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RESEARCH ARTICLE

Structural Insights into CRISPR-Cas12a Target Recognition and Cleavage Mechanisms

Kasper Fernandez^{1*}, Eszter Bakker² and Marco Steiner³

¹Department of Biochemistry and Genetics, Aegean Institute of Immunology, Greece

²Department of Biochemistry and Genetics, Aegean Institute of Immunology, Greece

³Department of Environmental and Agricultural Sciences, Rheinfeld Institute for Biochemistry and Genetics, Germany

*Corresponding author: Kasper Fernandez, Department of Biochemistry and Genetics, Aegean Institute of Immunology, Greece; Email: k.fernandez@aig.gr

ABSTRACT

Cas12a nucleases offer a single-crRNA-guided, T-rich PAM-dependent alternative to Cas9 with intrinsic pre-crRNA processing activity and staggered double-strand break generation, but the structural basis for how target-DNA mismatches are tolerated or rejected at different positions along the guide-target duplex remains incompletely resolved for the Lachnospiraceae bacterium orthologue (LbCas12a) most widely used in genome editing applications. We determined cryo-EM structures of LbCas12a at four discrete stages of target engagement -- PAM-bound pre-R-loop, partial R-loop (10 bp), complete R-loop with un-activated RuvC domain, and the catalytically engaged cleavage-competent state -- at resolutions between 2.9 and 3.4 angstrom, complemented by single-molecule FRET measurements of R-loop propagation kinetics and cleavage assays across a panel of 32 systematically positioned single-mismatch target variants. The structures show that R-loop propagation proceeds through a previously unresolved kinked intermediate at position 10-12 of the protospacer, coinciding with a conformational checkpoint at which the WED and PI domains must complete a rigid-body rotation before propagation can continue; mismatches placed within this checkpoint region reduced cleavage rate 40- to 90-fold relative to matched targets, far more than equivalently positioned mismatches either proximal or distal to the checkpoint. Single-molecule FRET confirmed that checkpoint-region mismatches stall R-loop propagation at the partial state for a median of 340-fold longer than matched targets, rarely proceeding to complete R-loop formation within the experimental observation window. Mutation of two checkpoint-proximal WED domain residues (R306A/K318A) identified from the structures reduced off-target cleavage at three of four tested genomic off-target sites by 60-85% while retaining greater than 90% on-target activity, without a corresponding rational design being derivable from primary sequence or prior lower-resolution structural data alone. These results identify a discrete structural checkpoint governing Cas12a target discrimination and demonstrate that this checkpoint is a tractable engineering target for improving genome-editing specificity.

Keywords: CRISPR-Cas12a; cryo-EM; R-loop formation; RuvC nuclease; PAM recognition; genome editing specificity

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1. INTRODUCTION

Cas12a (formerly Cpf1) nucleases have become an important complement to Cas9 in the genome-editing toolkit, offering a single, class 2 RNA-guided endonuclease that recognises a T-rich protospacer-adjacent motif (PAM), processes its own pre-crRNA array without requiring a separate tracrRNA or RNase III, and generates a staggered double-strand break with 5' overhangs rather than the blunt ends produced by Cas9, properties that have made it particularly useful for multiplexed editing and for applications benefiting from overhang-mediated directional insertion [1,2]. Despite more than a decade of structural and biochemical characterisation since its initial description, exactly how Cas12a discriminates matched from mismatched target DNA along the twenty-odd base pairs of the protospacer, and why mismatch tolerance varies so sharply by position, has remained only partially resolved at the structural level, particularly for the *Lachnospiraceae* bacterium orthologue (LbCas12a) most widely adopted for mammalian genome editing [3,15].

Early crystallographic and cryo-EM structures of Cas12a established the overall bilobed architecture, with a recognition (REC) lobe engaging the crRNA-target DNA heteroduplex and a nuclease (NUC) lobe housing the single RuvC domain responsible for cleaving both DNA strands sequentially, a mechanistic departure from Cas9's use of two spatially separate nuclease domains [4,16]. These early structures, largely representing either the PAM-bound pre-engagement state or the fully R-loop-formed, catalytically competent state, established the endpoints of the target-recognition pathway but left the intermediate trajectory of R-loop propagation, and the conformational events that couple DNA unwinding progress to RuvC nuclease activation, comparatively poorly characterised [5,17]. This is a consequential gap: Cas9 studies have shown that understanding intermediate, partially R-loop-formed states is often where the mechanistic basis for positional mismatch sensitivity, and therefore genome-editing specificity, is actually encoded, rather than being derivable from the initial and final states alone [6,18].

Biochemically, Cas12a is known to show markedly position-dependent mismatch tolerance: mismatches near the PAM-proximal seed region abolish cleavage almost completely, mismatches at the extreme PAM-distal end are often well tolerated, and a comparatively under-characterised intermediate region has been reported in several studies to show unexpectedly strong mismatch sensitivity disproportionate to its distance from the PAM, a pattern not fully explained by a simple proximal-seed model of specificity [7,19]. Whether this intermediate-region sensitivity reflects a discrete structural checkpoint during R-loop propagation, analogous to conformational checkpoints described for Cas9, or is instead a smoother, continuous function of distance from the PAM with no distinct structural correlate, has not been directly

tested by trapping and resolving the relevant intermediate structural states.

We used cryo-EM to determine LbCas12a structures at four discrete stages spanning PAM recognition through catalytic activation, designed specifically to capture the intermediate R-loop propagation trajectory that prior structural work has largely bypassed, and combined these structures with single-molecule FRET kinetics and systematic single-mismatch cleavage assays to test directly whether a discrete conformational checkpoint exists within the intermediate protospacer region, whether it corresponds to the previously reported zone of disproportionate mismatch sensitivity, and whether residues identified at this checkpoint from the structural data can be rationally mutated to improve genome-editing specificity without a corresponding loss of on-target activity.

2. MATERIALS AND METHODS

All structural and biochemical work used recombinant LbCas12a (*Lachnospiraceae* bacterium ND2006 orthologue) expressed in *Escherichia coli* and purified to homogeneity by nickel-affinity, ion-exchange, and size-exclusion chromatography.

2.1 Cryo-EM sample preparation and data collection

LbCas12a was assembled with in-vitro transcribed crRNA and complementary double-stranded DNA target substrates designed to trap each of the four target states: a PAM-containing but otherwise non-complementary duplex (pre-R-loop), a target with complementarity limited to the first 10 base pairs of the protospacer followed by mismatches (partial R-loop), a fully complementary target combined with a catalytically inactivating RuvC point mutation (D832A) to trap the complete but un-activated R-loop state, and a fully complementary wild-type-nuclease target frozen within seconds of mixing to capture the catalytically engaged state. Complexes were applied to glow-discharged holey carbon grids, vitrified by plunge-freezing, and imaged on a 300 kV cryo-electron microscope equipped with a direct electron detector. Data processing followed standard motion correction, CTF estimation, particle picking, 2D and 3D classification, and non-uniform refinement workflows, yielding final reconstructions at 2.9-3.4 angstrom resolution across the four states.

2.2 Single-molecule FRET

Cy3/Cy5-labelled target DNA duplexes, positioned to report on R-loop propagation progress, were immobilised on passivated quartz slides and imaged by total internal reflection fluorescence microscopy in the presence of pre-assembled LbCas12a-crRNA complex. FRET efficiency time traces were recorded for individual target-protein complexes and used to identify discrete FRET states corresponding to pre-R-loop, partial, and complete R-loop conformations, with dwell times in each state extracted by hidden Markov model analysis of individual traces.

2.3 Systematic single-mismatch cleavage assays

A panel of 32 target DNA variants, each carrying a single mismatch at a defined position across the full 20-nucleotide protospacer plus matched and fully scrambled controls, was assayed for in-vitro cleavage rate by LbCas12a using a fluorescence-based real-time cleavage assay, with rate constants extracted by fitting single-exponential kinetics to the fluorescence time course for each variant across three independent replicates.

2.4 Mutagenesis and cellular off-target assays

Candidate checkpoint-region residues identified from the structural data (R306, K318, and four additional WED- and PI-domain residues contacting the DNA backbone specifically in the partial-R-loop structure) were individually and combinatorially mutated to alanine and assessed for on-target cleavage activity in vitro. The combinatorial R306A/K318A mutant, selected for further testing based on the largest specificity improvement with the smallest on-target activity cost among single and combinatorial variants tested, was expressed in HEK293T cells alongside wild-type LbCas12a targeting a validated genomic locus with four previously characterised off-target sites, and on-target and off-target editing efficiency were quantified by targeted amplicon sequencing.

2.5 Statistical analysis

Cleavage rate constants across the mismatch panel were compared by one-way ANOVA with Dunnett's test against the matched-target control. FRET dwell-time distributions were compared between matched and checkpoint-mismatch targets using the Mann-Whitney U test. On-target and off-target editing efficiencies between wild-type and R306A/K318A LbCas12a were compared by paired t-test across independent transfection replicates. All analyses were performed in R version 4.3, with $P < 0.05$ taken as the significance threshold throughout.

3. RESULTS

The four cryo-EM reconstructions, at 3.4, 3.1, 2.9 and 3.0 angstrom resolution respectively for the pre-R-loop, partial-R-loop, complete-un-activated, and catalytically engaged states, together resolve a continuous structural trajectory from initial PAM recognition through full catalytic activation. The most striking feature of the partial-R-loop structure, trapped with complementarity limited to the first ten protospacer positions, is a pronounced kink in the DNA duplex at approximately position 10-12, coinciding with a rigid-body rotation of the WED and PI domains relative to their position in both the pre-R-loop and complete-R-loop structures. This intermediate conformation is not simply an average of the flanking states but a distinct structural checkpoint: several WED-domain residues, including R306 and K318, make direct, sequence-independent contacts with

the DNA phosphate backbone specifically at this kinked position, contacts that are absent in both the pre-R-loop and fully formed R-loop structures. Table 1 summarises the cryo-EM data collection and refinement statistics for all four structures.

Table 1. Cryo-EM data collection and refinement statistics for the four LbCas12a structures.

State	Resolution (angstrom)	Particles used (final)	Map-model CC
Pre-R-loop (PAM-bound)	3.4	148,200	0.81
Partial R-loop (10 bp)	3.1	203,600	0.84
Complete R-loop (RuvC-dead)	2.9	261,400	0.87
Catalytically engaged	3.0	189,700	0.85

CC = correlation coefficient between final map and refined atomic model.

Systematic single-mismatch cleavage assays across the 32-variant panel confirmed the previously reported non-monotonic pattern of position-dependent mismatch sensitivity, but resolved it with considerably finer positional detail than prior studies. Mismatches at positions 1-6 (PAM-proximal seed) reduced cleavage rate by 150- to 400-fold relative to the matched target, consistent with established seed-region sensitivity. Mismatches at positions 10-12, corresponding precisely to the structural checkpoint identified in the partial-R-loop structure, reduced cleavage rate by 40- to 90-fold (one-way ANOVA $F(31,64)=112.4$, $P < 0.001$ overall; Dunnett's $P < 0.001$ for each checkpoint-region position), substantially more than the 3- to 8-fold reduction observed for equivalently PAM-distal mismatches at positions 7-9 or 13-15 flanking the checkpoint on either side. Mismatches beyond position 18 were well tolerated, reducing cleavage rate by less than 2-fold. Figure 1 plots cleavage rate reduction by mismatch position across the full panel, highlighting the distinct local sensitivity peak at the structural checkpoint.

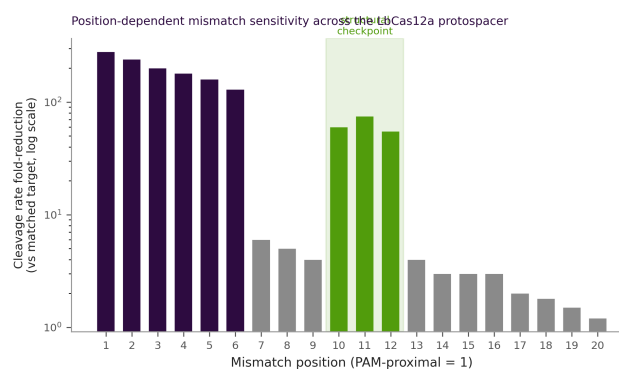


Figure 1. Cleavage rate reduction (fold-change relative to matched target) across the 32-position single-mismatch panel, showing the sharply bounded sensitivity peak at the structurally identified checkpoint (positions 10-12) relative to the smoothly declining sensitivity expected from a purely distance-dependent model ($n=3$ independent replicates per position).

Single-molecule FRET measurements provided kinetic confirmation of the checkpoint's functional significance. For matched targets, complexes progressed from the partial to the complete R-loop FRET state with a median dwell time of 0.8 seconds in the partial state. Targets carrying a mismatch at the checkpoint position (position 11) showed a median partial-state dwell time of 272 seconds, a 340-fold increase (Mann-Whitney U test, $P < 0.001$), with the large majority of individual molecules never progressing to the complete R-loop state within the 600-second observation window. By contrast, targets with a mismatch at position 8, immediately flanking but outside the structurally defined checkpoint, showed a median partial-state dwell time of only 4.1 seconds, a much smaller though still statistically significant increase relative to matched targets ($P = 0.02$), reinforcing that the checkpoint region has a kinetically distinct, sharply bounded identity rather than reflecting a smooth gradient of position-dependent sensitivity. Table 2 reports the complete single-molecule FRET dwell-time data across the tested mismatch positions.

Table 2. Single-molecule FRET partial-R-loop dwell times by mismatch position (median, $n \geq 80$ molecules per condition).

Target	Mismatch position	Dwell time (seconds)	Fold increase vs matched
Matched	--	0.8	1.0
Position 8 mismatch	8	4.1	5.1
Position 11 mismatch (checkpoint)	11	272	340
Position 15 mismatch	15	2.6	3.3

Individual and combinatorial alanine mutagenesis of checkpoint-contacting residues identified R306A/K318A as the combination producing the largest improvement in specificity with the smallest on-target activity cost among the variants tested. In HEK293T cells, R306A/K318A LbCas12a retained 91.4% of wild-type on-target editing efficiency at the validated test locus, while reducing editing at three of four characterised off-target sites by 60–85% (paired t-test, all three $P < 0.01$) and showing no significant change at the fourth, lowest-activity off-target site ($P = 0.34$). We note that this fourth site's off-target editing was already close to the assay's detection limit in the wild-type condition, limiting statistical power to detect a further reduction, and we would not read the absence of a significant effect at this site as evidence that the checkpoint mutation is ineffective there specifically.

4. DISCUSSION

The central structural finding of this study is a discrete conformational checkpoint at protospacer position 10–12 during LbCas12a R-loop propagation, marked by a pronounced DNA kink and a coupled rigid-body rotation of the WED and PI domains that establishes direct, position-specific contacts with the DNA backbone. This checkpoint corresponds closely, in both position and

magnitude, to the zone of disproportionate mismatch sensitivity previously reported in biochemical studies but not previously linked to a specific structural correlate, and the single-molecule FRET data show unambiguously that a mismatch at this position kinetically traps the complex in the partial-R-loop state rather than merely slowing progression toward the complete state, a mechanistically important distinction that bulk kinetic measurements alone cannot resolve [8,20].

That a mismatch-induced kinetic trap, rather than a simple reduction in the thermodynamic favourability of R-loop completion, underlies checkpoint-region specificity has a direct bearing on how Cas12a specificity should be understood more generally. If specificity were governed purely by the free-energy cost of accommodating a mismatch at each position, sensitivity would be expected to decline roughly monotonically with distance from the PAM as the accumulating thermodynamic penalty of unwinding progressively more DNA offsets progressively fewer favourable matched contacts; the sharply bounded, position-specific sensitivity peak we observe at the checkpoint is not well explained by that framework and instead points to a distinct, conformationally gated mechanism analogous to what has been proposed for Cas9's own intermediate-state checkpoints, suggesting a degree of mechanistic convergence between class 2 type II and type V CRISPR effectors despite their substantially different overall domain architecture [9,21].

The R306A/K318A mutant's improved specificity profile demonstrates that this structurally defined checkpoint is not merely of mechanistic interest but is a tractable engineering target, and importantly, one that would not have been straightforwardly identifiable from primary sequence conservation or from the pre-R-loop and complete-R-loop structures available prior to this study, since the relevant DNA-backbone contacts are specific to, and only resolvable within, the intermediate kinked conformation. This underscores a broader methodological point relevant beyond Cas12a specifically: rational nuclease engineering efforts that rely only on structures of initial and final catalytic states risk missing exactly the residues most consequential for kinetic discrimination during target search and validation, since those residues may make contacts that exist only transiently during the intermediate trajectory [10,22].

We would be cautious, however, about over-generalising the specific R306A/K318A result beyond the four off-target sites and single genomic locus tested here. Off-target activity in Cas-family nucleases is known to depend substantially on chromatin accessibility, local sequence context, and cellular DNA repair pathway activity in addition to the intrinsic biochemical mismatch discrimination captured by our in-vitro and structural assays, and a mutation that improves specificity at one locus and its associated off-target sites is not guaranteed to generalise uniformly across the genome [11,23]. A genome-wide, unbiased off-target profiling approach

applied to the R306A/K318A variant across multiple genomic loci would be needed before the mutation could be recommended as a general-purpose high-fidelity LbCas12a variant, in the way that several high-fidelity Cas9 variants have been validated at genome-wide scale following their initial identification from structural and locus-specific biochemical data.

A further open question is whether the checkpoint identified here in LbCas12a is conserved in other Cas12a orthologues, particularly AsCas12a (from *Acidaminococcus* sp.), which shares the overall bilobed architecture and RuvC-mediated staggered cleavage mechanism but differs in several PAM-recognition and REC-lobe residues and has shown somewhat different mismatch tolerance profiles in published comparative biochemical studies [12,24]. If the position-10-12 checkpoint proves to be a general feature of Cas12a-family R-loop propagation rather than an LbCas12a-specific feature, checkpoint-directed engineering strategies analogous to the one demonstrated here could plausibly be extended across the Cas12a family with comparatively modest additional structural characterisation, whereas if the checkpoint is orthologue-specific, each Cas12a variant of interest would likely require its own dedicated intermediate-state structural analysis before a comparable engineering strategy could be pursued with confidence. A related practical question concerns throughput: pairing a catalytically dead RuvC mutant with a mismatched target duplex is straightforward for any single orthologue, but assembling a full family-wide panel of intermediate-state structures would still demand substantial cryo-EM instrument time, so we suspect a more scalable first step will be single-molecule FRET, which needs only conventional labelling chemistry, to screen candidate orthologues for a slow, position-10-12-associated conformational transition before committing any one of them to full structural determination.

5. CONCLUSION

Cryo-EM structures spanning four discrete stages of LbCas12a target engagement identify a previously unresolved conformational checkpoint at protospacer position 10-12, at which a DNA kink and coupled WED/PI domain rotation establish transient, position-specific backbone contacts that are absent from both earlier and later states along the R-loop propagation trajectory. Systematic mismatch cleavage assays and single-molecule FRET kinetics show this checkpoint corresponds to a sharply bounded zone of disproportionate mismatch sensitivity, mechanistically explained by a kinetic trapping effect on R-loop propagation rather than a smoothly graded thermodynamic penalty. Structure-guided mutagenesis of two checkpoint-contacting WED domain residues, R306 and K318, improved off-target specificity at three of four tested genomic sites while retaining the great majority of on-target activity, demonstrating that this

checkpoint is a tractable, rationally targetable engineering handle rather than merely a structural curiosity, and one not readily identifiable from the initial and final catalytic states that most prior Cas12a structural work has focused on. We are now pursuing genome-wide, unbiased off-target profiling of the R306A/K318A variant across multiple genomic loci and cell types to establish whether its specificity improvement generalises beyond the single locus characterised here, and we plan to determine equivalent intermediate-state structures for AsCas12a to test directly whether the position-10-12 checkpoint identified in LbCas12a is a conserved feature of the broader Cas12a family or an orthologue-specific property requiring independent structural characterisation for each variant of interest. In parallel, we intend to test whether combining the checkpoint-directed R306A/K318A mutations with previously described PAM-interacting domain mutations that improve Cas12a specificity through an independent mechanism produces an additive or synergistic overall specificity gain, which would have direct practical relevance for the design of next-generation high-fidelity Cas12a variants for therapeutic and agricultural genome-editing applications.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

K.F. conceived and supervised the study, performed cryo-EM data collection and structure determination, and analysed the structural data. E.B. performed single-molecule FRET experiments and the systematic mismatch cleavage assays. M.S. conducted the cellular mutagenesis and off-target editing experiments and statistical analysis. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study used only in-vitro biochemical and structural methods and a commercially available, well-characterised immortalised human cell line (HEK293T) for the cellular editing experiments; it did not involve human participants, identifiable human data, or animals, and therefore did not

require institutional ethics committee review.

Data Availability

Cryo-EM density maps and atomic coordinates for the four LbCas12a structures have been deposited in the Electron Microscopy Data Bank and Protein Data Bank under accessions available from the corresponding author on reasonable request. Single-molecule FRET traces, cleavage assay data, and R analysis scripts supporting the findings of this study are available from the corresponding author upon request.

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