CutSmart buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, and 100 µg/mL BSA; NEB) and 0.5 mM DTT (1,4-dithiothreitol) with varying concentrations of recombinant KbCas12d or its mutant versions and RNA (as indicated in the results). Samples were incubated at 37°C for 30 minutes. To stop the reaction, 4× loading dye containing 10 mM Tris-HCl, pH 7.8, 40% glycerol, 40 mM ethylenediaminetetetraacetic acid, 0.01% bromphenol blue, and 0.01% xylocyanol was added. The electrophoretic analysis of the reaction products was performed on either 1.5% agarose gels or 5% denaturing gels (urea 8M).

Binding activity detection *in vitro*

The electrophoretic mobility shift assay (EMSA) was utilized to assess the DNA binding activity of dCasY D827A and dCasY E913A. The reactions were performed as described previously [11] on a DNA matrix obtained by amplification from the GRIN2B gene with a length of 132 bp limited by a primer containing a CY-3 tag. RNP complexes consisting of protein and RNA in a 1:5 ratio in different ratios (1:1; 1:3; 1:10) relative to 20 nM matrix DNA were incubated for 30 min at 37C. RNP complexes containing non-target RNA were used as negative controls. Samples were then applied to a 5% PAGE gel to visualize binding.

Optical tweezers setup

For single molecule manipulations a custom built dual trap optical tweezers setup based on the Axioimager.Z1 microscope was used as described previously [12,13]. In brief, two independent optical traps were generated using Nd:YVO4 1064 nm CW laser (5 W, Spectra Physics BL-106C) and oil immersion lens with high numerical aperture. Manipulations with single DNA molecules were carried out in a multichannel microfluidic systeml (Lumicks). Bead positions and applied tension were controlled with a 30 ms resolution using a custom LabView software.

Fluorescent images of dCasY-sgRNA-DNA complexes were collected with an EMCCD camera (Cascade II, Photometrics) using Micromanager software. Fluorescence light was excited with the DPSS 473 nm CW laser (100 mW, Lasever, LSR473U) and separated using filter set 10 (Carl Zeiss). Images were obtained with 1000 ms exposure time and electron multiplier gain of 3800. Saved TIFF files were further processed in FIJI ImageJ.

Single-molecule assay

Double stranded DNA molecules (48,5 kbp) with biotinylated ends were prepared from Bacteriophage lambda DNA as described previously [12] For single-molecule experiments 100 nM dCasY E913A was individually mixed with 200 nM of RNA1/2/3/4 in four separate tubes in 1x CutSmart buffer at room temperature. After short incubation at room temperature four solutions were mixed together. All experiments were done in 40 mM Tris-OAc (pH 8.0), 80 mM KOAc, 10 mM MgOAc, 0.02% BSA at 37°C. The inner surfaces of microfluidic system were passivated with 0.5% Pluronic F-127 and 0.1% BSA[14]Two channels of microfluidic cell were used to obtain single optically trapped DNA tether and thus were fed

with a 0.01% solution of a 2.1 µm streptavidin-coated polystyrene beads (Spherotech) and 1.25 pM solution of a double stranded DNA with biotinylated ends. After DNA was captured, its force-extension behavior was measured to verify that single DNA molecule is tethered between two beads. After this DNA tether was incubated in the channel containing 100 nM dCasY-sgRNA for 3 min and then was fluorescently visualized in protein free channel.

RESULTS

Production of catalytically inactive versions of the KbCas12d effector.

Bioinformatic analysis of the CRISPR-cas12d locus from *Catanobacterium* shows that the system includes a 1041 bp Cas1 gene encoding protein and a KbCas12d effector gene of 3378 bp. The system includes 14 spacers of 17-18 nucleotides in length separated by repeat sequences of 26 nucleotides in length. A sequence encoding scout RNA is located upstream of the CRISPR cassette. Transcription of the scout RNA and CRISPR RNA is in the same direction. (Figure. 1a).

To obtain catalytically inactive forms of the effector KbCas12d, its amino acid sequence was analyzed to identify possible functional domains. Using catalytically inactive mutant data for CRISPR-Cas12e [9] and CRISPR-Cas12a[10], the identified RuvC nuclease domain of all three systems were aligned to each other. The alignment (Figure. 1b) suggested substitutions in the amino acid sequence of the Ruvc domain of the KbCas12d system at positions D827 and E913 for alanine to create "dead" forms (DNA-binding but non-cutting versions of the protein).

Cloning, isolation and purification of mutant and wild-type proteins are described in the methods. As a result, recombinant proteins were successfully obtained in monomeric form and sufficient concentration.

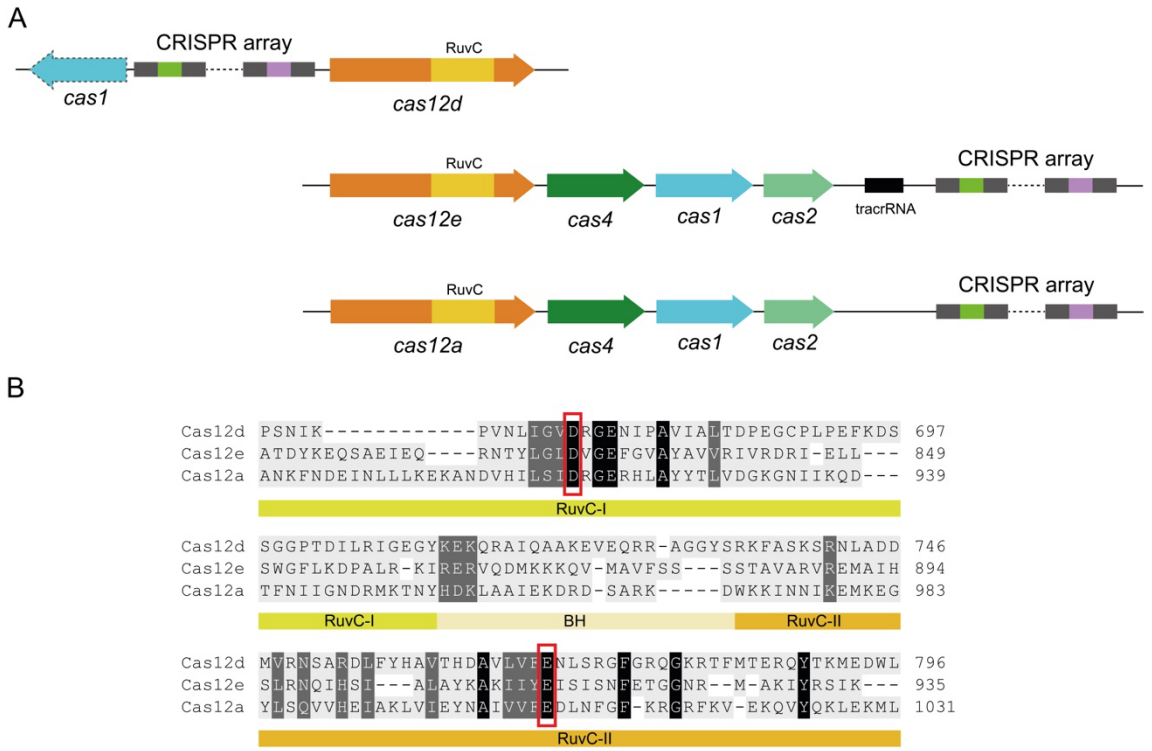

Figure 1: (a) Organization of type V systems: Cas12d, Cas12a and Cas12e. (b) Fragment of the alignment of the amino acid sequence of the effector domain of the KbCas12d system to the amino acid sequences of the RuvC domain of the DpbCas12e and AsCas12a. Known mutation sites for the AsCas12a protein are D917A and E1006A. Known mutation sites of DpbCas12e protein: D672A and E769A. Corresponding KbCas12d protein mutation sites: D827A and E913A.

Design of single guide RNA

To optimize the system, the optimal scout RNA sequence was selected. For this purpose, a number of scout RNA forms differing in length and secondary structure were synthesized based on scout RNA sequence data [8](Figure. A): (sequences are given in supplementary information)
With the synthesized scout RNA, crRNA sequence and recombinant KbCas12d protein, a 20µl volume of target DNA cutting reaction was performed at 37°C for 30 minutes (1X CutSmart buffer (NEB), 20 nM DNA, 4 µM scoutRNA/crRNA, 400 nM KbCas12d protein)
The experimental results showed that scout RNA1, the shortest form of the molecule, is optimal for DNA cutting. In complex with it and crRNA, the KbCas12d protein actively cuts a double-stranded DNA fragment carrying a protospacer sequence flanked by a PAM 5' TA 3'. This molecule was used as the basis for the design of a hybrid RNA, a fusion molecule of guide crRNA and scout RNA (sgRNA, single guide RNA, sgRNA) (Figure 2b). To create sgRNA, the 3'-end of the scout RNA was joined to the crRNA sequence via linkers by the "GAAA" sequence. The sgRNAs with different linker lengths were tested (Figure. 2b,c):

KbCas12d protein in complex with sgRNA 1, sgRNA 2, sgRNA 3 respectively was incubated with the DNA target used in the above experiments for 30 min at 37°C. The reaction products were applied to agarose electrophoresis to evaluate the cutting efficiency of the DNA fragment. A reaction mixture where a complex of crRNA and scout RNA was used as a positive control.
The result of the experiment showed that the use of sgRNA 1, sgRNA 2 led to efficient cutting of target DNA, along with the set of guide RNAs of two kinds - scout and crRNA. Further, sgRNA 2 was used in the experiments. Thus, it was possible to find a form of hybrid RNA (sgRNA) for efficient DNA cutting by the KbCas12d protein(Figure 2c). The used algorithm of sgRNA and crRNA joining can potentially be used for other representatives of the Cas12d family.

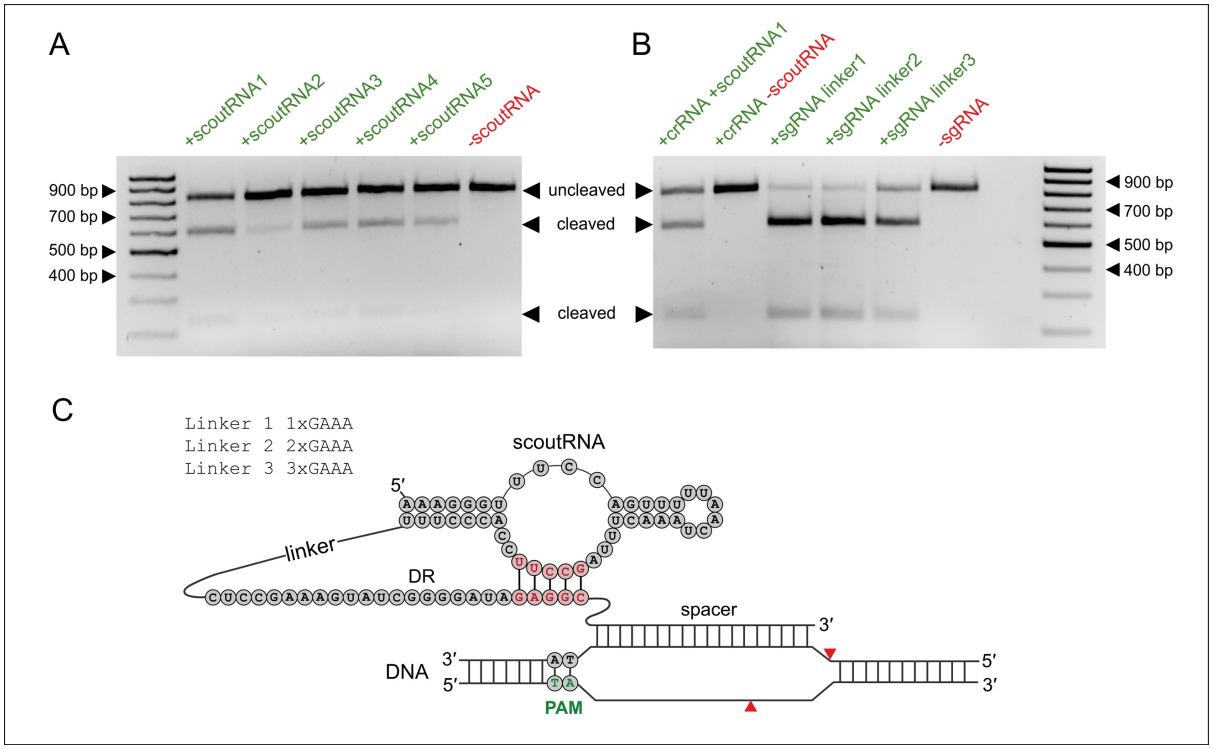

Figure 2: (a) Design of the KbCas12d sgRNA binding to target DNA. (b) *In vitro* testing of sgRNA with different linker lengths. A mixture of crRNA and scoutRNA was used as a positive control.

Testing the nuclease and binding activity of dKbCas12d D827A and dKbCas12d E913A proteins.

The nuclease activity of mutant forms of KbCas12d was tested. For this purpose, an in vitro DNA cutting reaction was performed with ratios of target DNA to ribonucleic acid complex (1:1, 1:10, and 1:20); samples were incubated for 30 min at 37°C. The reaction was then split into equal parts, and samples were plated on a native agarose gel (Figure 3a) as well as on PAGE under denaturing conditions (Figure 3b). Denaturing conditions were chosen to demonstrate the absence of DNA nicking (Figure 3b).
Next, the ability of the dKbCas12d D827A and dKbCas12d E913A mutant forms to bind to the target DNA via guide RNA was tested (Figure 3c). To assess DNA-binding abilities, KbCas12d in complex with guide RNAs were mixed with the DNA target in vitro using protein/RNA:DNA ratios of 1:1, 1:3, and 1:10 (see Methods). The 130 bp long target DNA contained a Cy3 fluorescent tag at the 5'-end. The reaction was run for 30 min at 37°C. Samples were then spread on a 5% polyacrylamide gel and dispersed in gel in an electrophoresis apparatus placed in ice. Gel-shifts (Figure 3c) demonstrate the change in mobility of DNA in complex with the effector ribonucleic acid complex.
The EMSA assay results demonstrate the ability of the mutant proteins to bind to the double-stranded DNA sequence of the target.

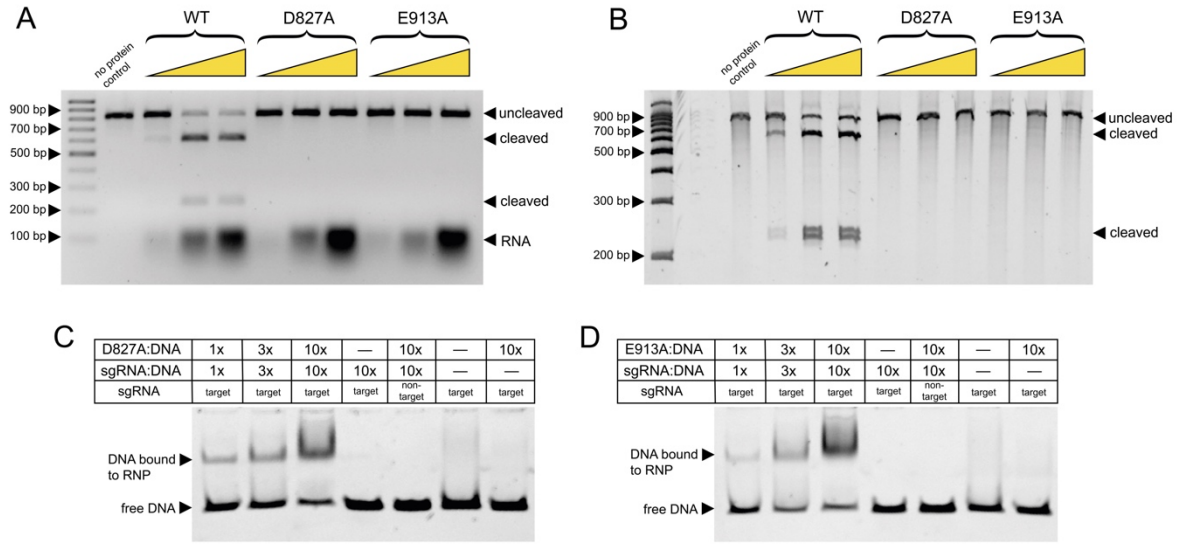

Figure 3: (a) Nuclease activity of wild-type KbCas12d protein and mutant KbCas12d D827A and dCasY E913A proteins when incubated at different ratios with target DNA (1:3, 1:10, and 1:20). Agarose gel electrophoresis under native conditions. (b) Nicking activity of wild-type KbCas12d protein and mutant KbCas12d D827A and dCasY E913A when incubated at different ratios with target DNA (1:3, 1:10, and 1:20). Polyacrylamide gel electrophoresis under denaturing conditions. (c) Binding of an effector complex consisting of the KbCas12d D827A and dCasY E913A mutant proteins and guide RNA to the target DNA.

Specificity of catalytic inactive KbCas12d binding to DNA at the single molecule level.

To assess the efficiency and specificity of DNA binding on extended molecules, we used a single-molecule laser tweezers approach. We designed four sgRNAs containing FAM-tagged UTPs targeting four equally spaced (approximately 5000 bp) regions of lambda phage DNA. Then we used a custom optical trapping system and a microfluidic chamber, (see methods) to capture lambda DNA between two microspheres, and incubated for 3 min in the channel with channel containing the mixture of four RNP, keeping the DNA between the traps in a relaxed state. We then transferred the system to a channel containing only the buffer and performed fluorescence imaging (Figure 4). We observed that RNP complexes (n=20) were often bound at specific target sites, albeit with low varying efficiency. Only in a couple of cases we were able to observe simultaneous landing of 3 RNP complexes at the same time. (Fig4) Since the amount of fluorescent UTPs can vary between molecules we observed different signal intensities from the targets. Data analysis revealed almost the equal binding probability of RNP to four specific targets T1/T2/T3/T4: 0.19 ± 0.1, 0.25 ± 0.18, 0.33 ± 0.17 and 0.28 ± 0.17 (mean ± SEM). Almost no non-specific interactions were observed during the experiments.

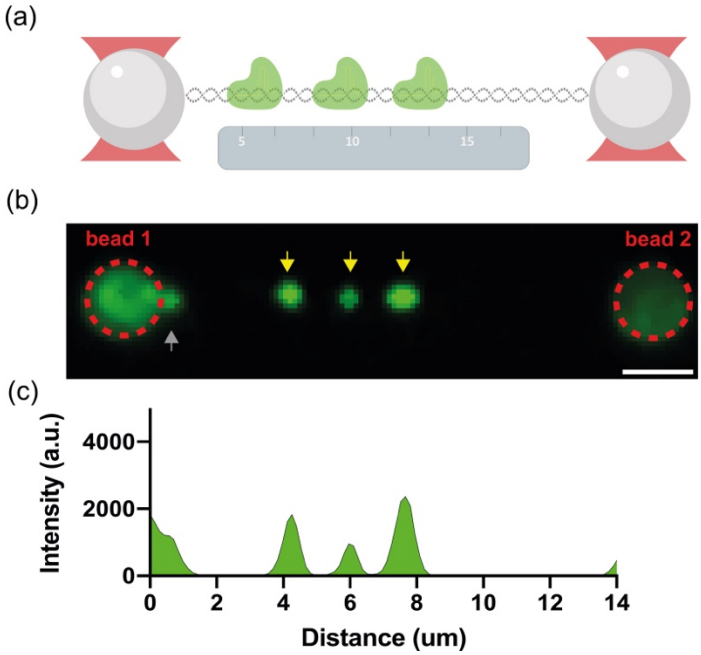

Figure 4. Single-molecule visualization of dCasY-sgRNA-DNA complexes. (a) Schematic of experimental workflow. (b) Representative fluorescent image, revealing three specific complexes (yellow arrows) and non-specific binding (gray arrow) on 48.5 kbp DNA containing four targets. (c) Corresponding intensity plot profile.

DISCUSSION

The study demonstrates catalytically inactive mutants of the Cas12d system, which are unable to perform DNA double stranded break but have the ability to bind to the target DNA in a directed manner. Interestingly, despite the significant differences in organization, the structure of the Cas12d RuvC nuclease domain aligns well with the amino acid sequences of the Cas12e and Cas12a systems. The structure of the protein itself may be closer to the Cas12e system, but this requires further structural studies. On the other hand there is a significant difference in the CRISPR-RNA structure, where a characteristic scout RNA should be present for the Cas12d system. Despite rather long and conserved scoutRNAs described in the literature we have shown that significantly shortened versions also show *in vitro* activity. Therefore, the identification of all features of the scout RNA remains to be elucidated. Nevertheless, we were able to create an efficient single-guide RNA in which both of crRNA and scout RNA are bound through a linker and demonstrate its activity to accomplish directed double-strand breaks for a wild-type system. Such a catalytically active effector-based system could be used in the future for genetic editing.
Using standard biochemical methods, we demonstrated the ability of the dKbCas12d D827A and dKbCas12d E913A mutants to bind target DNA using single guide RNA. Subsequently, we employed optical tweezers to evaluate the specificity and efficiency of the dKbCas12d E913A system at the single-molecule level. Our data presented good specificity, which is similar with another single-molecule research on Cas12a [15]. Also, according to the published data [16], Cas12a is more sensitive than Cas9 to the introduction of artificial mismatches in the guide RNA (16 vs. 9 PAM proximal matches). The Cas12d system is characterized by a spacer sequence comprised of 17-19 letters. Substituting any of these

letters may significantly impact the recognition and nuclease activity of the complex, [8]. Thus, the low binding efficiency of the complexes and the lack of off-target binding may be explained by the fact that a prolonged binding event, which we are able to capture, requires complementarity of all spacer nucleotides. While low off target binding efficiency appears due to the complex's rapid and specific scanning of the DNA molecule.
Many mechanisms of the CRISPR KbCas12d system remain to be thoroughly elucidated, but its unique features indicate a high potential for future use in diagnostic, medical and biotechnological applications.

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