

ATPase facilitates PlmCas12e-mediated gene editing through conformational modulation

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Abstract

19

20 CRISPR-Cas systems represent powerful and versatile tools for genome engineering, yet the editing efficacy of
21 compact effectors such as Cas12e is often constrained by the intrinsic energy barriers associated with
22 conformational activation. Here, we develop a systematic screening strategy to evaluate the performance of various
23 ATPases fused to SpCas9 and PlmCas12e effectors using diverse linker architectures, aiming to overcome
24 conformational activation barriers through ATPase-driven remodeling. One lead architecture, PlmCas12e fused
25 with DnaA at the N-terminus via a rigid linker, demonstrates significantly enhanced editing efficiencies across
26 exogenous and endogenous targets in HEK293T cells. *In vitro* biochemical assays further suggest enhanced intrinsic
27 catalytic activity of the fusion variant. Molecular dynamics simulations show that DnaA-fused variant exhibits a
28 more confined Free Energy Landscape (FEL) and reduced Root Mean Square Fluctuation (RMSF) in critical
29 domains. These findings support a model in which DnaA facilitates conformational pre-organization, thereby
30 lowering the entropic cost of activation for PlmCas12e to transition into a catalytically active state. Overall, our
31 study not only provides a robustly enhanced PlmCas12e variant for genome editing but also highlights
32 conformational modulation as a strategy for engineering CRISPR effectors.

33 **Keywords:** CRISPR-Cas, PlmCas12e, ATPase, genome editing, conformational modulation

34

35 **Introduction**

36 The rapid development of CRISPR-Cas systems has revolutionized the field of genome engineering, providing
37 powerful tools to manipulate the genetic code. Among the diverse array of CRISPR effectors, Cas12e (also known
38 as CasX), belonging to the Class 2 Type V system, has attracted great interest due to its small molecular size and
39 distinct DNA cleavage mechanism (Liu et al. 2019; Tsuchida et al. 2022). Despite these advantages, at many
40 targeting sites, wild-type Cas12e variants often exhibit lower editing potency compared to the gold-standard
41 *Streptococcus pyogenes* Cas9 (SpCas9) (Li et al. 2024). On the other hand, SpCas9 displays attenuated editing
42 efficiency at some clinical targeting sites as well (Canver et al. 2015; Chari et al. 2015). These limitations motivate
43 further protein engineering of Cas proteins to meet the demands of clinical and industrial applications.

44 To overcome the performance limitations of wild-type CRISPR effectors, researchers have developed several
45 robust strategies for protein and guide RNA (gRNA) optimization. Rational design based on high-resolution
46 structures has enabled the creation of high-fidelity variants, such as eSpCas9 and SpCas9-HF1, by neutralizing non-
47 specific DNA-binding charges to reduce off-target effects (Chen et al. 2022; Kleinstiver et al. 2016; Slaymaker et
48 al. 2016). Complementary to this, directed evolution, exemplified by methods like arginine scanning mutagenesis
49 or PACE (Phage-assisted continuous evolution), has been successfully employed to broaden the PAM recognition
50 profile and enhance catalytic activity (Hu et al. 2018; Huang et al. 2023; Jin et al. 2025). Beyond the protein, gRNA
51 engineering, including the incorporation of chemical modifications and the optimization of scaffold secondary
52 structures, have further improved the stability and activity of the RNP complex (Bin Moon et al. 2018; Chen et al.
53 2023; Kim et al. 2022; Park et al. 2018; Su et al. 2024). While these approaches primarily optimize local interactions,
54 a general design principle based on global conformational modulation has yet to be established for broad CRISPR
55 enhancement.

56 One of the bottlenecks limiting the editing efficiency of structurally dynamic Cas effectors is the energy
57 barriers associated with the transition from DNA binding to catalytic activation (Sternberg et al. 2015; Strohkendl
58 et al. 2018; Xing et al. 2024). We therefore hypothesize that the fusion of the ATPase proteins to the Cas effector
59 could facilitate this transition, leveraging the mechanical work driven by ATP hydrolysis. In fact, many accessory
60 proteins in nature, particularly ATPase-based DNA manipulation proteins, have evolved to facilitate DNA
61 unwinding, protein-DNA assembly, and conformational remodeling (Eustermann et al. 2024; Hauk and Berger
62 2016). For instance, the DnaA protein, a specialized ATPase, plays a pivotal role in the initiation of DNA replication
63 by binding to the *oriC* region and inducing local DNA strands separation (Messer et al. 1999; Fujikawa et al. 2003;
64 Duderstadt et al. 2011). Thus, we propose that the fusion of such DNA-remodeling related ATPases with CRISPR

65 effectors could potentially lower the activation energy of the CRISPR complex, either by enhancing DNA
66 accessibility or by stabilizing the effector's catalytically active conformation.

67 In this study, we systematically engineer Cas-ATPase fusion proteins by varying ATPase variant, fusion
68 orientation, and linker architecture. Our screening identified DnaA ATPase as a potent enhancer of PlmCas12e-
69 mediated genome editing activity. This enhancement was consistent across multiple genomic targets, suggesting a
70 general improvement in enzymatic functionality. *In vitro* assays also indicated enhanced cleavage efficiency of the
71 fusion variant. Molecular dynamics (MD) simulations suggests that DnaA fusion facilitates the conformational pre-
72 organization of the effector into a catalytically competent state, thereby reducing the entropic penalty during the
73 DNA cleavage process. Collectively, our work not only provides a robustly enhanced Cas12e variant but also
74 highlights the feasibility of conformational modulation in the optimization of genome editing tools.

75

76 **Results**

77 **Systematic screening identifies DnaA-fusion as a potent enhancer for PlmCas12e**

78 To investigate whether the integration of ATPase domains could modularly enhance CRISPR effector performance,
79 we firstly curated a library of ATPase proteins with diverse DNA-remodeling capabilities (Figure 1A, Table S1).
80 These ATPase proteins were tethered to either the N- or C-terminus of SpCas9 and PlmCas12e using a flexible
81 (GGSGGGS) or a rigid (PAPAPAP) linker. We then evaluated the designs in HEK293T cells using a GFP reporter
82 assay to quantify gene disruption efficiency (Figure 1B, S1A, B).

83 In the initial phase, we screened the editing efficacy of SpCas9 protein fused with six ATPases (424 aa–747 aa)
84 at two GFP targets. Most variants exhibited target-dependent improvements, and the constructs with rigid linkers
85 moderately performed better than those with flexible linkers (Figure S2). However, we did not see consistent
86 enhancement when we scaled up to five GFP targets (Figure S3A). Subsequent testing of SpCas9 proteins fused
87 with three additional small ATPases (188 aa–363 aa) across these five GFP targets yielded similar results (Figure
88 S3B).

89 In contrast to SpCas9, the fusion of ATPases to PlmCas12e, a compact Type V CRISPR effector, yields robust
90 and consistent enhancement in special designs (Figure 1C, D). Notably, a specific architecture, PlmCas12e fused
91 with an N-terminal DnaA domain via a rigid or a flexible linker (labeled as PlmCas12e-NR-DnaA and PlmCas12e-
92 NF-DnaA), exhibited a significant increase of the GFP-negative HEK293T cells. To further validate this finding,
93 we performed a repeat experiment and further evaluated the editing efficiency of the DnaA fusion variants using
94 both next-generation sequencing (Figure 1E, S4A) and the GFP reporter assay (Figure S4B). The enhancement was
95 consistent across replicates and detection methods. Furthermore, the fusion variant with a rigid linker (PlmCas12e-
96 NR-DnaA) consistently outperformed the flexible-linker construct (DnaA-NF-PlmCas12e), suggesting that precise
97 spatial positioning is critical for the synergy between the DnaA domain and the PlmCas12e scaffold.

99 **PlmCas12e-DnaA exhibits enhanced editing efficiency across multiple genomic targets**

100 The editing superiority of the PlmCas12e-NR-DnaA configuration was further validated across a broader range of
101 genomic targets (Figure 2A, B). ATPase-fused PlmCas12e exhibited significantly higher editing efficiency at the
102 EGFP reporter gene compared to wild-type PlmCas12e (Figure 2C). This enhancement was further corroborated at
103 endogenous genomic loci. In HEK293T cells, PlmCas12e-NR-DnaA exhibited robust indel formation across seven
104 native targets, with an average 1.34-fold increase of editing efficiency compared to the wild-type control (Figure
105 2D, E).

106 To determine whether the enhancement of gene editing efficiency depends on the intrinsic catalytic activity of
107 DnaA, we introduced point mutations into the Walker A and Walker B motifs of DnaA (K178A/T179A and
108 D235A/D236A), which are known to abolish ATP binding and hydrolysis (Hou et al. 2022; Katayama et al. 2010).
109 Both DnaA mutant variants exhibited a marked reduction in gene editing activity, reverting to levels lower than the
110 wild-type PlmCas12e (Figure 2F). This result indicates the necessity of a functional ATPase core domain, suggesting
111 that ATP hydrolysis is required for the enhanced editing efficiency.

112 Having established that ATPase activity underlies the enhanced editing efficiency, we next asked whether this
113 improvement extends across the genome. Using the GenomePAM approach, a GUIDE-seq-based assay targeting
114 high-copy-number repetitive sequences in mammalian cells (Yu et al. 2025), we systematically profiled editing
115 activity at thousands of genomic sites. We selected the Rep-1RC (5'-GGCCAGGCACAGTGGCTCAC-3') sequence
116 in Alu elements as the spacer, which has 8,471 occurrences across the human genome. The results showed that the
117 total number of effectively edited Alu genome targets (on-target sites) generated by PlmCas12e-NR-DnaA was
118 significantly higher than that of the wild-type control group, which was consistent with the single-locus assays
119 (Figure 2G). Under the same sequencing depth, the number of on-target sites generated by the PlmCas12e-NR-
120 DnaA fusion variant was higher than that of the wild-type PlmCas12e (Figure 2H), and the total number of effective
121 on-target reads captured also showed a notable increase (Figure 2I). Meanwhile, analysis of the edited sequences
122 using GenomePAM revealed that the fusion protein maintained the canonical PAM recognition preference of
123 PlmCas12e (Figure S5A–F). Together, these results demonstrate that the DnaA fusion enhances editing efficiency
124 in a genome-wide manner.

125

126 **PlmCas12e-NR-DnaA exhibits enhanced DNA cleavage activity *in vitro***

127 To determine whether the enhanced editing efficiency observed in cells stems from improved chromatin
128 accessibility or intrinsic enzymatic optimization, we next performed *in vitro* cleavage assays using purified
129 components. Wild-type PlmCas12e (PlmCas12e-WT) and recombinant PlmCas12e-NR-DnaA proteins were
130 reconstituted with sgRNA to form ribonucleoprotein (RNP) complexes, and time-course cleavage assays were
131 carried out using a linearized double-stranded DNA substrate containing the cognate spacer and PAM (Figure 3A).

132 The PlmCas12e-NR-DnaA variant exhibited superior enzymatic performance compared to the WT enzyme
133 (Figure 3B). The PlmCas12e-WT RNP showed slower cleavage rate and incomplete substrate depletion within the
134 tested timeframe, while the PlmCas12e-NR-DnaA rapidly achieved a maximal cleavage plateau with a higher
135 fraction of converted DNA. This result indicates that the enhancement arises, at least in part, from intrinsic

136 enzymatic properties. Given that the rate-limiting step for Cas12 effectors typically involves the energy-dependent
137 formation of the R-loop and the subsequent conformational rearrangement required to activate the RuvC catalytic
138 center (Stella et al. 2018; Strohkendl et al. 2024; Swarts and Jinek 2019), our observation suggests that the fused
139 DnaA domain might lower the energetic barrier for these transitions, which facilitates a more rapid progression to
140 the catalytically competent state, even in the absence of cellular factors.

141

142 **Molecular dynamics reveals DnaA-driven conformational modulation**

143 To elucidate the mechanism underlying the superior activity of the DnaA fusion protein, we performed all-atom
144 molecular dynamics (MD) simulations using the AlphaFold 3-predicted ternary structures of PlmCas12e-WT and
145 PlmCas12e-NR-DnaA in complex with sgRNA and target DNA (Hu et al. 2025). Root Mean Square Deviation
146 (RMSD) analysis indicated that both systems reached equilibrium after 150 ns (Figure 4A, S6A).

147 We first analyzed the protein-DNA interaction interface. Intriguingly, when considering only the Cas12e
148 effector module, the fusion variant exhibited significantly fewer intermolecular hydrogen bonds and a lower binding
149 free energy (ΔG_{bind}) compared to the WT enzyme (Figure 4B, C). However, the inclusion of the DnaA domain
150 reversed this trend, resulting in a higher total interaction strength for the fusion complex. Structural analysis
151 confirmed that the DnaA DNA-binding domain (DBD) forms stable, canonical interactions with the DNA substrate
152 during the simulation, adopting a conformation similar to the crystal structure of the *E. coli* DnaA-DBD-DNA
153 complex (Fujikawa et al. 2003) (Figure 4D). This is consistent with a model in which DnaA functions as a primary
154 anchor, effectively recruiting the DNA to protein while relieving the Cas12e effector from rigid interfacial
155 constraints.

156 This “anchoring” effect efficiently enhanced the structural stability of the complex. The RMSD of the DNA
157 substrate within the fusion complex was approximately one third lower than that in the WT complex (Figure 4E),
158 indicating that DnaA acts as a conformational scaffold to minimize DNA fluctuation. Consistent with this, Root
159 Mean Square Fluctuation (RMSF) analysis revealed that the fusion protein exhibits reduced flexibility in critical
160 regions, specifically the Non-Target Strand Binding (NTSB) domain and the RuvC catalytic pocket (including the
161 key catalytic residue D922) (Figure 4F), which are both crucial for Cas12e effector catalytic activity (Li et al. 2024).

162 Additionally, to characterize the thermodynamic properties of the enzymes, we constructed the Free Energy
163 Landscape (FEL) as a function of RMSD and Radius of Gyration (Rg) (Figure S6B, C). The WT enzyme exhibited
164 a rugged landscape featuring two distinct, deep energy basins separated by energy barriers, indicating that the protein
165 oscillates between multiple metastable states (Figure 4G). In contrast, the Cas12e core of the fusion variant

166 converged into a single, focused free energy basin (Figure 4H). Collectively, these data suggest that DnaA may
167 facilitate conformational pre-organization, narrowing the conformational search space and stabilizing the catalytic
168 center in an active state (Frederick et al. 2007), thereby lowering the entropy penalty for DNA cleavage and
169 enhancing the gene editing efficiency of PlmCas12e.

170

171 **Discussion**

172 In this study, we developed an engineered CRISPR-Cas12e system by tethering a DnaA ATPase domain to its N-
173 terminus, significantly enhancing genome editing efficiency both in cells and *in vitro*. Our multi-dimensional
174 analysis suggests that the DnaA domain acts as a conformational scaffold, optimizing the free energy landscape of
175 PlmCas12e and facilitating its transition into a catalytically active state. Together, our results reveal conformational
176 modulation as an underexplored axis for CRISPR engineering.

177 Our approach adds a unique dimension to the rapidly evolving field of CRISPR optimization. Traditional
178 strategies have predominantly relied on structure-guided rational design and directed evolution, which focus on
179 local amino acid substitutions to improve DNA binding or PAM recognition (Liu et al. 2022). More recently, AI-
180 driven approaches have transformed the field by enabling the identification of distal mutations with long-range
181 effects on enzymatic activity (Fei et al. 2025; Thomson et al. 2025). While AI performs well at navigating the vast
182 sequence space, they primarily operate at the level of sequence-function relationships. In contrast, our work
183 highlights a complementary biophysical perspective, demonstrating that modular fusion of an accessory domain can
184 reshape the thermodynamic landscape of a CRISPR effector. This framework suggests that incorporating
185 conformational dynamics into computational design may further improve the prediction and optimization of
186 complex domain-fused architectures by AI models (Cavanagh et al. 2026).

187 Despite the promising results, we acknowledge several limitations in the current study that warrant further
188 investigation. First, our screening library was relatively constrained, focusing on a selected set of well-characterized
189 ATPases. Expanding this library through high-throughput synthesis or metagenomic mining may reveal better fusion
190 partners. Second, while we validated PlmCas12e-NR-DnaA across several endogenous loci, broader genomic
191 characterization, including large-scale off-target assessments and testing in diverse cell types, is necessary to
192 establish its clinical utility. Third, it remains to be seen whether this DnaA-mediated enhancement is universally
193 applicable to other compact Cas12e orthologs or even other CRISPR families like Cas12a. Furthermore, our
194 mechanistic insights into conformational optimization were primarily derived from *in silico* molecular dynamics

195 simulations. Although the molecular simulation results provide a convincing theoretical basis, they lack direct
196 experimental structural evidence. Future studies employing Cryo-EM (Cryogenic Electron Microscopy) or single-
197 molecule fluorescence resonance energy transfer (FRET) assays will be crucial to visualize the precise structural
198 transitions and confirm the improved conformational transition of the fusion complex during DNA engagement
199 (Dagdas et al. 2017; Ha et al. 2024).

200 In summary, we have successfully engineered a PlmCas12e-NR-DnaA variant with enhanced editing
201 capabilities, driven by optimized conformational dynamics. This study not only provides an optimized tool for gene
202 editing but also highlights the potential of using accessory domains to modulate the energy landscapes of enzymes.
203 Looking forward, combining the recruitment of natural functional domains with precise structural and
204 conformational optimization could facilitate the rational design of gene editors with improved efficiency and
205 specificity, thereby broadening their potential in therapeutic and biotechnological applications.

206

207 **Materials and methods**

208 **Strains and cultural conditions**

209 The cloning strain used in this study was *E. coli* DH5 α chemically competent cells from Sangon Biotech. Liquid
210 cultures were grown in LB medium (containing 10 g/L NaCl, 5 g/L yeast extract, and 10 g/L tryptone) or in TB
211 medium (containing 24 g/L yeast extract, 12 g/L tryptone, 12.54 g/L K₂HPO₄, 2.31g/L KH₂PO₄). In contrast, solid
212 cultures were maintained on LB agar plates (supplemented with 10 g/L NaCl, 5 g/L yeast extract, and 10 g/L tryptone,
213 and 15 g/L agar). For protein purification, *E. coli* Rosetta (DE3) competent cells from Sangon Biotech were used.
214 Stock solution of ampicillin (100 mg/L) and kanamycin (50 mg/L) were prepared, and unless otherwise indicated,
215 this stock solution was added to the culture medium at a 1:1000 dilution.

216 **Plasmids construction**

217 For mammalian genome editing experiments, the plasmids pBLO62.5 (Addgene #123124) (Liu et al. 2019) and
218 pBFC0398 (Sun et al. 2023) served as the expression backbones for PlmCas12e and SpCas9, respectively. Both
219 vectors were codon-optimized for human cell expression and flanked by SV40 nuclear localization sequences (NLS)
220 at both termini. To construct sgRNA expression vectors, oligonucleotide pairs containing the target spacer sequences
221 were annealed, phosphorylated, and cloned into BbsI-digested backbones via Golden Gate assembly. For the
222 construction of fusion proteins, coding sequences of ATPase domains were PCR-amplified and inserted into the N-
223 or C-terminus of the Cas effectors using Gibson assembly (One Step Seamless Cloning Mix, CWBIO). For bacterial

224 protein expression and purification, *E. coli* codon-optimized genes encoding PlmCas12e variants were subcloned
225 into a modified pET28b vector containing an N-terminal hexahistidine tag followed by a SUMO tag. Point mutations
226 in the PlmCas12e-NR-DnaA variants were introduced via PCR-based site-directed mutagenesis using mutagenic
227 primers. To generate templates for in vitro gRNA transcription, a DNA fragment containing the T7 promoter and
228 the sgRNA scaffold was inserted into the pUC19 vector. A linear dsDNA template (approximately 0.7 kb) was
229 subsequently PCR-amplified from this plasmid to serve as the substrate for in vitro transcription.

230 **Cell culture and genome editing assay**

231 HEK293T cells and HEK293T-GFP reporter cells were cultured in DMEM (Gibco) supplemented with 10% FBS
232 (VWR). Cells were seeded into 96-well plates and transfected at 60-70% confluency using YALEPIC DNA/RNA
233 Transfection Reagent-LFDR (Yali Biotech) with 200 ng of plasmid DNA. To select successfully transfected
234 populations, puromycin (1.5 µg/mL) was added to cell culture medium 24 hours post-transfection. After 48 hours
235 of selection, the cell culture medium was replaced with fresh medium without puromycin for an additional 24 hours.
236 For GFP reporter assay, cells were harvested and washed twice with 1× PBS containing 0.5% FBS. GFP
237 fluorescence intensity was quantified using a BD LSRFortessa. For subsequent NGS evaluation, genomic DNA was
238 extracted using 50 µL of Lysis Buffer for Direct PCR (Genesand). The lysates were incubated at room temperature
239 for 5 min, followed by incubation at 50°C for 30 min and 95°C for 3 min. All spacer sequences used in this study
240 are listed in Table S2 and Table S3.

241 For GenomePAM assay, plasmids were transfected into HEK293T cells via Lipofectamine 3000 transfection
242 reagent (Thermo Fisher Scientific) in strict accordance with the manufacturer's operating instructions. Prior to
243 transfection, cells were seeded in 24-well plates at a density of 1×10^5 cells per well. For each well, the transfection
244 mixture was prepared as follows: 500 ng of Cas expression plasmid, and 5 pmol of annealed dsODN were first
245 combined with 1.5 µL of P3000 reagent in 25 µL of Opti-MEM reduced-serum medium. This mixture was then
246 mixed thoroughly with a second solution containing 1.5 µL of Lipofectamine 3000 reagent diluted in 25 µL of Opti-
247 MEM reduced-serum medium, yielding a 50 µL final volume of DNA–lipid transfection complex. The complex
248 was incubated at room temperature for 10 min to allow for lipid–nucleic acid complex formation. Subsequently, the
249 resultant transfection complex was added dropwise to individual wells, and the plates were incubated in a standard
250 cell culture incubator for 72 h.

251 **Next-generation sequencing and data analysis**

252 Target genomic regions were amplified from genomic DNA samples using 2× Phanta Max Master Mix (Vazyme)
253 in a 25 µL reaction volume. Thermal cycling was performed as follows: 98°C for 30 s; 20 cycles of 98°C for 15 s,

254 59°C for 15 s, and 72°C for 15 s; followed by a final extension at 72°C for 5 min. Illumina adapters and barcodes
255 were added via a second round of PCR using 2× Q5 High-Fidelity Hot-Start Master Mix (New England Biolabs).
256 The resulting amplicons were purified, pooled, and sequenced on an Illumina NextSeq platform with 160-bp paired-
257 end reads. Raw sequencing data were processed using Trim Galore (v0.6.10) to remove adapters and low-quality
258 reads, and paired-end reads were subsequently merged using FLASH (v2.2.00). Indel quantification was performed
259 using CRISPResso2 (v2.3.2) (Clement et al. 2019) with a quantification window centered at the cleavage site. The
260 editing frequency was calculated based on the percentage of reads containing indels. The sequences of PCR primers
261 used for NGS library preparation are listed in Table S3.

262 **Protein expression and purification**

263 Plasmids encoding His-SUMO-tagged proteins were transformed into *E. coli* Rosetta (DE3) competent cells by heat
264 shock at 42°C for 90 s, followed by incubation on ice for 20 min. Cells were recovered in 900 µL of LB medium at
265 37°C for 1 h and plated onto LB agar plates containing 50 µg/mL kanamycin. After overnight incubation at 37°C,
266 single colonies were picked and inoculated into 100 mL of LB medium for future culture. After approximately 6 h,
267 20 mL of the culture was transferred into 750 mL of TB medium supplemented with 50 µg/mL kanamycin and 6
268 mL of 50% glycerol. When the OD₆₀₀ reached 1.0-1.2, protein expression was induced with 0.4 mM IPTG, and cells
269 were grown overnight at 16°C. Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM
270 HEPES pH 7.5, 500 mM NaCl, 10% glycerol, 50 mM imidazole, 0.5mM ATP, 1 mM TCEP). The suspension was
271 lysed by sonication, and the lysate was clarified by centrifugation at 15,000 r.p.m. for 80 min at 4°C. The supernatant
272 was incubated with pre-equilibrated Ni-NTA agarose beads (QIAGEN) by passing through the resin three times to
273 maximize binding. After washing, the His-tagged protein was eluted using lysis buffer supplemented with 300 mM
274 imidazole. To remove the His-SUMO tag, the eluate was incubated with Ulp1 protease at 4°C for 30 min. The
275 mixture was then loaded onto a 5 mL HiTrap Heparin HP column (GE Healthcare) and eluted with a linear NaCl
276 gradient from 200 mM to 1.5 M. Peak fractions were pooled and further purified by size-exclusion chromatography
277 using a Superdex Increase 200 10/300 GL column (GE Healthcare) equilibrated with SEC buffer (20 mM HEPES
278 pH 7.5, 150 mM NaCl, 10% glycerol, 0.5mM ATP, 1 mM TCEP). Fractions containing purified protein, as
279 confirmed by SDS-PAGE, were aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C. Protein sequences
280 are listed in Table S4.

281 **RNA preparation**

282 The sgRNA was produced using *in vitro* transcription (IVT) as described previously (Sun et al. 2023). Briefly, DNA
283 template was incubated with 0.5 mg/mL T7 polymerase and 4 mM NTP mix in IVT buffer (30 mM Tris-HCl pH

284 8.1, 25 mM MgCl₂, 0.01% Triton, 2 mM spermidine) for 3 h at 37°C. The reaction mixture was resolved on a 10%
285 denaturing urea-polyacrylamide gel (Urea-PAGE). The target RNA band was excised, crushed, and eluted in soaking
286 buffer (0.38 M NaAc pH 5.2, 0.8 mM EDTA, 0.8% SDS) overnight at 4°C. The eluted RNA was buffer-exchanged
287 and concentrated using a 3 kDa MWCO concentrator (Merck Millipore). For precipitation, 0.3 volumes of 3 M
288 NaOAc and 3 volumes of chilled 100% ethanol were added, and the sample was incubated at -80°C for 2 h. After
289 centrifugation at 12,000 × g for 30 min, the pellet was washed twice with 70% ethanol and resuspended in nuclease-
290 free water. To ensure proper folding, the RNA was diluted in refolding buffer (50 mM KCl, 5 mM MgCl₂), incubated
291 at 72°C for 5 min, and slowly cooled to room temperature before being frozen and stored at -80°C. SgRNA
292 sequences are listed in Table S5.

293 ***In vitro* cleavage assay**

294 Ribonucleoprotein (RNP) complexes were assembled by incubating 500 nM of purified Cas protein with 750 nM
295 sgRNA at 25°C for 20 min in cleavage buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT,
296 1 mM ATP). Subsequently, 30 nM dsDNA substrates were added and mixed with RNP on ice. The reaction mixtures
297 were incubated at 37°C, and aliquots were withdrawn at the time points indicated in the figure legends. Reactions
298 were quenched by the addition of 25 mM EDTA and 200 µg/mL RNase A (Beyotime), followed by incubation at
299 37°C for 1 h. Then, 1 mg/mL Proteinase K (Solarbio) was added, and samples were incubated for an additional 30
300 min at 50°C. Reaction products were separated by electrophoresis on a 2% agarose gel and subsequently quantified
301 using ImageJ software (version 1.53k).

302 **Double-stranded oligodeoxynucleotide preparation**

303 Double-stranded oligodeoxynucleotide (dsODN) oligos used for GenomePAM assay were purchased from
304 GENEWIZ and subjected to high-performance liquid chromatography (HPLC) purification. Individual oligos
305 (dsODN-Top and dsODN-Bot) were resuspended in 1× Tris-EDTA (TE) buffer (Thermo Fisher Scientific) to
306 achieve a final concentration of 100 µM. Subsequently, the oligos were annealed at a working concentration of 10
307 µM in 1× annealing buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 7.4) using a thermocycler. The thermal
308 cycling protocol was set as follows: 95°C for 3 min; followed by 70 cycles of 95°C for 1 min with a ramp rate of -
309 1°C per cycle; and a final hold at 4°C. The sequences of dsODN-Top and dsODN-Bot are listed in Table S6.

310 **GenomePAM assay**

311 Genomic DNA (gDNA) was extracted 72 hours post-transfection, and 500 ng of the purified gDNA was utilized for
312 GenomePAM sequencing library construction in accordance with our previously published protocol (Yu et al. 2025).
313 Briefly, the library preparation workflow comprises enzymatic fragmentation, end repair, A-tailing, adaptor ligation,

314 and two sequential rounds of anchored nested polymerase chain reaction (PCR). Library quantification was
315 performed using KAPA Library Quantification kits, followed by sequencing on an Illumina NextSeq 1000 System
316 with a 300-cycle kit (2× 150-bp paired-end reads). Sequencing was conducted following the standard Illumina
317 workflow, with no modifications to indexing cycles or the requirement for customized sequencing primers. Raw
318 sequencing data (FASTQ files) were processed and analyzed using the GenomePAM bioinformatics pipeline. To
319 assess PlmCas12e cleavage dynamics as a function of increasing sequencing depth, datasets were subsampled at
320 read counts ranging from 100,000 to 1,000,000 reads for subsequent GenomePAM analysis.

321 **Molecular dynamics simulation and analysis**

322 The initial ternary complex structures of PlmCas12e-WT and PlmCas12e-NR-DnaA were predicted using
323 AlphaFold 3 (Abramson et al. 2024), with the top-ranked model (model_0) selected as the starting conformation for
324 simulations (Table S7). MD simulations were performed using GROMACS (version 2025.1, CUDA-accelerated)
325 employing the Amber99BSC1 force field and the TIP3P water model. Each complex was solvated in a cubic box,
326 maintaining a minimum distance of 1.5 nm between the solute surface and the box edges. The systems were
327 neutralized and adjusted to an ionic concentration of 0.15 M by the addition of Na⁺ and Cl⁻ ions. All simulations
328 applied periodic boundary conditions in three dimensions. Long-range electrostatic interactions were calculated
329 using the Particle Mesh Ewald (PME) method, while short-range electrostatic and van der Waals interactions were
330 cut off at 1.4 nm. The LINCS algorithm was used to constrain bonds involving hydrogen atoms. Energy
331 minimization was performed using the steepest descent algorithm until the maximum force converged to <1,000 kJ
332 mol⁻¹ nm⁻¹. The systems were then equilibrated in two phases: a 100 ps NVT ensemble equilibration followed by a
333 100 ps NPT ensemble equilibration. During equilibration, temperature was maintained at 310 K using the V-rescale
334 thermostat, and pressure was maintained at 1.0 bar using the Parrinell–Rahman barostat. Following equilibration,
335 300 ns production runs were conducted. To ensure statistical significance and independent sampling, three
336 independent replicates were performed for each system. Each replicate was prepared individually from the solvation
337 step, followed by independent energy minimization and equilibration (NVT and NPT) phases. In the NVT
338 equilibration, initial velocities were generated using random seeds based on a Maxwell-Boltzmann distribution at
339 310 K.

340 Trajectory data were analyzed using standard GROMACS modules. RMSD and RMSF data were calculated
341 using the `gmx rms` and `gmx rmsf` commands, respectively. Hydrogen bonds were analyzed using the `gmx hbond`
342 command. Binding free energies were calculated using the `gmxMMPBSA` (v16.0) tool (Valdés-Tresanco et al. 2021)
343 at 310 K. Free Energy Landscapes (FEL) were constructed using the `gmx sham` module with the temperature

344 parameter set to 310 K, projected onto the coordinates of RMSD and Rg. Data visualization was performed using
345 Python 3.9 with the matplotlib (v3.5.2) package.

346 **Statistics analysis**

347 GraphPad Prism 10.6 software was used to analyze the data. Unless otherwise specified in figure legends, numerical
348 values are presented as means \pm SD. Significant differences between controls and treatments were tested using the
349 two-tailed Student's *t* test. $P < 0.05$ was considered statistically significant.

350 **Data availability**

351 All data supporting the findings of this study are available in the main text and the supplementary materials.

352 **Compliance and ethics**

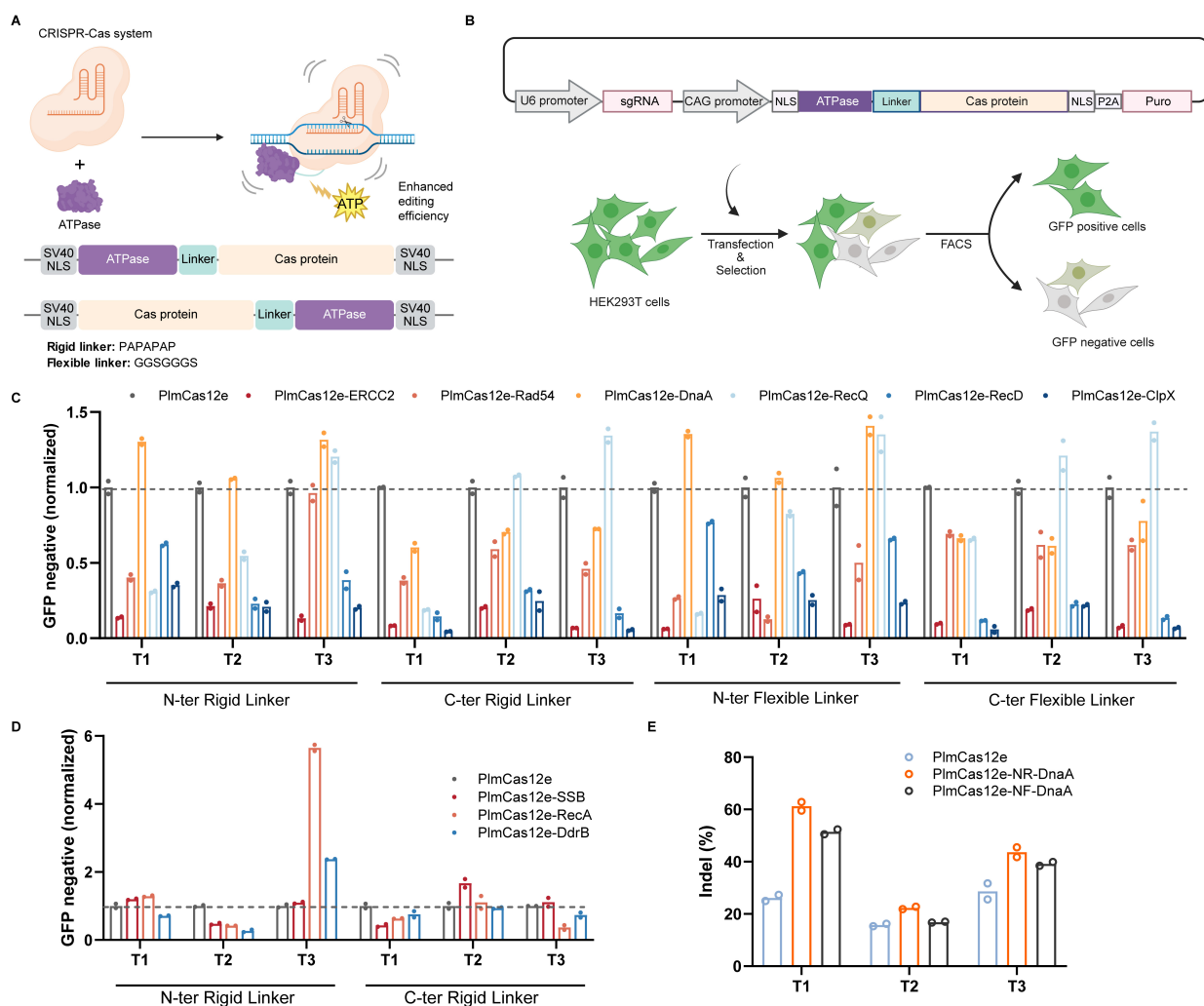
353 Tsinghua University has filed a patent that includes work described in this paper.

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356 Foundation of China (32425030), the Agriculture Science and Technology Major Project and the New Cornerstone
357 Science Foundation through the XPLOER PRIZE (J.J.G.L.).

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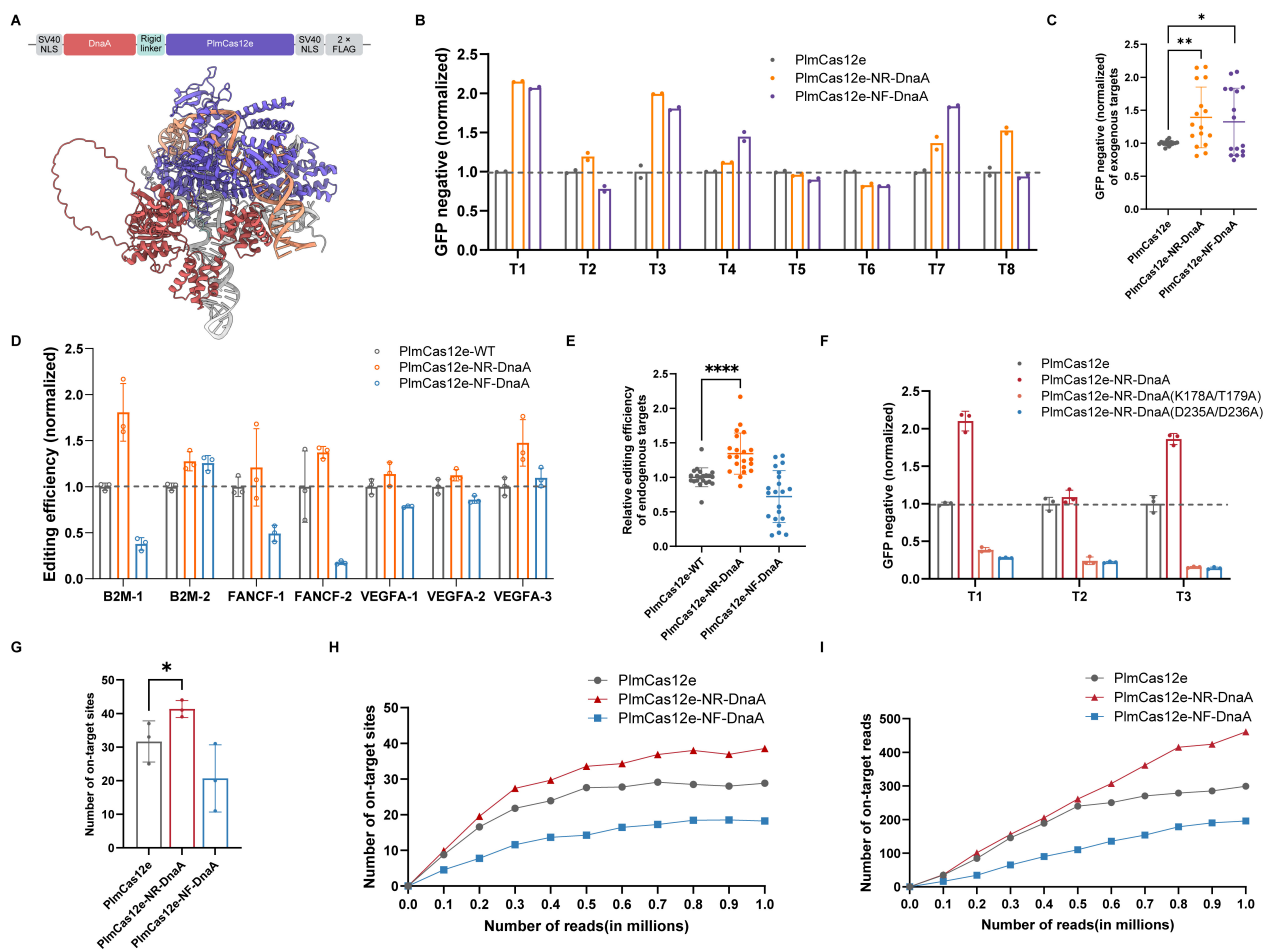
359 Figures



360

361 **Figure 1** Initial editing efficiency screening of PlmCas12e-ATPase constructs. A, Schematic of Cas-ATPase fusion
 362 architectures designed for enhanced editing efficiency. B, Illustration of GFP reporter system for gene editing
 363 efficiency evaluation. C, Normalized frequency of GFP-negative cells for six ATPase variants fused to the N- or C-
 364 terminus of PlmCas12e via rigid or flexible linkers, targeting three EGFP loci. D, Normalized frequency of GFP-
 365 negative cells for three small ATPase variants fused to the N- or C-terminus of PlmCas12e via rigid linkers, targeting
 366 three EGFP loci. E, High throughput sequencing indicated indels generated by PlmCas12e-DnaA variants. Data are
 367 presented as the mean from two biological replicates.

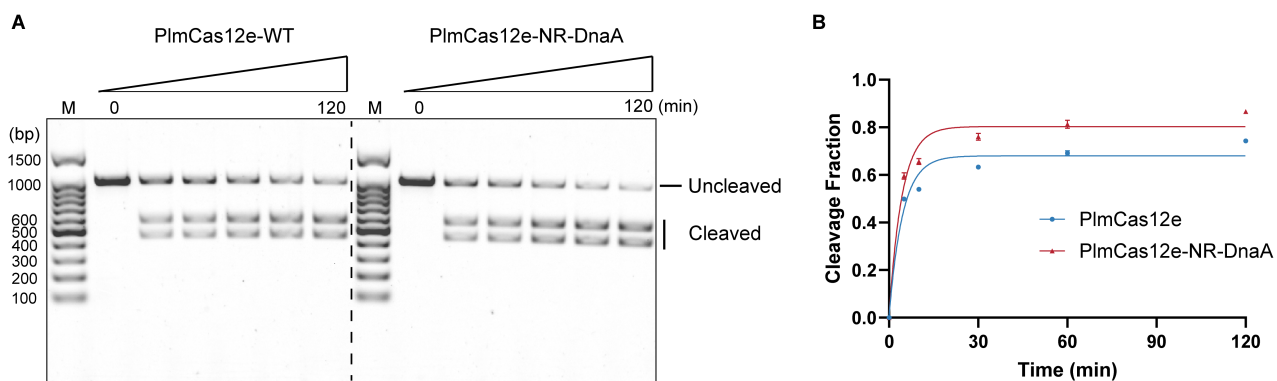
368



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370 **Figure 2** Enhanced editing efficiency of PlmCas12e-NR-DnaA at both exogenous and endogenous targets. A,
 371 Schematic representation of the PlmCas12e-NR-DnaA fusion construct. The ternary complex structure was
 372 predicted using AlphaFold 3. B, Normalized frequency of GFP-negative cells for PlmCas12e-DnaA variants
 373 targeting eight distinct EGFP sites in HEK293T cells. Data are presented as the mean from two biological replicates.
 374 C, Aggregate analysis of normalized GFP-negative cells across the eight exogenous targets shown in (B). D,
 375 Normalized indel frequencies generated by PlmCas12e variants at seven endogenous genomic loci, determined by
 376 high-throughput sequencing. E, Aggregate statistics of normalized indel frequencies across the seven endogenous
 377 targets shown in (D). F, Normalized frequency of GFP-negative cells for PlmCas12e and DnaA ATPase-deficient
 378 mutants (Walker A/B) targeting three EGFP sites. G, Number of effectively edited sites targeting Alu elements in
 379 HEK293T cells. H, I, The number of on-target sites (H) and total on-target reads (I) detected in the GenomePAM
 380 assay, plotted against varying raw sequencing depths (from 0.1 to 1 million reads). Data in D–I are presented as
 381 mean \pm SD from three biological replicates. P values were calculated using a two-tailed Student's t test. * $P < 0.05$,
 382 ** $P < 0.01$, **** $P < 0.0001$.

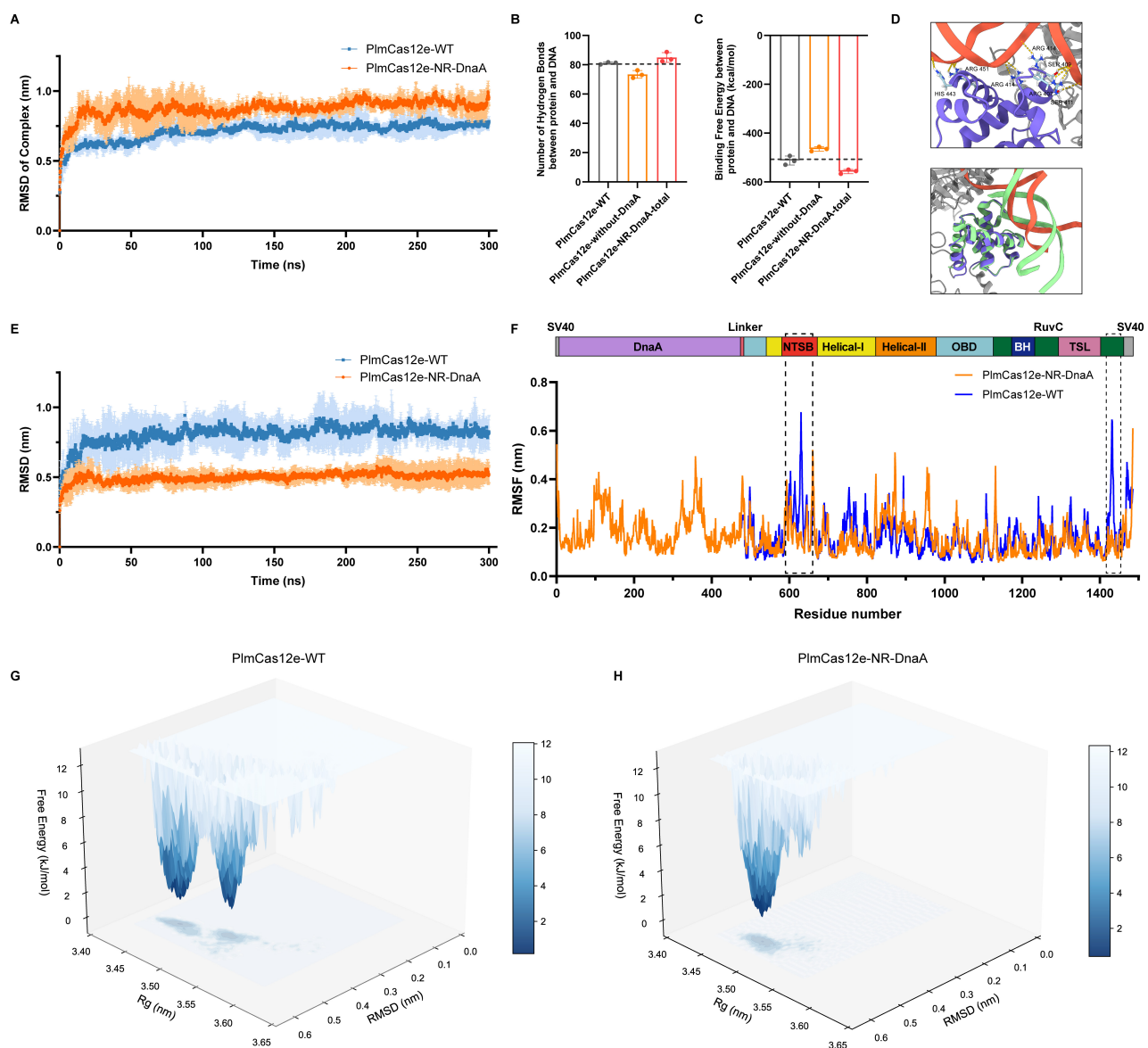
383



384

385 **Figure 3** DnaA fusion enhances the DNA cleavage activity of PlmCas12e *in vitro*. A, Representative agarose gel
386 showing the time-course cleavage of the linear dsDNA substrate by PlmCas12e-WT and PlmCas12e-NR-DnaA
387 RNP complexes. Time points: 0, 5, 10, 30, 60, 120 min. M, Marker. B, Quantification of dsDNA cleavage rate. The
388 fraction of cleaved DNA substrate is plotted against time. Data were fitted into a one-phase decay model. Data are
389 presented as mean \pm SD from three biological replicates.

390



391

392 **Figure 4** Molecular dynamics simulations reveal conformational optimization mediated by DnaA. A, RMSD
 393 trajectories for PlmCas12e-WT and PlmCas12e-NR-DnaA ternary complex in the MD simulation, the systems
 394 reached equilibrium after 150 ns. B, Average number of hydrogen bonds formed between the protein components
 395 and DNA, calculated over the final 150 ns of the simulation. C, Binding free energy between protein and DNA
 396 calculated over the final 150 ns of the simulation. D, Structural illustrations of the DnaA-DNA interface. Top:
 397 Snapshot from the 300 ns MD simulation showing hydrogen bond networks (yellow dashed lines) between the
 398 DnaA-DBD (purple) and DNA (orange). Bottom: Structural alignment of the simulated DnaA-DBD with the *E. coli*
 399 DnaA-DBD-DNA crystal structure (PDB: 1J1V, green). E, RMSD trajectories for DNA substrates within the
 400 PlmCas12e-WT and PlmCas12e-NR-DnaA complexes during the MD simulation. F, Per-residue RMSF plot for
 401 PlmCas12e-WT and PlmCas12e-NR-DnaA in the MD simulation over the last 150 ns. Key domains (NTSB, RuvC)
 402 showing reduced fluctuation are highlighted in dashed box. G, H, The free energy landscape against RMSD and Rg

403 for Cas effector protein of PlmCas12e-WT (G) and PlmCas12e-NR-DnaA (H) during a 300 ns MD simulation. Data
404 in B, C are presented as mean \pm SD from three independent replicates.

405

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