# A tryptophan 'gate' in the CRISPR-Cas3 nuclease controls ssDNA entry into the nuclease site, that when removed results in nuclease hyperactivity.

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#### **Supplementary materials**

#### Strains and plasmids

The *E. coli* K-12 strains used in this study are described in the **Table S1**. Plasmids used were: pKOV (Link et al. 1997), pAH4 (*cas3* cloned into Bad-HisA using XhoI and EcoRI), pIIB39 (mutagenized pAH4 in W406A residue) using primers listed in Table 2 (oligos), pEB526 (Cas3 cloned in pUC19) (1). Plasmid pCas3 is described in (2).



**Figure S1. Fork DNA substrate.** DNA oligos MW12 and MW14 were annealed to form a DNA fork substrate with 25 base pairs double-stranded region and extended two single-stranded 25 nt arms. A Cy5 fluorescent dye oligonucleotide labelling was incorporated at 5'-end of MW12 that has a maximal absorbance at 646 nm.



**Figure S2. CD monitoring of wild type Cas3 thermal denaturation. (i).** Replica1. CD monitoring of Cas3 thermal denaturation. Changes at 222 nm in temperature range 30-55 °C presented as mean residue ellipticity (MRE) *vs* Temperature ([Q]<sub>222</sub>*vs*T): • experimental data — Boltzmann fit of experimental data; Boltzmann sigmoid value x0=34.68 °C; IΔMRE<sub>exp</sub>I=IMRE<sub>30</sub>-MRE<sub>55</sub>I=1056.56

degcm<sup>2</sup>dmol<sup>-1</sup>, 19.2 %  $\downarrow$ . (ii). Replica2. CD monitoring of Cas3 thermal denaturation. Changes at 222 nm in temperature range 20-55 °C presented as mean residue ellipticity (MRE) *vs* Temperature ([Q]<sub>222</sub>*vs*T): • experimental data — Boltzmann fit of experimental data; Boltzmann sigmoid value x0=35.27 °C; I $\Delta$ MREexpl=IMRE<sub>20</sub>-MRE<sub>55</sub>I= 1552.74 degcm<sup>2</sup>dmol<sup>-1</sup>, 20.2 %  $\downarrow$ . (iii). Replica3. CD monitoring of Cas3 thermal denaturation. Changes at 222 nm in temperature range 30-55 °C presented as mean residue ellipticity (MRE) *vs* Temperature ([Q]<sub>222</sub>*vs*T): • experimental data — Boltzmann fit of experimental data; Boltzmann sigmoid value x0=34.29 °C; I $\Delta$ MREexpl=IMRE<sub>30</sub>-MRE<sub>55</sub>I= 771.81 degcm<sup>2</sup>dmol<sup>-1</sup>, 10.1 %  $\downarrow$ .



**Figure S3. EMSAs showing wild-type Cas3 and Cas3**<sup>W406A</sup> **DNA-protein complexes.** EMSAs show that wild-type Cas3 and Cas3<sup>W406A</sup> form stable DNA-protein complex (panels i and ii), but other mutant proteins do not (panels iii, iv and v). Increasing concentrations of Cas3 and mutant proteins (0, 0.4 0.8, 1.6 and 3.3  $\mu$ M) were incubated with DNA fork (20 nM). Stable DNA-protein complex is indicated.



**Figure S4. CD monitoring of Cas3**<sup>W406A</sup> **thermal denaturation.** Changes at 222 nm in temperature range 30-55 °C presented as mean residue ellipticity (MRE) vs Temperature ([Q]<sub>222</sub>vsT): • experimental data — Boltzmann fit of experimental data; Boltzmann sigmoid value x0=37.20 °C; I $\Delta$ MREexpI=IMRE<sub>30</sub>-MRE<sub>55</sub>I=1730.4 degcm<sup>2</sup>dmol<sup>-1</sup>, 14.0 % $\downarrow$ .



Figure S5. The distances between the centre of phenyl rings of Trp-230 and Trp-406 and the most prominent interactions that Trp-406 forms with the aliphatic part of sidechains of residues Val-415, Gln-426 and Arg-440 when entering a hydrophobic pocket at higher temperatures. The data shown

is from simulations of wild-type Cas3 protein complex with Mg<sup>2+</sup> and ATP at 28 °C, 30 °C, 37 °C, and 44 °C. **A.** The distance between the sidechains of residues Trp-230 and Trp-406. **B.** The distance between the sidechains of residues Trp-406 and Val-415. **C.** The distance between the sidechain of Trp-406 and the aliphatic part of Gln-426 sidechain. **D.** The distance between the sidechain of Trp-406 and the aliphatic part of Arg-440 sidechain. **E.** The radius of gyration for the sidechains of Trp-406 and Val415 and the aliphatic part of Gln-425 and Arg-440 sidechains.

Α.	Model/1-888	1	ME <mark>P</mark> FKY <mark>ICHYWGK</mark> SSKSLTK <mark>G</mark> NDIHLLIYHCLDVAAVADCWWDQSVV LQNTF	52
	4Q2C/1-914	1	D <mark>PWIFWAKWG</mark> S <mark>G P</mark> DL <mark>GWHPLLCHMLDVAAV</mark> TLQMWRRVL <mark>P</mark> AAWKARI	47
	Model/1-888	53	C R N E M L S K Q R V K A W L L F F I A L H D I G K F D I R F Q Y K S A E S W L - K L N P A T P S L N G P S	105
	4Q2C/1-914	48	S G V L G V G Q E D A E R W L A F F A G G H D I G K A S P A F Q L Q L R P E Q G R E L V A R R L R D A G L P	101
	Model/1-888	106	T QMC R <mark>K F N H G</mark> A A <mark>G L</mark> YWF N Q D S L S E Q S L <mark>G</mark> D F F S F F D A A <mark>P H P</mark> Y E <mark>S</mark> WF PWV E A V T G H	159
	4Q2C/1-914	102	- L F N A <mark>R A P H G</mark> T I S A N V L E T V L A D V F G L S <mark>G</mark> R <mark>S</mark> A R WV A F A V G G H	142
	Model/1-888	160	HGFILHSQDQDKSRWEMPASLASYAAQDKQAREEWISVLEALFLTPAGLSINDI	213
	4Q2C/1-914	143	HGFVPSYDEVRRDL-D-QQAVGWGMWDAAREVLLCRLADALGLPGSSRPTVE	192
	Model/1-888	214	<mark>P P</mark> D C S S L L A <mark>G F C S L A DWL G S</mark> WT T T N T F L F N E D A <mark>P</mark> S D I NA L R T <mark>Y F Q</mark> D R Q Q D A S	265
	4Q2C/1-914	193	S T <mark>P</mark> D A F M L A G L V S V A DW I G S N E E Y F <mark>P Y</mark> A A Q S A L Q V <mark>P</mark> Q L D A E A Y L E R A M R Q A E	244
	Model/1-888	266	RVLELSGLVSNK RCYE <mark>G</mark> VHALLDNGY <mark>Q</mark> PRQLQVLVDALP VAPGLTVIEA	314
	4Q2C/1-914	245	RAMASLGWVGW <mark>RP</mark> ASGSMRLTELFPYIRQPTTVQAAAEELAGEVKS <mark>P</mark> SITIIEA	298
	Model/1-888	315	P T G S G K T E T A L A YAWK L I D Q Q I A D S V I F A L P T Q A T A N AML T R ME A S A S H L F S S -	367
	4Q2C/1-914	299	P M G E G K T E A AML L A D T F S T A H G M S G C Y F A L P T MA T S N Q M F G R V T D Y L R H R Y P E D	352
	Model/1-888	368	- PNLILAHGNSRFNHLFQSIKSRAITEQGQEEAWVQCCQWLSQ	409
	4Q2C/1-914	353	VVVVNLVHGHSDLSALLQELRQKGEEIFQLQGVYDEALGDEQLGAVVAGQWFTR	406
	Model/1-888	410	S N <mark>KK</mark> VFLGQIGVC <mark>TIDQ</mark> VLISVLPVKHRFI <mark>R</mark> GLGIGRSVLIVDEVHAYDTYMNG	463
	4Q2C/1-914	407	- GKRALLPPYGVGTVDQALLAVLQVKHVFVRLFALSTKTVIVDEVHAYDVYMTT	459
	Model/1-888	464	L L E A Y L K A Q A D Y G G S Y I L L <mark>S A T L P</mark> MK Q <mark>K</mark> Q K L L D T Y G L H T D <mark>P</mark> V E NNS A Y P L	513
	4Q2C/1-914	460	L L H R L L E W L G A L S V <mark>P V V V L S A T L P</mark> S A R <mark>R</mark> R E L V K A Y A R G A G WQ A E R D L <mark>P P</mark> A G Y P R	513
	Model/1-888	514	I NWR <mark>G</mark> V NGA Q R F D L L A H P E Q L P P R F S I Q P E P I C L A DML P D L TML E RMI A A A N A G	567
	4Q2C/1-914	514	I T Y A A A E D V R G I H F A P - S E A S R R K V A L RW V S A P E H E A L G Q L L A E A L S Q G	561
	Model/1-888	568	AQVCLICNLVDVAQVCYQRLKELNNTQVDIDLFHARFTLNDRREKENRV	616
	4Q2C/1-914	562	GCAAIICNTVPRAQALYSALREVFPGLAEDGMPELDLLHARYPYEEREVREART	615
	Model/1-888	617	I S N F <mark>G K N G K R</mark> N V <mark>G</mark> R I L V A <mark>T Q</mark> V V <mark>E Q S</mark> L D V D F DWL I T Q H C P A D L L F <mark>Q R</mark> L G R L H R H H	670
	4Q2C/1-914	616	L <mark>G</mark> R F S <mark>R N G R R P H</mark> R A I L V A T Q V I E Q S L D L D F D L M V T D L A P V D L V L Q R M G R L H R H P	669
	Model/1-888	671	RK <mark>Y R P</mark> AGFE I PVATILL PDGEG <mark>YG</mark> - RHEH I YS NV RVMWRTQQH I EEL N	717
	4Q2C/1-914	670	VHD <mark>PLRP</mark> ERLRS <mark>PELWVV</mark> SPQVMGDV <mark>P</mark> IFDRGSASVYDEH - TLLRSWLALRDR -	721
	Model/1-888	718	GA S L F F P D A Y R QWL D S I Y D D A E MD E P EWV G N G MD K F E S A E C E K R F K A R	765
	4Q2C/1-914	722	- D T L Q L P E D I E E L V E Q V Y S D G R V P Q G A S E E L R S L W E R T F K A Q Q K V L R E D S L Q A K	774
	Model/1-888	766	K V L QWA E E Y S L <mark>QD</mark> ND E T I L A V <mark>T R</mark> D <mark>G E MS</mark> L P L L P Y V Q T S S G K	806
	4Q2C/1-914	775	Y R <mark>Y</mark> I K <mark>G P G</mark> Y N S I W <mark>G I V</mark> T A S V E <mark>E D</mark> A P E L H <mark>P</mark> A L QA L <b>T R</b> L A <mark>E P S V</mark> S A V C L V A <mark>G S G G P</mark>	828
	Model/1-888	807	QLLDGQVY EDLSHEQQ <mark>YEALALNRVNVP</mark> FT - WKRSFSEV VD - EDGLLWLE	854
	4Q2C/1-914	829	CLPDGTPVDLDT <mark>PP</mark> DAAMAERLLRRSVAITDARVLD <mark>P</mark> LLDVPVPKGWERSSLLR	882
	Model/1-888	855	<mark>G</mark> KQNLD <mark>GWVWQG</mark> NSIVITYTGDEGMTRVIPAN <mark>P</mark> K	888
	4Q2C/1-914	883	GYR <mark>P</mark> LVFD <mark>ASG-RAMVGRWIVRIDPELGI</mark> VVES	914

в.	Model/1-888 4QQW/1-929	1 1	ME <mark>P</mark> FK <mark>Y</mark> I <mark>PL</mark> D	C H <mark>YWG K</mark> L R FWA <mark>K</mark>	S S K S L E R <mark>G</mark>	T K <mark>G</mark> N D - L R <mark>G</mark> K	HLLI T <mark>YP</mark> LV	YHCLD CHSLD		CWWD <mark>Q</mark> SV /LWN <mark>E</mark> YL	V <mark>L</mark> QN T S <mark>PGL</mark> RD T	FC 53 IA 48
	Model/1-888 4QQW/1-929	54 49	RNEMLSK SSMETDE	QRVKAV EHA <mark>GH</mark> C	VLLFFI CIAFWA	A L HD I <mark>G L HD</mark> I	GKFDI GKLTR	R F Q Y K S E F Q Q Q	S A E S W L H	K <mark>LNP</mark> AT <mark>P</mark> A <mark>YP</mark>	S L N <mark>G P</mark> S T <mark>G</mark> E E -	QM 108 L S 95
	Model/1-888 4QQW/1-929	109 96	C R K F N <mark>H G</mark> G E Q R S <mark>H</mark> A	A A <mark>G</mark> L YV A A T <mark>G</mark> KV	VF NQD S VL <mark>P</mark> F A L	L S E Q S - <mark>P</mark> S L <mark>G</mark>	LGDFF YP	S F F D A /	A <mark>P</mark> HPYES N- <mark>GG</mark> LN	SWF <mark>PWVE</mark> /T <mark>GLVAQ</mark>	AVT <mark>GHHG</mark> ML <mark>GGHHG</mark>	F I 163 T F 139
	Model/1-888 4QQW/1-929	164 140	<mark>L H</mark> S QDQD <mark>H P</mark> H <mark>P</mark> S F Q	K S <mark>R</mark> WEN S - <mark>R</mark> N P L	I <mark>P</mark> ASLA - AEF <mark>G</mark>	S Y A <mark>A</mark> Q F S S <mark>P</mark> H	QDKQA <mark>R</mark> IWEKQ <mark>R</mark>	E E <mark>W I</mark> S HA L L H	V E A L F I V F D A T C	TPAGLS RPTPP-	I ND I <mark>P P</mark> D - DML D <mark>G P</mark>	CS 218 TA 190
	Model/1-888 4QQW/1-929	219 191	<mark>S</mark> LLA <mark>G</mark> FC SVVC <mark>G</mark> LV	S L A <mark>D</mark> WL I L A <mark>D</mark> WL	<mark>G</mark> SWTT V <mark>S</mark> QED	T N T F L F L L	F N E D A E R L T S	<mark>P</mark> SI L <mark>P</mark> AD <mark>G</mark>	DINAL <mark>R</mark> SASALR	TYFQDRQ HF <mark>E</mark> TSL	QD <mark>A</mark> S R <mark>V L</mark> R R <mark>I P</mark> S L L	EL 270 DA 243
	Model/1-888 4QQW/1-929	271 244	S <mark>GL</mark> VSNK A <mark>GL</mark> R <mark>P</mark> IT	R <mark>CY</mark> E <mark>G</mark> V V <mark>PP</mark> ATF	HALLD TES <mark>F</mark> P	N <mark>G</mark> YQ <mark>P</mark> HLSK <mark>P</mark>	RQLQV N <mark>GLQ</mark> A	L V D A L S L A K H	PCLCT	A <mark>PGLTVI</mark> SPGLVLI	E <mark>A P</mark> T <mark>G</mark> S <mark>G</mark> T <mark>A P</mark> M <mark>G</mark> E G	KT 321 KT 298
	Model/1-888 4QQW/1-929	322 299	E T A L A Y A E A A Y H V A	WK <mark>L</mark> IDO DL <mark>LG</mark> KA	QQIADS T <mark>G</mark> R <mark>PG</mark>	V I F A L R F L A L	PTQAT PTMAT	A NAML A DQMH	RMEAS RLKEY	SHLFS- RYRVEN	<mark>SP</mark> TDL <mark>P</mark> R <mark>S</mark> S	NL 370 TL 353
	Model/1-888 4QQW/1-929	371 354	ILA <mark>HG</mark> NS ALL <mark>H</mark> SMA	R <mark>F N H</mark> L F W L N P D Y	QSIKS A <mark>P</mark> ADL	RAI PGVSK	- T E Q <mark>G</mark> V L S N L	Q E E A W G H R D P	QCCQWI	SQSN <mark>KK</mark> M <mark>G</mark> -R <mark>KR</mark>	V F L <mark>G</mark> Q I <mark>G</mark> G L L A <mark>PW</mark> A	VC 422 VG 407
	Model/1-888 4QQW/1-929	423 408	TIDQVLI TIDQALM	SVL <mark>P</mark> VK AVLRAK	HRFIR HNALR	<mark>g l g l g</mark> l <mark>f g l</mark> a	R S V L I G K V V V	V <mark>DE</mark> VH/ V <mark>DE</mark> AH/	A Y D T YMI A V D P YMO	N <mark>GLLEAV</mark> QVLLEQL	L <mark>K A</mark> Q A D V L <mark>R</mark> WL <mark>G</mark> T L	<mark>GG</mark> 477 DV 462
	Model/1-888 4QQW/1-929	478 463	SVILL <mark>S</mark> A PVVLL <mark>S</mark> A	<mark>T L P</mark> MK ( <mark>T L HH</mark> S I	QK QK L L A N S L V	D T <mark>Y G</mark> L K A <mark>Y</mark> L E	HTD <mark>G</mark> AR <mark>G</mark> R	RWN R S	- <mark>P</mark> - <mark>V</mark> E N I E <mark>P</mark> QPV S I	N S A <mark>Y P</mark> L I E V S <mark>Y P G</mark> W	NWR <mark>GV</mark> N <mark>G</mark> LHVDARI	AQ 523 GK 517
	Model/1-888 4QQW/1-929	524 518	R F V T R S S D V	DLLA <mark>H</mark> F DPLPIA	P E Q <mark>L P P</mark> T T <mark>P</mark> R K	R F S I Q P L E V R	PEPIC LVDVP	LADM- VKEGA	P - DL T NR S T V	ILERMIA AKELT <mark>P</mark>	AANA <mark>G</mark> AQ LVKQ <mark>GG</mark> C	VC 571 AA 572
	Model/1-888 4QQW/1-929	572 573	LICNLVD IICTTVA	VAQVC EAQGV	Q R L K <mark>E</mark> D L L S Q	L NN WFATL	TQ <mark>V</mark> GEDAP		HA <mark>RF</mark> TLI IS <mark>RFP</mark> NI	ND <mark>R</mark> REKE RQ <mark>R</mark> TEIT	N R <mark>V I</mark> S N F A T <mark>I V</mark> D L F	GK 622 GK 627
	Model/1-888 4QQW/1-929	623 628	N <mark>G</mark> K E <mark>G</mark> A Q S <mark>G</mark> R	<mark>R</mark> - NVGF <mark>RP</mark> TR <mark>G</mark> A	R I L VA <mark>T</mark> V L VA <mark>T</mark>		S L D V D L D L D	F <mark>D</mark> WL I V <mark>D</mark> LMI	QHC PAI DLAPV	DLLF <mark>QR</mark> L SLLL <mark>QR</mark> A	<mark>G R L H R H</mark> H G <mark>R CW R H</mark> E	RK 672 HL 682
	Model/1-888 4QQW/1-929	673 683	Y <mark>RP</mark> A GIIN <mark>R</mark> PQ	<mark>g f</mark> e i <mark>p</mark> v Wakq <mark>p</mark> e	ATILL LVVLT	PDGEG PEQNG	DADRA	- <mark>YG</mark>   PWF <mark>P</mark> R :	R H E H I Y S SWT S V Y I	SNVR <mark>VM</mark> W PL-ALLQ	<mark>R T</mark> Q Q <mark>H I</mark> E <mark>R T</mark> Y T L L R	EL 716 RR 736
	Model/1-888 4QQW/1-929	717 737	NGASLFF NGAPVQI	PDAYR PEDVQ	WLDSI LVDDV	YDDAE YDDDS	MDE <mark>PE</mark> L-AED	WV <mark>G</mark> N <mark>G</mark> LE-AD	M <mark>DK</mark> FES/ MERMG-I	ECEK <mark>R</mark> F ELAQ <mark>RG</mark>	KA <mark>R</mark> KVL - LA <mark>R</mark> NAV I	768 <mark>P</mark> D 788
	Model/1-888 4QQW/1-929	769 789	PDDAEDN	A E E <mark>Y S</mark> L L N <mark>G L T</mark> E	Q - DND F S F DV	E T I L A D E HV L	VTRDG ATRFG	EM <mark>SLP</mark> A <mark>GSV</mark> R	L L <mark>P Y</mark> V Q <sup>-</sup> / L C Y Y V I	T <mark>S</mark> S <mark>G</mark> KQL D <mark>T</mark> A <mark>G</mark> NRW	L <mark>D G Q V</mark> Y E L <mark>D P E C</mark> T V	D - 816 E F 843
	Model/1-888 4QQW/1-929	817 844	P E Q <mark>G</mark> T <mark>G</mark> R	L S H E E <mark>G</mark> R F T N	E Q Q <mark>Y</mark> E A 1A D <mark>C</mark> R D	LALNR LVART	VNV <mark>P</mark> F I <mark>P</mark> VRM	T - <mark>W</mark> K R S I <mark>G PW</mark> A S (	6 F <mark>S E</mark> QL T E DNI	VV - DED I <mark>PP</mark> EAWR	<mark>G</mark> L L W E S F Y L R D	852 LV 898
	Model/1-888 4QQW/1-929	853 899	- <mark>L E GK</mark> QN L <mark>I P</mark> Q <mark>R</mark> V T	L <mark>DGW</mark> D <mark>EGA</mark> VL	VWQGN PTETG	S I V I T G R EWL	YT <mark>G</mark> DE LD <mark>P</mark> CK	GMTRV GLI	I <mark>P</mark> AN <mark>P</mark> K			888 929

**Figure S6. Sequence alignment of** *E. coli* **Cas3 with the templates provided by SwissModel server. A.** Sequence alignment of *E. coli* Cas3 and *T. terrenum* Cas3 (PDB ID: 4Q2C). **B.** Sequence alignment of *E. coli* Cas3 and *T. fusca* Cas3 (PDB ID: 4QQW).





**Figure S7. Root mean square deviations (RMSD) of the protein backbone Cα atoms. A.** RMSD for simulation of wild-type protein in complex with Mg<sup>2+</sup> and ATP at 301 K. **B.** RMSD for simulation of wild-type protein in complex with Mg<sup>2+</sup> and ATP at 303 K. **C.** RMSD for simulation of wild-type protein in complex with Mg<sup>2+</sup> and ATP at 310 K. **D.** RMSD for simulation of wild-type protein in complex with Mg<sup>2+</sup> and ATP at 310 K. **D.** RMSD for simulation of wild-type protein in complex with Mg<sup>2+</sup> and DNA at 303 K. **F.** RMSD for simulation of wild-type protein in complex with Mg<sup>2+</sup> and DNA at 303 K. **F.** RMSD for simulation of W230A mutant in complex with Mg<sup>2+</sup> and DNA at 303 K. **H.** RMSD for simulation of W230A mutant in complex with Mg<sup>2+</sup> and DNA at 310 K. **I.** RMSD for simulation of W406A mutant in complex with Mg<sup>2+</sup> and DNA at 303 K. **J.** RMSD for simulations of W406A mutant in complex with Mg<sup>2+</sup> and DNA at 303 K. **J.** RMSD for simulations of W406A mutant in complex with Mg<sup>2+</sup> and DNA at 303 K. **J.** RMSD for simulations of W406A mutant in complex with Mg<sup>2+</sup> and DNA at 303 K. **J.** RMSD for simulations of W406A mutant in complex with Mg<sup>2+</sup> and DNA at 303 K. **J.** RMSD for simulations of W406A mutant in complex with Mg<sup>2+</sup> and DNA at 303 K.

## Table S1. List of *E. coli* K-12 strains used in this study.

Bacterial strain	Relevant genotype	Source or reference		
	F⁻rrnB ∆lacZ4748 (::rrnB-3) hsdR514	(3)		
BW25113	$\Delta$ (araBAD)567 $\Delta$ (rhaBAD)568 rph-1 $\lambda^2$			
	Bacterial strains related to BW25113			
BW39121	+ ∆hns::kan	(4)		
IIB1040	+ λc + λT3 $\Delta cas1::kan \Delta hns::cat$	(5)		
IIB1043	+λc + λT3 <i>Δcas1</i> ::FRT	(5)		
IIB1309	+λc + λT3 $\Delta cas1$ ::FRT $\Delta hns$ ::kan	P1. BW39121 x IIB1043		
IIB1342	+λc + λT3 $\Delta cas1$ ::FRT $\Delta hns$ ::kan cas3W406A	Gene replacement of <i>cas3</i> with allele <i>cas3<sup>W406A</sup></i> using pKOV – see main methods		

Oligonucleotide Name	Sequence from 5' to 3'					
Primers for amplyfing ygcB from genomic DNA						
ygcB_Forward	ATCGCTCGAGATAGAACCTTTTAAATATA					
ygcB_Reverse	ATCGGCATGCTCGAATTCTTATTTGGGATTTGCAGGGA					
Primers for DNA substrate prepar	ration					
Cas3HD_Forward	GCCGCTCGAGGAACCTTTTAAATATATATGCCATT					
W42A_Reverse	GCAACAGCAGCAACATCA					
Primers for site-direct mutagenesis						
W149A_Forward	TTATGAGTCCgcgTTTCCATGGGTAGAGGC					
W149A_Reverse	GGATGAGGAGCGGCATCA					
W152A_Forward	CTGGTTTCCAgctGTAGAGGCCG					
W152A_Reverse	GACTCATAAGGATGAGGAG					
W230A_Forward	GCTTGCTGACgctTTAGGCTCCTGG					
W230A_Reverse	GAGCAAAAACCTGCTAAC					
W406A_Forward	GTGTTGTCAGgctTTGTCACAAAGCAATAAGAAAG					
W406A_Reverse	TGAACCCACGCTTCTTCT					
Primers for allele replacement						
FP-cas	TAAAAAAACAGGGAGGCTATTAGAATTAACCATGGGGGGGTTC					
RP-vector	ATCACTGAGATCATGTTGTAGCGCCCTTATTTGGGATTTGCAGGGATG					
FP-promoter	CTATTGCTGGTTTANTCGGTACCCCAAGACATGTGTATATCACTG					
RP-promoter	GAACCCCCCATGGTTAATTCTAATAGCCTCCCTGTTTTTTAG					
pKOV-F	GCAAATTCGACCCGGTCGTC					
pKOV-R	GTTCCTGACCGATAACATCACAGA					
Upcas3	CGATATTTATGAGCAGCATC					
Downcas3	GATGTACATTGTGCACCTTC					
Sequencing primer						
Cas3700	GGTTAGGCTCCTGGACTACAAC					
Oligos for construct DNA fork substrate						
MW12	CY5-GTCGGATCCTCTAGACAGCTCCATGATCACTGGCACTGGTAGAATTCGGC					
MW14	CAACGTCATAGACGATTACATTGCTACATGGAGCTGTCTAGAGGATCCGA					
CRISPR RNA targeting sequence	AGGCCCGCACCGATCGCCCTTCCCAACAGTTG					

## Table S2. List of oligonucleotides used in this study.

Table S3. Summary of molecular dynamics simulations conducted for different systems of *E. coli* Cas3 protein.

	wild	-type	W230A	W406A		
	Mg²⁺, ATP	Mg²⁺, DNA	Mg²⁺, DNA	Mg²⁺, DNA		
Temperature / K	Time / ns					
301	110	Х	Х	Х		
303	300	120	120	120		
310	300	120	120	120		
317	110	Х	Х	Х		

#### Supplementary References

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