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**Supplementary Table 1. Oligonucleotides used in this study.**

Name	Assay	Sequence (5' to 3')
oGS1 (dsODN Fwd)	GUIDE-seq	/5Phos/GTTTAATTGAGTTGTCATATGTTAATA ACGGT*A*T
oGS2 (dsODN Rev)		/5Phos/ATACCGTTATTAACATATGACAACCTC AATTAA*A*C
T <sub>6</sub> -RT primer	Quantify sgRNA expression	TGGAGTTCAGACGTGTGCTCTTCCGATCTTT TTTT
sgRNA Fwd		GGCCTTTTTACGGTTCCTGGCCTT
sgRNA Rev		TGGAGTTCAGACGTGTGCTCTTCC
HBB Fwd	CRISPRa qPCR	GCACGTGGATCCTGAGAACT
HBB Rev		ATTGGACAGCAAGAAAGCGAG
HBG Fwd		GCTGAGTGAACCTGCACTGTGA
HBG Rev		GAATTCTTTGCCGAAATGGA
IL1RN Fwd		GGAATCCATGGAGGGAAGAT
IL1RN Rev		TGTTCTCGCTCAGGTCAGTG
GAPDH Fwd	qPCR	GTCTCCTCTGACTTCAACAGCG
GAPDH Rev		ACCACCCTGTTGCTGTAGCCAA
DNA-Fwd	Cryo-EM	AAAACAGGTTTTTGGCTCTCAAGACCCACAAT CCAGGCCGGAA
DNA-Rev		TTCCGGCCTGGATTGTGGGTCTTGAGAGCAA AAACCTGTTTT
Full-length sgRNA	Cryo-EM and gene editing in cells	GGGAUUCGUCGGUUCAGCGACGAUAAGCC GAGAAGUGCCAAUAAAACUGUUAAGUGGU UUGGUAACGCUCGGUAAGGUAGCCAAAAG GCUGAAACUCCGUGCACAAAGACCGCACGG ACGCUUCACAUUAGCUCAUAAACAAGGG UUUGCGAGCUAGCUUGUGGAGUGUGAACN NNNNNNNNNNNNNNNNNNNNNN
sgRNA-v2	Gene editing in cells	GGGAUUCGUCGGUUCAGCGACGAUAAGCC GAGAAGUGCCAAUAAAACUGUUAAGUGGU UUGGUAACGCUCGGUAAGGUAGCCUUCGG GCAAGACCACUGAACNNNNNNNNNNNNNNNN NNNNNN

/5Phos/ indicates 5' phosphorylation. Asterisks indicate phosphorothioate linkages.

**Supplementary Table 2. Cas12f target sequences.**

<b>Target name</b>	<b>Sequence (5' to 3')</b>	<b>Assay</b>	<b>5' PAM</b>
<i>HBB</i>	GTAGCAATTTGTACTGATGG	CRISPRa	TTTA
<i>HBG</i>	CATTGAGATAGTGTGGGGAA	CRISPRa	TTTG
<i>IL1RN</i>	GTTTCTGCTAGCCTGAGTCA	CRISPRa	TTTG
<i>APOB</i>	CTGTCGACACCCAGAATCAT	DNA Cleavage	TTTG
<i>CARS</i>	CAACAGCCTCACCAGGAACA	DNA Cleavage	TTTA
<i>CLTA4</i>	CCTGGAGATGCATACTCACA	DNA Cleavage	TTTG
<i>DNMT1</i>	TGTGGCCACAAGGCTCAGTT	DNA Cleavage	TTTG
<i>HEXA</i>	AGTATACGCTTCCACAGAAA	DNA Cleavage	TTTG
<i>INIP</i>	AGAGCAGCGATTGTAAGGAG	DNA Cleavage	TTTA
<i>MRPL39</i>	ATTTACAGGACTTTGTAA	DNA Cleavage	TTTA
<i>NOTCH1</i>	GCATCAGCTGGCACTCGTCC	DNA Cleavage	TTTG
<i>PDCD1</i>	CTGTGAGCTCTAGTCCCCAC	DNA Cleavage	TTTG
<i>POLRMT</i>	AGGACTATGTGTGGCCAGTG	DNA Cleavage	TTTA
<i>PRNP</i>	TGGCCACATGGAGTGACCTG	DNA Cleavage	TTTG
<i>TP53-1</i>	AGGCATCACTGCCCCCTGAT	DNA Cleavage	TTTG
<i>TP53-2</i>	ATAAGAGGTCCCAAGACTTA	DNA Cleavage	CTTG
<i>TP53-3</i>	CTTACCTCGCTTAGTGCTCC	DNA Cleavage	CTTG
<i>TP53-4</i>	CCTCTTTCCTAGCACTGCC	DNA Cleavage	CTTG
<i>VEGFA-1</i>	CTCTCAAGACCCACAATCCA	DNA Cleavage	TTTG
<i>VEGFA-2</i>	AAGAAGGGATGTGGTGCATT	DNA Cleavage	ATTG

**Supplementary Table 3. Cas12a target sequences.**

<b>Target name</b>	<b>Sequence (5' to 3')</b>	<b>Assay</b>	<b>5' PAM</b>
<i>HBB</i>	GTAGCAATTTGTA CTGATGGTAT	CRISPRa	TTTA
<i>HBG</i>	CATTGAGATAGTGTGGGGAAGGG	CRISPRa	TTTG
<i>IL1RN</i>	GTTTCTGCTAGCCTGAGTCACCC	CRISPRa	TTTG
<i>APOB</i>	CTGTCGACACCCAGAATCATGGC	DNA Cleavage	TTTG
<i>CARS</i>	CAACAGCCTCACCAGGAACAAGG	DNA Cleavage	TTTA
<i>CLTA4</i>	CCTGGAGATGCATACTCACACAC	DNA Cleavage	TTTG
<i>DNMT1</i>	TGTGGCCACAAGGCTCAGTTCTC	DNA Cleavage	TTTG
<i>HEXA</i>	AGTATACGCTTCCACAGAAAGGA	DNA Cleavage	TTTG
<i>INIP</i>	AGAGCAGCGATTGTAAGGAGAGG	DNA Cleavage	TTTA
<i>MRPL39</i>	ATTTACAGGACTTTGTAAAGG	DNA Cleavage	TTTA
<i>NOTCH1</i>	GCATCAGCTGGCACTCGTCCACA	DNA Cleavage	TTTG
<i>PDCD1</i>	CTGTGAGCTCTAGTCCCCACTGT	DNA Cleavage	TTTG
<i>POLRMT</i>	AGGACTATGTGTGGCCAGTGAGG	DNA Cleavage	TTTA
<i>PRNP</i>	TGGCCACATGGAGTGACCTGGGC	DNA Cleavage	TTTG
<i>TP53-1</i>	AGGCATCACTGCCCCCTGATGGC	DNA Cleavage	TTTG
<i>TP53-2</i>	ATAAGAGGTCCCAAGACTTAGTA	DNA Cleavage	CTTG
<i>TP53-3</i>	CTTACCTCGCTTAGTGCTCCCTG	DNA Cleavage	CTTG
<i>TP53-4</i>	CCTCTTTCCTAGCACTGCCAAC	DNA Cleavage	CTTG
<i>VEGFA-1</i>	CTCTCAAGACCCACAATCCAGGC	DNA Cleavage	TTTG
<i>VEGFA-2</i>	AAGAAGGGATGTGGTGCATTTGG	DNA Cleavage	ATTG

**Supplementary Table 4. Cas9 target sequences.**

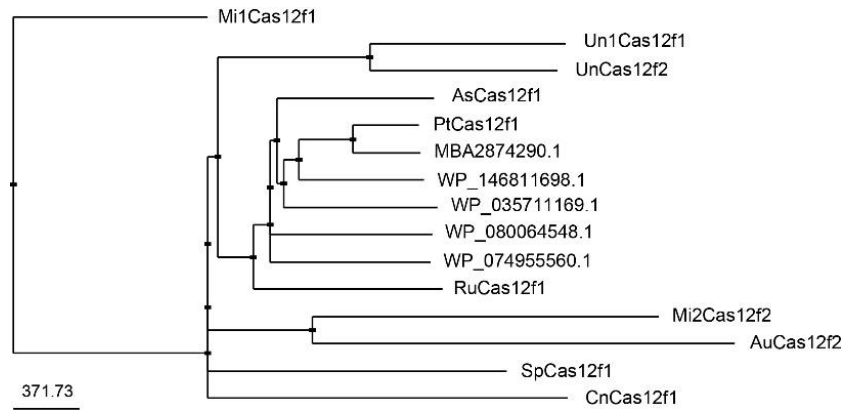
<b>Target name</b>	<b>Sequence (5' to 3')</b>	<b>Assay</b>	<b>3' PAM</b>
<i>HBB</i>	AGCAATTTGTACTGATGGTA	CRISPRa	TGG
<i>HBG</i>	CATTGAGATAGTGTGGGGAA	CRISPRa	GGG
<i>ILIRN</i>	GCTAGCCTGAGTCACCCTCC	CRISPRa	TGG
<i>CARS</i>	CAACAGCCTCACCAGGAACA	DNA Cleavage	AGG
<i>HEXA</i>	GAGTATACGCTTCCACAGAA	DNA Cleavage	AGG
<i>INIP</i>	AGAGCAGCGATTGTAAGGAG	DNA Cleavage	AGG
<i>PDCD1</i>	TGTCTTGCTGGAAAATGTGG	DNA Cleavage	AGG
<i>POLRMT</i>	AGGACTATGTGTGGCCAGTG	DNA Cleavage	AGG
<i>TP53-1</i>	ATGGCAAATGCCCAATTGC	DNA Cleavage	AGG
<i>VEGFA-1</i>	GACCCAGAGATGCATAAAAC	DNA Cleavage	AGG
<i>VEGFA-2</i>	AAGAAGGGATGTGGTGCATT	DNA Cleavage	TGG

**Supplementary Table 5. Cryo-EM data collection, refinement, and validation statistics.**

	AsCas12f-gRNA-DNA complex (EMDB-27801) (PDB 8DZJ)
<b>Data collection and processing</b>	
Magnification	81,000
Voltage (kV)	300
Electron exposure (e-/Å <sup>2</sup> )	50
Defocus range (µm)	-1.0 to -2.5
Pixel size (Å)	0.5323
Symmetry imposed	C1
Initial particle images (no.)	5,285,777
Final particle images (no.)	490,190
Map resolution (Å)	2.9
FSC threshold	0.143
Map resolution range (Å)	2.7 to 7.1
<b>Refinement</b>	
Model resolution (Å)	3.1
FSC threshold	0.5
Map sharpening <i>B</i> factor (Å <sup>2</sup> )	-78.8
Model composition	
Non-hydrogen atoms	9030
Protein residues	613
Nucleotide	190
Ligands	Zn: 1
<i>B</i> factors (Å <sup>2</sup> )	
Protein	19.0
Nucleotide	46.3
Ligand	57.1
R.m.s. deviations	
Bond lengths (Å)	0.003
Bond angles (°)	0.603
Validation	
MolProbity score	1.61
Clashscore	12.59
Poor rotamers (%)	0.38
Ramachandran plot	
Favored (%)	98.41
Allowed (%)	1.49
Disallowed (%)	0.00

**Supplementary Table 6. Numbers of potential off-target sites predicted by Cas-OFFinder for all AsCas12f targets.**

<b>Rank</b>	<b>Target site</b>	<b>Number of potential off-target sites</b>
1	<i>MRPL39</i>	1538095
2	<i>TP53-2</i>	1113028
3	<i>APOB</i>	898574
4	<i>HEXA</i>	771008
5	<i>PDCD1</i>	551945
6	<i>INIP</i>	537243
7	<i>NOTCH1</i>	505045
8	<i>PRNP</i>	503098
9	<i>CLTA4</i>	479644
10	<i>CARS</i>	461355
11	<i>POLRMT</i>	444226
12	<i>VEGFA-2</i>	404473
13	<i>VEGFA-1</i>	373137
14	<i>TP53-1</i>	348930
15	<i>DNMT1</i>	334536
16	<i>TP53-3</i>	325648
17	<i>TP53-4</i>	240052



**Supplementary Fig. 1** | Phylogenetic tree of Cas12f family proteins. Hypothetical proteins are denoted by Genbank protein accession numbers.



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AsCas12f1/1-422          1 M I ----- K V Y R Y E I V K P L ----- D L ----- 15
Un1Cas12f1/1-529       1 M A K N - T I T K T L K L R I V R P Y ----- N S A E V E K I V A D E K N N R E K I A L E K N K D K V K 47
UnCas12f2/1-500        1 M E V Q K T V M K T L S L R I L R P L ----- Y S Q E I E K E I K E E K E R R K Q A G ----- 39
PtCas12f1/1-424        1 M K Y ----- T K V M R Y Q I I K P L ----- N A ----- 17
RuCas12f1/1-440        1 M V ----- K V V K I H L I S E Q F D K A G N R I ----- 21
MBA2874290.1/1-424     1 M K Y ----- T K V M R Y Q I I K P L ----- N A ----- 17
WP_146811698.1/1-422   1 M K H ----- T K V M R Y Q I I K P L ----- N D ----- 17
WP_035711169.1/1-424   1 M K ----- K T V R L Q I V K P M ----- D E ----- 15
WP_080064548.1/1-424   1 M A ----- T K V M R Y Q I I K P L ----- D C ----- 16
WP_074955560.1/1-428   1 M K L ----- V K T M R Y Q I I K P L ----- S C ----- 17

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Un1Cas12f1/1-529       48 E A C S K H L K V A A Y C T T Q V E R N A C L F C K A R K L D D K F Y Q K L R G Q F P D A V F W Q E I S E I 101
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PtCas12f1/1-424        18 ----- E W D E L G M V 25
RuCas12f1/1-440        22 ----- D Y E E V N K I 29
MBA2874290.1/1-424     18 ----- E W D E L G M V 25
WP_146811698.1/1-422   18 ----- T W E T L G H V 25
WP_035711169.1/1-424   16 ----- D W E I L G R V 23
WP_080064548.1/1-424   17 ----- N W D L F G K V 24
WP_074955560.1/1-428   18 ----- D W D T L G T V 25

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Un1Cas12f1/1-529       102 F R Q L Q K Q A A E I Y N Q S L I E L Y Y E I F ----- I K G K --- G I A N A S S V E 138
UnCas12f2/1-500        69 L N Q L Q R E I A K V Y N H A I - S E L Y I A T ----- I A Q G N K S N K H Y I S S I V Y N R A Y 112
PtCas12f1/1-424        26 L R D I Q K E T R A A L N K T I - Q L C W E Y Q G F S A D Y K Q I H G Q Y P K P K --- D V L G Y T S M H 74
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MBA2874290.1/1-424     26 L R D I Q K E T R A A L N K T I - Q L C W E Y Q G F S A D Y K Q I H G Q Y P K P K --- D V L R Y T S M H 74
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Un1Cas12f1/1-529       139 H Y L S D V C Y T R A A E L F K N A A I A S G L R S K I K S N F R L K E L K N M K S G L P T T K S D N F P I 192
UnCas12f2/1-500        113 G Y F Y N A ----- Y I A L G I C S K V E A N F R S N E L L T Q Q S A L P T A K S D N F P I 154
PtCas12f1/1-424        75 G Y A Y D R L K N E F S K I A S S N L S Q T I K R A V D K W N S D L K E I L R G D R S I P N F R K D - C P I 127
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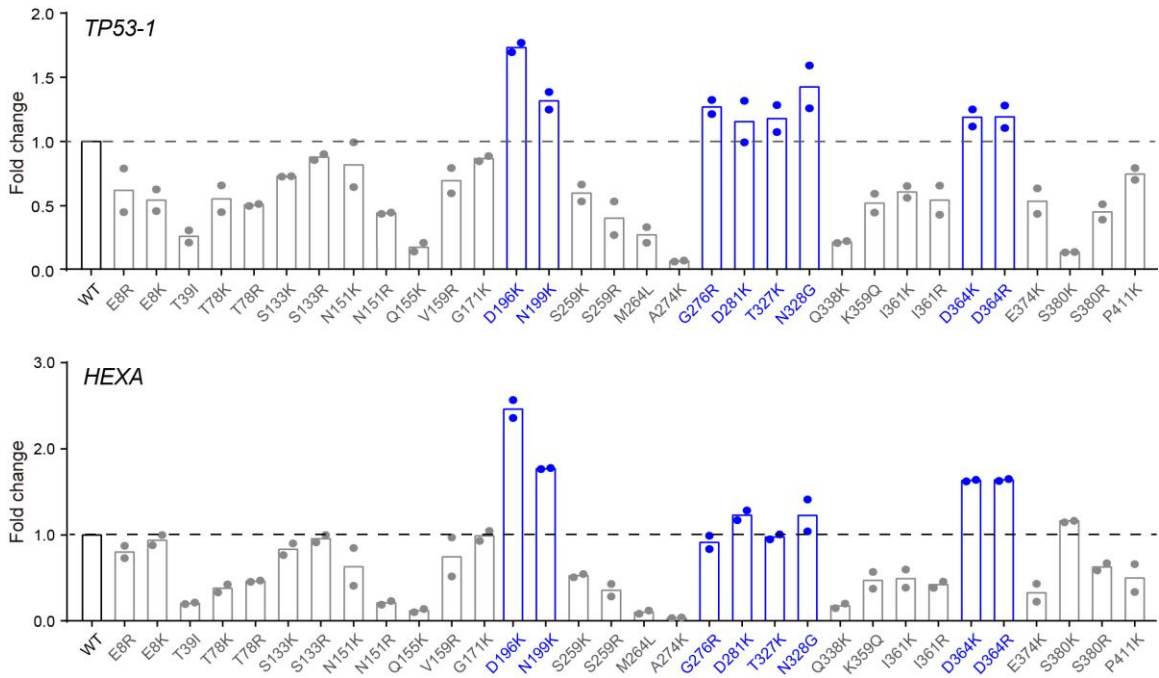
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RuCas12f1/1-440        131 D L H K D S ----- I K L I Y E - - N N E F Y V R L A L L K K A E F A K Y G F K D G - - F R F K - 170
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Un1Cas12f1/1-529       243 V Q K S P K P I - S L L L S T Q R R K R N K G W S K D E G T E A E I K K V M N G D Y Q T S Y I E V K R G S K 295
UnCas12f2/1-500        202 K K G G Q K P V L K L I L S T F R R Q R N K G W A K D E G T D A E I R K V T E G K Y Q V S Q I E I N R G K K 255
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RuCas12f1/1-440        171 ----- M Q V K D N S T K T I L E R C F D E V Y K I N A S K L L Y D - - 200
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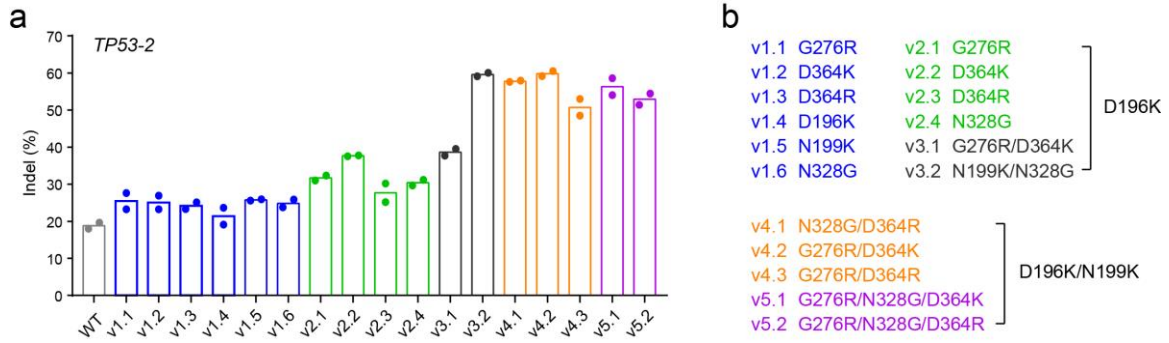
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WP_074955560.1/1-428	198	-KRNRKWFVNLAYHFEARP-EQLDKTKILGVDLGVVFPVYMAVA-DGHFRAGIP	248
AsCas12f1/1-422	247	GGEIEINFRRQVESRRISMLRQKYGAGGARGGHGRDKRIKPIEQLRDKIANFRDT	300
Un1Cas12f1/1-529	348	DNDLFHFHFKMFAARRILLKKNRH---KRAGHGAKNKLKPIITILTEKSERFRKK	398
UnCas12f2/1-500	308	SNDVFKFSKQVFAFRRLLSKNSL---KRKGHGAHAKLEPITEMTEKNDKFRKK	358
PtCas12f1/1-424	248	GGEIERFRRQVEKRRKRELLNQGKYCGDGRKGGHYATRTKSIESISDKIARFRDT	301
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AsCas12f1/1-422	301	TNHRYSRYIVDMAIKEGCGTIQMEDLTNIRDIGSRFL-----QNWTYYDLQKKI	349
Un1Cas12f1/1-529	399	LIERWACEIADFFIKNKVGTQOMENLESMKRKEDSYFNIRLGRGFWPYAEMQNKI	452
UnCas12f2/1-500	359	IIERWAKEVTNFFVKNQVGIQIJDLSMTKDRDHFNFNYLRGFWPYYQMQLTI	412
PtCas12f1/1-424	302	CNHKYSRFIVDMALKHNCGIQMEDLTGISK-ESTFL-----KNWTYYDLQKKI	349
RuCas12f1/1-440	307	TNHKYSRALIEYAVKKGCGTIQMEKLTGITSKSDRFL-----KDWTYYDLQTKI	355
MBA2874290.1/1-424	302	CNHKYSRFIVDMALKHNCGIQMEDLTGISK-ESTFL-----KNWTYYDLQKKI	349
WP_146811698.1/1-422	302	VNHRYSRYVVDMAIKHRCGTIQMEDLSGIAA-EDTFL-----KRWSYYDLQKKI	349
WP_035711169.1/1-424	303	KNHYSRYVVDMAEKHECATIQLEELKGIHQ-DDAFL-----KRWSYHDLQEKI	350
WP_080064548.1/1-424	301	INHYSRYKAVVEFAIKNGCGIQMEDLKGINT-DNVFL-----KNWTYYDLQKQV	348
WP_074955560.1/1-428	303	INHYSRYVYVETARKLGCQVIQMEDLTGIRE-ENLFL-----ANWPHYDLQRKI	350
AsCas12f1/1-422	350	IYKAE EAGIKV IKIDPQYTSQRCS E--CGNIDSGN-----RIGQAIFKCRACG	395
Un1Cas12f1/1-529	453	EFKLKQYGI ERKVPNNTSKTCSK--CGHLNNYFNFEYRKKNKFPFKCEKCN	504
UnCas12f2/1-500	413	ENKLEKYGIEVKKRVQAKYTSQLCSNPNCRYWNNYFNFEYRKKVKNKFPFKCEKCN	466
PtCas12f1/1-424	350	EYKAREAGIQVIKIEPQYTSQRCSK--CGYIDKEN-----RQEATFKCIECG	395
RuCas12f1/1-440	356	ENKAKEVGINVVYIAPKYTSQRCSK--CGYIHKDN-----RPNQAKFRCLCED	401
MBA2874290.1/1-424	350	EYKAREAGIQVIKIEPQYTSQRCSK--CGYIDKEN-----RQEATFKCIECG	395
WP_146811698.1/1-422	350	EYKAKEAGIQVYIKPDYTSQRCSK--CGHIERDN-----RTEQATFECKSCG	395
WP_035711169.1/1-424	351	TYKAE EKG IQVIKVDPPQKTSQRCHH--CGNIDSN-----RKEQASFLCTSCG	396
WP_080064548.1/1-424	349	KYKAELEGIEVKLIDPQYTSQRCSK--CGYIHRDN-----RPEQAKFKCIDCG	394
WP_074955560.1/1-428	351	EYKAREYGI EYRVPQYTSQRCS D--CGYIHPDN-----RPEQAKFRCLACG	396
AsCas12f1/1-422	396	YEANADYNAARNI AIPNIDKIIAES I-----K	422
Un1Cas12f1/1-529	505	FKENADYNAALN ISNP K LKSTKEE-----P	529
UnCas12f2/1-500	467	LEISADYNAARNLSTPDI EK FVAKATKGIN----LPE-K	500
PtCas12f1/1-424	396	FKTNADYNAARNI AIPNIDK IIRKTLKM-----Q	424
RuCas12f1/1-440	402	FESNADYNASQNI GIKNIDK IIEKDLQKQES EVQV NENK	440
MBA2874290.1/1-424	396	FKTNADYNAARNI AIPNIDK IIRKTLKM-----Q	424
WP_146811698.1/1-422	396	FKTNADFNAARNI ATKDIEKIIAETLK-----	422
WP_035711169.1/1-424	397	METNADFNAAKNISIPGIEQIIQTEMK-----S	424
WP_080064548.1/1-424	395	FEVNADYNASLNIATPDI DK ILEFLKCE-----T	424
WP_074955560.1/1-428	397	FETNADYNAARNIATEGIEELIAAALNKAS----V--V	428

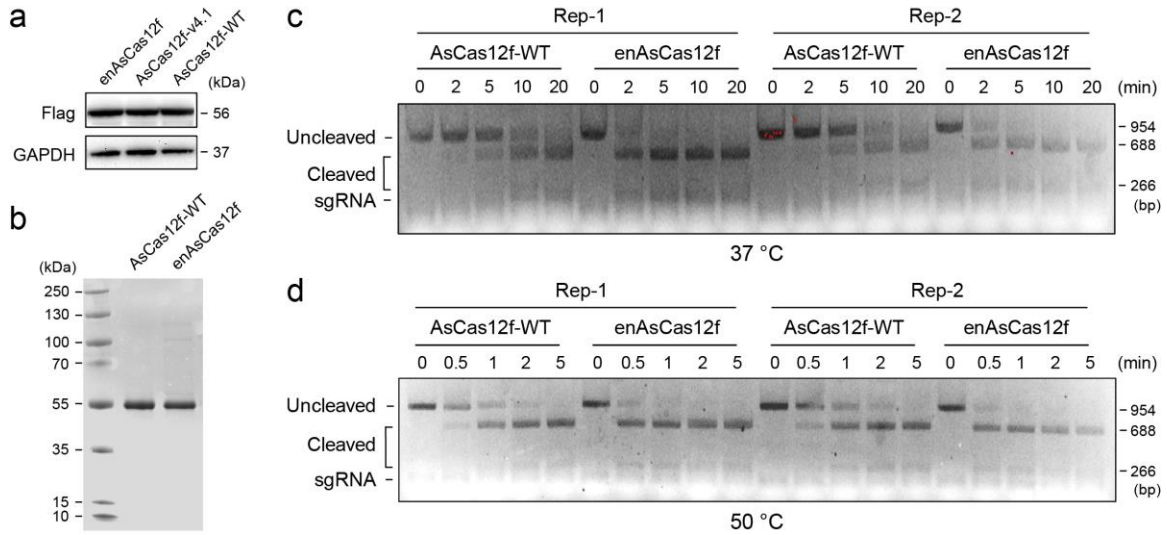
**Supplementary Fig. 2 | Full sequence alignment of AsCas12f and other Cas12f family proteins.** The alignment was performed using the T-Coffee multiple sequence alignment program.



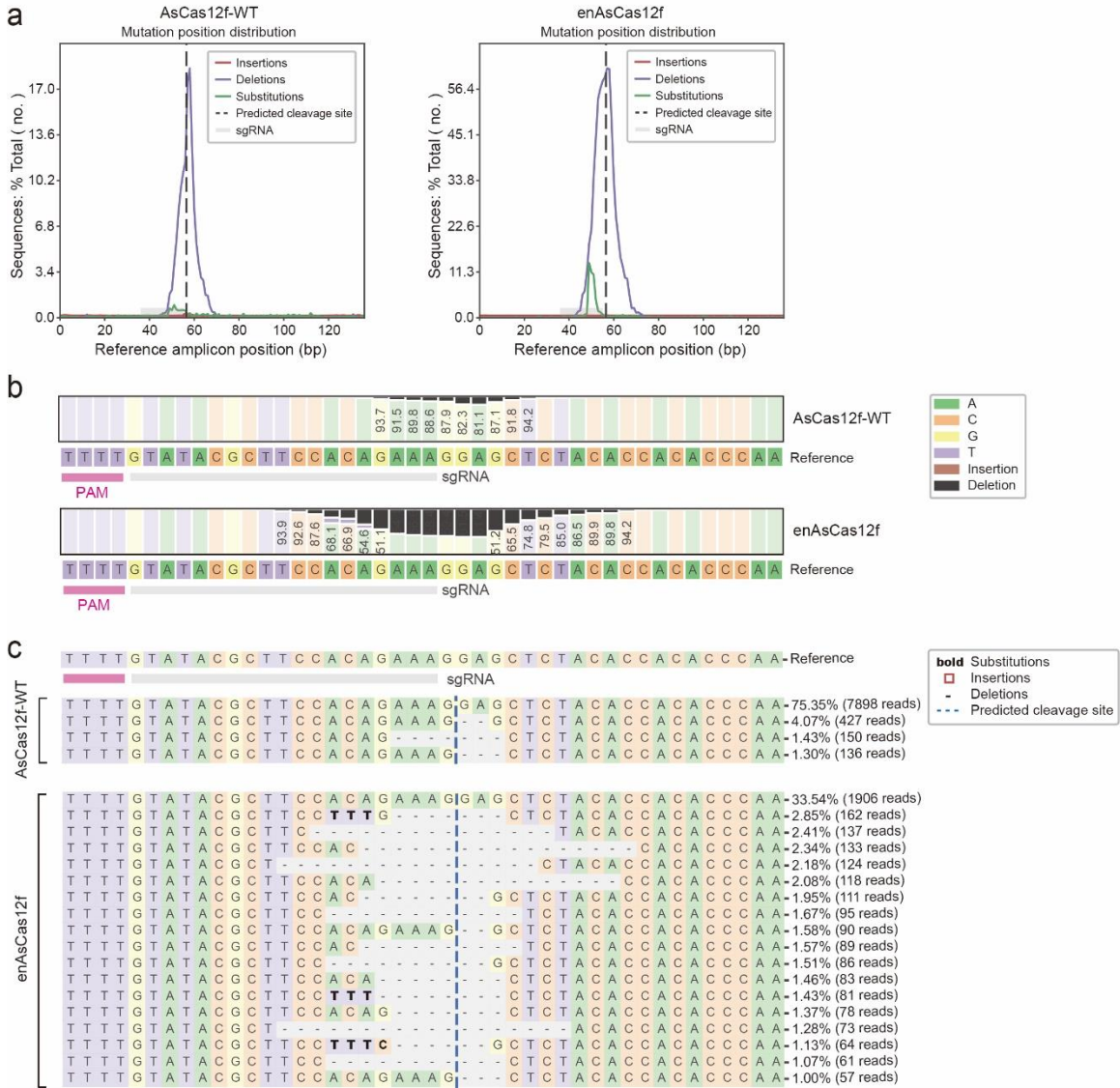
**Supplementary Fig. 3 | Gene-editing performance of AsCas12f variants that bear single-point mutations at *TP53-1* (top) and *HEXA* (bottom) loci in HEK293T cells. Fold changes are calculated relative to the indel levels mediated by the wild-type protein.**



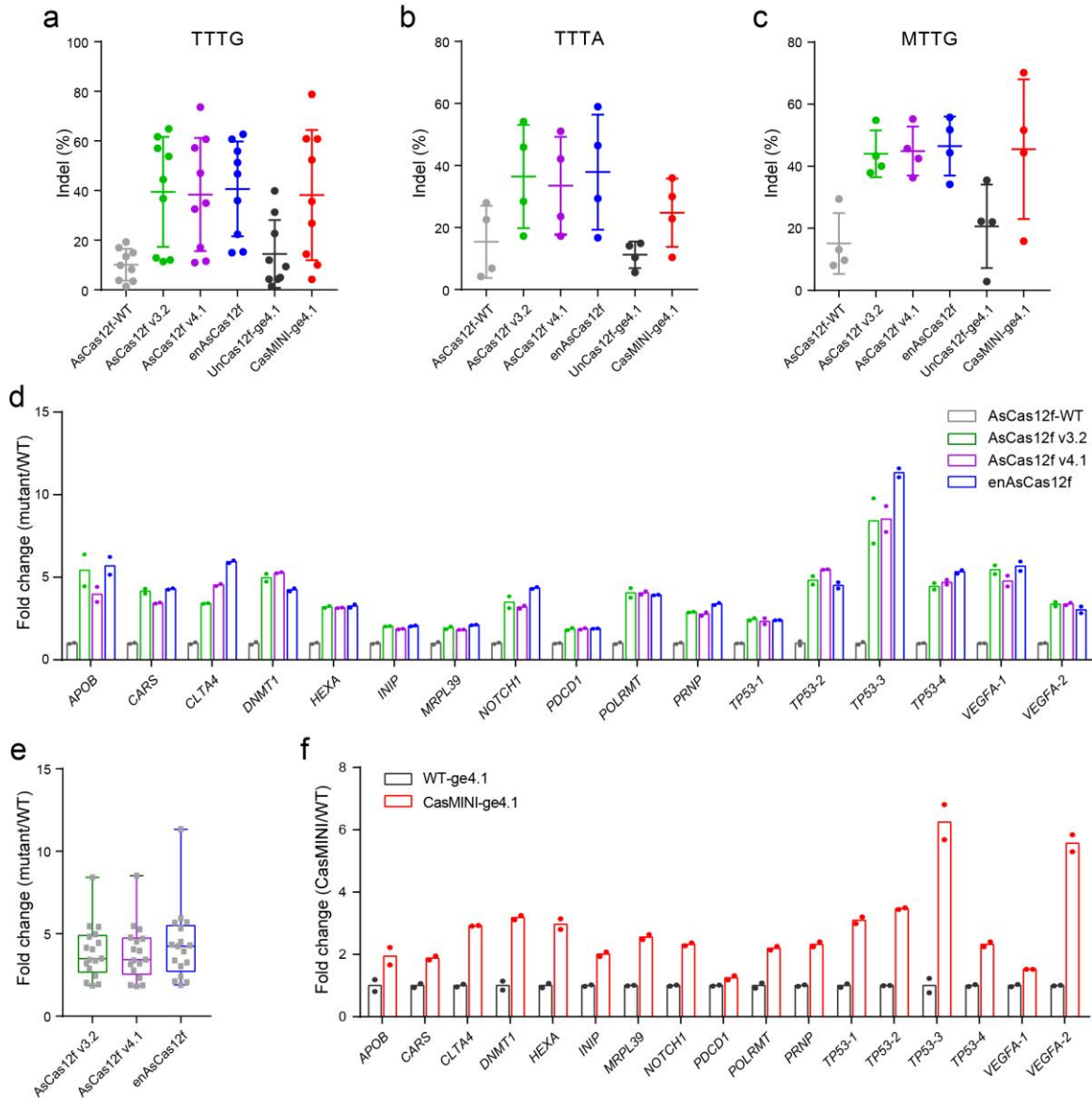
**Supplementary Fig. 4 | AsCas12f variants with increased gene-editing efficiency. a,** Indel level at the *TP53-2* locus generated by AsCas12 variants that bear one, two, three, four, or five single-point mutations. **b,** List of mutations included in each AsCas12f variant in (a) and Fig. 1 (d)-(e).



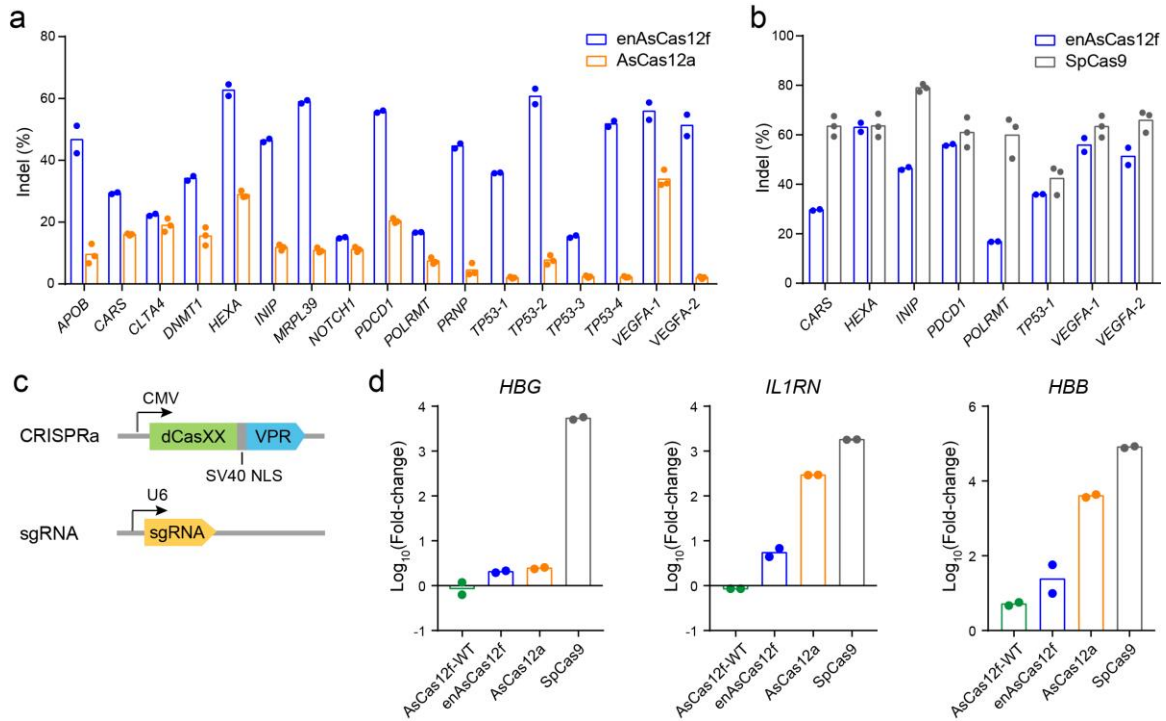
**Supplementary Fig. 5 | *In vitro* DNA cleavage by wild-type AsCas12f and enAsCas12f.**  
**a**, Western blot showing the protein level of Flag-tagged AsCas12f variants in HEK293T cells. **b**, SDS-PAGE analysis of wild-type AsCas12f and enAsCas12f proteins used for *in vitro* DNA cleavage experiments. **c**, **d**, Gel electrophoresis monitoring *in vitro* DNA cleavage by wild-type AsCas12f and enAsCas12f over time courses at 37 °C (**c**) and 50 °C (**d**). Expected DNA sizes are noted on the right of the gel images.



**Supplementary Fig. 6 | Representative deep sequencing data processed by CRISPEResso2 showing DNA-editing patterns generated by wild-type AsCas12f and enAsCas12f. a, Metaplots showing the positions of insertions, deletions, and substitutions from samples edited by wild-type AsCas12f (left) and enAsCas12f (right). b, Frequencies of insertions, deletions, and substitutions observed near the PAM sequence from samples edited by wild-type AsCas12f (top) and enAsCas12f (bottom). c, Raw sequencing reads from samples edited by wild-type AsCas12f (top) and enAsCas12f (bottom). Sequence variants with  $\geq 1\%$  of total reads are shown.**

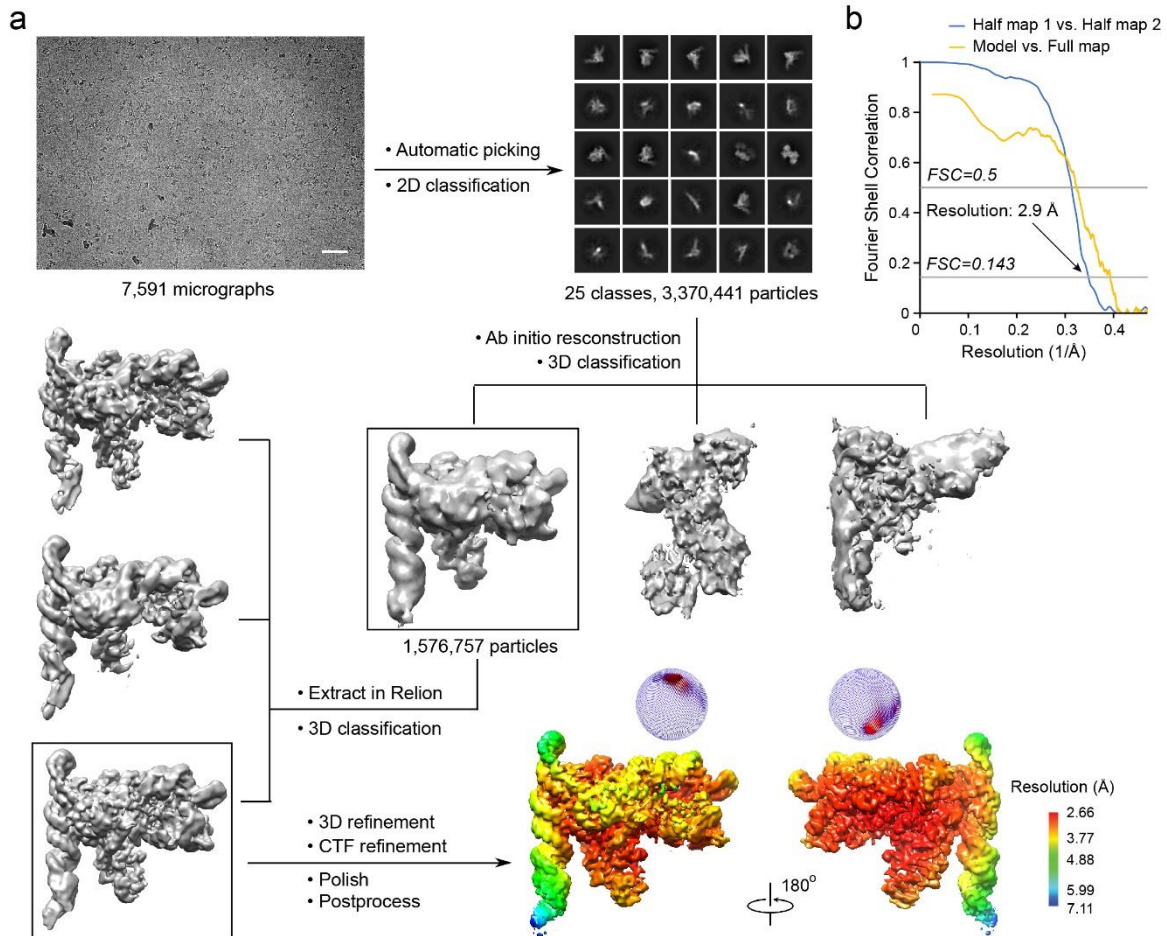


**Supplementary Fig. 7 | Performance of engineered AsCas12f across a wide range of target sites with different PAM sequences.** **a-c**, Frequencies of indels delivered by denoted AsCas12f and UnCas12f systems at sites of TTTG (**a**), TTTA (**b**), and MTTG (**c**) PAM sequences (M = A/C). n = 9 target sites for (**a**), n = 4 target sites for (**b**) and (**c**). Error bars represent mean  $\pm$ SD. **d**, Fold changes of indel frequencies generated by engineered AsCas12f variants compared to wild-type AsCas12f. **e**, Box-and-whisker plot showing fold change mediated by engineered AsCas12f variants. All data points (n = 17 target sites) were plotted, with the centerline showing the median and the whiskers showing the minimum to the maximum. The boundaries of the boxes indicate the 25th and 75th percentiles. **f**, Fold changes of indel frequencies generated by CasMINI compared to wild-type UnCas12f paired with ge4.1.

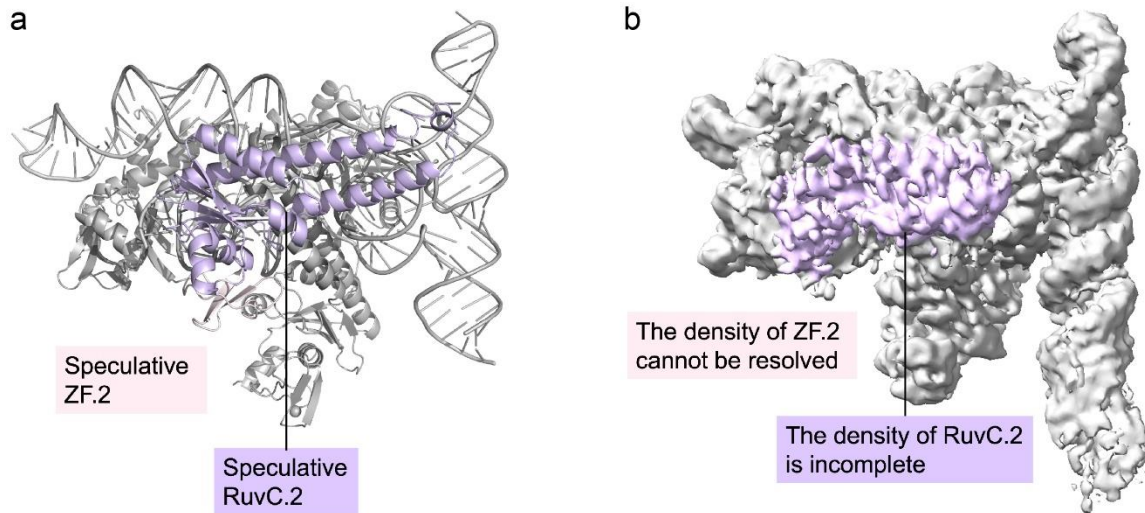


**Supplementary Fig. 8 | Comparison between enAsCas12f, AsCas12a, and SpCas9. a,** Indel frequencies mediated by enAsCas12f and AsCas12a in HEK293T cells. **b,** Indel frequencies mediated by enAsCas12f and SpCas9 in HEK293T cells. To satisfy different PAM requirements, SpCas9 sgRNAs for *PDCD1*, *TP53-1*, and *VEGFA-1* were designed to recognize sites proximal to the ones targeted by AsCas12f sgRNAs. **c,** Schematic construct design for CRISPRa mediated by different Cas proteins. **d,** Gene activation effects by different CRISPRa constructs in HEK293T cells. Transcription activation was measured by the relative RNA level of *HBG*, *IL1RN*, and *HBB* normalized to *GAPDH*. Fold changes are normalized to RNA levels in cells transfected with a non-target sgRNA.

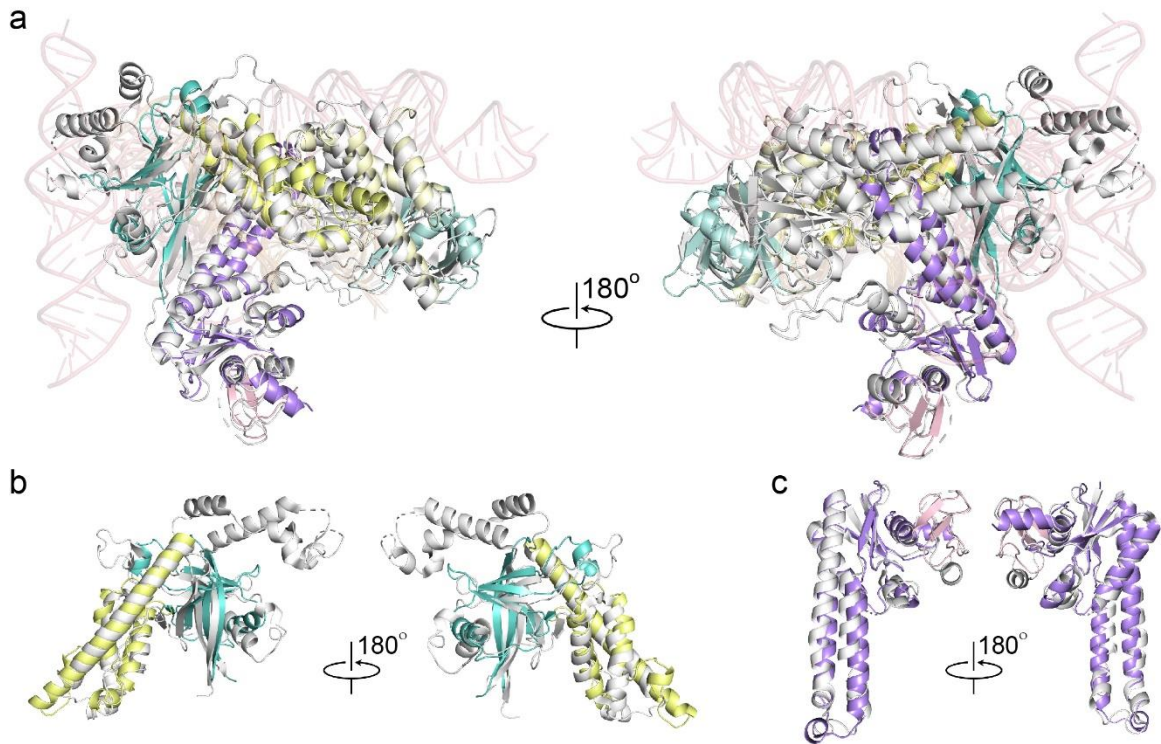




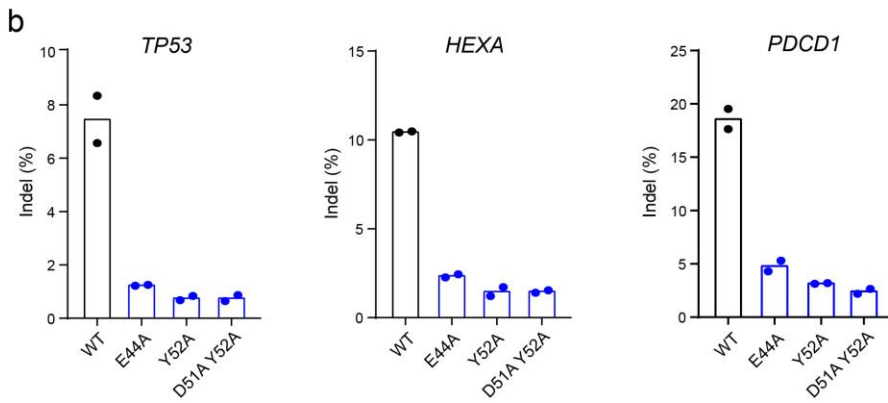
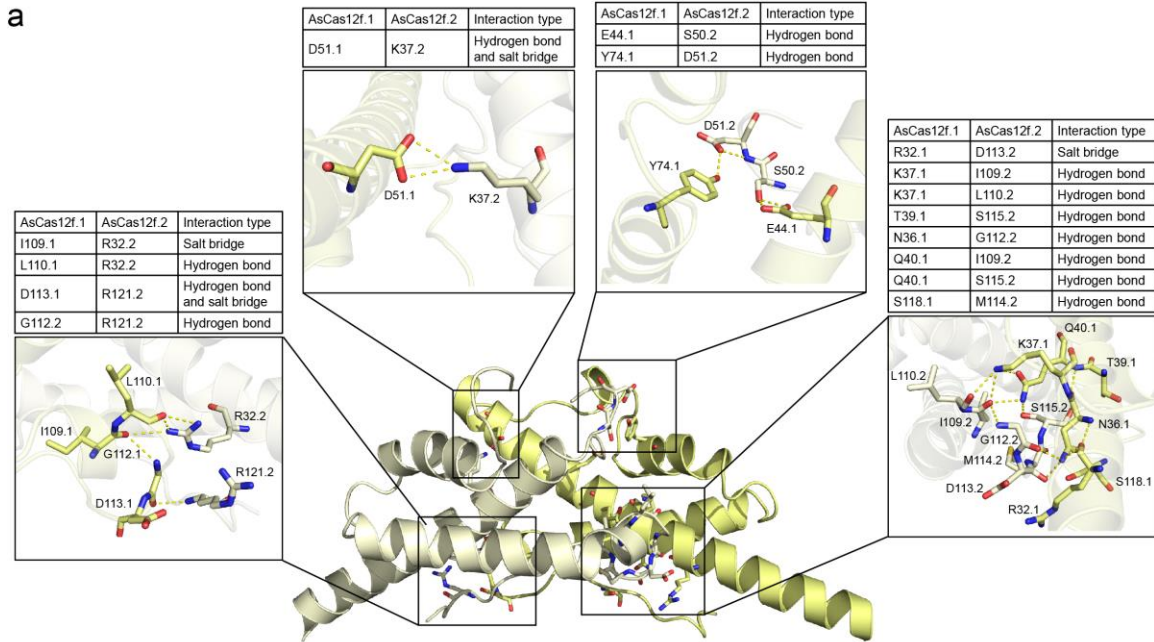
**Supplementary Fig. 9 | Single-particle cryo-EM analysis of the AsCas12f-gRNA-DNA complex.** **a**, Data processing workflow. A representative micrograph from 7,591 micrographs is shown along with a 50 nm scale bar. After the first heterogeneous refinement in CryoSPARC, the particles were exported to RELION for further processing. **b**, Fourier shell correlation curves of the half map 1 versus the half map 2 (blue), and the refined model versus the full map (yellow). The overall resolution of the reconstruction was estimated by the Fourier shell correlation (FSC) = 0.143 criterion.



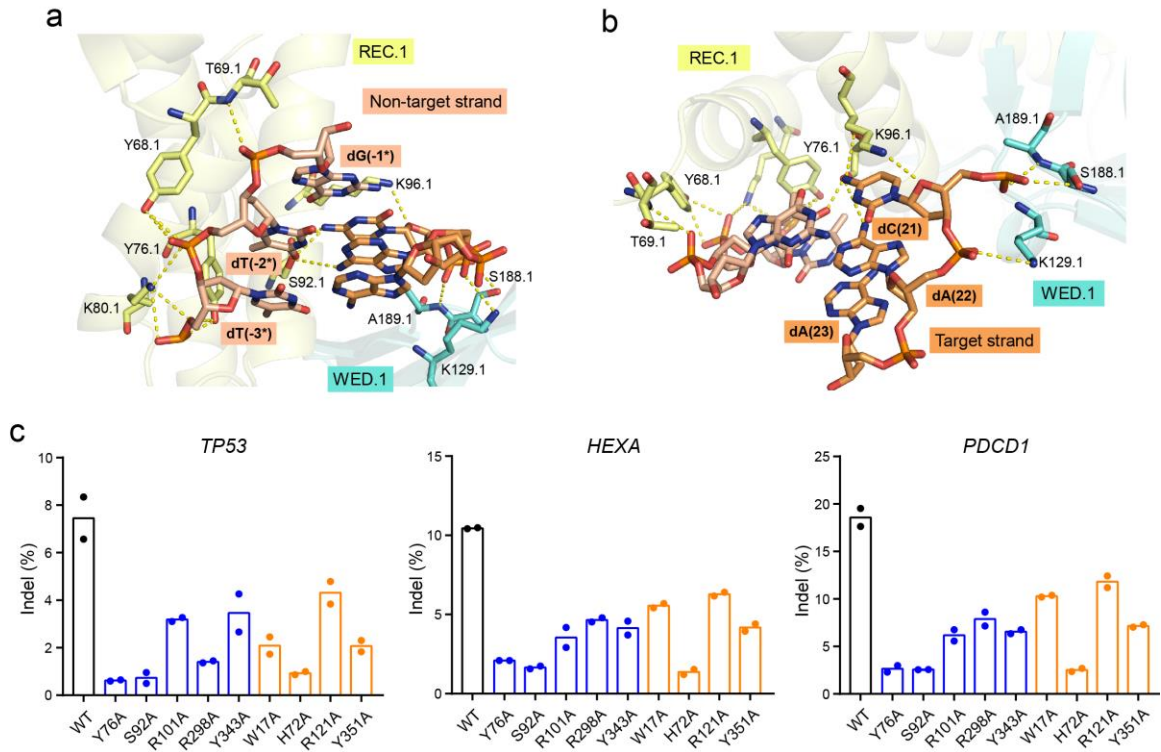
**Supplementary Fig. 10 | The C-lobe of AsCas12f.2 was not fully resolved. a,** The AsCas12f-sgRNA-DNA structure with speculative RuvC.2 and ZF.2 domains. **b,** Unsharpened AsCas12f-sgRNA-DNA cryo-EM map (contoured at a level of 0.01) with incomplete RuvC.2 domain density colored in light purple.



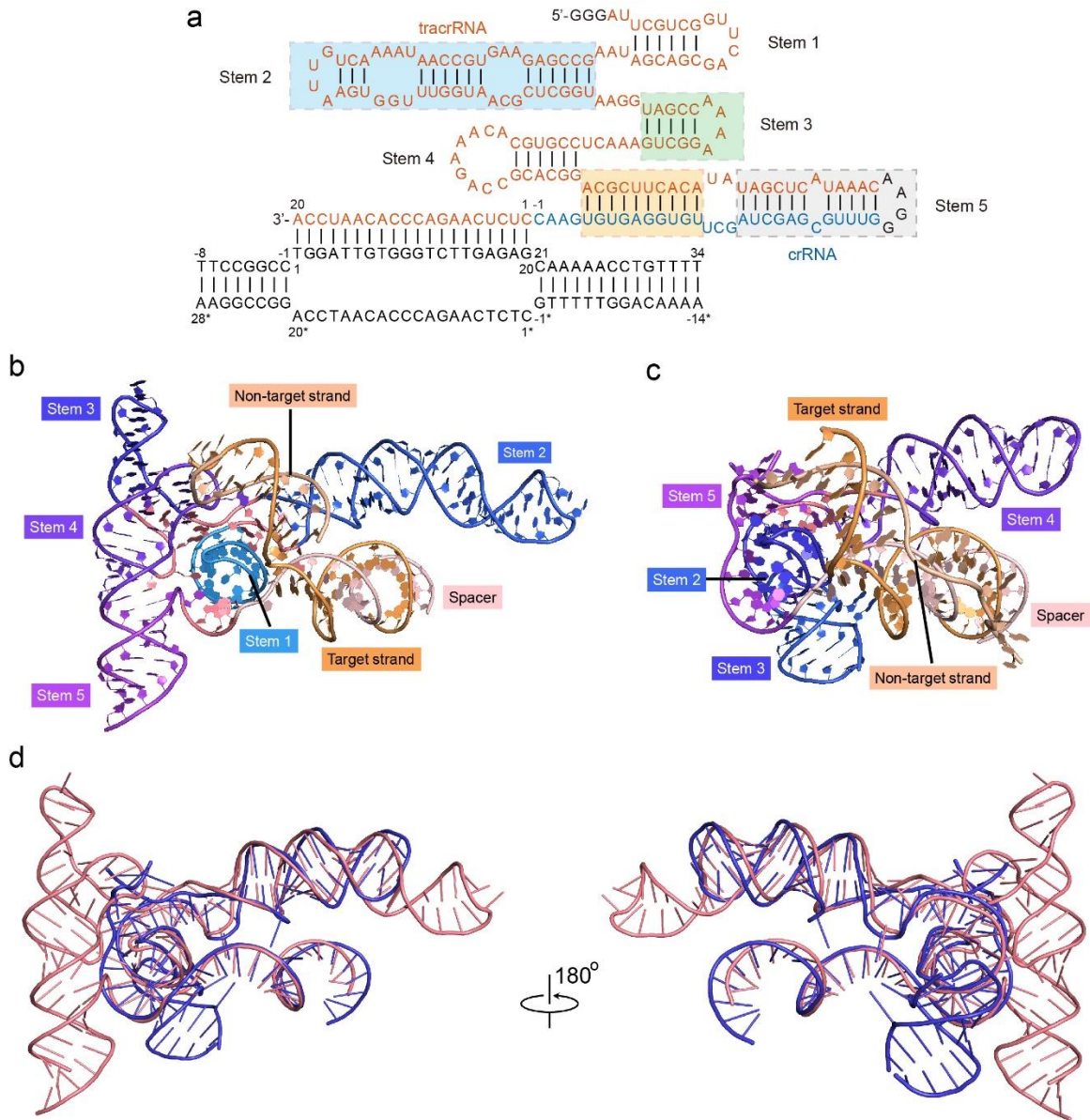
**Supplementary Fig. 11 | Comparison of the AsCas12f the UnCas12f complexes. a,** Superimposition of two complexes based on the protein part. UnCas12f is colored grey. The C-lobe of UnCas12f.2 (321 - 529) was hidden for comparison. RMSD = 6.117Å (all atom). **b,** The N-lobe of AsCas12f.1 superimposed with the N-lobe of UnCas12f.1 (grey). RMSD = 6.117 Å (all-atom). **c,** The C-lobe of AsCas12f.1 superimposed with the C-lobe of UnCas12f.1 (grey). RMSD = 2.344 Å (all-atom).



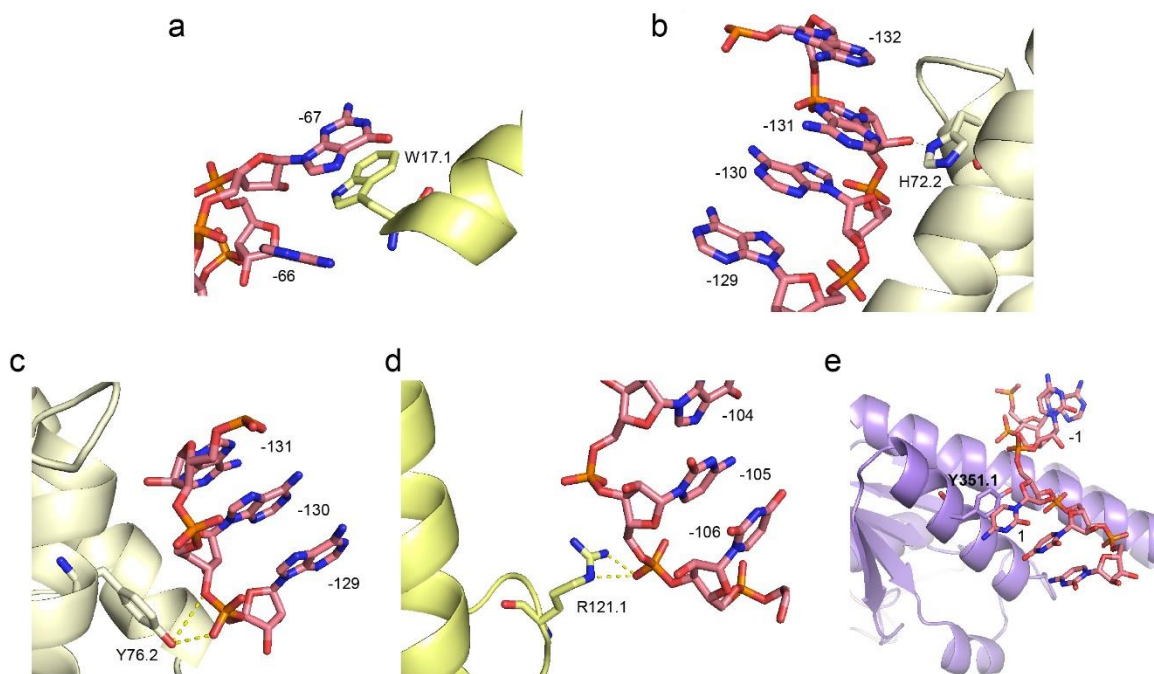
**Supplementary Fig. 12 | Dimerization is essential for the activity of AsCas12f. a,** Zoom-in views of the dimer interfaces. **b,** Indel frequencies generated by wild-type AsCas12f and AsCas12f variants bearing mutations that disrupt the dimer interfaces.



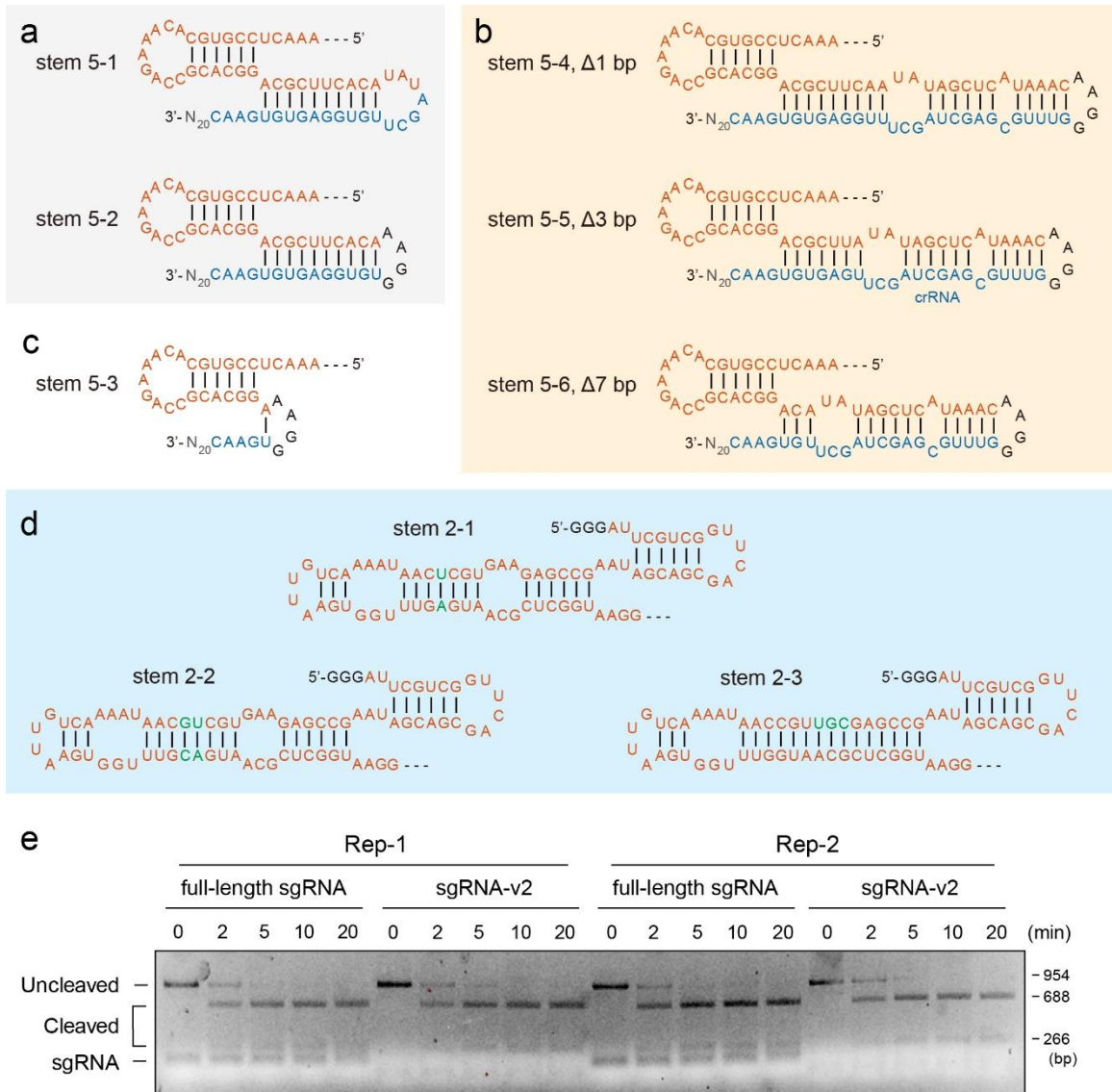
**Supplementary Fig. 13 | PAM and sgRNA recognition by AsCas12f. a, b**, Recognition of the non-target strand (**a**) and the target strand (**b**). Numberings of sgRNA and DNA are shown in Supplementary Figure 12. **c**, Indel frequencies generated by wild-type AsCas12f and AsCas12f variants bearing mutations that disrupt interactions between the protein and DNA (blue) or sgRNA (yellow).



**Supplementary Fig. 14 | Comparison of AsCas12f and UnCas12f gRNAs. a**, Secondary structure scheme of the wild-type AsCas12f sgRNA and regions interacting with the target DNA. **b-c**, Structure of the sgRNA and the target dsDNA in AsCas12f-sgRNA-DNA (**b**) and UnCas12f-sgRNA-DNA (**c**) complexes. **d**, Superimposition of UnCas12f sgRNA (violet) and AsCas12f sgRNA (red).

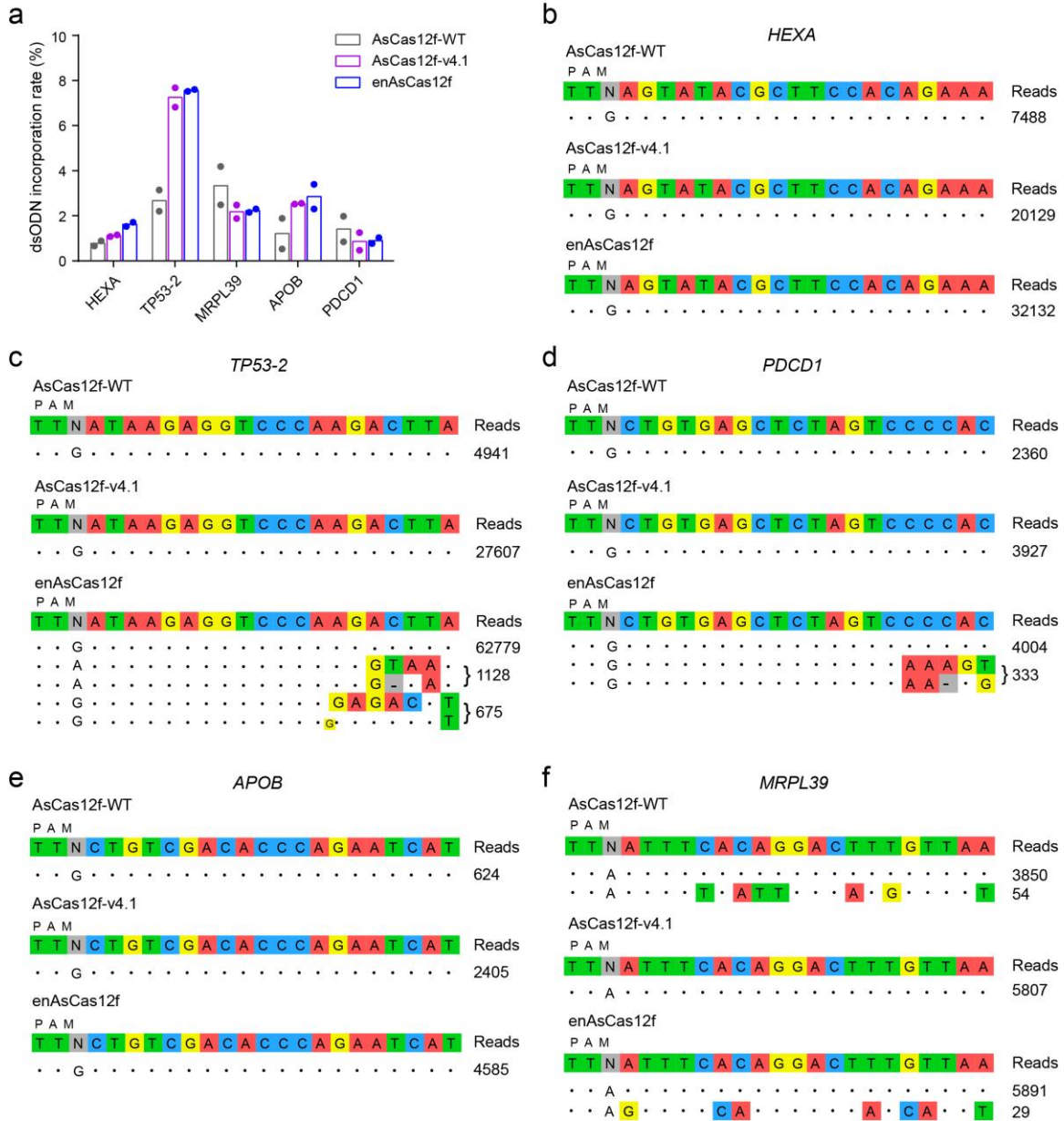


**Supplementary Fig. 15 | AsCas12f-gRNA interactions.** **a**, W17.1 forms a  $\pi - \pi$  interaction with G(-67) of the gRNA. **b**, H72.2 forms a hydrogen bond with A(-131) at  $O^2$  position. **c**, Y76.2 forms a hydrogen bond with the phosphate backbone of A(-129) of the gRNA. **d**, R121.1 forms a hydrogen bond with the phosphate backbone of C(-105) of the gRNA. **e**, Y351.1 forms a  $\pi - \pi$  interaction with C(1) of the gRNA.

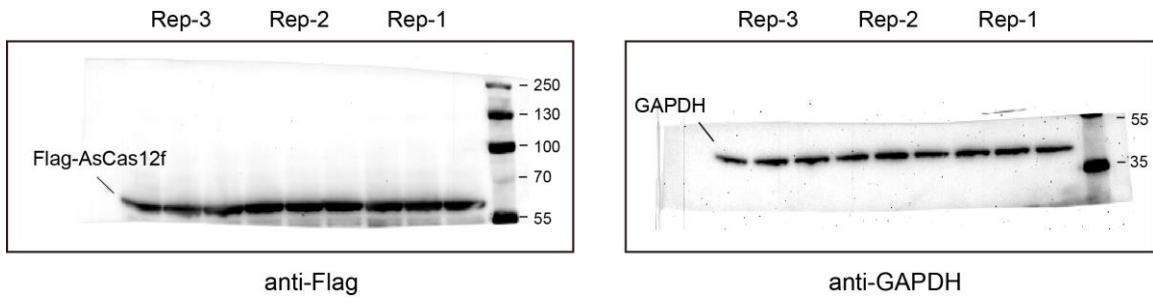


**Supplementary Fig. 16 | Schematics showing modifications on the AsCas12f gRNA. a-c,** Truncations of stem 5 (colored in the grey and yellow boxes in Supplementary Fig. 12a). **d,** Modifications of stem 3 (colored in the blue box in Supplementary Fig. 12a). **e,** Gel electrophoresis monitoring *in vitro* DNA cleavage over time courses using full-length sgRNA and sgRNA-v2. The assay was conducted using enAsCas12f at 37 °C. Expected DNA sizes are noted on the right of the gel images.





**Supplementary Fig. 17 | Genome-wide specificity of wild-type and engineered AsCas12f variants.** **a**, On-target GUIDE-seq tag integration efficiency measured by targeted amplicon sequencing. **b-f**, Off-target editing sites for wild-type AsCas12f, AsCas12f-v4.1, and enAsCas12f with gRNAs targeting *HEXA* (**b**), *TP53-2* (**c**), *PDCD1* (**d**), *APOB* (**e**), and *MRPL39* (**f**) loci, reported by GUIDE-seq in HEK293T cells. Mismatch positions are highlighted in colors. GUIDE-seq experiments were performed in duplicates, with the read counts of one replicate shown to the right of the corresponding sequences. Results from the other replicate are shown in Fig. 5. Full-length sgRNAs were applied in all GUIDE-seq experiments. In (f), the two off-target integrations detected in AsCas12f-WT and enAsCas12f samples are regarded as false positives because they are not detected in the other replicate nor other conditions for the same target.



**Supplementary Fig. 18 | Uncropped scans for blots shown in Supplementary Fig. 5a.**