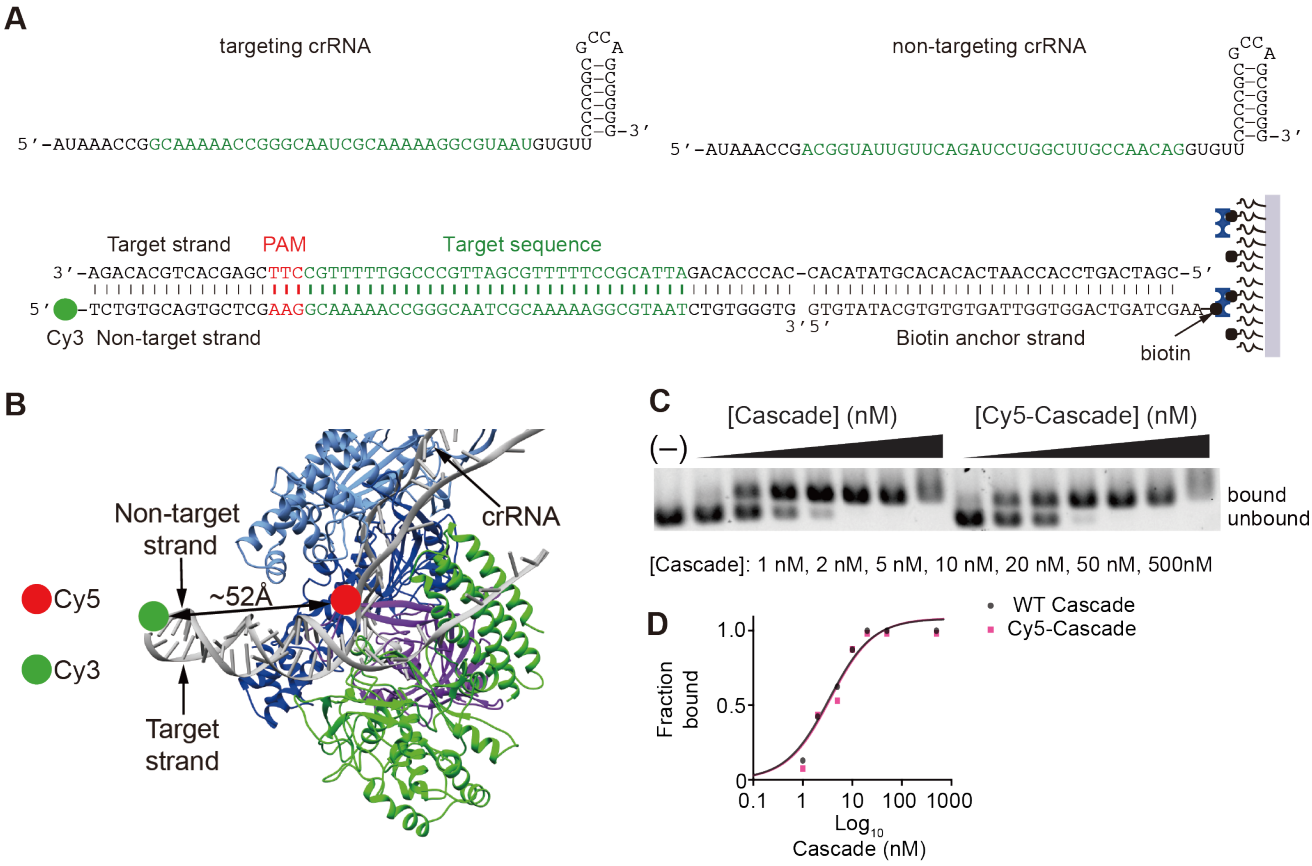

Figure S1. Development of smFRET assay. Related to Figure 1.

(A) Schematic of two crRNAs correspond to targeting Cascade and non-targeting Cascade and a surface-immobilized, Cy3 labelled dsDNA that contains the target sequence (green) and the PAM (red). A 33-nt biotinylated adaptor is used as an anchor for surface immobilization. (B) Cy3 (green dot) and Cy5 (red dot) labelling locations shown in the Cascade-dsDNA bound crystal structure (PDB ID: 5H9F). (C) Electrophoretic mobility shift assay for WT Cascade and Cy5 labelled Cascade (labelling efficiency > 90%) (1, 2, 5, 10, 20, 50, and 500 nM) binding to plasmid containing target sequence. (D) Fraction of DNA bound versus Cascade concentration were fit with a binding isotherm (solid lines).

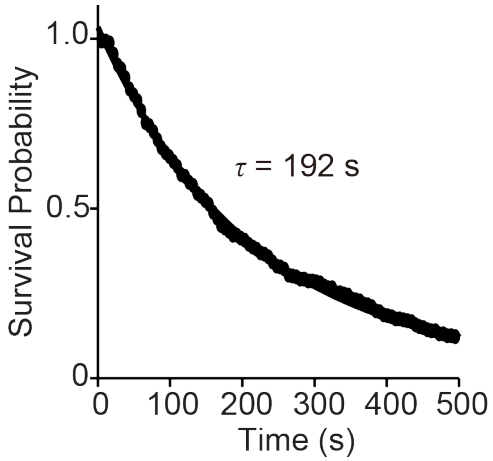

Figure S2. Photobleaching rate for Cy3-labeled dsDNA. Related to Figures 1 and 2

Mean donor photobleaching time was obtained from fitting donor survival probability to a single-exponential decay equation.

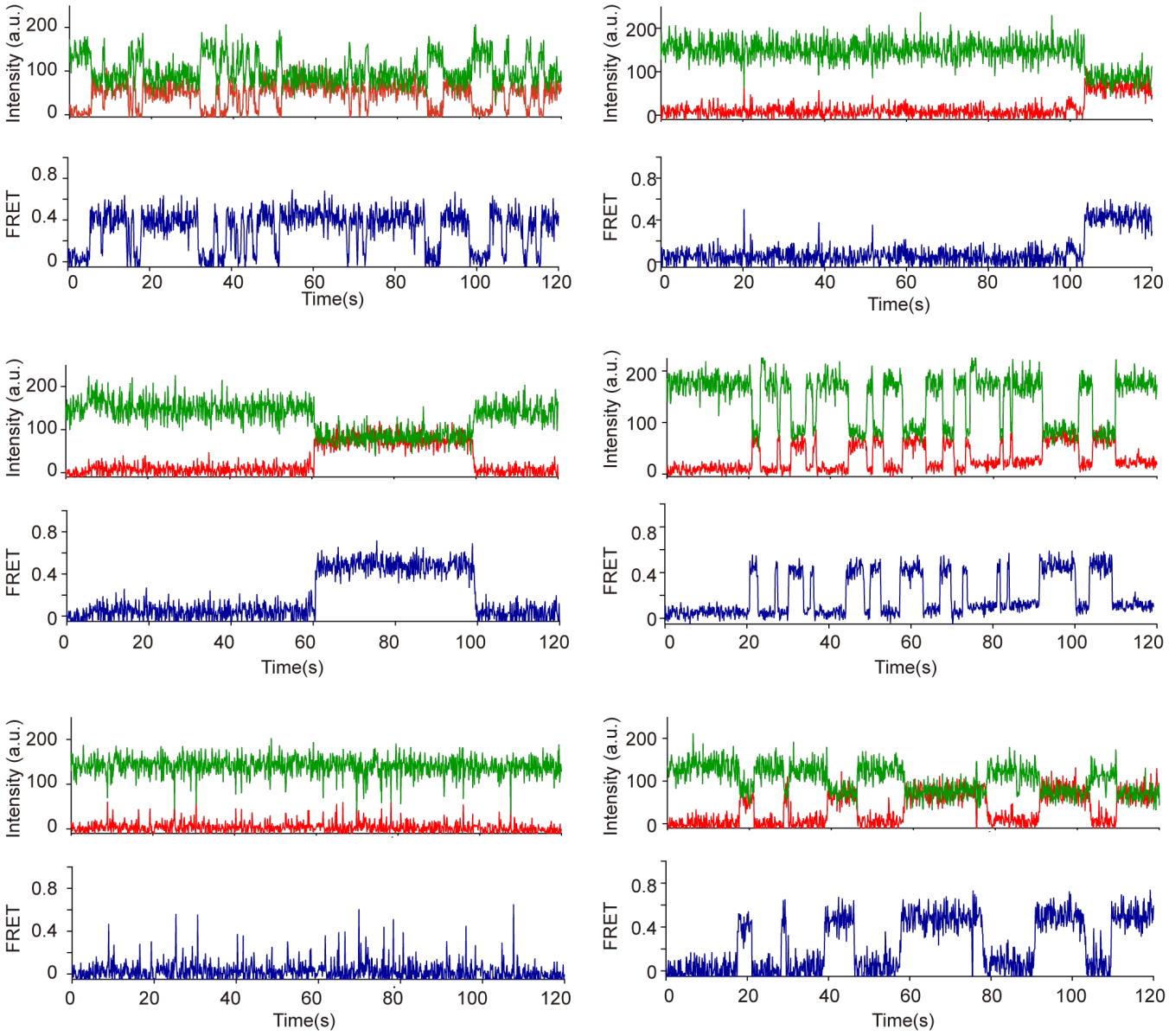

Figure S3. Representative smFRET trajectories of targeting Cascade with dsDNA$_{target}$. Related to Figure 1.

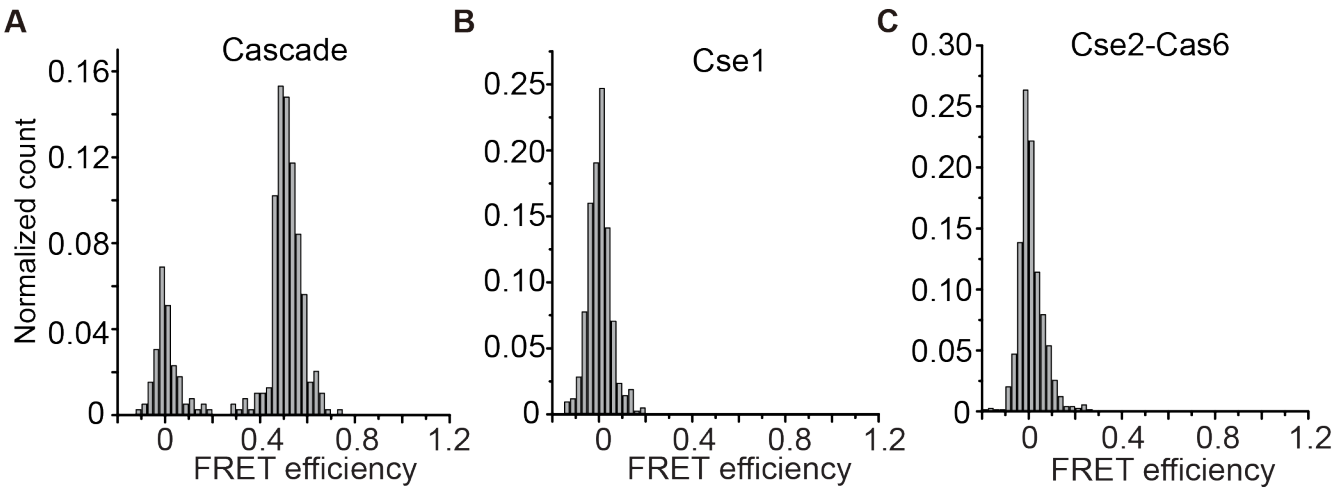

Figure S4. FRET distribution for whole Cascade, Cse1, and Cse2-Cas6e with dsDNA$_{trunc}$. Related to Figure 1.

(A-C) FRET histograms for FRET events existed in all extracted traces of Cascade with Cy5 labelled Cse2-Cas6e (A), Cy5 labelled Cse1 (B), and Cy5 labelled Cse2-Cas6e (C). The number of molecules used for FRET histograms was 392 (Cascade), 378 (Cse1), and 400 (Cse2).

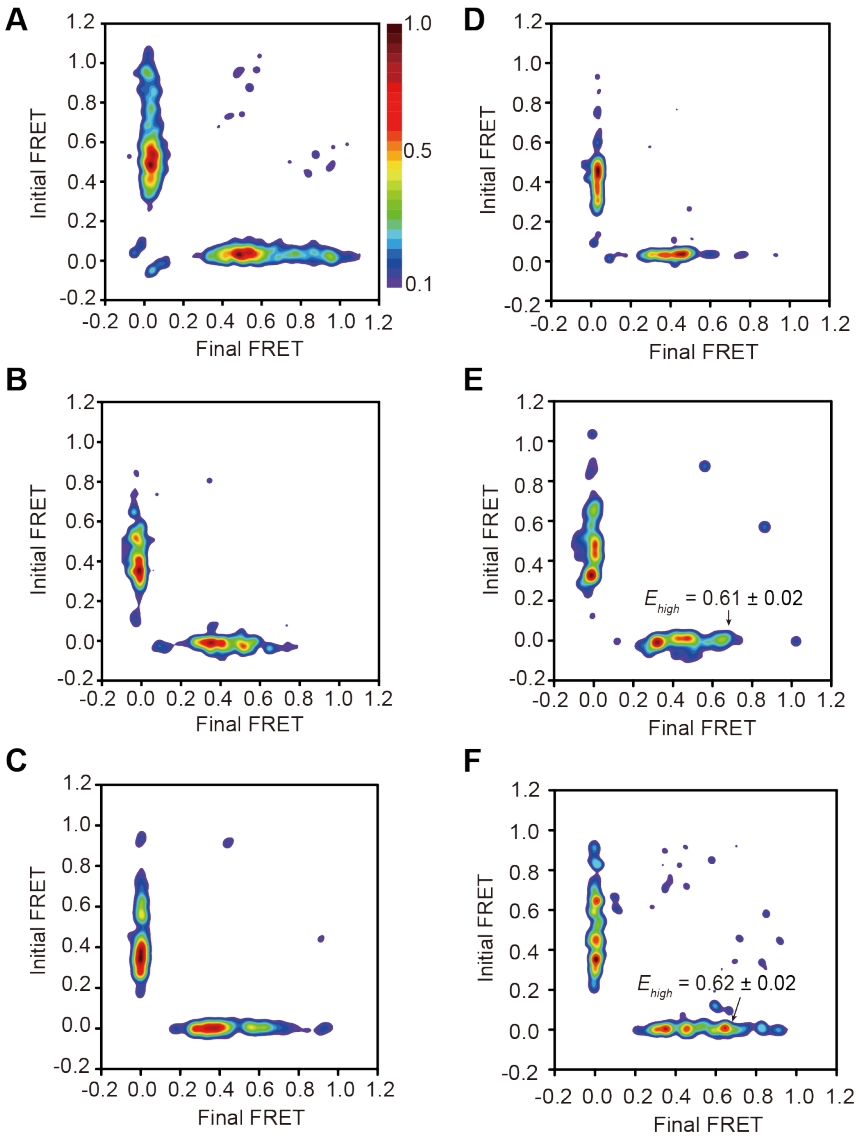

Figure S5. Transition density plots for Cascade binding to different dsDNA substrates. Related to Figure 2.

Transition density plots for (A) $dsDNA_{target}$ with non-targeting Cascade, (B) $dsDNA_{0PAM}$ with non-targeting Cascade, (C) $dsDNA_{0PAM}$ with targeting Cascade, (D) $dsDNA_{1PAM}$ with non-targeting Cascade, (E) $dsDNA_{3PAM}$ with non-targeting Cascade, and (F) $dsDNA_{1PAM\text{-}12bp}$ with non-targeting Cascade. The number of molecules for each TDP ranged from 1050 to 4,775.

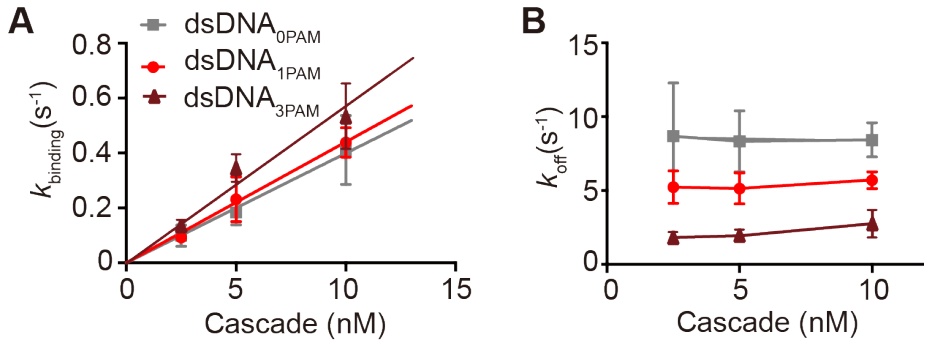

Figure S6. FRET appearance and disappearance at different Cascade concentrations. Related to Figure 2.

(A) $k_{binding}$ for $dsDNA_{0PAM}$, $dsDNA_{1PAM}$ and $dsDNA_{3PAM}$ at three different Cascade concentrations (2.5 nM, 5 nM and 10 nM). (B) k_{off} for $dsDNA_{0PAM}$, $dsDNA_{1PAM}$ and $dsDNA_{3PAM}$ at three different Cascade concentrations (2.5 nM, 5 nM and 10 nM).

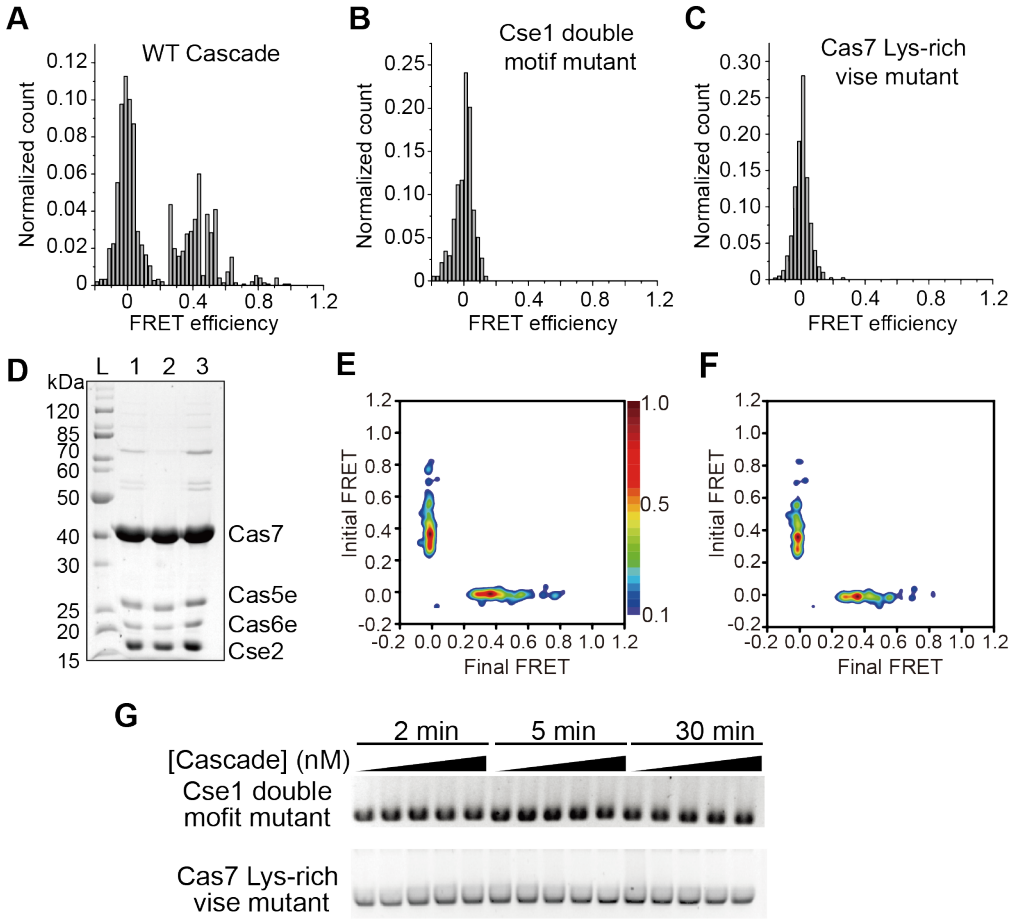

Figure S7. Cse1 and Cas7 motif mutations abolish target binding. Related to Figure 4.

(A-C) FRET distribution for (A) WT Cascade, (B) G160A and lysine-rich β hairpin (K289A, K290A, and K296A) double mutation in Cse1, (C) Cas7 quadruple Lys-rich vise mutation (K137A, K138A, K141A and K144A) binding to $dsDNA_{1PAM}$. The number of molecules used for FRET histograms was 1517 (WT Cascade), 850 (Cse1 double mutant), and 744 (Cas7 Lys-rich vise mutant). (D) Coomassie blue-stained SDS-PAGE gel analysis of Cse2-Cas6e. Gel lanes: (1) WT Cse2-Cas6e; (2) WT Cse2-Cas6e with K137A Cas7; (3) WT Cse2-Cas6e with K137A, K138A, K141A and K144A Cas7. (E-F) Transition density plots for Cascade with (E) Cse1 G160A mutation and (F) Cascade with Cse1 Lys-rich β hairpin mutation (K289A, K290A, and K296A) binding to $dsDNA_{1PAM}$. (G) Electrophoretic mobility shift assay for G160A and lysine-rich β hairpin (K289A, K290A, and K296A) double mutation in Cse1 and quadruple Lys-rich vise mutation (K137A, K138A, K141A and K144A) in Cas7 at different time point (2, 5, and 30 min) and various Cascade concentrations (1, 5, 10, 50, and 500 nM).

Table S1: Plasmids used in this study Related to Experimental procedures.

Plasmids	Description	Primers	Source
EcCse1-pSV272	*cse1* expression vector with N-terminal His_6-MBP-TEV site tag in pSV272		(Sashital et al., 2012)
pWUR547	*E.coli* R44 CRISPR, 7×spacer number 2 in pACYCDuet-1 expression vector used for non-targeting Cascade		(Brouns et al., 2008)
pX965	*E. coli* CRISPR 2 spacer 1 (S2.1) CRISPR template in pACYC-Duet used for targeting Cascade		(Xue et al., 2016)
pX1154	*cse1* with K289A, K290A, and K296A expression vector with N-terminal His_6-MBP-TEV site tag in pSV272	XCY1106+XCY1136	This study
pX1484	*cse1* with G160A expression vector with N-terminal His_6-MBP-TEV site tag in pSV272	XCY1075+XCY1076	This study
pX996	Cys-free *cas6e* in pCDF-1b with no tag		(Xue et al., 2016)
pX1169	Minimal-Cys *cse2-cas7-cas5e* with N-terminal His_6-TEV site tag is pET-52b(+)		(Xue et al., 2016)
pX1187	E102C in *cas5e* in minimal Cys *cse2-cas7-cas5e* with N-terminal His_6-TEV site tag is pET-52b(+)	XCY713+XCY718	This study

pX1496	*cas7* with K137A, K138A, K141A, K144A in minimal-Cys *cse2-cas7-cas5e* expression vector pX1169	XCY1102+XCY1103	This study
pX1500	E102C in *cas5e* and *cas7* with K137A, K138A, K141A, K144A in minimal-Cys *cse2-cas7-cas5e* expression vector pX1169	XCY1102+XCY1103	This study
pX1534	E102C in *cas5e* and *cas7* with K137A in minimal-Cys *cse2-cas7-cas5e* expression vector pX1169	XCY1124+XCY1126	This study
pX1796	*cse1* with G160A, K289A, K290A, and K296A expression vector with N-terminal His_6-MBP-TEV site tag in pSV272	XCY1106+XCY1136 XCY1075+XCY1076	This study

Table S2: Oligonucleotides used for plasmid construction and EMSA. Related to Figure 3 and Experimental procedures.

Name	Sequence (5′-3′)	Description
XCY798	TGTACGATTCAAACATGGCGCGAATATCTGAGTGATGCC	F-E102C minimal-Cys *cas5e*
XCY713	ATGACTTTTCAAACCACGGTAATCTTCTCGCGCTCCAAGG	R-E102C minimal-Cys *cas5e*
XCY1075	GGTTTTAAAAGCGGTTTACGTGGAGGAAC	F-G160A *cse1*
XCY1076	TGCACCAAAACCTGGTGCCTGATTCGCCTG	R-G160A *cse1*
XCY1136	GGGGAGGTTGAGGAAGCATTTCTTGCTTTCACCACCTC	F-K289A, K290A, K296A, *cse1*
XCY1106	TGCAGCGACTGTTACCAGACAAGGGGAATG	R-K289A, K290A, K296A, *cse1*
XCY1102	GCTGTTCTTGCTGAAGATATTGCCGCCATACG	F- K137A, K138A, K141A, K144A *cas7*
XCY1103	GAGCAGAGCAGCATCATCCAGATTATCAGCCTC	R-K137A, K138A, K141A, K144A *cas7*
XCY601	GGATCCGAATTCGAGCTCGCGAAGGCAAAAACCGGGCAATCGCAAAAAGGCGTAATCGCCTGCAGGTCGACAAGCTTG	F-Spacer 2.1 non-target strand
XCY602	CAAGCTTGTCGACCTGCAGGCGATTACGCCTTTTTGCGATTGCCCGGTTTTTGCCTTCGCCGAGCTCGAATTCGGATCC	R-Spacer 2.1 non-target strand
XCY1124	GAGCAGCTTAGCATCATCCAGATTATCAGCCTC	F-K137A Cas7
XCY1126	AAAGTTCTTAAGGAAGATATTGCCGCCATACG	R-K137A Cas7
XCY1187	CCCGCCGCCGCCGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGCCGCCGCCGCCGT	F-Competitor with 16 AAG
XCY1188	ACGGCGGCGGCGGCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCGGCGGCGGCGGG	R-Competitor with 16 AAG

XCY1189	CCCGCCGCCGCCGAAGCCGAAGCCGAAGCCGAAGCCGAAGCCGAAGCCGAAGCCGCCGCCGCCGCCGT	F-Competitor with 8 AAG
XCY1190	ACGGCGGCGGCGGCGGCTTCGGCTTCGGCTTCGGCTTCGGCTTCGGCTTCGGCTTCGGCGGCGGCGGG	R-Competitor with 8 AAG
XCY1191	CCCGCCGCCGCCGCCGCCGAAGCCGAAGCCGCCGCCGAAGCCGAAGCCGCCGCCGCCGCCCCCGCCGCCGT	F-Competitor with 4 AAG
XCY1192	ACGGCGGCGGGGGCGGCGGCGGCGGCGGCTTCGGCTTCGGCGGCGGCTTCGGCTTCGGCGGCGGCGGCGGG	R-Competitor with 4 AAG
XCY1197	GTCCGCCGCCGCCGCCGCACGCCGCCGCCGCCGCCGCACGCCGCCGCCGCCGCCGCCGCACGCCGCCA	R-Competitor with 0 AAG
XCY1198	TGGCGGCGTGCGGCGGCGGCGGCGGCGGCGGCGGCGTGCGGCGGCGGCGGCGTGCGGCGGCGGCGGAC	R-Competitor with 0 AAG

Table S3: Oligonucleotides used for smFRET. Related to Figure 1-2, 4, and Figure S1, 3-7

Name	Sequence (5′-3′)	Description
XCY1018	GTGTATACGTGTGTGATTGGTGGACTGATC GAA/3Bio/	Biotin anchor
XCY1019	/5Cy3/TCTGTGCAGTGCTCGAAGGCAAAAAC CG	Truncated non-target strand with AAG PAM and S2.1 target, Cy3 labelled at 5′ end
XCY1407	/5Cy3/TCTGTGCAGTGCTCGAAGGCAAAAAC CGGGCAATCGCAAAAAGGCGTAATCTGTGG GTG	Full length non-target strand with AAG PAM and S2.1 target, Cy3 labelled at 5′ end
XCY1066	CGATCAGTCCACCAATCACACACGTATACA CCACCCACAGATTACGCCTTTTTGCGATTGC CCGGTTTTTGCCTTCGAGCACTGCACAGA	Target strand S2.1 AAG, annealed with X1018 and X1019 for $dsDNA_{trunc}$ or X1018 and X1407 for $dsDNA_{target}$
XCY1111	/5Cy3/ACGCCGCCGCCGCCGCCGCCACCGCC GCCGCATCGCACCAACCCAGTAATCTGTGG GTG	All CCG, Cy3 labelled at 5′ end
XCY1112	ATCAGTCCACCAATCACACACGTATACACCA CCCACAGATTACTGGGTTGGTGCGATGCGG CGGCGGTGGCGGCGGCGGCGGCGGCGT	All CCG, annealed with X1018 and X1111 for $dsDNA_{0PAM}$
XCY1156	/5Cy3/ACGCCGCCGCCGCCGAAGCGCCGCCG CCGCCGCCTCGCACCAACCCAGTAATCTGT GGGT	One AAG PAM at 16th position, Cy3 labelled at 5′ end
XCY1157	ATCAGTCCACCAATCACACACGTATACACAC CCACAGATTACTGGGTTGGTGCGAGGCGGC GGCGGCGGCGCTTCGGCGGCGGCGGCGT	One AAG PAM at 16th position, annealed with X1156 and X1018 for $dsDNA_{1PAM}$
XCY1092	/5Cy3/ACGCCGCCGCCAAGCCAAGCAAGCC GCCGCCATCGCACCAACCCAGTAATCTGTG GGTG	Three AAG PAMs, Cy3 labelled at 5′ end

XCY1093	ATCAGTCCACCAATCACACACGTATACACCA CCCACAGATTACTGGGTTGGTGCGATGGCG GCGGCTTGCTTGGCTTGGCGGCGGCGT	Three AAG PAMs, annealed with X1092 and X1018 for $dsDNA_{3PAM}$
X1409	/5Cy3/ACGCCGCCGCCAAGCCCGCGCCGCCG CCGCCGCCTCGCACCAACCCAGTAATCTGT GGGT	One AAG PAM at 12th position, Cy3 labelled at 5′ end
X1410	ATCAGTCCACCAATCACACACGTATACACAC CCACAGATTACTGGGTTGGTGCGAGGCGGC GGCGGCGGCGCGGGCTTGGCGGCGGCGT	One AAG PAM at 12th position, annealed with X1409 and X1018 for $dsDNA_{1PAM-12bp}$

Supplemental Experimental Procedures

Plasmid Construction

K289, K290, and K296 in *cse1* were mutated to alanine using primers XCY1106 and XCY1136 in EcCse1-pSV272. G160 in *cse1* was mutated to alanine using XCY1075 and XCY1076 in EcCse1-pSV272. K137, K138, K141, and K144 in *cas7* were mutated to alanine using XCY1102 and XCY1103 in pX1169 to produce pX1500. K137 in *cas7* was mutated to alanine using XCY1124 and XCY1126 in pX1169 to produce pX1534. E102C was introduced to the *cas5e* gene in the plasmid pX1169 or pX1500 for site-specific labeling of Cas5e using XCY713 and XCY798.

Protein purification and Dye labeling

Minimal-Cys Cse2-Cas6e with different crRNA sequences containing E102C Cas5e was labeled by Cy5-maleimide (Lumiprobe) as previously described (Xue et al., 2016). All labeling reactions were performed in sample storage buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 5% glycerol, and 1 mM TCEP). Cse2-Cas6e (25 μM) and Cy5 (200 μM) were mixed in 5% DMSO. Reactions were incubated in the dark at 4 ºC for 1 hr, then quenched by adding 10 mM DTT. Labeled Cse2-Cas6e was separated from free dye using a 10K protein concentrator (Corning), concentrated to 2 μM, aliquoted and stored at -80 ºC. Each labeled Cse2-Cas6e aliquot was only thawed one time. The labelling efficiency of Cse2-Cas6e was calculated by measuring the concentration of Cse2-Cas6e at 280 nm and the dye concentration at 650 nm after labelling. Labelling efficiency of Cse2-Cas6e was consistently greater than 90%.

Preparation of dsDNA used for smFRET and EMSA

Oligos were dissolved in ddH2O to 100 μM. Two complementary oligos used for EMSA experiments were mixed in annealing buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA) in 1:1 ratio. Two complementary oligos and a biotinylated anchor (X1018) used for surface immobilization were mixed in annealing buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA) in 1:1:1 ratio (Fig. S1A). Samples were incubated at 95ºC for 5 min in a heat block covered by aluminum foil to avoid light. The samples were slow-cooled by turning off the heat block until the block decreased to room temperature (2-3 h). Samples were purified on a 6% native polyacrylamide gel run at 4 ºC.

Supplemental References

Brouns, S.J.J., Jore, M.M., Lundgren, M., Westra, E.R., Slijkhuis, R.J.H., Snijders, A.P.L., Dickman, M.J., Makarova, K.S., Koonin, E. V., and Van Der Oost, J. (2008). Small Crispr Rnas Guide Antiviral Defense in Prokaryotes. Science (80). *321*, 960–964.

Sashital, D.G., Wiedenheft, B., and Doudna, J.A. (2012). Mechanism of Foreign DNA Selection in a Bacterial Adaptive Immune System. Mol. Cell *46*, 606–615.

Xue, C., Whitis, N.R., and Sashital, D.G. (2016). Conformational Control of Cascade Interference and Priming Activities in CRISPR Immunity. Mol. Cell *64*, 826–834.