Electronic Supplementary Material (ESI) for Chemical Science.

Supplementary Data

Nonspecific interactions between Cas12a and dsDNA located downstream of the PAM mediate target search and assist AsCas12a for DNA cleavage

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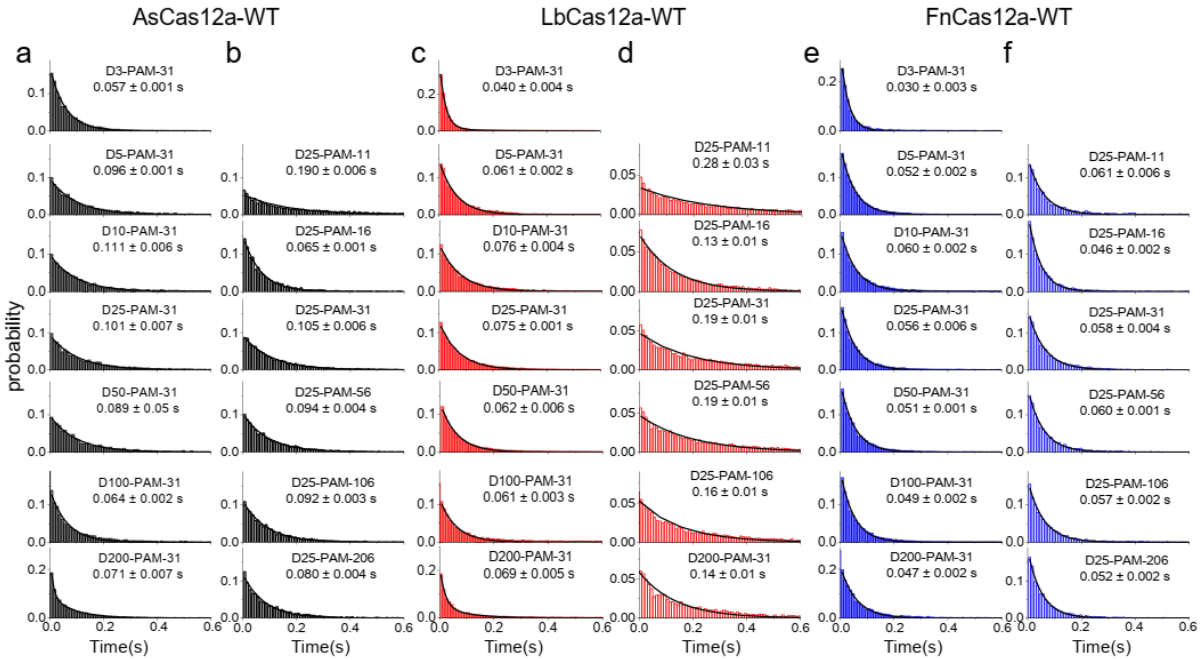

Figure S1. Dwell times of Cas12a/RNA complex on specific dsDNAs. (a-b) Distributions of dwell time of AsCas12a-WT on DN-PAM-31 dsDNAs (a) and D25-PAM-N dsDNAs (b), respectively. (c-d) Distributions of dwell time of LbCas12a-WT on DN-PAM-31 dsDNAs (c) and D25-PAM-N dsDNAs (d), respectively. (e-f) Distributions of dwell time of FnCas12a-WT on DN-PAM-31 dsDNAs (e) and D25-PAM-N dsDNAs (f), respectively.

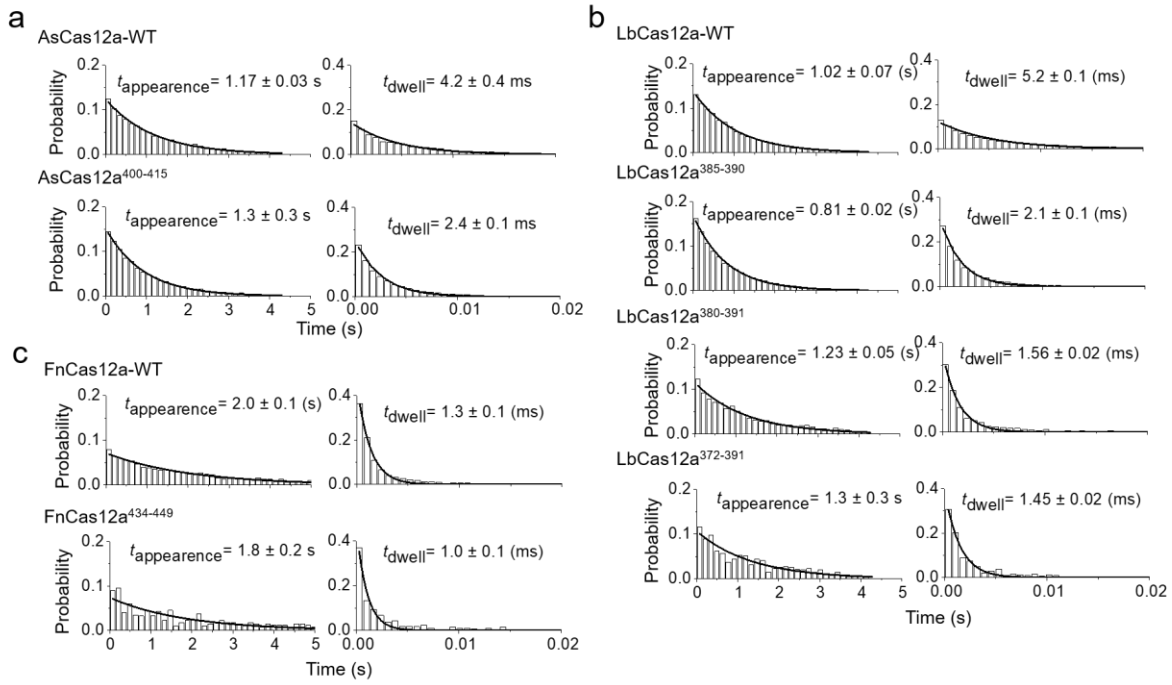

Figure S2. Appearance time and dwell time of Cas12a/RNA complex on dsDNA containing no PAM and no target site, which correspond to their binding rate and dissociation rate, respectively. 0.7 ms per frame was used to capture transient binding events. (a) Appearance time (left) and dwell time (right) of AsCas12a-WT (upper) and AsCas12a$^{400-415}$ (bottom) on dsDNA containing no PAM and no target site. (b) Appearance time (left) and dwell time (right) of LbCas12a-WT, LbCas12a$^{385-390}$, LbCas12a$^{380-391}$ and LbCas12a$^{372-391}$ on nonspecific dsDNA. (c) Appearance time (left) and dwell time (right) of FnCas12a-WT and FnCas12a$^{434-449}$ no nonspecific dsDNA. Sequence of one single strand was TACAGATCTACTAGTGATCTATGACTGATCTGTACATGATCTACA. 10nM Cas12a proteins were used.

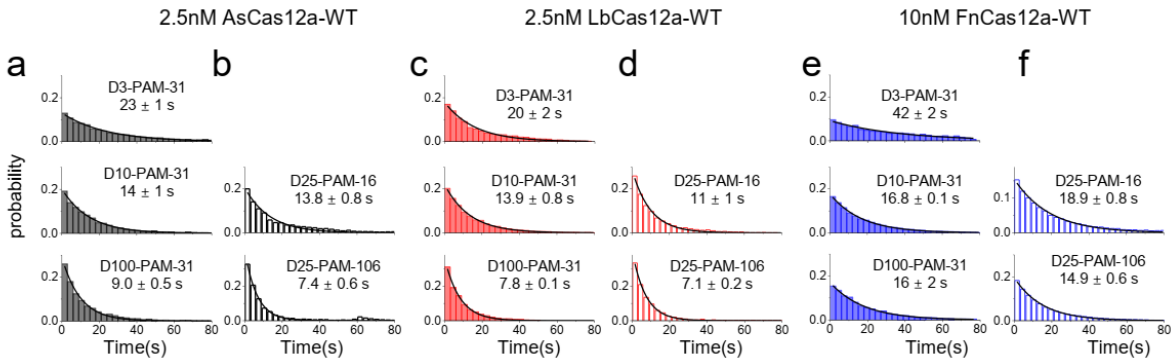

Figure S3. Single-molecule fluorescence assays quantifying 1D diffusion of AsCas12a, LbCas12a and FnCas12a. (a-b) Distributions of appearance time of AsCas12a-WT on DN-PAM-31 dsDNAs (a) and D25-PAM-N dsDNAs (b). (c-d) Distributions of appearance time of LbCas12a-WT on DN-PAM-31 dsDNAs (c) and D25-PAM-N dsDNAs (d). (e-f) Distributions of appearance time of FnCas12a-WT on DN-PAM-31 dsDNAs (e) and D25-PAM-N dsDNAs (f). This data is supplementary to figure 1.

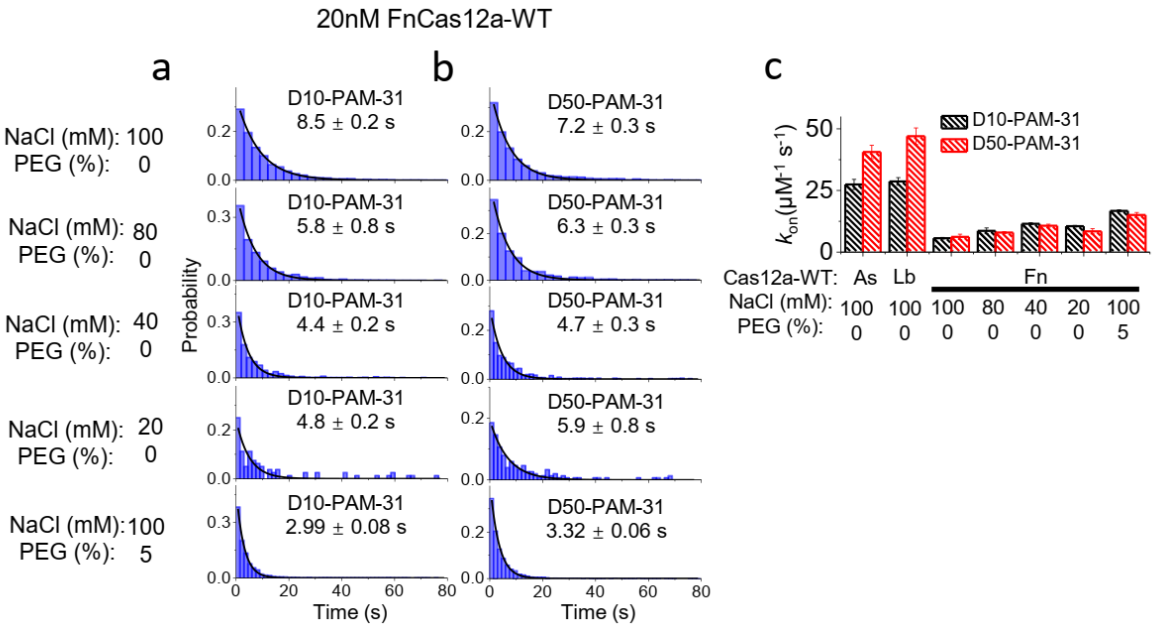

Figure S4. Effect of salt concentration and crowding reagent on target search of FnCas12a. (a-b) Distributions of appearance time of FnCas12a-WT on D10-PAM-31 (a) and D50-PAM-31 (b) dsDNAs in buffer (50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) supplied with 20-100 mM NaCl or in buffer (50 mM Tris-Cl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) supplied with 5% PEG-8000 (w/v). Distributions of one repeat were shown and fitted by the single exponential decay. Values were mean ± SEM from two replicates. (c) The apparent target search rates of FnCas12a-WT on D10-PAM-31 and D50-PAM-31 dsDNAs under various conditions, compared with those of AsCas12a-WT and LbCas12a-WT. Values were mean ± SEM from two replicates.

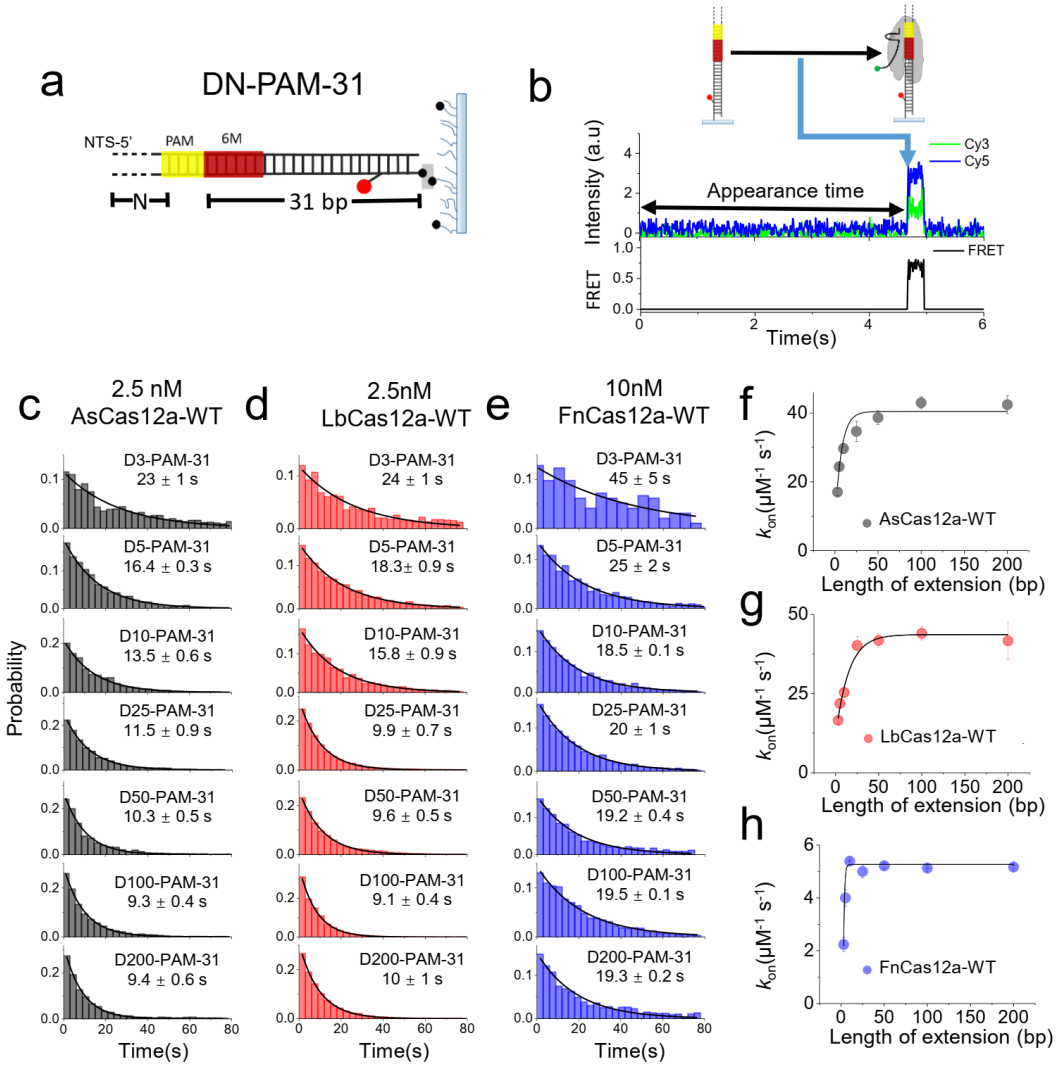

Figure S5. Single-molecule FRET assays quantifying 1D diffusion of AsCas12a, LbCas12a and FnCas12a on DN-PAM-31 dsDNAs. (a) Design of DN-PAM-31 dsDNAs. The distance between PAM and the 3' end of NTS is always 31 bp and the distance between PAM and the 5' end of NTS varies from 3 bp to 200 bp. There are 6 matched base pairs between crRNA and target DNA shown in red. PAM is shown in yellow. (b) A representative single-molecule FRET trace recording the appearance time of Cas12a from sample injection until finding its target indicated by the blue arrow. crRNA was labeled with Cy3 (green dots) on its 3' end and Cy5 (red dots) was labeled at the target strand located 23 nt downstream of the PAM. Cy3-labeled crRNA/Cas12a binding to target site of Cy5-labeled dsDNAs results in ~0.68, ~0.48 and ~0.38 FRET states for AsCas12a, LbCas12a and FnCas12a, respectively. (c-e) Distributions of appearance time of AsCas12a-WT (c), LbCas12a-WT (d) and FnCas12a-WT (e) on DN-PAM-31 dsDNAs from one repeat, which were fitted by single exponential decay. Values were mean ± SEM from three replicates. (f-h) The apparent target search rates of AsCas12a-WT (f), LbCas12a-WT (g) and FnCas12a-WT (h) on DN-PAM-31 dsDNAs. Values were presented as mean ± SEM from three replicates.

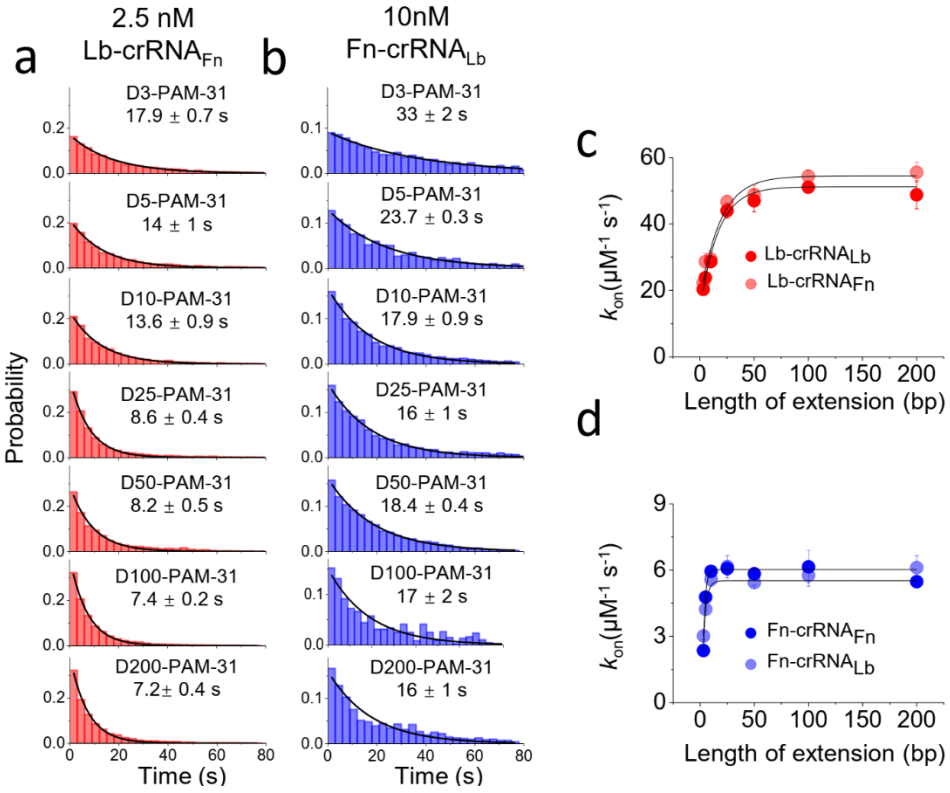

Figure S6. Effect of crRNA on target search dynamics of Cas12a. (a) Distributions of appearance time of LbCas12a/crRNA$_{Fn}$ (Lb-crRNA$_{Fn}$), formed by incubated LbCas12a with crRNA$_{Fn}$, on DN-PAM-31 dsDNAs, which were fitted by the single exponential decay. Distributions of one repeat were shown. Values were mean ± SEM from three replicates. (b) Distributions of appearance time of FnCas12a/crRNA$_{Lb}$ (Fn-crRNA$_{Lb}$), formed by incubated FnCas12a with crRNA$_{Lb}$, on DN-PAM-31 dsDNAs. (c-d) The apparent target search rates of Lb-crRNA$_{Fn}$ (c) and Fn-crRNA$_{Lb}$ (d) on DN-PAM-31 dsDNAs, compared with that of Lb-crRNA$_{Lb}$ and Fn-crRNA$_{Fn}$ respectively. Values were mean ± SEM from three replicates.

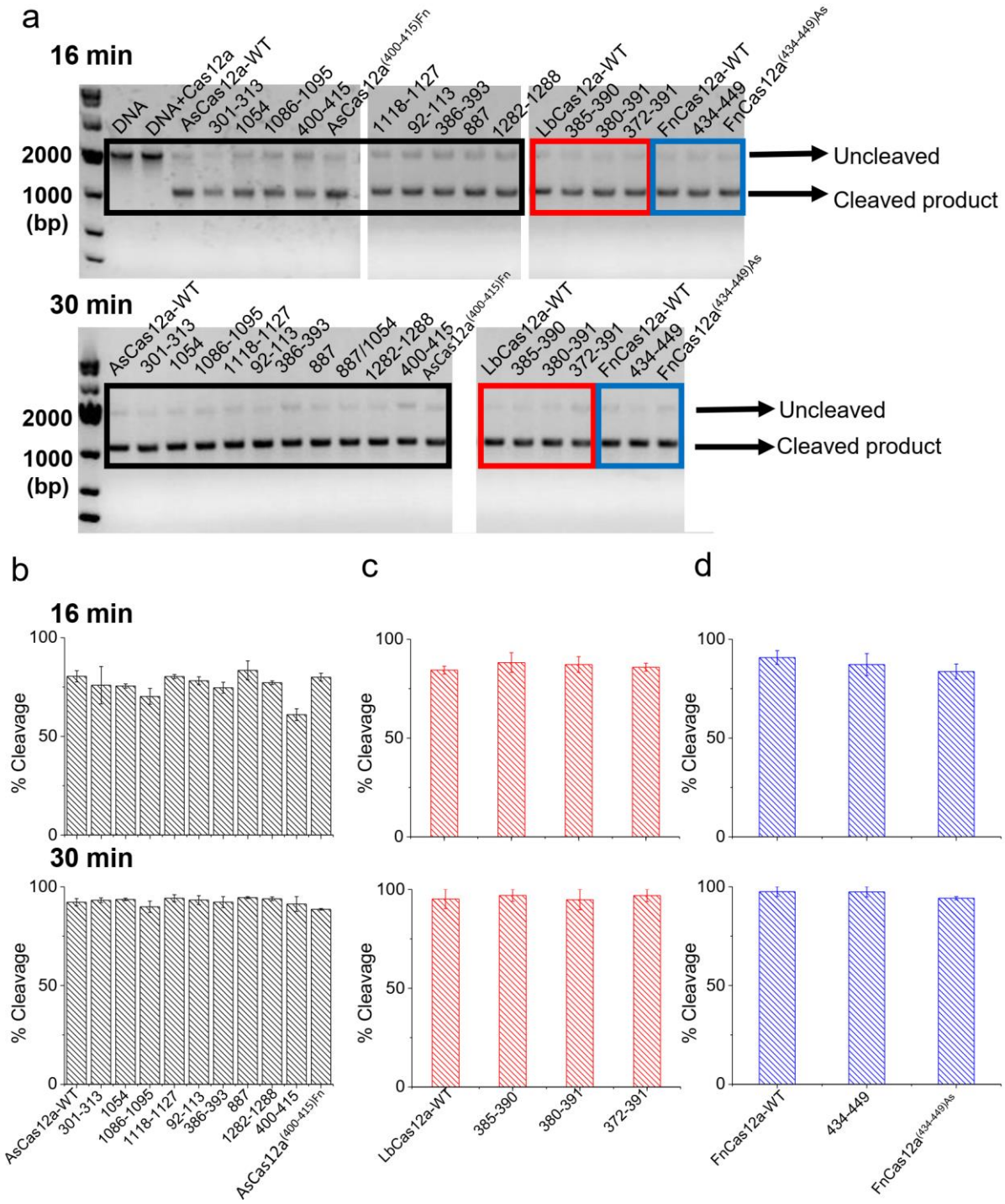

Figure S7. The dsDNA cleavage activity of Cas12a mutants. (a) DNA cleavage assays of Cas12a variants toward 100 ng dsDNA (2281bp) were carried out at 25°C for 16 min and 30 min. Cas12a was incubated with pre-annealed crRNAs (1:2 molar ratio) and diluted to 100 nM. The uncleaved and cleaved bands were resolved by 1% agarose gels. (b-d) The dsDNA cleavage percentage of 100 nM pre-formed AsCas12a (b), LbCas12a (c) and FnCas12a (d) mutants for 16 min (upper) or 30 min (bottom). Values were quantified from gel images in (a) using imageJ and Gaussian fitting and presented as mean ± SEM from three replicates.

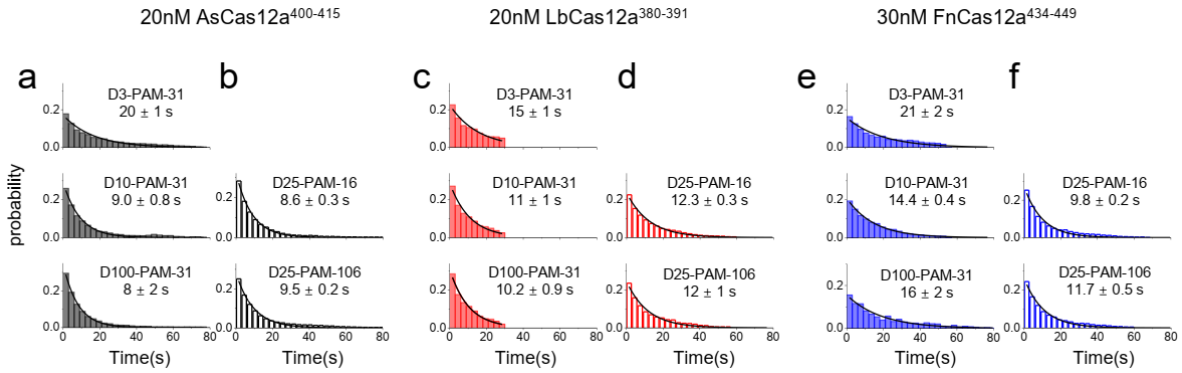

Figure S8. Single-molecule fluorescence assays quantifying 1D diffusion of AsCas12a$^{400-415}$, LbCas12a$^{380-391}$ and FnCas12a$^{434-449}$. (a-b) Distributions of appearance time of AsCas12a$^{400-415}$ on DN-PAM-31 dsDNAs (a) and D25-PAM-N dsDNAs (b). (c-d) Distributions of appearance time of LbCas12a$^{380-391}$ on DN-PAM-31 dsDNAs (c) and D25-PAM-N dsDNAs (d). (e-f) Distributions of appearance time of FnCas12a$^{434-449}$ on DN-PAM-31 dsDNAs (e) and D25-PAM-N dsDNAs (f). This data is supplementary to figure 2.

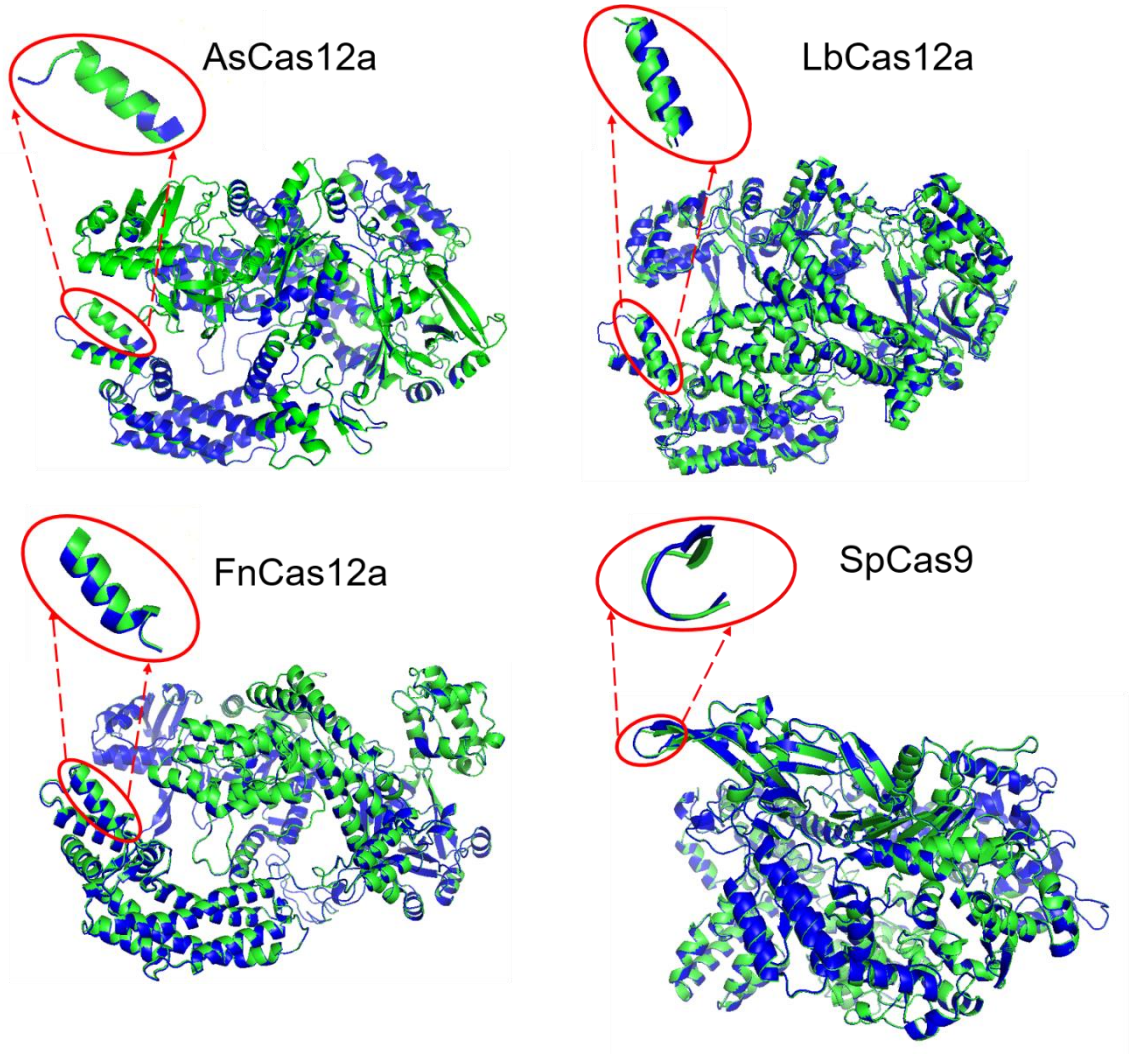

Figure S9. AlphaFold to predict the effects of mutations on the structures of Cas12a proteins and Cas9. Cas protein wild types and mutants are shown in green and blue, respectively. The regions enlarged by the red ellipse are key amino acids affecting 1D diffusion of the Cas proteins.

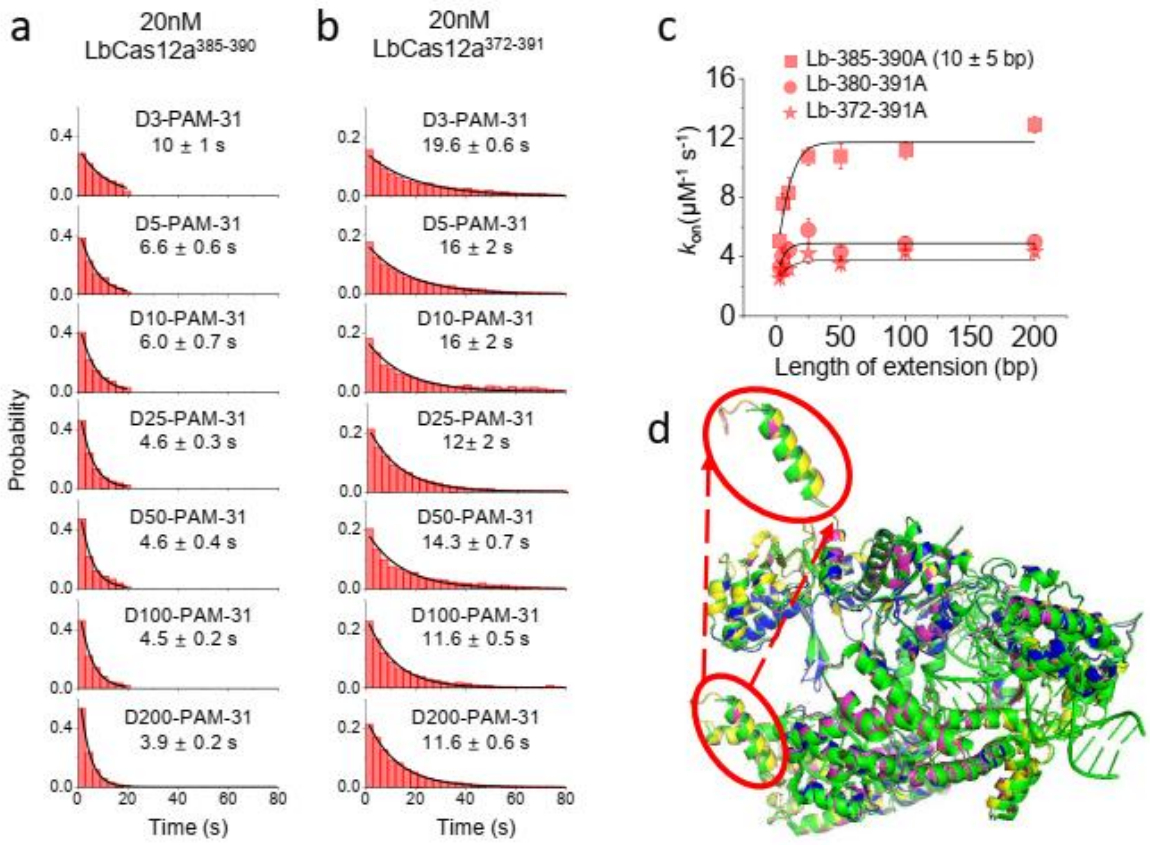

Figure S10. Target search dynamics of LbCas12a mutants on DN-PAM-31 dsDNAs. (a-b) Distributions of appearance time of LbCas12a$^{385\text{-}390}$ (a) and LbCas12a$^{372\text{-}391}$ (b) on DN-PAM-31 dsDNAs, which were fitted by the single exponential decay. Distributions of one repeat were shown. Values were mean ± SEM from three replicates. (c) The apparent target search rates of LbCas12a$^{385\text{-}390}$, LbCas12a$^{380\text{-}391}$ and LbCas12a$^{372\text{-}391}$on DN-PAM-31 dsDNAs. Values were mean ± SEM from three replicates. (d) AlphaFold to predict the effects of mutations on the structures of LbCas12a. LbCas12a-WT, LbCas12a$^{385\text{-}390}$, LbCas12a$^{380\text{-}391}$ and LbCas12a$^{372\text{-}391}$ are shown as green, yellow, blue and purple, respectively. The region enlarged by the red ellipse are alpha helix affecting 1D diffusion of the LbCas12a.

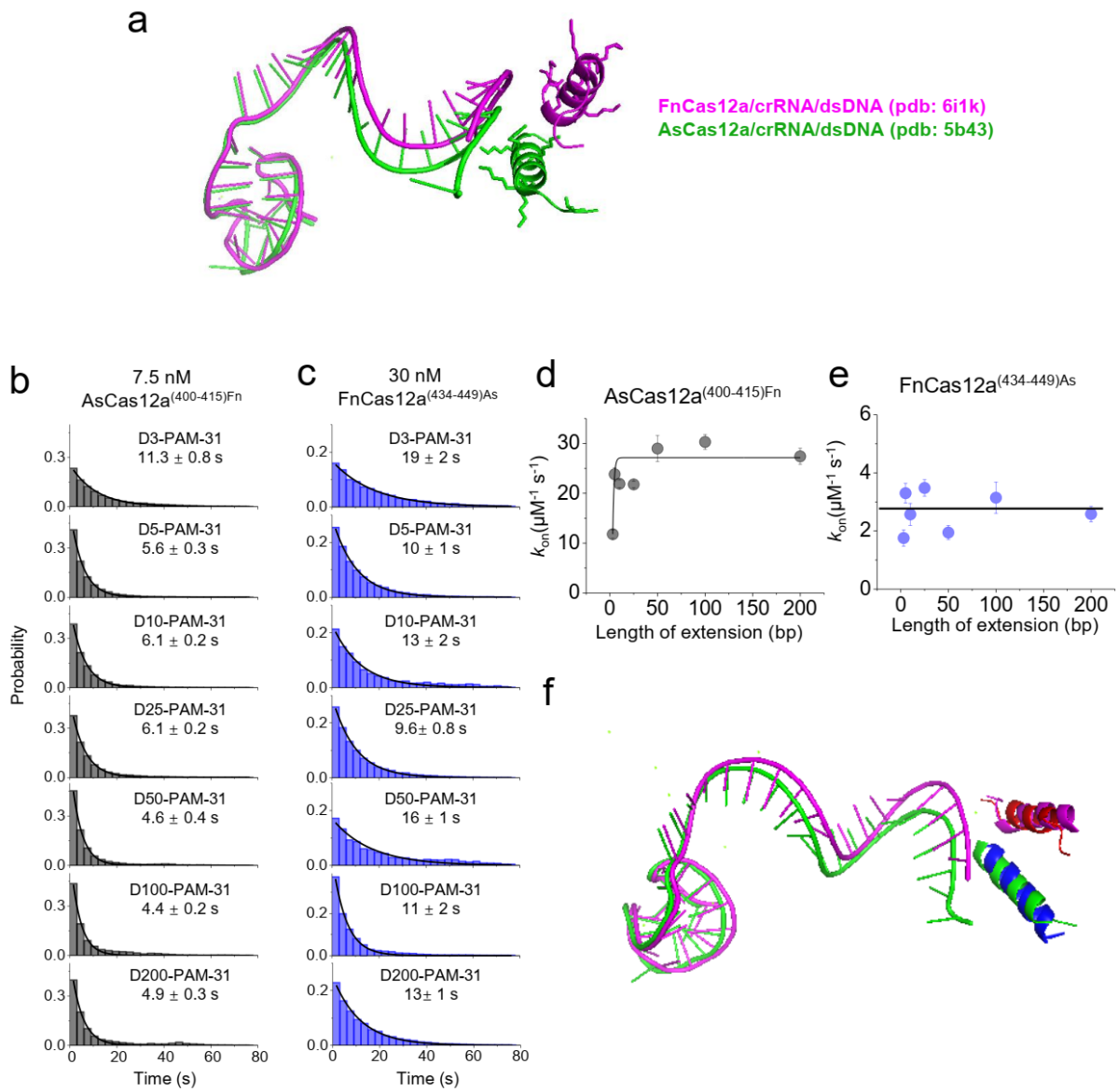

Figure S11. Target search dynamics of AsCas12a and FnCas12a chimera proteins. (a) Structures showing interactions of AsCas12a residues 400-415 (pdb: 5b43) and FnCas12a residues 434-449 (pdb: 6i1k) with their corresponding crRNAs. Positively charged residues are shown in sticks. (b-c) Distributions of the appearance time of AsCas12a$^{(400\text{-}415)Fn}$ (b) and FnCas12a$^{(434\text{-}449)As}$ (c) on DN-PAM-31 dsDNAs, which were fitted by the single exponential decay. Distributions of one repeat were shown. Values were mean ± SEM from two replicates. (d-e) The effective apparent target search rates of AsCas12a$^{(400\text{-}415)Fn}$ (d) and FnCas12a$^{(434\text{-}449)As}$ (e) on DN-PAM-31 dsDNAs. Values were mean ± SEM from two replicates. (f) AlphaFold to predict the structure information of two chimera proteins. FnCas12a-WT, FnCas12a$^{(434\text{-}449)As}$, AsCas12a-WT and AsCas12a$^{(400\text{-}415)Fn}$ are shown as purple, red, green and blue, respectively.

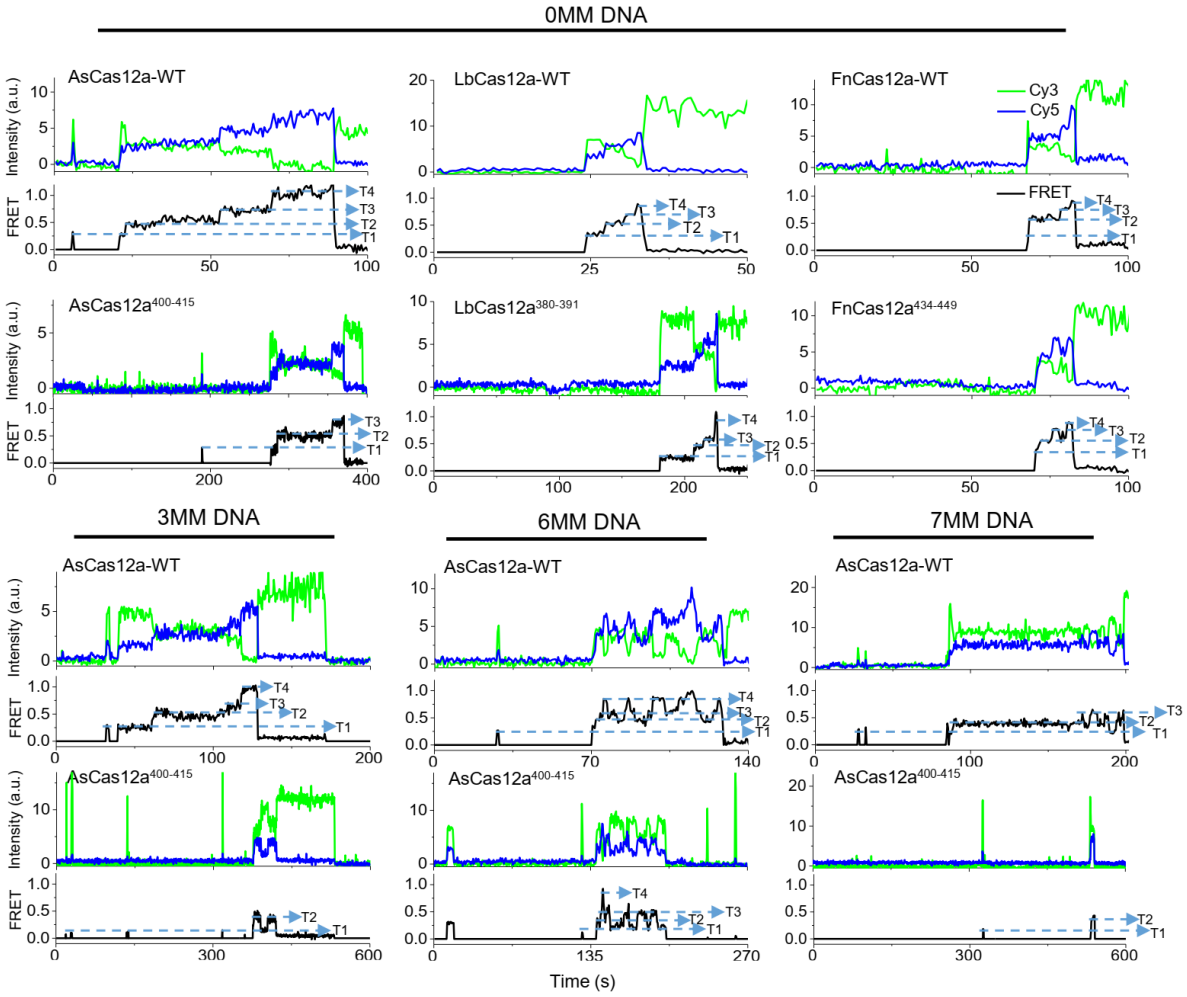

Figure S12. Representative smFRET traces capturing formation and conformational dynamics of Cas12a/crRNA/dsDNA complex. Green, blue and black curves are Cy3 signal, Cy5 signal and apparent FRET efficiencies, respectively. Under 532 nm excitation, spontaneous appearance of Cy3, Cy5 and FRET signals represents the formation of Cas12a/crRNA/dsDNA ternary complex. Simultaneous disappearance of Cy3, Cy5 and FRET signals implies that Cy3-labeled Cas12a/crRNA dissociated from immobilized Cy5-labeled DNA, whereas disappearance of Cy5 and FRET accompanied by increasing of Cy3 intensity corresponds to dissociation of the cleaved PAM-distal DNA fragment.

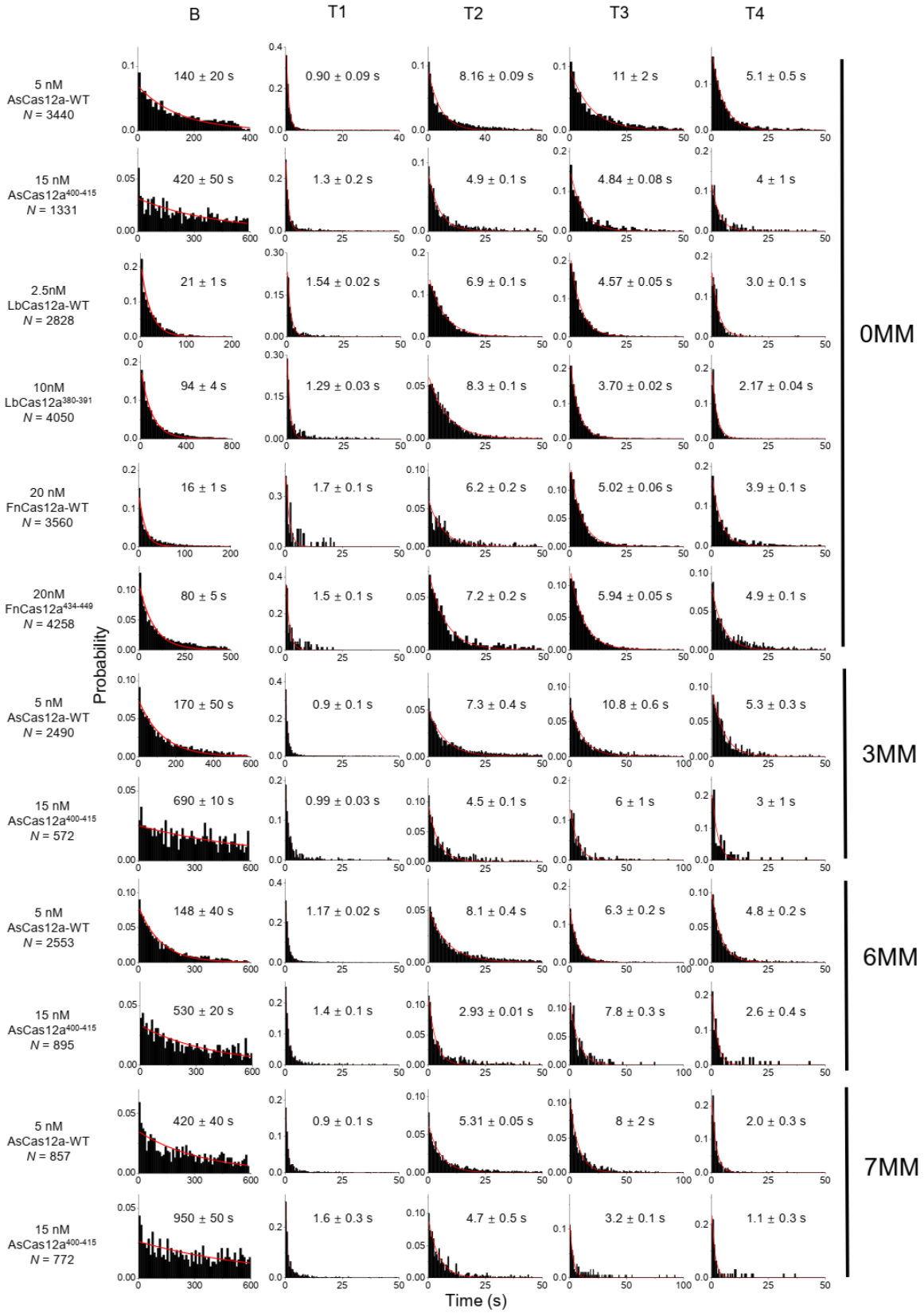

Figure S13. Dwell times of different states of Cas12a binary and ternary complex. Distributions of dwell times for T1 – T4 states were extracted from HMM modeling and were fitted by the single-exponential decay curves to quantify average dwell times. Number of events (*N*) from representative experiment were shown.

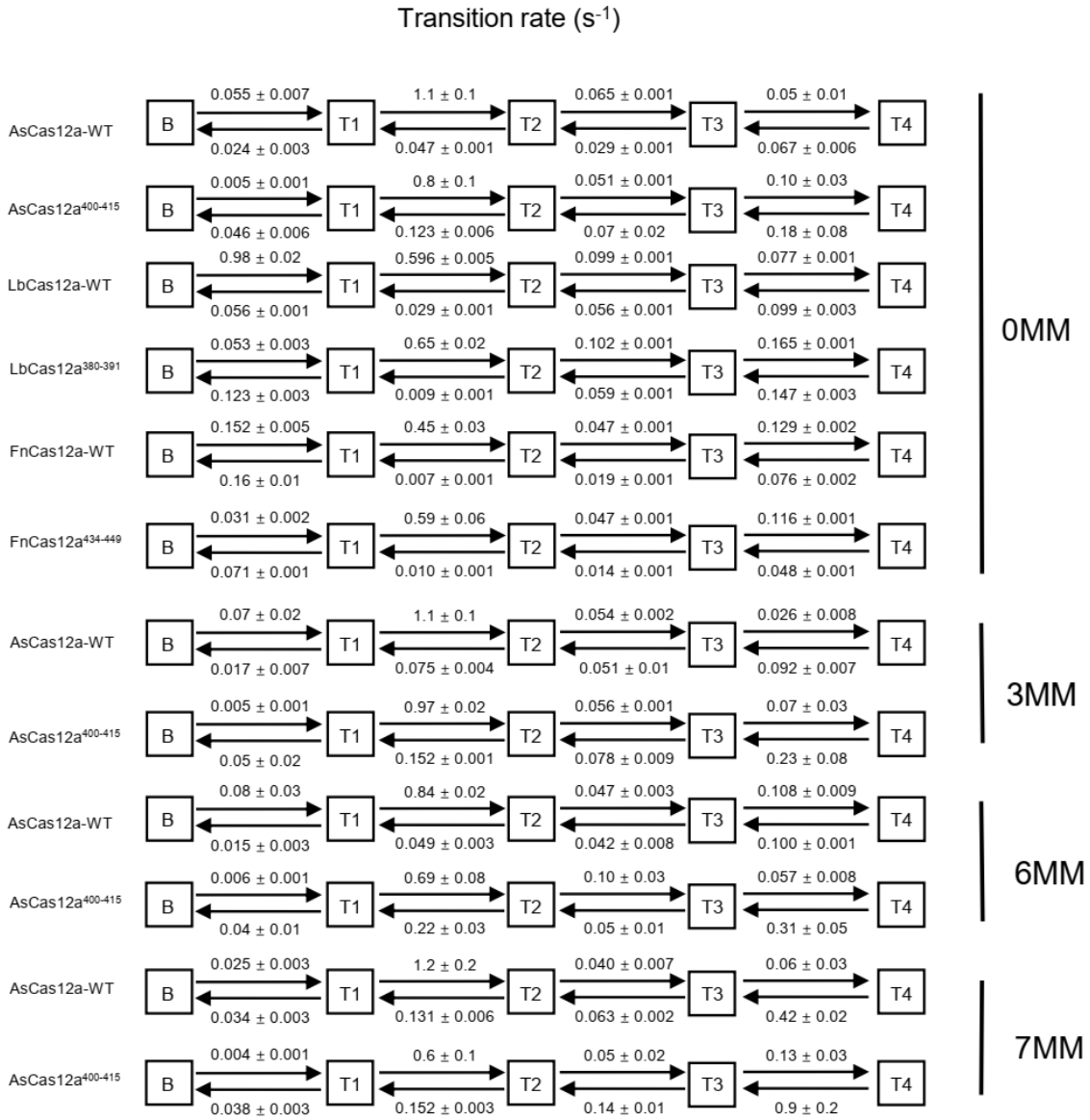

Figure S14. Kinetic schemes of Cas12a/crRNA/dsDNA formation and conformational changes. Kinetic schemes showing transition pathways and rates among different states. The rates were extracted from Figure S13.

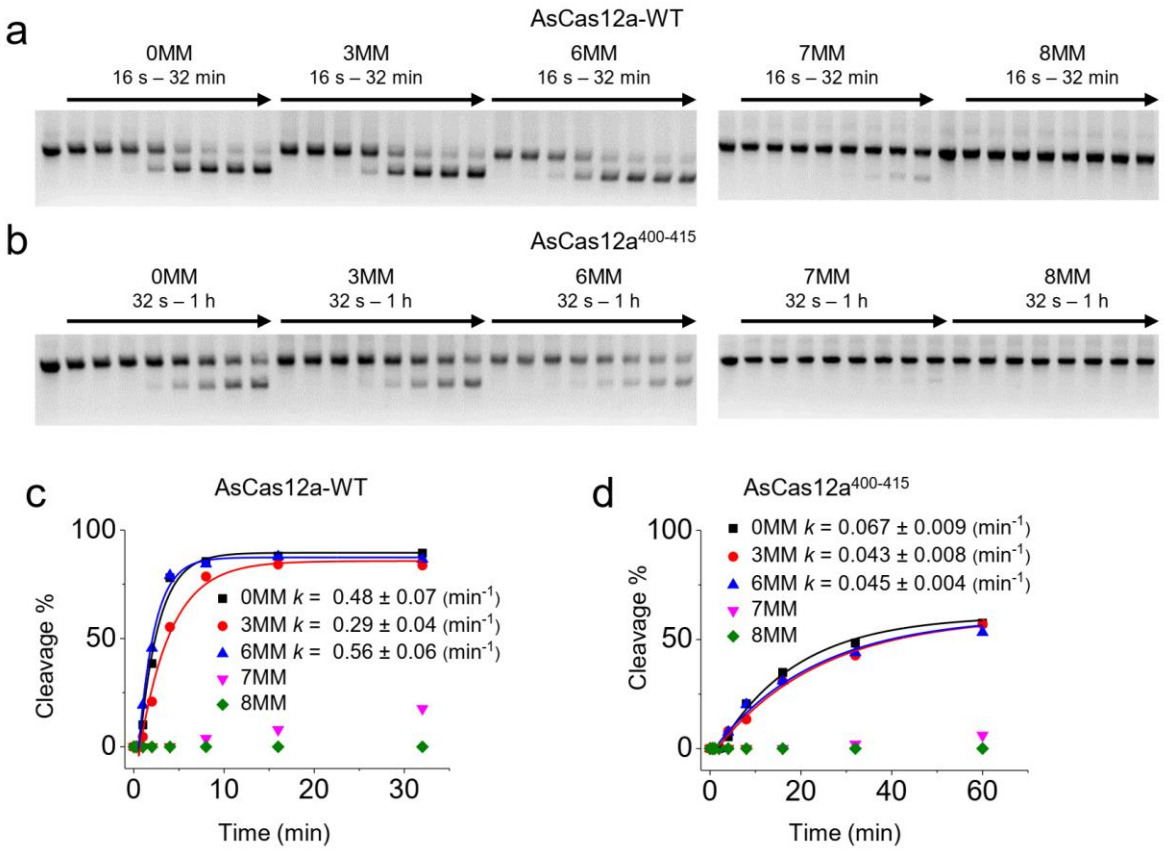

Figure S15. Time-dependent dsDNA cleavage assays for AsCas12a. (a) Time-dependent cleavage of fully (0MM dsDNA) and partially matched dsDNAs (3MM, 6MM, 7MM and 8MM dsDNAs) by AsCas12a-WT. The uncleaved and cleaved products were separated by 1% agarose gel. (b) Time-dependent cleavage of fully and partially matched dsDNAs by AsCas12a$^{400\text{-}415}$. (c-d) Time-dependent cleavage percentages for AsCas12a-WT (c) and AsCas12a$^{400\text{-}415}$ (d) were fitted by the single-exponential decay to quantify the cleavage rates. The cleavage rate of Cas12a on 7MM DNA cannot be accurately quantified because the majority of DNA is not cleaved even with the longest incubation time.

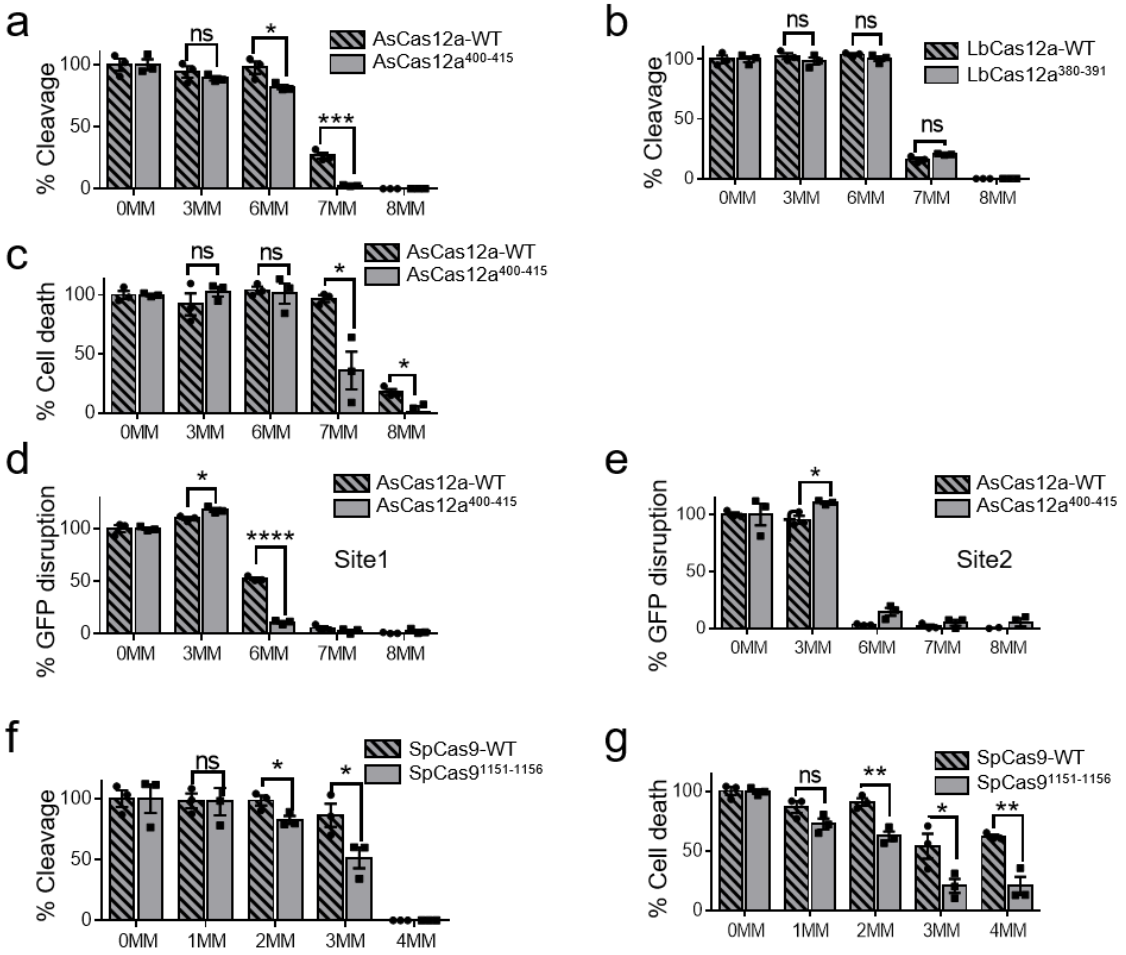

Figure S16. Specificity of Cas12a and SpCas9 *in vitro* and *in vivo*. Cleavage, GFP disruption and cell death percentages using fully matched targets were normalized to 100%. (a-b) Cleavage percentage for wide type or mutant of AsCas12a (a) and LbCas12a (b). (c) Cell death percentage using AsCas12a-WT and AsCas12a$^{400\text{-}415}$. (d-e) GFP disruption percentage using AsCas12a-WT and AsCas12a$^{400\text{-}415}$ targeting site 1 (d) and site 2 (e) located within GFP. (f) Cleavage percentage of SpCas9-WT and SpCas9$^{1151\text{-}1156}$. (g) Cell death percentage of SpCas9-WT and SpCas9$^{1151\text{-}1156}$. Significances were determined via a 2-tailed Student's t-test between two groups. ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

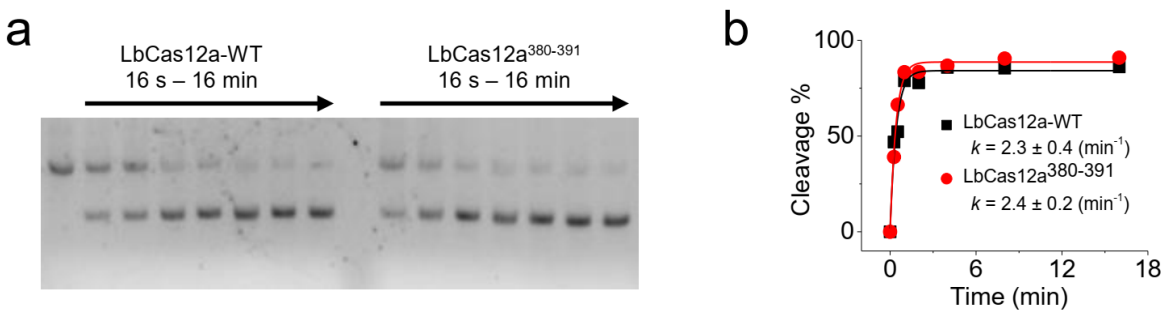

Figure S17. Time-dependent dsDNA cleavage assays for LbCas12a. (a) Time-dependent cleavage of fully matched dsDNAs (0MM dsDNA) by LbCas12a-WT and LbCas12a$^{380\text{-}391}$. The uncleaved and cleaved products were separated by 1% agarose gel. (b) Time-dependent cleavage percentages were fitted by the single exponential decay to quantify the cleavage rates.

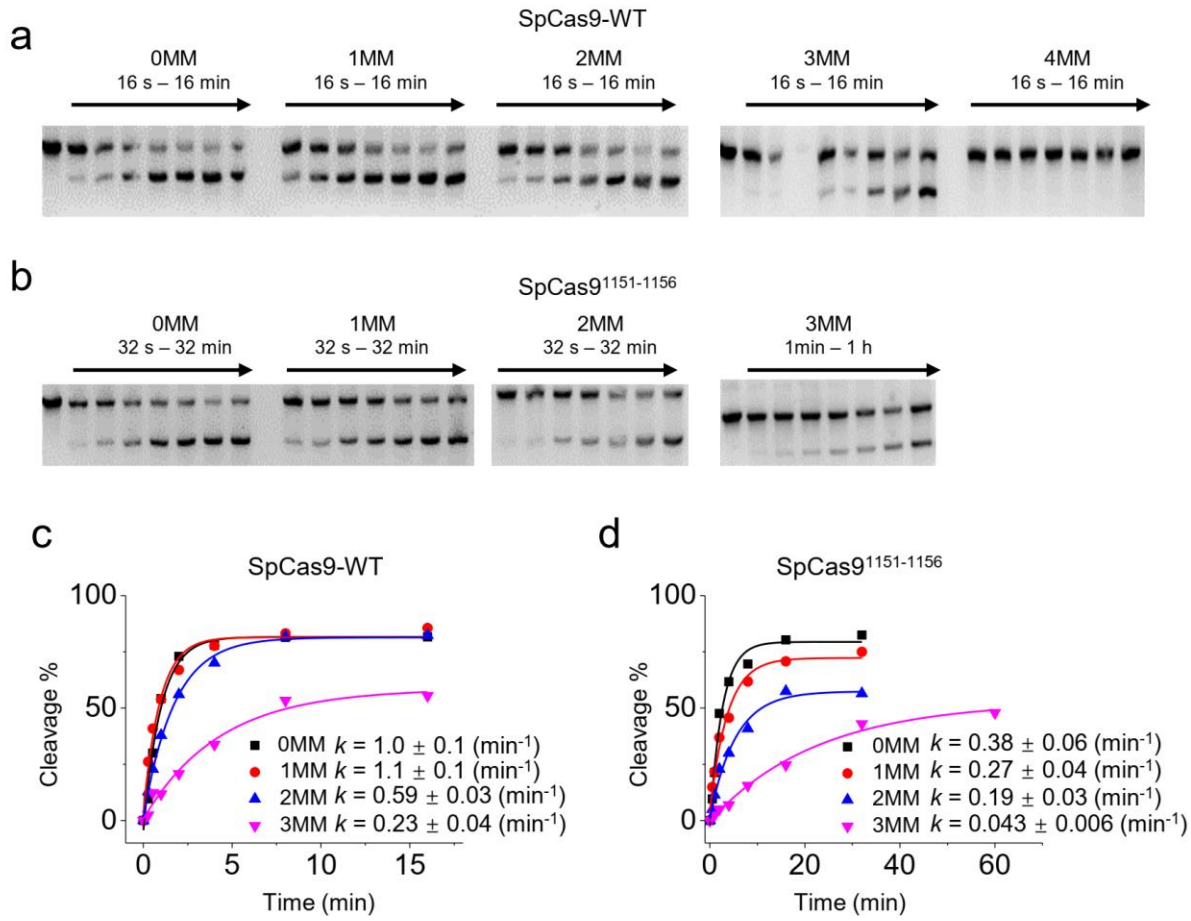

Figure S18. Time-dependent dsDNA cleavage assays for SpCas9. (**a**) Time-dependent cleavage of fully (0MM dsDNA) and partially matched dsDNAs (1MM, 2MM, 3MM and 4MM dsDNAs) by SpCas9-WT. The uncleaved and cleaved products were separated by 1% agarose gel. (**b**) Time-dependent cleavage of fully and partially matched dsDNAs by SpCas9$^{1151-1156}$. (**c-d**) Time-dependent cleavage percentages for SpCas9-WT (**c**) and SpCas9$^{1151-1156}$ (**d**) were fitted by the single exponential decay to quantify the cleavage rates.

Table S1. DNA substrates used for single-molecule fluorescence assay.

Name	Sequence (5' to 3')
D3-PAM-31	AGGTTTACTGATGCAGGTACAGACAATGAACGGAATTC -Biotin
D5-PAM-31	ACAGGTTTACTGATGCAGGTACAGACAATGAACGGAATTC-Biotin
D10-PAM-31	TTGGCACAGGTTTACTGATGCAGGTACAGACAATGAACGGAATTC-Biotin
D25-PAM-31	TCGATCCGCAGTCTCTTGGCACAGGTTTACTGATGCAGGTACAGACAATGA ACGGAATTC-Biotin
D50-PAM-31	ACGGCCAGTCCGTCTCTATCCGGTCTCGATCCGCAGTCTCTTGGCACAGGTT TACTGATGCAGGTACAGACAATGAA CGGAATTC-Biotin
D100-PAM-31	CATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTTGTAAAACGAC GGCCAGTCCGTCTCTATCCGGTCTCGATCCGCAGTCTCTTGGCACAGGTTTA CTGATGCAGGTACAGACAATGAACGGAATTC-Biotin
D200-PAM-31	TACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACAT TTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATT AACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTTGTAAAACGACGGC CAGTCCGTCTCTATCCGGTCTCGATCCGCAGTCTCTTGGCACAGGTTTACTG ATGCAGGTACAGACAATGAACGGAATTC-Biotin
D25-PAM-11	Biotin-TCGATCCGCAGTCTCTTGGCTCAGGTTTACTGAT GCAGGT
D25-PAM-16	Biotin-TCGATCCGCAGTCTCTTGGCTCAGGTTTACTGATGCAGGT ACAGA
D25-PAM-31	Biotin- TCGATCCGCAGTCTCTTGGCTCAGGTTTACTGATGCAGGTACAGACAATG AGCGGAATTC
D25-PAM-56	Biotin- TCGATCCGCAGTCTCTTGGCTCAGGTTTACTGATGCAGGTACAGACAAT GAGCGGAATTCGAGTACAAACGTCAGCACGTGTGTG
D25-PAM-106	Biotin- TCGATCCGCAGTCTCTTGGCTCAGGTTTACTGATGCAGGTACAGACAAT GAGCGGAATTCGAGTACAAACGTCAGCACGTGTGTGGCGGAGCGAGGAGC TGCTGTCCCCGTGGGAGCCGGCCTCAGAGGTAGCTC
D25-PAM-206	Biotin- TCGATCCGCAGTCTCTTGGCTCAGGTTTACTGATGCAGGTACAGACAAT

	GAGCGGAATTCGAGTACAAACGTCAGCACGTGTGTGGCGGAGCGAGGAGC TGCTGTCCCCGTGGGAGCCGGCCTCAGAGGTAGCTCCATGACCCAGACACC AGTGGGGGATGTCAGTGTTGGGGGAAAGTAGAAGCTTGGACCGTGCGAGT TACTGCCAACCGAGACCCAACCGAGACGGGTCATA

For DN-PAM-31 series and D25-PAM-N series, PAM sequence and matched bases between target DNA and crRNA in PAM proximal end are underlined. Only non-target strand sequences are listed in this Table. Cy5 labeling positions for single-molecule FRET measurements are highlight in yellow.

Table S2. RNA sequences used in this study.

Name	Sequence (from 5' to 3')
$crRNA_{Lb}$	UAAUUUCUACUAAGUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-NH2
$crRNA_{Fn/As}$	UAAUUUCUACUCUUGUAGAUCUGAUGGUCCAUGUCUGUUACUC-NH2
$sgRNA_{Cas9}$	GACGCAUAAAGAUGAGACGCGUUUUAGAGCUAUGCUGUUUUGGAAACAAAA CAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGG CACCGAGUGGUGCUUUUUUU

Spacer sequences for crRNA and sgRNA are underlined. crRNAs were labeled with Cy3 at their 3' end before used in single-molecule fluorescence experiments. Cas12a and Cas9 were incubated with their corresponding RNAs, unless otherwise specified.

Table S3. Cas12a and Cas9 mutants.

Cas12a and Cas9 mutants		
Cas12a and Cas9 mutants	Mutated amino acid	Domain containing mutated amino acids
AsCas12a$^{92\text{-}113}$	R92A/R103A/R113A	REC1
AsCas12a$^{301\text{-}313}$	R301A/K307A/R313A	REC1
AsCas12a$^{386\text{-}393}$	R386A/R392A/R393A	REC2
AsCas12a$^{400\text{-}415}$	K400A/K403A/K406A/K408A /R411A/K414A	REC2
AsCas12a^{887}	K887A	RuvC-I
AsCas12a^{1054}	K1054A	RuvC-II
AsCas12a$^{1086\text{-}1095}$	K1086A/K1089A/R1094A/K1095A	Nuc
AsCas12a$^{1118\text{-}1127}$	K1118A/R1121A/R1127A	Nuc
AsCas12a$^{1282\text{-}1288}$	K1282A/K1285A/K1287A	RuvC-III
LbCas12a$^{385\text{-}390}$	R385A/R386A/K387A /K390A	REC2
LbCas12a$^{380\text{-}391}$	K380A/R385A/R386A/K387A /K390A/K391A	REC2
LbCas12a$^{372\text{-}391}$	K372A/K373A/K374A/K380A /R385A/R386A/K387A /K390A/K391A	REC2
FnCas12a$^{434\text{-}449}$	K435A/K436A/K443A/K444A /K447A/K449A	REC2
Cas9$^{1151\text{-}1156}$	K1151A/G1152A/K1153A/S1154A /K1155A/K1156A	PI

Cas12a chimeras			
Cas12a chimeras	Helix that exchanged	Original sequence	Mutated sequence
AsCas12a$^{(400\text{-}415)Fn}$	400-415	KITKSAKEKVQRSLKH	SKKEQELIAKKTEKAK
FnCas12a$^{(434\text{-}449)As}$	434-449	SKKEQELIAKKTEKAK	KITKSAKEKVQRSLKH

Table S4. DNA sequences used in single-molecule FRET measurements to capture dynamics of Cas12a ternary complex.

Name	Sequence (from 5' to 3')
biotin handle	CCCTGGTCCGGTGGTCCGCCTGCTGGTCCC-biotin
0MM-NTS	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGTTACTCCGTCAGT
0MM-TS	ACTGACGGAGTAACAGACATGGACCATCAGTAAACCTGT GC
3MM-NTS	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGTTAgagCGTCAGT
3MM-TS	ACTGACGctcTAACAGACATGGACCATCAGTAAACCTGTGC
6MM-NTS	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTGaatgagCGTCAGT
6MM-TS	ACTGACGctcattCAGACATGGACCATCAGTAAACCTGTGC
7MM-NTS	CGGACCACCGGACCAGGGGCACAGGTTTACTGATGGTCCATGTCTcaatgagCGTCAGT
7MM-TS	ACTGACGctcattgAGACATGGACCATCAGTAAACCTGTGC

The fully- or partially-matched dsDNAs were immobilized to the slide surface by biotin handle. PAM and protospacer sequences at the NTS are underlined. Mismatches towards crRNA are shown in lower case. Cy5 labeled positions are highlight in yellow.

Table S5. Plasmid for Cas12a *in vitro* cleavage assay.

Name	Sequence (from 5' to 3')
Plasmid sequences containing target sequence (from 5' to 3')	AAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACG TCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGC CCTTTCGTTGTAAAACGACGGCCAGTCCGTCTCTATCCGGTCTCGATCCGCAGTCT CTTGGCACAGGTTTACTGATGGTCCATGTCTGTTACTCCGGAATTCGAGTACAAAC GTCAGCACGTGTGTGGCGGAGCGAGGAGCTGCTGTCCCCGTGGGAGCCGGCCTC AGAGGTAGCTCCATGACCCAGACACCAGTGGGGGATGTCAGTGTTGGGGGAAAG TAGAAGCTTGGACCGTGCGAGTTACTGCCAACCGAGACCCAACCGAGACGGGTC ATAGCTGTTTCCAGTGTGCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTT CGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTACCCACAG AATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCC AGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTG ACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGAC TATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCG ACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCT TTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGC TGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAA CTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCC ACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGA AGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTG CTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAA CCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAA AAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGG AACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCA CCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT AAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATC TGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATA CGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATAATACCGCGGGACCCACGCT CACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCA GAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAA GCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATCGCTAC AGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCC AACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTC CTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCCGTGTTATCACTCATGG TTATGGCAGCACTACATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTG TGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAG

	TTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAA AAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACC GCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCAT CTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGC AAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTT CAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAAT GTATTTAG

For plasmid, only non-target strand of dsDNA is shown. PAM and target sequence are underlined. A series of DNAs with different number of mismatched bases at PAM-distal ends used in Figs. 4a-d were obtained from this plasmid and the target sequences of them are (from 5'-3'): CTGATGGTCCATGTCTGTTACTC (0MM), CTGATGGTCCATGTCTGTTAgag (3MM), CTGATGGTCCATGTCTGaatgag (6MM), CTGATGGTCCATGTCTcaatgag (7MM), CTGATGGTCCATGTCacaatgag (8MM). Mismatches towards crRNA are shown in lower case.

Table S6. Fully-matched and partially-matched crRNA spacer sequences used in *in vivo* assays.

Proteins	Gene locis		crRNA spacer sequences (from 5' to 3')
Cas12a	*zrap*		CUCACGGCGGACACGGUAUGUGG (0MM)
			CUCACGGCGGACACGGUAUGacc (3MM)
			CUCACGGCGGACACGGUtacacc (6MM)
			CUCACGGCGGACACGGatacacc (7MM)
			CUCACGGCGGACACGcatacacc (8MM)
	GFP	Site 1	CGUCGCCGUCCAGCUCGACCAGG (0MM)
			CGUCGCCGUCCAGCUCGACCtcc (3MM)
			CGUCGCCGUCCAGCUCGuggtcc (6MM)
			CGUCGCCGUCCAGCUCcuggtcc (7MM)
			CGUCGCCGUCCAGCUgcuggtcc (8MM)
		Site 2	CUCAGGGCGGACUGGGUGCUCAG (0MM)
			CUCAGGGCGGACUGGGUGCUgtc (3MM)
			CUCAGGGCGGACUGGGUcgagtc (6MM)
			CUCAGGGCGGACUGGGacgagtc (7MM)
			CUCAGGGCGGACUGGcacgagtc (8MM)
Cas9	*zrap*		AAUGAUGGCGCUUUCAGCAA (0MM)
			uAUGAUGGCGCUUUCAGCAA (1MM)
			uuUGAUGGCGCUUUCAGCAA (2MM)
			uuaGAUGGCGCUUUCAGCAA (3MM)
			uuacAUGGCGCUUUCAGCAA (4MM)

Mismatched bases in crRNA or sgRNA spacer toward target gene at the PAM distal end are shown in lower case.

Table S7. Plasmid sequence for Cas9 *in vitro* cleavage assay.

Plasmid sequences containing fully complementary target sequence (from 5' to 3')
AAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAA CCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTTGTAAAACGA CGGCCAGTCCGTCTCTATCCGGTCTCGATCCGCAGTCTCTTGGCACAGGTCTAGAGACGAGCAG AAATCTCTGCTGACGCATAAAGATGAGACGCTGGAGTACAAACGTCAGCTCATATGACCGTGCG AGTTACTGCCAACCGAGACCCAACCGAGACGGGTCATAGCTGTTTCCAGTGTGCCGCTTCCTCG CTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGG TAATACGGTTACCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGC AAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGA CGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATA CCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGAT ACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTC AGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACC GCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTG GCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGA AGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCC AGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGT GGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGA TCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAG ATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAG TATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGAT CTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGG CTTACCATCTGGCCCCAGTGCTGCAATAATACCGCGGGACCCACGCTCACCGGCTCCAGATTTAT CAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCT CCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGC AACGTTGTTGCCATCGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAG CTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGC TCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCCGTGTTATCACTCATGGTTATGGC AGCACTACATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTC AACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGG GATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCG AAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACT GATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGC CGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATT ATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAG

Only non-target (NT) strand of dsDNA is shown. PAM and target sequences are underlined. Target sequence was mutated to cACGCATAAAGATGAGACGC, ctCGCATAAAGATGAGACGC, ctgGCATAAAGATGAGACGC, or ctgcCATAAAGATGAGACGC to generate plasmids containing 1-4 mismatched bases, respectively.