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Supplemental Information

Conformational Dynamics and Cleavage Sites of Cas12a Are Modulated by Complementarity between crRNA and DNA

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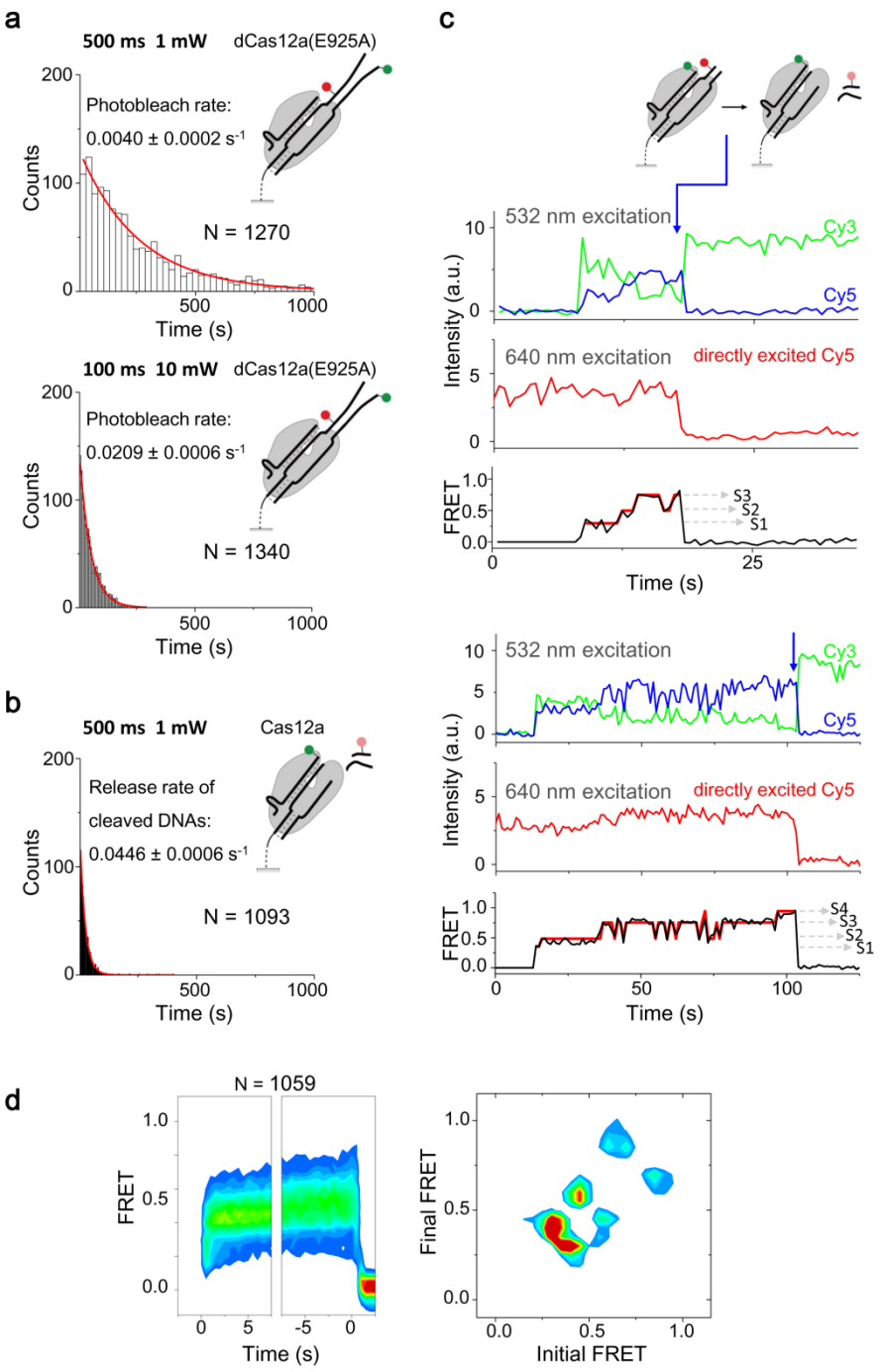

Figure S1. Photobleaching rates, appearance cleavage rates, smFRET trajectories and crRNA-NTS FRET of Cas12a complexes, related to Figure 1. a, Distributions of FRET duration time of labeled dsDNA within dCas12a (E925A) ternary complexes which are unable to cleave DNA. With 500 ms exposure time and 1 mW laser excitation power, FRET disappearance rates caused by Cy3 and Cy5 photobleaching are 0.0036 ± 0.0002 s^{-1} (N = 890) and 0.0052 ± 0.0004 s^{-1} (N = 380), respectively. The overall FRET disappearance rate is 0.0040 ± 0.0002 s^{-1} (top panel). With 100 ms exposure time and 10 mW laser excitation power, FRET disappearance rates caused

by Cy3 and Cy5 photobleaching are 0.020 ± 0.001 s^{-1} (N = 960) and 0.029 ± 0.003 s^{-1} (N = 380), respectively. The overall FRET disappearance rate is 0.021 ± 0.001 s^{-1} (bottom panel). **b**, FRET disappearance rate caused by release of cleaved DNA from Cas12a ternary complexes containing Cy3-labeled crRNA and Cy5-labeled dsDNA using 500 ms exposure time and 1 mW laser excitation power. With these instrumental settings, contributions of photobleaching is < 10%. **c**, Representative smFRET trajectories of DNA cleavage events under alternating-laser excitation of 532 nm and 640 nm lasers, from which disappearance of Cy3 and Cy5 are captured independently. FRET disappearance from either S3 or S4 is accompanied by Cy5 signal disappearance, which indicates that termination of FRET is caused by release of cleaved Cy5-labeled DNA fragment. Such phenomena suggest that both S3 and S4 states are DNA pre-cleavage states. **d**, Time-dependent FRET probability density plot and transition density plot of Cas12a complexes on 23m-dsDNA captured using FRET pairs located on crRNA and NTS. Cy3 is labeled at 3' end of crRNA, Cy5 is labeled at the NTS 30 nt away from the PAM.

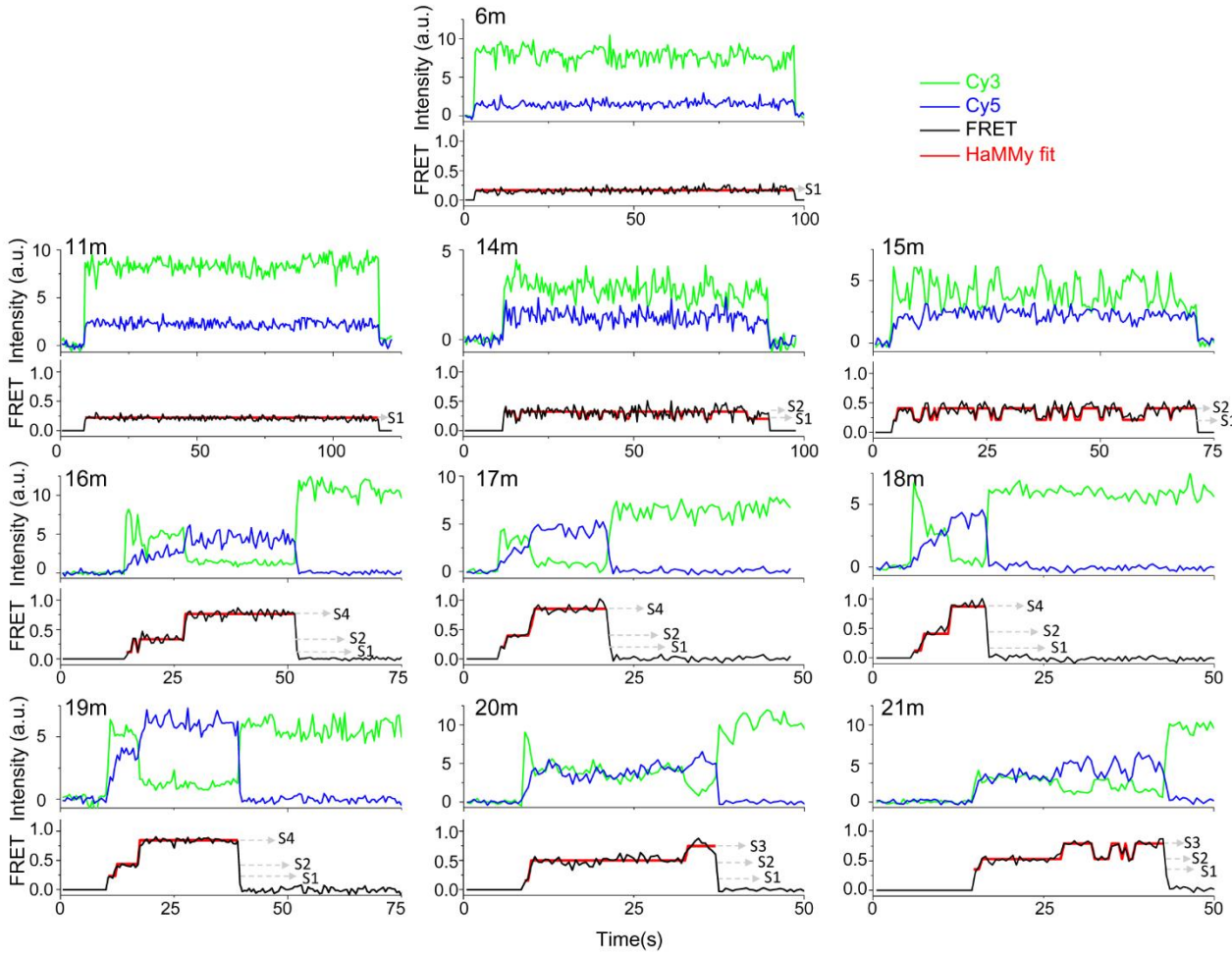

Figure S2. Representative smFRET trajectories of Cas12a ternary complexes on partial cognate dsDNAs, related to Figure 2. Representative smFRET traces of Cas12a complexes on 6m-, 11m-, 14m-, 15m-, 16m-, 17m-, 18m-, 19m-, 20m- and 21m-dsDNAs. Black lines are apparent FRET efficiencies and red lines are hidden Markov modeling of FRET. Under 532 nm excitation, spontaneous appearance of Cy3 and Cy5 (due to FRET) signals represents the formation of Cas12a ternary complex on immobilized dsDNA. Simultaneous disappearance of FRET and Cy3 is caused by dissociation of Cy3-labeled Cas12a/crRNA from immobilized Cy5-labeled DNA, whereas disappearance of FRET accompanied by increasing of Cy3 intensity corresponds to dissociation of the cleaved Cy5-labeled PAM-distal DNA fragment.

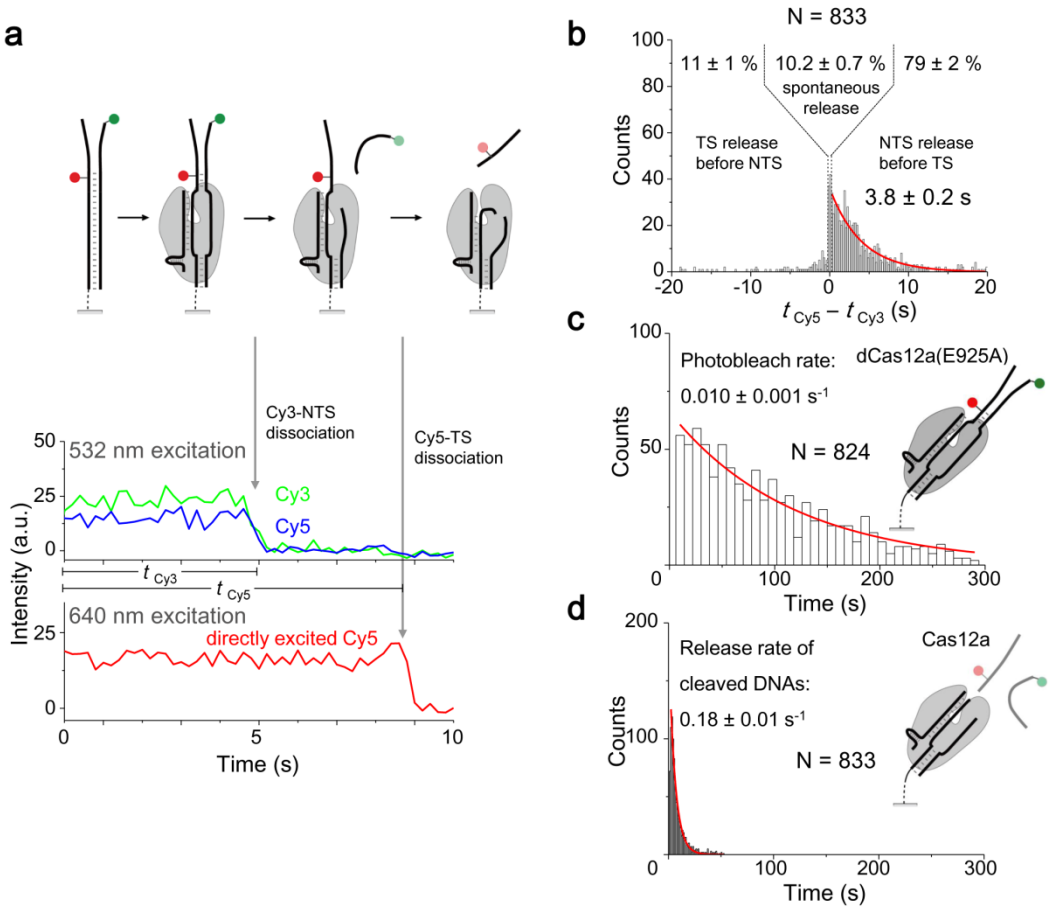

Figure S3. Release of cleaved DNA fragments examined using fluorophore labeled NTS and TS, related to Figure 2. a, smFRET trajectories and cartoons illustrating dissociation of Cy3-labeled NTS and Cy5-labeled TS. Under alternating-laser excitation of 532 nm and 640 nm lasers, disappearance of Cy3 and Cy5 can be captured independently. Times from injecting unlabeled Cas12a/crRNA into flow channels containing immobilized dsDNAs until disappearance of Cy3 and Cy5 are extracted from individual single-molecule trajectories and defined as t_{Cy3} and t_{Cy5}, respectively. **b**, Distribution of t_{Cy5} - t_{Cy3}. 79% ± 2% of events have Cy5-NTS disappear before Cy3-TS, whereas only 11% ± 1% of events have Cy3-TS disappear before Cy5-NTS. Single-exponential fitting shows that, for the majority of molecules, cleaved TS is released 3.8 ± 0.2 s after cleaved NTS. **c**, Photobleaching rate measured using dCas12a. Power of 532 nm and 640 nm lasers are 5 mW and 3 mW, respectively, with 100 ms exposure time. **d**, Apparent release rate of cleaved Cy3-NTS fragments after formation of Cas12a ternary complexes, which is an order of magnitude faster than photobleaching rate.

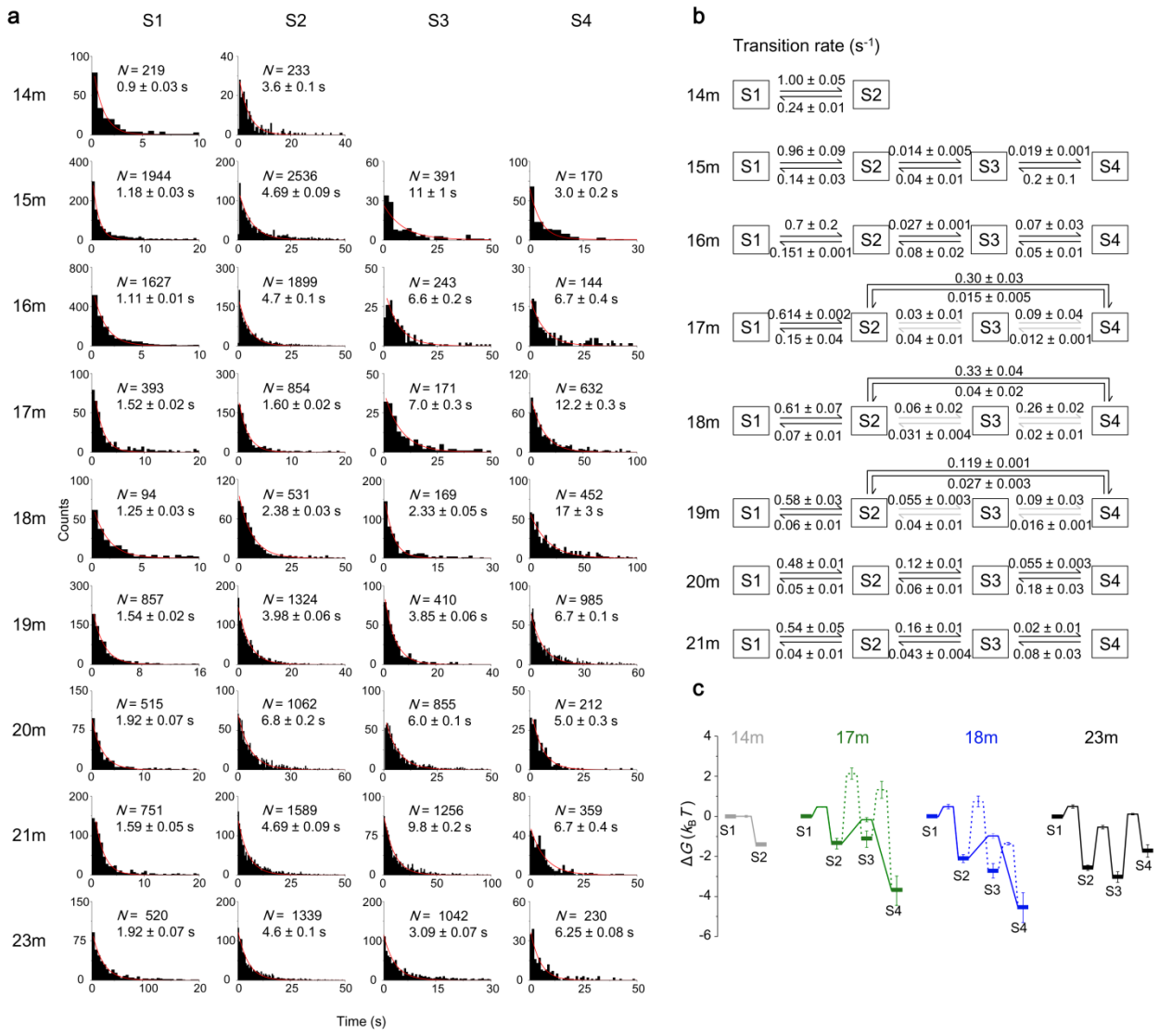

Figure S4. Kinetic schemes and energy landcapes of Cas12a ternary complexes, related to Figure 2. a, Distributions of dwell time extracted from HMM modeling. Average dwell times fitted from single-exponential decay curve and number of events (*N*) from a representative experiment were shown. Three to five repeats were used when calculating transition rates among FRET states shown in b. **b**, Kinetic schemes showing transition pathways and rates among different FRET states. Arrows in black and grey indicate major and minor transition pathways, respectively. **c**, Energy landscapes of Cas12a complexes on 14m-, 17m-, 18m- and 23m-dsDNAs. Bold horizontal lines represent four conformational states captured by smFRET, and thin horizontal lines represent energy barriers along transition pathways. For 17m- and 18m-dsDNA which contains parallel reaction pathways, the major pathway is shown in solid lines and the minor pathway is in dash lines. k_B is Boltzmann constant and T is temperature.

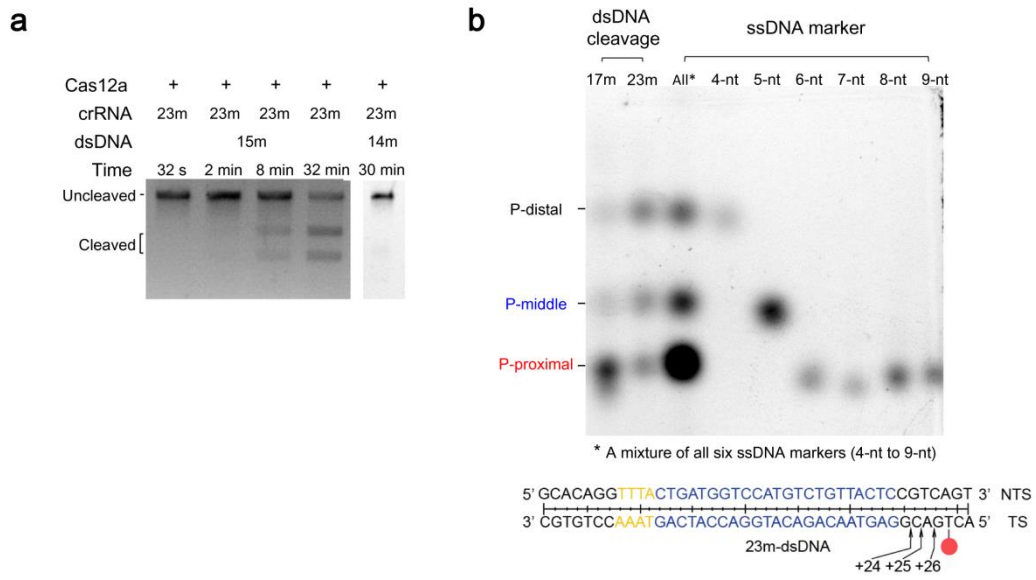

Figure S5. Cleavage of dsDNAs, related to Figure 3 and 4. a, Cleavage of 15m- and 14m-dsDNAs. 15m-dsDNA is cleaved much slower than dsDNA containing more matches between crRNA and dsDNA as shown in Fig. 2c. Prolonged cleavage time (8 min and 32 min) results in detectable dsDNA cleavage. 14m-dsDNA cannot be cleaved within 30 min. **b**, Cleavage sites on target strand (TS) of dsDNA. Cy5 labeled single-stranded DNAs (ssDNAs) of different length were resolved on 20% urea-PAGE gel to illustrate TS cleavage sites as shown in Fig. 3b. The band named 'P-distal' represented a 4-nt-length product, indicating a cleavage site at position + 26 of TS; 'P-middle' product resulted from cleavage at position + 25, and 'P-proximal' originated from cleavage at position about + 24.

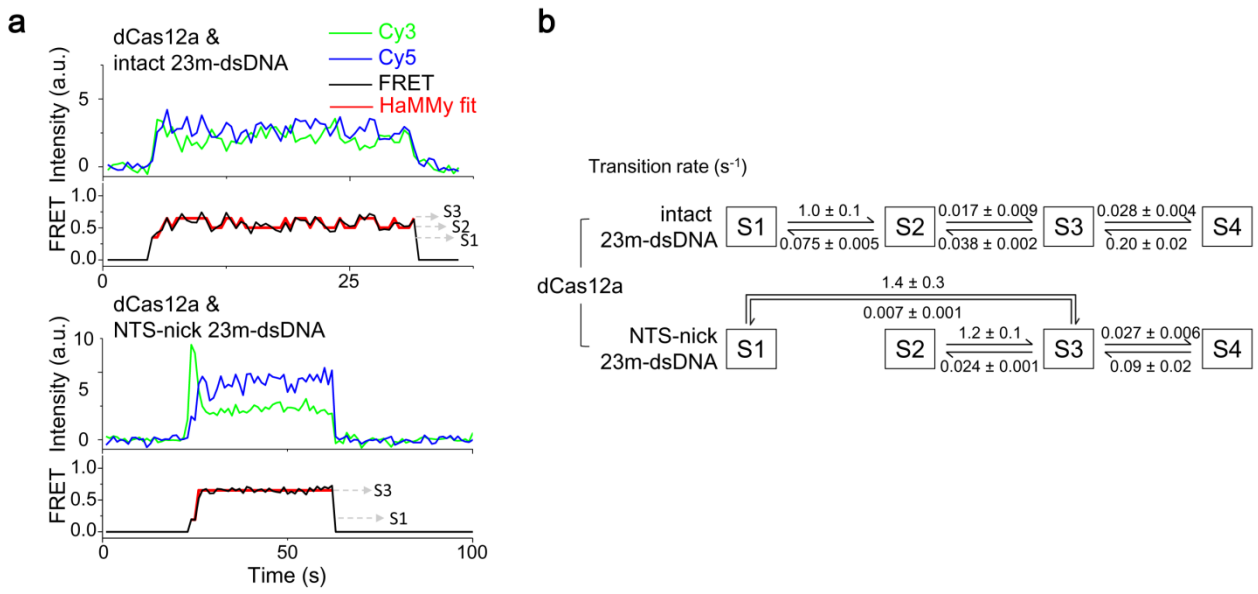

Figure S6. Representative smFRET trajectories and kinetic schemes of dCas12a ternary complexes, related to Figure 5. a, Representative smFRET trajectories of dCas12a complexes on intact and NTS-nick dsDNAs. **b**, Kinetic schemes showing transition pathways and rates of dCas12a complexes on intact and NTS-nick dsDNAs.

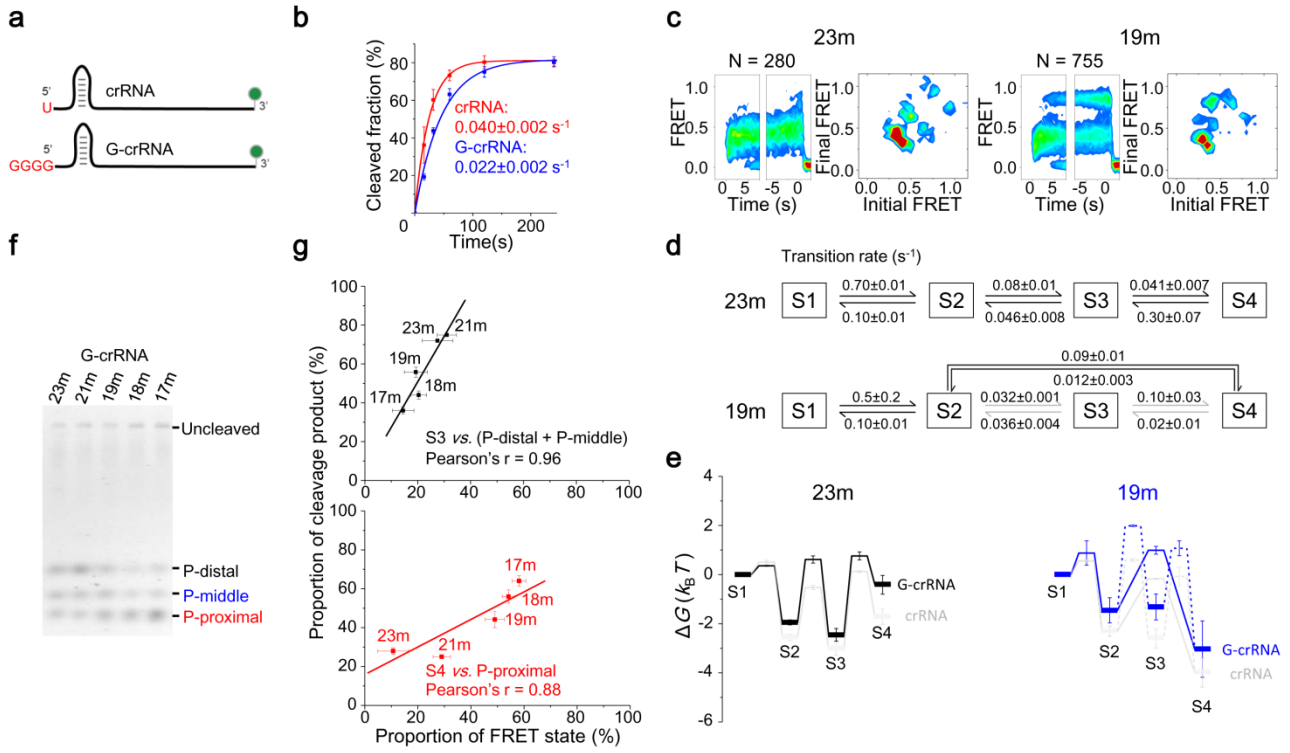

Figure S7. Conformational dynamics and cleavage patterns of Cas12a complexes guided by G-crRNA, related to Figure 2 and 3. a, Cartoon illustrating difference between crRNA and G-crRNA. Four G nucleotides at the 5' end of G-crRNA replace a single U at the 5' end of crRNA used in all other experiments. The other sequences remain the same. **b**, Time-dependent 23m-dsDNA cleavage using crRNA and G-crRNA. **c-e**, Time-dependent FRET probability density plots and transition density plots (**c**), transition pathways (**d**) and energy landscapes (**e**) of Cas12a/G-crRNA complexes on 23m- and 19m-dsDNAs. Energy landscapes of Cas12a/crRNA/dsDNA complexes are shown in grey for comparison. **f**, Cleavage of full and partial cognate dsDNAs labeled at their TS PAM-distal ends. **g,** Linear correlation between proportion of FRET states in the last frame and proportion of cleavage products shown in **f.** They display a strong positive correlation with Pearson's r = 0.88 ~ 0.96.

Table S1. RNA sequences, related to Figure 1, 2, 3, 4 and 5.

Name	Sequence (from 5' to 3') [a]	Related figures
crRNA (Takara) [b]	UAAUUUCUACUAAGUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-NH_2	Figs. 1, 2, 4d, 5 and S7b
23m-crRNA (transcribed *in-vitro*)	*GGUUUCAAAGAUUAAA*UAAUUUCUACUAAGUGUAGAUCUGAUGGUCCAUGUCUGUUACUC	Figs. 3b, 4
14m-crRNA (transcribed *in-vitro*)	*GGUUUCAAAGAUUAAA*UAAUUUCUACUAAGUGUAGAUCUGAUGGUCCAUGUgacaaugag	Figs. 4b and 4c
G-crRNA (transcribed *in-vitro*) [b]	GGGGAAUUUCUACUAAGUGUAGAUCUGAUGGUCCAUGUCUGUUACUC	Fig. S7

[a] Stem-loop sequences are highlighted in grey, spacer sequences are underlined in which mismatches are shown in lower case, transcribed RNA sequences which will be cleaved by Cas12a to generate matured crRNA are italicized (Singh et al., 2018). 14m-crRNA contains 14 matched bases towards 23m-dsDNA at its PAM-proximal end. The other crRNAs are fully matched towards 23m-dsDNA.

[b] crRNA (Takara) and G-crRNA were labeled with Cy3 at their 3' end before used in smFRET experiments.

Table S2. DNA sequences, related to Figure 1, 2, 3, 4 and 5.

Name	Sequence (5' to 3')
biotin handle	CCCTGGTCCGGTGGTCCGCCTGCTGGTCCC-biotin
dsDNA with 6 or 11 mismatched bases at the PAM-proximal end	
PAMprox6mm_NTS	CGGACCACCGGACCAGGGGCACAGGTTTAgactacGTCCATGTCTGTTACTCCGTCAGT
PAMprox6mm_TS	AC T(-Cy5)GACGGAGTAACAGACATGGACgtagtcTAAACCTGTGC
PAMprox11mm_NTS	CGGACCACCGGACCAGGGGCACAGGTTTAgactaccaggtTGTCTGTTACTCCGTCAGT
PAMprox11mm_TS	AC T(-Cy5)GACGGAGTAACAGACAacctggtagtcTAAACCTGTGC
TS labeled full and partial cognate dsDNAs, related to Figs. 1, 2, 3, S5b and S7	
23m-NTS (S)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGTTACTCCGTCAGT
23m-TS (S)	ACT(-Cy5)GACGGAGTAACAGACATGGACCATCAGTAAACCTGTGC
21m-NTS (S)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGTTACagCGTCAGT
21m-TS (S)	ACT(-Cy5)GACGctGTAACAGACATGGACCATCAGTAAACCTGTGC
20m-NTS (S)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGTTAgagCGTCAGT
20m-TS (S)	ACT(-Cy5)GACGctcTAACAGACATGGACCATCAGTAAACCTGTGC
19m-NTS (S)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGTTtgagCGTCAGT
19m-TS (S)	ACT(-Cy5)GACGctcaAACAGACATGGACCATCAGTAAACCTGTGC
18m-NTS (S)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGTatgagCGTCAGT
18m-TS (S)	ACT(-Cy5)GACGctcatACAGACATGGACCATCAGTAAACCTGTGC
17m-NTS (S)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGaatgagCGTCAGT
17m-TS (S)	ACT(-Cy5)GACGctcattCAGACATGGACCATCAGTAAACCTGTGC
16m-NTS (S)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTcaatgagCGTCAGT
16m-TS (S)	ACT(-Cy5)GACGctcattgAGACATGGACCATCAGTAAACCTGTGC
15m-NTS (S)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCacaatgagCGTCAGT
15m-TS (S)	ACT(-Cy5)GACGctcattgtGACATGGACCATCAGTAAACCTGTGC
14m-NTS (L)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTgacaatgagCGTCAGTCGAGGCT CTCGAC
14m-TS (L)	GTCGAGAGCCTCGACT(-Cy5)GACGctcattgtcACATGGACCATCAGTAAACCTGTG
11m-NTS (S)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCAacagacaatgagCGTCAGT
11m-TS (S)	ACT(-Cy5)GACGctcattgtctgtTGGACCATCAGTAAACCTGTGC

6m-NTS (S)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGcaggtacagacaatgagCGTCAGT
6m-TS (S)	ACT(-Cy5)GACGctcattgtctgtacctgCATCAGTAAACCTGTGC
NTS labeled full cognate dsDNAs, related to Fig. S1d and 4c	
23m-NTS (L)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGTTACTCCGTCAGT(-Cy5)CGAGGCTCTCGAC
23m-TS (L)	GTCGAGAGCCTCGACTGACGGAGTAACAGACATGGACCATCAGTAAACCTGTG
dsDNA with both NTS and TS labeled, related to Fig. S3	
Cy3-23m-NTS (L)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGTTACTCCGAGTCAGCTCCGAGAGCTG(-Cy3)
Cy5-23m-TS (L)	GTCGAGAGCCTCGACT(-Cy5)GACGGAGTAACAGACATGGACCATCAGTAAACCTGTG
Non-specific ssDNA and pre-unwound dsDNA, related to Figs. 4a-b	
14m activator	ctcattgtcACATGGACCATCAGTAAACCTGTG
23m activator	GAGTAACAGACATGGACCATCAGTAAACCTGTG
non-specific ssDNA	(Cy5-)TCTGCGTATTTCTTGAGACGCTGGAGTACAATCGTCTGAGACGCTGGTCAGCTCTCTGA
NTS_23m	(Cy5-)AGTCTCTTGGCACAGGtttaCTGATGGTCCATGTCTGTTACTCcggaattcgagtacaa
TS_23m	(AF488-)ttgtactcgaattccgGAGTAACAGACATGGACCATCAGtaaaCCTGTGCCAAGAGACT
TS_14m_9mm	(AF488-)ttgtactcgaattccgctcattgtcACATGGACCATCAGtaaaCCTGTGCCAAGAGACT
NTS_17m_6mm	(Cy5-)AGTCTCTTGGCACAGGtttaCTGATGGTCCATGTCTGaatgagcggaattcgagtacaa
6m+8mm+9m_NTS	(Cy5-)AGTCTCTTGGCACAGGtttaCTGATGcaggtacaCTGTTACTCcggaattcgagtacaa
6m+17mm_TS	(AF488-)ttgtactcgaattccgctcattgtctgtacctgCATCAGtaaaCCTGTGCCAAGAGACT
6m+8mm+3m+6mm_NTS	(Cy5-)AGTCTCTTGGCACAGGtttaCTGATGcaggtacaCTGaatgagcggaattcgagtacaa
Full and partial cognate dsDNAs containing pre-cleaved NTS (NTS-nick) or pre-cleaved TS (TS-nick), related to Figs. 4c-d and 5	
23m-NTS (L)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGTTACTCCGTCAGTCGAGGCTCTCGAC
Cy5-23m-NTS (L)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGTTACTCCGTCAGT(-Cy5)CGAGGCTCTCGAC
23m-NTS(A) (L)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGT
23m-NTS(B) (L)	ACTCCGTCAGTCGAGGCTCTCGAC

Cy5-23m-TS (L)	GTCGAGAGCCTCGACT(-Cy5)GACGGAGTAACAGACATGGACCATCAGTAAACCTGTG
23m-TS (L)	GTCGAGAGCCTCGACTGACGGAGTAACAGACATGGACCATCAGTAAACCTGTG
23m-TS(A) (L)	GAGTAACAGACATGGACCATCAGTAAACCTGTG
23m-TS(B) (L)	GTCGAGAGCCTCGACTGACG
20m-NTS(A) (L)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGT
20m-NTS(B) (L)	AgagCGTCAGTCGAGGCTCTCGAC
Cy5-20m-TS (L)	GTCGAGAGCCTCGACT(-Cy5)GACGctcTAACAGACATGGACCATCAGTAAACCTGTG
18m-NTS(A) (L)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGT
18m-NTS(B) (L)	tgagCGTCAGTCGAGGCTCTCGAC
Cy5-18m-TS (L)	GTCGAGAGCCTCGACT(-Cy5)GACGctcatACAGACATGGACCATCAGTAAACCTGTG
17m-NTS(A) (L)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGa
17m-NTS(B) (L)	tgagCGTCAGTCGAGGCTCTCGAC
Cy5-17m-TS (L)	GTCGAGAGCCTCGACT(-Cy5)GACGctcattCAGACATGGACCATCAGTAAACCTGTG
14m-NTS(A) (L)	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTgaca
14m-NTS(B) (L)	tgagCGTCAGTCGAGGCTCTCGAC
Cy5-14m-TS (L)	GTCGAGAGCCTCGACT(-Cy5)GACGctcattgtcACATGGACCATCAGTAAACCTGTG
ssDNA marker, related to Fig. S5b	
9nt marker	AC T(-Cy5)GACGGA
8nt marker	AC T(-Cy5)GACGG
7nt marker	AC T(-Cy5)GACG
6nt marker	AC T(-Cy5)GAC
5nt marker	AC T(-Cy5)GA
4nt marker	AC T(-Cy5)G

'*N*m' stands for that the number of PAM-proximal matched bases is *N* and mismatches are shown in lower case. Internal labeled bases are highlighted in yellow. '(S)' and '(L)' represent two different DNA series used to form dsDNAs with a total length of 59 bp and 72 bp, respectively. 72 bp-length dsDNAs were used to introduce NTS-nicks and TS-nicks. The PAM and target sequence were remained the same within these two series of dsDNAs.

Table S3. Sequence of linearized dsDNA for *in-vitro* cleavage, related to Figs. 2c and S5.

Full cognate sequence (from 5' to 3')
CGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAG
TAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATCGCTACAGGCATCGTGGTGTCACGCTCGTCGT
TTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAA
AAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCCGTGTTATCACTCATGGT
TATGGCAGCACTACATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACT
CAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAA
TACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCA
AGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTT
TACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGC
GACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCT
CATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGA
AAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGA
GGCCCTTTCGTTGTAAAACGACGGCCAGTCCGTCTCTATCCGGTCTCGATCCGCAGTCTCTTGGCACAGGT
TTACTGATGGTCCATGTCTGTTACTCCGGAATTCGAGTACAAACGTCAGCACGTGTGTGGCGGAGCGAGG
AGCTGCTGTCCCCGTGGGAGCCGGCCTCAGAGGTAGCTCCATGACCCAGACACCAGTGGGGGATGTCAGT
GTTGGGGGAAAGTAGAAGCTTGGACCGTGCGAGTTACTGCCAACCGAGACCCAACCGAGACGGGTCATA
GCTGTTTCCAGTGTGCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTA
TCAGCTCACTCAAAGGCGGTAATACGGTTACCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGA
GCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGC
CCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGA
TACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCT
GTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGT
AGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGT
AACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGA
TTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAG
AAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGA
TCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAA
AAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTA

AGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTA
AATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTAT
CTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGG
AGGGCTTACCATCTGGCCCCAGTGCTGCAATAATACCGCGGGACCCACGCTCACCGGCTCCAGATTTATC
AGCAATAAACCAGCC

PAM sequence is highlighted in cyan, and target sequence (23m) is in yellow. A series of DNAs with different number of matched bases at PAM-proximal ends are used and they target sequences are (from 5' to 3'): CTGATGGTCCATGTCTGTTACTC (23m), CTGATGGTCCATGTCTGTTACag (21m), CTGATGGTCCATGTCTGTTtgag (19m), CTGATGGTCCATGTCTGTatgag (18m), CTGATGGTCCATGTCTGaatgag (17m), CTGATGGTCCATGTCTcaatgag (16m), CTGATGGTCCATGTCacaatgag (15m), CTGATGGTCCATGTgacaatgag (14m). Mismatches towards crRNA are shown in lower case.

Transparent Methods

Plasmid construction, protein expression and purification

The LbCas12a expression plasmid constructed on pGEX-6P-1 was a generous gift from Dr. Zhiwei Huang (Dong et al., 2016), which contains an N-terminal GST tag and a precise protease cleavage site (HRV 3C site). Point mutations were introduced by site-directed mutagenesis and confirmed by DNA sequencing.

E. Coli BL21 (DE3) strain was transformed with LbCas12a expression plasmids, and grown in 2YT medium (100 μg/ml ampicillin) at 37 ℃ to an OD_{600} of 0.6 ~ 0.8. Protein expression was induced by the addition of isopropyl β-d-1-thiogalactopyranoside (IPTG) to a final concentration of 0.7 mM with additional culture for 12-16 h at 16 ℃. Cells were collected and resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM $MgCl_2$, 1 mM freshly prepared TCEP) with 1 mM protease-inhibitor PMSF (Sigma). After sonication, cell debris was removed by a centrifugation step at 14,000 rpm for 45 min at 4 ℃. Supernatant was collected and incubated with glutathione sepharose 4B (GS4B) beads (GE Healthcare) at 4 ℃ for 30 min. Protein was then eluted by GSH elution buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM $MgCl_2$, 1 mM TCEP, 20 mM GSH), collected, incubated overnight (16 h) with HRV 3C protease (protein : protease =25 : 1) to remove GST tag.

Cation exchange was used for further purification. Firstly, adjust the sodium concentration to 125 mM and pH to 6.0. LbCas12a protein was purified by Source S (GE Healthcare) column using buffer A (50 mM MES pH 6.0, 150 mM NaCl, 1 mM TCEP) and buffer B (50 mM MES pH 6.0, 1 M NaCl, 1 mM TCEP). The integrity and purity of protein were verified by SDS-PAGE electrophoresis. Protein was concentrated in lysis buffer, flash-frozen in aliquots, and stored at -80 ℃.

in-vitro RNA transcription and purification

crRNAs without chemical modifications were transcribed *in vitro*. Template dsDNA containing a T7 promoter followed by a sequence corresponding to crRNA transcript was prepared through a PCR reaction with two single-stranded DNAs (Sangon Biotech, Shanghai, China). dsDNAs with correct length were recovered from agarose gels. The HiScribe T7 kit (NEB) was used for *in-vitro* transcription. crRNAs were purified by gel electrophoresis on a 10% denaturing (8 M urea) polyacrylamide gel. Gel bands containing crRNAs were excised and soaked in soaking buffer (1

mM EDTA, 100 mM NaOAc pH 5.2) at 50 ℃ for 2 h. crRNAs were precipitated by ethanol precipitation, resuspended in nuclease-free water (Life tech), and stored at -80 ℃.

To avoid RNA degradation, nuclease-free water was used in all RNA-related experiments. Before used in cleavage assays, crRNAs were annealed by a pre-heat at 95 ℃ for 10 min and a subsequent gradual temperature decrease (-5 ℃/min) to room temperature.

Amine-derivated DNA/RNA labeling

Amine-derivated DNA (Sangon Biotech, Shanghai, China) or crRNA (Takara) was dissolved to 0.5~1 mM in 10 mM $NaHCO_3$ (Sigma) buffer. Cy3-NHS or Cy5-NHS (Lumiprobe) was then added to a final concentration of 10 mM. The labeling mixture was incubated in the dark at 25 ℃ for at least 2 h. Labeled nucleic acids were purified via ethanol precipitation for three times. Pellet was dried in the air and dissolved in nuclease-free water (Life tech). Concentrations of nucleic acids, Cy3 and Cy5 were estimated by A_{260}, A_{549} and A_{649}, respectively.

3' labeling of RNA

RNA 3' labeling procedure was modified from a previous method (Odom et al., 1980), based on selective periodate oxidation of RNA at its 3' end and reaction of the oxidized product with hydrazide. Detailed procedure was describe before. (Yang et al., 2018)

In-vitro cleavage assay

Cas12a and annealed-crRNAs were pre-incubated to form binary complexes at 37 ℃ for 10 min and applied to target dsDNAs in cleavage buffer (50 mM KOAc, 20 mM Tris-OAc pH 7.9, 10 mM $MgOAc_2$) at 25 ℃. The reactions were stopped by the addition of 6× DNA loading buffer (NEB) or 2× formamide loading. Standard errors of the mean were calculated from three or more replicates.

For non-specific single-stranded DNA (ssDNA) cleavage in Fig. 4a, 375 nM Cas12a, 250 nM crRNA and 250 nM ssDNA activator were incubated at 37 ℃ for 10 min before applied to non-specific ssDNA at 25 ℃ for 20 min. For dsDNA cleavage assays in Figs. 2c and S5a, 75 nM Cas12a and 50 nM crRNA were applied to dsDNA. In Fig. 4b, 375 nM Cas12a and 250 nM crRNA were used to cleave 20 nM labeled dsDNAs for 20 min. In Figs. 3b, 4c, S5b and S7f, 150 nM Cas12a and 100 nM crRNA were used. Uncleaved and cleaved bands of unlabeled linearlized

dsDNA target were separated by 2% agarose gel. Labeled short dsDNAs were resolved on denaturing PAGE gels.

smFRET experiment

All smFRET experiments were performed at 25 ℃ using a home-built objective-type TIRF microscope in cleavage buffer with an oxygen scavenging system containing 3 mg/mL glucose, 100 μg/mL glucose oxidase (Sigma-Aldrich), 40 μg/mL catalase (Roche), 1 mM cyclooctatetraene (COT, Sigma-Aldrich), 1 mM 4-nitrobenzylalcohol (NBA, Sigma-Aldrich), 1.5 mM 6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Trolox, Sigma-Aldrich). Details of the TIRF microscope were described before. (Peng et al., 2017)

Cas12a or dCas12a was pre-incubated with crRNAs at ~1 μM at 37 ℃ for 10 min and then diluted to working concentrations. Target dsDNAs were firstly immobilized on the cover slide via biotin-streptavidin interaction. Laser powers were adjusted to prolong the time window of signal detection. Fluorescent signals were recorded at 500 ms/frame and started several seconds before flowing binary complexes. A mixture of 2.5 nM labeled crRNA and 15 nM Cas12a or dCas12a was used to capture dynamics of ternary complexes, whereas a mixture of 1 μM crRNA and 1 μM Cas12a was used to examine release of labeled non-target and target strands.

smFRET data analysis

Collected movies were analyzed by a custom-made software program. Fluorescence spots were fitted by a 2-D Gaussian function within a 9-pixel by 9-pixel area, matching the donor and acceptor spots using a variant of the Hough transform (Illingworth and Kittler, 1987). The background subtracted total volume of the 2-D Gaussian peak was used as raw fluorescence intensity *I*.

FRET traces displayed anti-correlation behaviors between donor and acceptor fluorescent signals were picked and further analyzed by a Hidden Markov Model based software (McKinney et al., 2006). Four FRET states from low to high FRET values were identified as S1 - S4. Transition rates (k) among FRET states were extracted from their dwell times. Relative free energies were calculated through

$$\Delta G_b - \Delta G_a = -k_B T \cdot \ln\left(\frac{k_{a\to b}}{k_{b\to a}}\right)$$

in which k_B is the Boltzmann constant, T is the temperature, $k_{a\to b}$ and $k_{b\to a}$ are transition rates from state a to b and from state b to a, respectively. S1 was set as the ground state $(\Delta G_1 = 0)$. The energy barrier from state a to b was calculated as $-k_B T \ln(k_{a\to b})$.

Supplemental Reference

Singh, D., Mallon, J., Poddar, A., Wang, Y., Tippana, R., Yang, O., Bailey, S., and Ha, T. (2018). Real-time observation of DNA target interrogation and product release by the RNA-guided endonuclease CRISPR Cpf1 (Cas12a). *Proceedings of the National Academy of Sciences of the United States of America* **115,** 5444-5449. doi:10.1073/pnas.1718686115