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Supplemental Information

Two Distinct DNA Binding Modes Guide Dual Roles of a CRISPR-Cas Protein Complex

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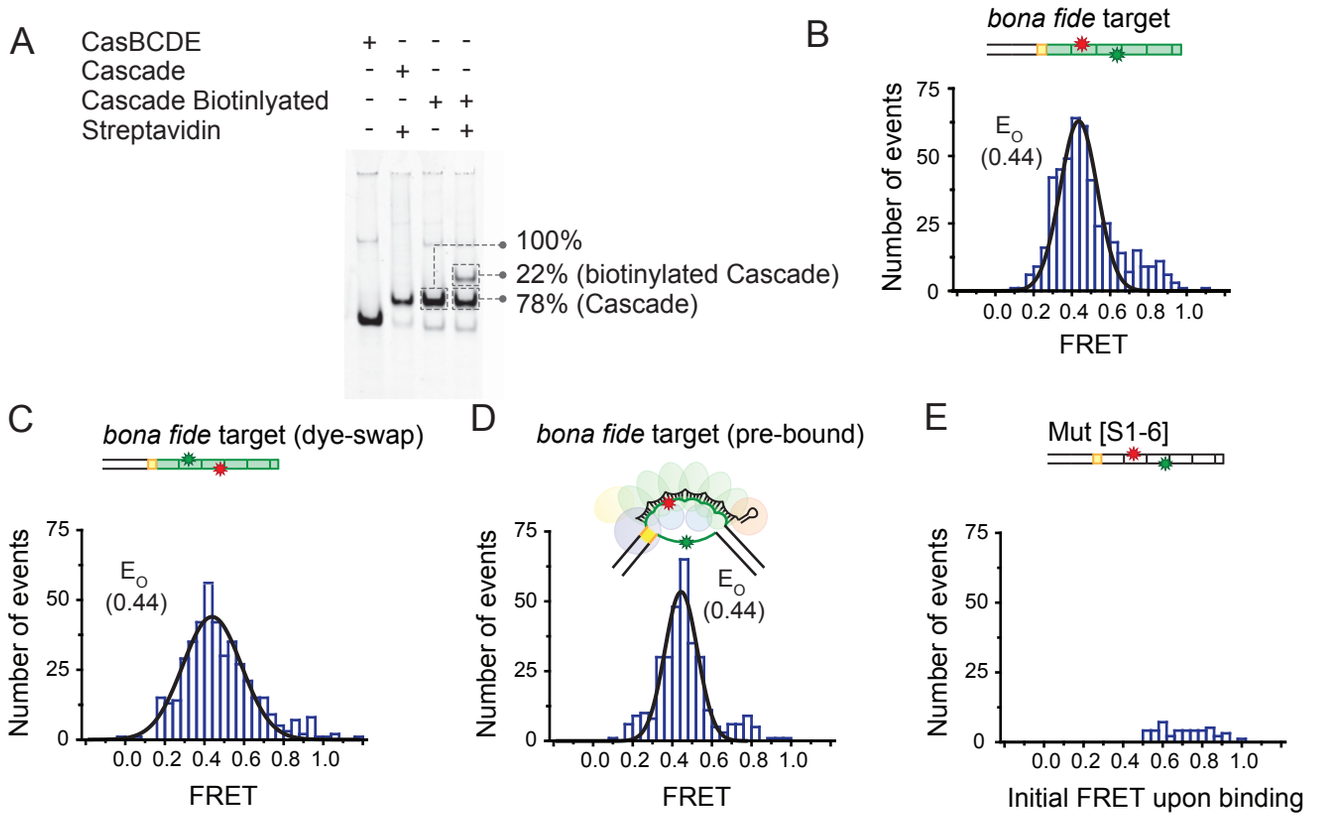

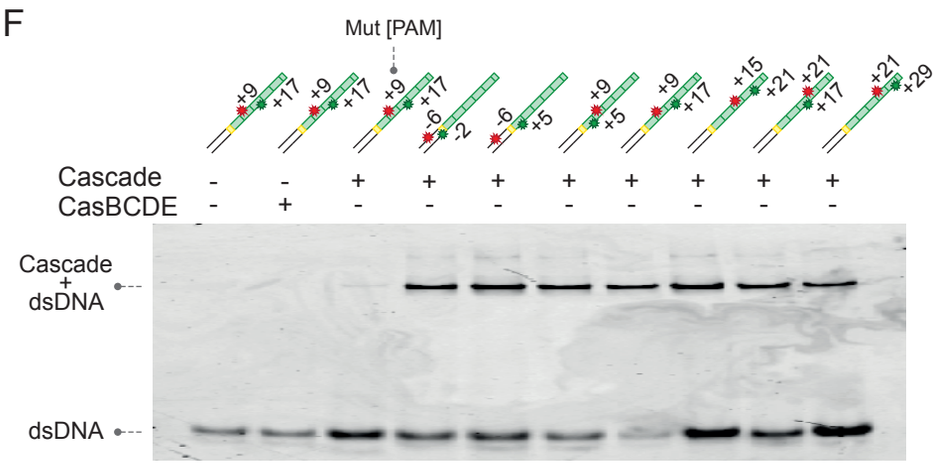

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Figure S1

Figure S1: Cascade biotinylation efficiency; dye-labeling and surface-immobilization controls for single-molecule assay, related to Figure 1.

(A) Efficiency of site-specific labeling of Cascade with a biotin on the Cse1 subunit. 50 nM of Cascade from the labeling reaction (see **Extended Experimental Procedures**) was incubated with 5 nM of a Cy5-labeled complementary oligo (TJ3_15 *bona fide* (+9), **Table S2**) and 500 nM Streptavidin in Cascade buffer. After 30 minutes of incubation at the room temperature, the protein/DNA solution was run on a 5% TBE PAGE gel at 100V for 1 hour, and imaged with Typhoon Trio scanner. Labeling efficiency was quantified with Imagequant software (GE Healthcare). Wild-type refers to unmodified Cascade. CasBCDE refers to wild-type Cascade lacking the Cse1 subunit. **(B)** A FRET histogram obtained after equilibration of a *bona fide* DNA target (target strand labeled with Cy7 (red star), non-target strand labeled with Cy3 (green star)) with immobilized Cascade. Peak centered at E_O (0.44) was derived from Gaussian fit (black line) (same data as Figure 1E, for reference). **(C)** Similar to [B] with Cy7 and Cy3 exchanged: target strand labeled with Cy3 (green star), non-target strand labeled with Cy7 (red star). Peak centered at E_O (0.44) was derived from Gaussian fit (black line). Data obtained from 5 fields of view (~200 molecules per field). **(D)** A FRET histogram obtained after immobilization of biotinylated Cascade and *bona fide* target DNA (Cy3 target strand, Cy7 non-target strand) which were pre-incubated in Cascade buffer for 30 min at room temperature (23 ± 1 °C) prior to immobilization. Peak centered at E_O (0.44) was derived from Gaussian fit (black line). Data obtained from 5 fields of view. **(E)** A histogram of initial FRET upon binding of a construct without complementarity (Mut [1-6]) to the crRNA. **(F)** EMSA of dsDNA constructs with different labeling positions (indicated by numbering, where the PAM sequence occupies positions *-1*, *-2*, and *-3*. Target strand was labeled with Cy7 (red star) and the non-target strand was labeled with Cy3 (green star). CasBCDE refers to wild-type Cascade lacking the Cse1 subunit. Cascade (50 nM) and dye-labeled dsDNA or ssDNA (5 nM) were incubated in Cascade buffer for 30 minutes at room temperature (23± 1 °C) and subsequently run on a 5% polyacrylamide TBE gel (Bio-Rad) at 100 V for 1 hour. This gel was imaged with a Typhoon Trio scanner (GE Healthcare).

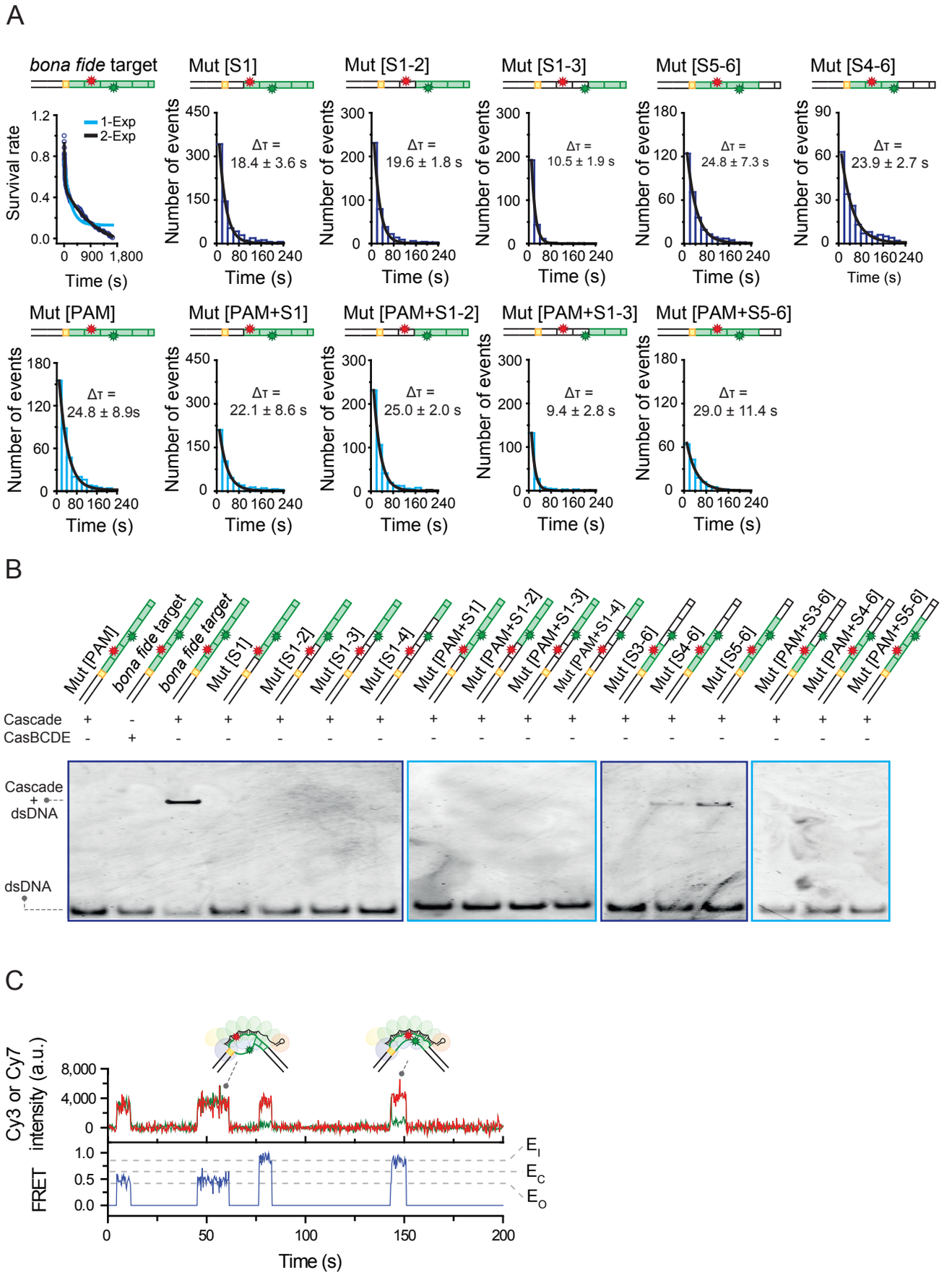

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Figure S2

Figure S2: Binding dwell-time distributions and EMSAs of all DNA constructs, related to Figure 3.

(A) Dwell-time (Δτ) histograms of *bona fide* and mutated target dsDNA binding to Cascade. The graph of the *bona fide* target shows the survival rate of events that start at E_I (0.84). Data was fitted using a single (light blue line) and a double (black line) exponential curve. The double exponential fit resulted in two characteristic times (25.9 and 1040 sec). The dwell time of the binding events of the mutant constructs was determined from a single exponential fit (black line). Error represents the standard deviation of 3 data sets from 3 different days. **(B)** EMSAs of DNA constructs. Mutants with an interference permissive PAM (5'-CAT-3') or an escape mutant PAM (5'-CGT-3') are indicated with a purple or light blue box, respectively. CasBCDE refers to wild-type Cascade lacking the Cse1 subunit. Cascade (50 nM) and dye-labeled dsDNA or ssDNA (5 nM) were incubated in Cascade buffer for 30 minutes at room temperature (23± 1 °C) and subsequently run on a 5% polyacrylamide TBE gel (Bio-Rad) at 100 V for 1 hour. This gel was imaged with a Typhoon Trio scanner (GE Healthcare). **(C)** A representative time trace exhibiting the short-lived binding of Mut[S5-6] exhibits two FRET states, E_O (0.44), E_O (0.65) and E_I (0.84). The duration of each state is measured as the dwell time (Δτ).

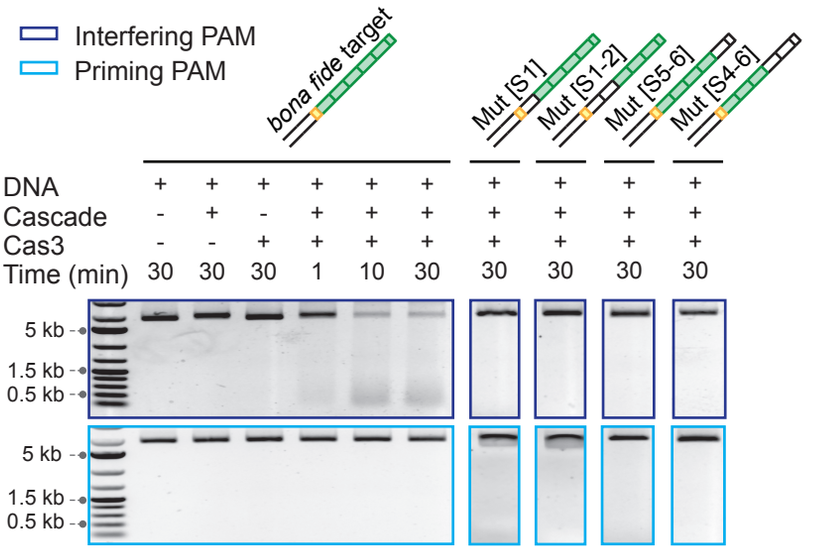

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Figure S3

Figure S3. DNA degradation requires *bona fide* target, related to Figure 4

CRISPR interference reconstituted in vitro. Target plasmids (3.5 nM) harboring different target sequences (*bona fide*, Mut[S1], Mut[S1-2], Mut[S5-6], and Mut[S4-6], see schematics at top) with an interfering (purple outline, top row) or a priming (light blue outline, bottom row) PAM were incubated with Cascade (35 nM) for 15 min prior to addition of Cas3 (70 nM), which subsequently incubated for 1, 10, or 30 min. Reactions were run on a 0.8% agarose gel and stained for imaging. Degradation of the *bona fide* target can be seen a diffuse band at the bottom of the gel that increases in intensity with time.

SUPPLEMENTAL TABLES

Table S1: Oligos used for target DNA immobilization, related to Figure 1 and Figure 4

Construct name (label pos.)	Sequence (5' → 3') [a]	Description [b]
TJ3_15-18 *bona fide* target (+9)	TTTGGTCTGCTCAATTTTGACAGCCCACATGGCATTCCACT /iAmMC6T/ATCACTGGCATCCTTCCACACTCCTG	Oligo for immobilisation
nTJ3_15 *bona fide* (+17)	CAGGAGTGTGGAAGGATGCCAGTGATAAGTGGAA/iAmMC6T/GCCATGTGGGCTGTC	Oligo for immobilisation
80nt biotin linker	AAAATTGAGCAGACCAAA$(PolyT)_{62}$ - Biotin	Oligo for immobilisation
BG3903	CACCGGCCATGGCACTGTGCACACCATCGCGGAATTTGCTTATTGATAACTGGATCCCTGTACGCC	Fw for N-term FGE tag CseI (NcoI)
BG3904	CCGGTGGGTACCTCAGCCATTTGATGGCCCTCCTTGCGGTTTTAACTCCC	Rv CseI (KpnI)
BG4225	TTTGAATTCGCGCTGCATGCCTATTTG	Fw KmR in pRSF-1b (EcoRI)
BG5157	TTTTCCATGGGACAGCCCACATGGCATTCCACTTATCACTGGCATCCTTCCACACTCCTGTTAGAAAAACTCATCGAGCATC	Rv KmR in pRSF-1b with J3 protospacer (NcoI)
BG5158	TTTTCCATGGACGGTGAGACATGGCATTCCACTTATCACTGGCATCCTTCCACACTCCTGTTAGAAAAACTCATCGAGCATC	Rv KmR in pRSF-1b with J3 protospacer (Mut^{S5-6}) (NcoI)
BG5159	TTTTCCATGGACGGTGAGCACGCACATTCCACTTATCACTGGCATCCTTCCACACTCCTGTTAGAAAAACTCATCGAGCATC	Rv KmR in pRSF-1b with J3 protospacer (Mut^{S4-6}) (NcoI)
BG5160	TTTTCCATGGGACAGCCCACATGGCATTCCACTTATCACTGGCGTCCTTCCACACTCCTGTTAGAAAAACTCATCGAGCATC	Rv KmR in pRSF-1b with J3 protospacer (Mut^{PAM}) (NcoI)
BG5161	TTTTCCATGGACGGTGAGACATGGCATTCCACTTATCACTGGCGTCCTTCCACACTCCTGTTAGAAAAACTCATCGAGCATC	Rv KmR in pRSF-1b with J3 protospacer ($Mut^{PAM\ S5-6}$) (NcoI)
BG5162	TTTTCCATGGACGGTGAGCACGCACATTCCACTTATCACTGGCGTCCTTCCACACTCCTGTTAGAAAAACTCATCGAGCATC	Rv KmR in pRSF-1b with J3 protospacer ($Mut^{PAM\ S4-6}$) (NcoI)
BG5163	TTTTCCATGGGACAGCCCACATGGCATTCCACTTATACGGCCCATCCTTCCACACTCCTGTTAGAAAAACTCATCGAGCATC	Rv KmR in pRSF-1b with J3 protospacer (Mut^{S1}) (NcoI)
BG5164	TTTTCCATGGGACAGCCCACATGGCATTCCCGGTGCACGGCCCATCCTTCCACACTCCTGTTAGAAAAACTCATCGAGCATC	Rv KmR in pRSF-1b with J3 protospacer (Mut^{S1-2}) (NcoI)
BG5165	TTTTCCATGGGACAGCCCACATGGCATTCCACTTATACGGCCCGTCCTTCCACACTCCTGTTAGAAAAACTCATCGAGCATC	Rv KmR in pRSF-1b with J3 protospacer ($Mut^{PAM\ S1}$) (NcoI)
BG5166	TTTTCCATGGGACAGCCCACATGGCATTCCCGGTGCACGGCCCGTCCTTCCACACTCCTGTTAGAAAAACTCATCGAGCATC	Rv KmR in pRSF-1b with J3 protospacer ($Mut^{PAM\ S1-2}$) (NcoI)
BG5301	AAGGTTGGTGGGTTGTTTTTATGG	Fw Oligonucleotide annealing to the CRISPR 2.1 leader
BG5302	GGATCGTCACCCTCAGCAGCG	Rv Oligonucleotide annealing to spacer g8

a. Underlined sequence indicates Nco1 restriction site.
b. Fw stands for forward, Rv stands for reverse, FGE stand for formylglycine generating enzyme

Table S2: DNA oligos used in single-molecule studies of PAM-distal and PAM-proximal mutations, related to Figure 3

Construct name (label pos.)	Sequence (5' → 3') [a]
TJ3_15 *bona fide* (+9)	GACAGCCCACATGGCATTCCACT/iAmMC6T/ATCACTGGCATCCTTCCACACTCCTG
nTJ3_15 *bona fide* (+17)	CAGGAGTGTGGAAGGATGCCAGTGATAAGTGGAA/iAmMC6T/GCCATGTGGGCTGTC
TJ3_15 Mut^{s5-6} (+9)	ACGGTGAGACATGGCATTCCACT/iAmMC6T/ATCACTGGCATCCTTCCACACTCCTG
nTJ3_15 Mut^{s5-6} (+17)	CAGGAGTGTGGAAGGATGCCAGTGATAAGTGGAA/iAmMC6T/GCCATGTCTCACCGT
TJ3_15 Mut^{s4-6} (+9)	ACGGTGAGCACGCACATTCCACT/iAmMC6T/ATCACTGGCATCCTTCCACACTCCTG
nTJ3_15 Mut^{s4-6} (+17)	CAGGAGTGTGGAAGGATGCCAGTGATAAGTGGAA/iAmMC6T/GTGCGTGCTCACCGT
TJ3_15 Mut^{s3-6} (+9)	ACGGTGAGCACGCAGACCGGACT/iAmMC6T/ATCACTGGCATCCTTCCACACTCCTG
nTJ3_15 Mut^{s3-6} (+17)	CAGGAGTGTGGAAGGATGCCAGTGATAAGTCCGG/iAmMC6T/CTGCGTGCTCACCGT
TJ3_15 Mut^{s1-6} (+9)	ACGGTGAGCACGCAGACCGGCGG/iAmMC6T/GCACGGCCCATCCTTCCACACTCCTG
nTJ3_15 Mut^{s1-6} (+17)	CAGGAGTGTGGAAGGATGGGCCGTGCACCGCCGG/iAmMC6T/CTGCGTGCTCACCGT
TJ3_15 Mut^{PAM} (+9)	GACAGCCCACATGGCATTCCACT/iAmMC6T/ATCACTGGCGTCCTTCCACACTCCTG
nTJ3_15 Mut^{PAM} (+17)	CAGGAGTGTGGAAGGACGCCAGTGATAAGTGGAA/iAmMC6T/GCCATGTGGGCTGTC
TJ3_15 Mut$^{PAM+s5-6}$ (+9)	ACGGTGAGACATGGCATTCCACT/iAmMC6T/ATCACTGGCGTCCTTCCACACTCCTG
nTJ3_15 Mut$^{PAM+s5-6}$ (+17)	CAGGAGTGTGGAAGGACGCCAGTGATAAGTGGAA/iAmMC6T/GCCATGTCTCACCGT
TJ3_15 Mut$^{PAM+s4-6}$ (+9)	ACGGTGAGCACGCACATTCCACT/iAmMC6T/ATCACTGGCGTCCTTCCACACTCCTG
nTJ3_15 Mut$^{PAM+s4-6}$ (+17)	CAGGAGTGTGGAAGGACGCCAGTGATAAGTGGAA/iAmMC6T/GTGCGTGCTCACCGT
TJ3_15 Mut$^{PAM+s3-6}$ (+9)	ACGGTGAGCACGCAGACCGGACT/iAmMC6T/ATCACTGGCGTCCTTCCACACTCCTG
nTJ3_15 Mut$^{PAM+s3-6}$ (+17)	CAGGAGTGTGGAAGGACGCCAGTGATAAGTCCGG/iAmMC6T/CTGCGTGCTCACCGT
TJ3_15 Mut^{s1} (+9)	GACAGCCCACATGGCATTCCACT/iAmMC6T/ATACGGCCCATCCTTCCACACTCCTG
nTJ3_15 Mut^{s1} (+17)	CAGGAGTGTGGAAGGATGGGCCGTATAAGTGGAA/iAmMC6T/GCCATGTGGGCTGTC
TJ3_15 Mut^{s1-2} (+9)	GACAGCCCACATGGCATTCCCGG/iAmMC6T/GCACGGCCCATCCTTCCACACTCCTG
nTJ3_15 Mut^{s1-2} (+17)	CAGGAGTGTGGAAGGATGGGCCGTGCACCGGGAA/iAmMC6T/GCCATGTGGGCTGTC
TJ3_15 Mut^{s1-3} (+9)	GACAGCCCACATGGGACCGGCGG/iAmMC6T/GCACGGCCCATCCTTCCACACTCCTG
nTJ3_15 Mut^{s1-3} (+17)	CAGGAGTGTGGAAGGATGGGCCGTGCACCGCCGG/iAmMC6T/CCCATGTGGGCTGTC
TJ3_15 Mut^{s1-4} (+9)	GACAGCCCCACGCAGACCGGCGG/iAmMC6T/GCACGGCCCATCCTTCCACACTCCTG
nTJ3_15 Mut^{s1-4} (+17)	CAGGAGTGTGGAAGGATGGGCCGTGCACCGCCGG/iAmMC6T/CTGCGTGGGGCTGTC
TJ3_15 Mut^{PAM+s1} (+9)	GACAGCCCACATGGCATTCCACT/iAmMC6T/ATACGGCCCGTCCTTCCACACTCCTG
nTJ3_15 Mut^{PAM+s1} (+17)	CAGGAGTGTGGAAGGACGGGCCGTATAAGTGGAA/iAmMC6T/GCCATGTGGGCTGTC
TJ3_15 Mut$^{PAM+s1-2}$ (+9)	GACAGCCCACATGGCATTCCCGG/iAmMC6T/GCACGGCCCGTCCTTCCACACTCCTG
nTJ3_15 Mut$^{PAM+s1-2}$ (+17)	CAGGAGTGTGGAAGGACGGGCCGTGCACCGGGAA/iAmMC6T/GCCATGTGGGCTGTC

TJ3_15 Mut$^{PAM+s1-3}$ (+9)	GACAGCCCACATGGGACCGGCGG/iAmMC6T/GCACGGCCCGTCCTTCCACACTCCTG
nTJ3_15 Mut$^{PAM+s1-3}$ (+17)	CAGGAGTGTGGAAGGACGGGCCGTGCACCGCCGG/iAmMC6T/CCCATGTGGGCTGTC
TJ3_15 Mut$^{PAM-s1-4}$ (+9)	GACAGCCCCACGCAGACCGGCGG/iAmMC6T/GCACGGCCCGTCCTTCCACACTCCTG
nTJ3_15 Mut$^{PAM+s1-4}$ (+17)	CAGGAGTGTGGAAGGACGGGCCGTGCACCGCCGG/iAmMC6T/CTGCGTGGGGCTGTC

a. "/iAmMC6T/" refers to an amino-modified thymine base at the indicated position

Table S3, Plasmid constructs, related to Figure 4

Plasmid	Description and order of genes (5' → 3')	Restriction sites	Primers	Source
pWUR408	*cse1* in pRSF-1b, no tags			*(Brouns et al., 2008)*
pWUR564	CRISPR containing J3 spacer in pACYCDuet-1 Derivative of pWUR477			*(Westra and Brouns, 2012)*
pWUR610	pUC-3xJ3; pUC19 three copies of the J3-protospacer, corresponding to a sequence derived from phage Lambda.			*(Westra et al., 2012)*
pWUR630	CRISPR poly J3, GA0936818 (Table S4) in pACYCDuet-1	NcoI/KpnI		*(Westra et al., 2012)*
pWUR656	*cse2* with Strep-tag II (N-term)-*cas7-cas5-cas6e* in pCDF-1b	NcoI/NotI		*(Jore et al., 2011)*
pWUR706	*cse1* containing an N-terminal LCTPSR FGE recognition sequence		BG3903/ BG3904	This study
pWUR738	J3 target plasmid. derivative of pGFPuv	NcoI(BspHI)/EcoRI	BG4225/ BG5157	This study
pWUR739	Mutant J3 target plasmid (Mut^{S5-6}) derivative of pGFPuv (Clontech)	NcoI(BspHI)/EcoRI	BG4225/ BG5158	This study
pWUR740	Mutant J3 target plasmid (Mut^{S4-6}) derivative of pGFPuv (Clontech)	NcoI(BspHI)/EcoRI	BG4225/ BG5159	This study
pWUR741	Mutant J3 target plasmid (Mut^{PAM}) derivative of pGFPuv (Clontech)	NcoI(BspHI)/EcoRI	BG4225/ BG5160	This study
pWUR742	Mutant J3 target plasmid ($Mut^{PAM\ S5-6}$) derivative of pGFPuv (Clontech)	NcoI(BspHI)/EcoRI	BG4225/ BG5161	This study
pWUR743	Mutant J3 target plasmid ($Mut^{PAM\ S4-6}$) derivative of pGFPuv (Clontech)	NcoI(BspHI)/EcoRI	BG4225/ BG5162	This study
pWUR744	Mutant J3 target plasmid (Mut^{S1}) derivative of pGFPuv (Clontech)	NcoI(BspHI)/EcoRI	BG4225/ BG5163	This study
pWUR745	Mutant J3 target plasmid (Mut^{S1-2}) derivative of pGFPuv (Clontech)	NcoI(BspHI)/EcoRI	BG4225/ BG5164	This study
pWUR746	Mutant J3 target plasmid ($Mut^{PAM\ S1}$) derivative of pGFPuv (Clontech)	NcoI(BspHI)/EcoRI	BG4225/ BG5165	This study
pWUR747	Mutant J3 target plasmid ($Mut^{PAM\ S1-2}$) derivative of pGFPuv (Clontech)	NcoI(BspHI)/EcoRI	BG4225/ BG5166	This study
pWUR748	pMAT11-MBP-Cas3	EcoRI/XhoI		*(Mulepati and Bailey, 2013)*
Plasmid #16132	pBAD/myc-his A Rv0712 (FGE)			Addgene

EXTENDED EXPERIMENTAL PROCEDURES

Protein preparation. Cascade was expressed in *E. coli* BL21 (DE3) using plasmids listed in **Table S3** and purified as described (Jore et al., 2011). Elution buffer contained 20 mM HEPES pH 7.5, 75 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$ (storage buffer) with 4 mM desthiobiotin. Primers for cloning are listed in **Table S1**. The nuclease-helicase Cas3 was produced and purified as described previously (Mulepati and Bailey, 2013) with the following modifications. BL21-AI cells were used for over expression, and protein expression was induced with 0.5 mM IPTG and 0.2% L-Arabinose. The purification process was stopped after size exclusion chromatography and before the proteolytic removal of the Maltose Binding Protein (MBP) using the Tobacco Edge Virus protease (Hochstrasser et al., 2014). MBP-Cas3 was flash frozen in liquid nitrogen and stored at -80 °C.

For site-specific Cascade labeling, plasmid pWUR706 (Cse1 with an N-terminal LCTPSR FGE recognition motif) was co-expressed with plasmid #16132 (*fge* gene, Addgene) (Carrico et al., 2007), pWUR656 (CasBCDE) and pWUR630 (CRISPR J3). A solution of 45 µL purified Cascade (1.5 mg/ml) was mixed with 45 µL potassium acetate (0.5 M (pH 5.5)) and 40 µL Hydrazide-LC-Biotin (50 mM in DMSO, Thermo Scientific) and incubated overnight at room temperature. Labeled Cascade was purified by size exclusion chromatography (Superdex-200 HR 10/30 (GE Healthcare)). Fractions were concentrated using Vivaspin (50 kDa) spin columns and stored at 4 °C in storage buffer or at -20 °C in storage buffer containing 50% glycerol.

DNA preparation. All the target dsDNA substrates that we used were 50 base pairs in length, bearing a protospacer, PAM, and a 15 bp sequence upstream of the PAM (**Figure 1B, Table S2**). These synthetic DNA targets (Integrated DNA Technologies) were internally labeled with a monoreactive acceptor dye (Cy7, GE Healthcare) at dT-C6 on the target strand (complementary to the crRNA) and a monoreactive donor dye (Cy3, GE Healthcare) at dT-C6 on the non-target strand (**Figure 1B**). After labeling, the ssDNA strands were annealed, which was followed by PAGE purification of the dsDNA constructs. To determine the initial FRET values of these constructs (**Figure 1F**), an additional 18 bp flank was added target strand downstream of the protospacer (**Table S1**). This flank allowed for hybridization with a 80 nt biotinylated poly(T)-linker that was used for immobilization of these constructs on a PEG-passivated surface.

Single-molecule fluorescence. The fluorescent label Cy3 was imaged using prism-type total internal reflection microscopy, through excitation by a 532nm (Compass 215M-50, Coherent). Cy7 was detected via FRET with Cy3, but if necessary, Cy7 was directly excited using a 640nm solid-state laser (CUBE 640-100C, Coherent). Fluorescence signals from single molecules were collected through a 60x water immersion objective (UPlanSApo, Olympus) with an inverted microscope (IX71, Olympus). Scattering of the 532nm laser beam was blocked with a 550nm long-pass filter (LP03-532RU-25, SemRock). When the 640nm laser was used, 640nm laser scattering was blocked with a notch filter (633 ± 12.5nm, NF03-633E-25, SemRock). Subsequently, signals of Cy3 and Cy7 were spectrally split with a dichroic mirror (λ_{cutoff} = 645 nm, Chroma) and imaged onto to halves of an electron multiplying CCD camera (iXon 897, Andor Technology). Given the reduced detection efficiency of the camera for Cy7 compared to Cy3 (~50%, Andor Technology), the measured Cy7 signal was multiplied by 2 prior to further analysis.

To eliminate non-specific surface adsorption of proteins and nucleic acids to a quartz surface (Finkenbeiner), piranha-etched slides were PEG-passivated over two rounds of PEGylation as described previously (Chandradoss et al., 2014). After assembly of a microfluidic flow chamber, slides were incubated for 1 minute with 20 µL streptavidin (0.1 mg/ml, S-888, Invitrogen) followed by a washing step with 100 µL of the Cascade buffer (50 mM HEPES (pH 7.5, AM9851, Ambion), 75 mM NaCl (AM9760G, Ambion), 2mM $MgCl_2$ (AM9530G, Ambion). Cascade molecules were end-specifically immobilized through biotin-streptavidin linkage by incubating the chamber with 100 µL of 1 nM biotinylated Cascade for 5 minutes. Remaining unbound Cascade molecule were flushed away with 100 µL Cascade buffer that was substituted with 60 nM J3-CasBCDE to reconstitute any Cse1 that lacks of CasBCDE subunits. After 5 minutes of incubation, unbound J3-CasBCDE were flushed away with 100 µL Cascade imaging buffer (50 mM HEPES (pH 7.5) ,75 mM NaCl, 2mM $MgCl_2$, 0.1 mg/mL glucose oxidase (G2133, Sigma), 4 µg/ml Catalase (10106810001, Roche) and 1 mM Trolox (((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 238813, Sigma). Next, 3 nM labelled dsDNA substrate was introduced in the chamber while imaging at room temperature (23± 1 °C) to monitor strand opening in real time.

A series of CCD images were acquired with the AndorSolis software (Andor Technology) at a time resolution of 0.3 or 1.5 sec. Fluorescence time traces were extracted with an algorithm written in IDL (ITT Visual Information Solutions) that picked fluorescence spots above a threshold with a defined Gaussian profile. The extracted time traces were analysed using custom written MATLAB algorithms (MathWorks) that selectively picked anti-correlated traces above a defined threshold. These selected traces were further analyzed using a custom written MATLAB algorithm to extract dwell times and initial FRET values upon binding. To obtain histograms for initial FRET values upon binding (**Figure 1F, 2B,**

S1E, 3B and 3E), the first five frames (1.5 s) of each binding event were averaged and plotted using MATLAB. Histograms were aligned by setting the donor-only signal to zero. Donor-only and low-FRET events (falling outside the most sensitive distance-range of FRET) were subsequently removed by discarding events with an acceptor intensity below 20% of the mean total-dye intensity (sum of the donor and acceptor) over the event.

To measure the initial FRET values upon binding, Cy3 molecules were excited an area of 50 x 50 μm^2 with a 16% of the full laser power (4 mW) green laser (532nm), while the time resolution was set to 0.3 seconds. Under these imaging conditions we obtained a high signal-to-noise ratio that allowed us to visualize kinetic intermediates while imaging over time periods of 8 min. In contrast, for dwell time measurements, Cy3 molecules were excited with 2% of the full laser power (1 mW) green laser (532nm) to minimize photobleaching of the donor and acceptor dye during our observation time. Meanwhile, the time resolution was set between 1.0 and 1.5 seconds to collect a large enough number of photons per time bin despite the weak excitation. Under these imaging conditions we obtained a signal-to-noise ratio that allowed us to visualize kinetic intermediates while imaging over long periods of time (30 min).

Target degradation assays. All oligonucleotides are listed in **Table S1**. Target plasmids (pWUR738-pWUR747) were constructed using plasmid pGFPuv-Kan as a backbone (Fineran et al., 2014). PCR amplicons of the J3 target were cloned into BspHI and EcoRI sites of the pGFP-Kan plasmid, and confirmed sequencing (GATC-Biotech). Plasmids were prepared using GeneJET Plasmid Miniprep Kits (Thermo Scientific) and DNA from PCR and agarose gels was purified using the Thermo Scientific GeneJET PCR Purification and Gel Extraction Kits. Plasmid DNA (3.5 nM) was mixed with purified Cascade (35 nM or 70 nM) in a buffer containing 5 mM HEPES, pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, 10 µM $CoCl_2$, and 2 mM ATP, and incubated at 37 ˚C for 15 min. After incubation purified Cas3 protein was added (70 nM) and incubated at 37 ˚C for 1, 10 or 30 minutes. Reactions were stopped by addition of 6x DNA Loading Dye (Thermo scientific). Samples were run on 0.8% TAE agarose gels containing SYBR Safe (Invitrogen) for 1h at 100 V. DNA band intensities were quantified using GeneTools Software (Syngene)

Direct interference and priming

The construction of *E. coli* strain KD263 was described previously (Shmakov et al., 2014). The strain contains the *cas3* gene under the control of the inducible lacUV5 promoter and the *cse1 – cas2* operon under control of the inducible araBp8 promoter. The KD263 strains harbors a single CRISPR cassette containing the g8 spacer targeting bacteriophage M13. Plasmid pWUR564 containing the J3 spacer under control of the native CRISPR 2.1 promoter (Westra et al., 2010) was introduced by transformation. *E. coli* strains were grown at 37 °C in Luria Broth (LB; 5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl) at 180 rpm or on LB-agar plates containing 1.5% (w/v) agar. When required, medium was supplemented with the following: ampicillin (Amp; 100 µg/L), chloramphenicol (Cam; 25 µg/mL), or kanamycin (Kan; 50 µg/mL). Bacterial growth was assessed spectrophotometrically at 600 nm (OD_{600}). To induce *cas* gene expression, IPTG (isopropyl β-D-1 thiogalactopyranoside) and L-arabinose were added to the final concentration of 1 mM each when an OD_{600} of approximately 0.4 was reached.

Direct interference was assessed by determining the transformation efficiency of target plasmid series pWUR738-pWUR747 to *E. coli* strain KD263 containing pWUR564. *Cas* gene expression was induced 30 minutes prior to making cells chemically competent. Priming was assessed using plasmid loss assays as described (Fineran et al., 2014). Briefly, *E. coli* transformants containing the target plasmid (pWUR738-747) were grown for 24 h in 5 mL LB in 15-mL tubes (Greiner) at 37 °C with shaking at 180 rpm. For further passaging, 100 µL of culture was subcultured into 5 mL LB in 15-mL tubes for a further 24 h at 37 °C at 180 rpm. Dilutions were plated on LBA and loss of fluorescence of individual colonies detected under mild UV light as described (Fineran et al., 2014). GFP-negative colonies were screened for spacer integration by colony PCR using DreamTaq Green DNA polymerase (Fermentas). Briefly, acquisition of spacers in the former CRISPR 2.1 locus containing the g8 spacer was assessed by PCR using primers BG5301 and BG5302 for strains KD263. PCR products were visualized on 2% ($wt \cdot vol^{-1}$) agarose gels and stained with SYBR-safe (Invitrogen). Newly acquired spacers were sequenced using primer BG5301 (**Table S1**). Spacer sequences were strand specifically mapped onto the target plasmid sequence to verify priming.

SUPPLEMENTAL REFERENCES

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