Characterizing a more efficient AsCas12a

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INTRODUCTION

Gene editing is a promising new approach to tackle treatments and cures for genetic diseases. In particular, CRISPR (Clustered Regular Interspaced Palindromic Repeats)/Cas has emerged as an exciting therapeutic modality due to its inherent ability to specifically target and modify specific sites within the genome. The two most common Cas enzymes used for therapeutic studies are Cas9 and Cas12a. Cas12a exhibits important differences from Cas9 that make it an exciting enzyme to further characterize as a gene editing tool. Specifically, Cas12a recognizes a different protospacer-adjacent motif (PAM), uses a shorter guide RNA (gRNA) and generates sticky ends instead of blunt ends at the cleavage site.[1,2,3,4] Additionally, Cas12a is less tolerant of mismatches within the R-loop compared to Cas9, making it a more specific enzyme.[5] While already a very specific enzyme, recent work has been performed to improve its editing efficiency, resulting in an engineered variant termed AsCas12a Ultra.[6] Here, we describe recent work performed to characterize why AsCas12a Ultra is more efficient than the WT AsCas12a enzyme, and specifically highlight why this new variant has promising therapeutic potential.

Figure 1. Cas12a (Cpf1) is a nuclease with high sequence-specificity

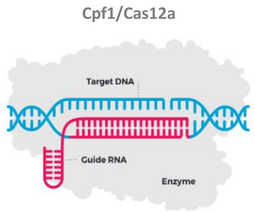


Figure 1. Cartoon of Cas12a (Cpf1)/DNA complex. Protein (grey) with crRNA (pink) complexed with complementary DNA duplex (blue, hydridized strand shown in pink). Figure copied from the Editas website (https://www.editasmedicine.com/gene-editing-pipeline/).

- AsCas12a recognizes a T-rich PAM sequence (TTTV). This is in contrast to the G-rich PAM (NGG) recognized by Cas9.[3]
- The Cas12a-associated gRNA is much shorter compared to Cas9 gRNA.[3] Additionally, since the Cas12a gRNA 3' end is PAM-proximal, while in Cas9 it is PAM-distal, odds of synthesis-related errors or mismatches in the RNA/DNA duplex is low, since gRNA synthesis occurs 3'-5'.
- AsCas12a has higher specificity to its target DNA compared to Cas9 due to its longer seed sequence and requirement for a perfectly matched RNA/DNA duplex for cleavage activity.[5,7]

Figure 2. AsCas12a Ultra is more efficient than WT AsCas12a

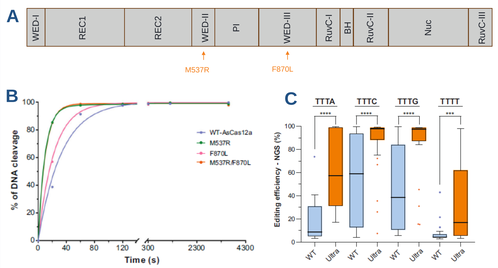


Figure 2. AsCas12a is a more efficient nuclease. **A.** Domain outline of AsCas12a with specific mutations to generate AsCas12a Ultra indicated in orange. **B**. DNA cleavage activity of WT AsCas12a (blue) compared to AsCas12a Ultra (orange) as well as to each single mutation (green and pink for M537R and F870L, respectively) (Figure from Zhang et al. Nat Com. 2021). **C**. Editing efficiency of WT (blue) vs Ultra AsCas12a (orange) with varying PAM sequences (Figure from Zhang et al. Nat Com. 2021).

- AsCas12a Ultra (M537R/F870L) is a more efficient nuclease and allows for cleavage at TTTN PAM sites compared to only TTTV for WT AsCas12a.[6]
- <u>Key question: how do M537R and F870L mutations result in a more efficient nuclease and how do they allow editing at TTTT PAM sites?</u>

RESULTS

Figure 3. Structure of AsCas12a Ultra in complex with DNA

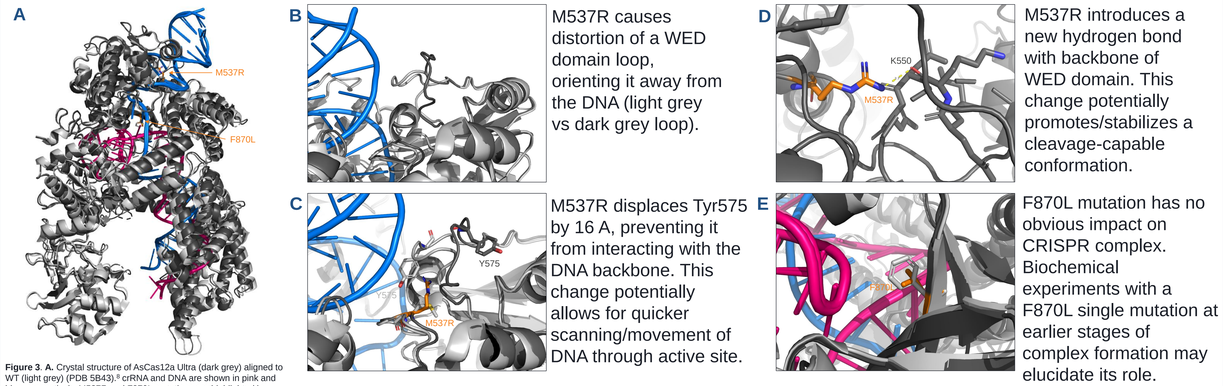


Figure 3. **A.** Crystal structure of AsCas12a Ultra (dark grey) aligned to WT (light grey) (PDB 5B43).[6] crRNA and DNA are shown in pink and blue, respectively. M537R and F870L mutations are highlighted in orange and indicated by arrows. **B**. Closeup view of DNA-contacting loop displaced in Ultra. **C**. Closeup of displaced Y575 in Ultra. **D**. Hydrogen bond between M537R and helix of the WED domain. **E.** Closeup of F870L mutation environment.

M537R causes distortion of a WED domain loop, orienting it away from the DNA (light grey vs dark grey loop).

M537R displaces Tyr575 by 16 A, preventing it from interacting with the DNA backbone. This change potentially allows for quicker scanning/movement of DNA through active site.

M537R introduces a new hydrogen bond with backbone of WED domain. This change potentially promotes/stabilizes a cleavage-capable conformation.

F870L mutation has no obvious impact on CRISPR complex. Biochemical experiments with a F870L single mutation at earlier stages of complex formation may elucidate its role.

<u>M537R results in decreased contact with DNA and F870L has no obvious effect in our crystal structure. How do these mutations affect activity?</u>

Figure 4. AsCas12a Ultra does not impact Non-template strand (NTS) and Template strand (TS) cleavage rates

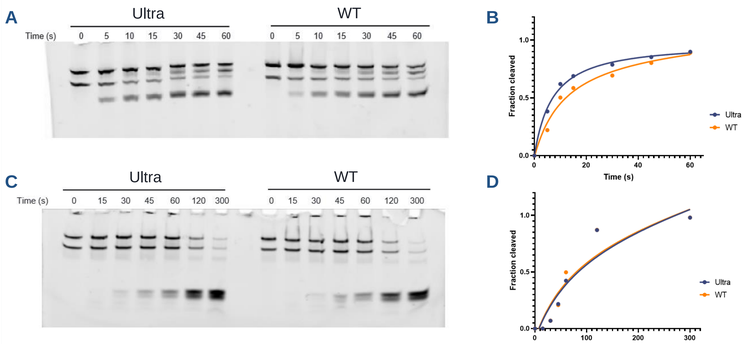
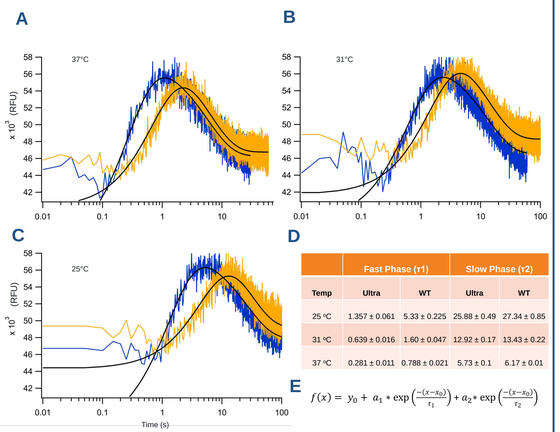


Figure 4. Cleavage kinetics of AsCas12a WT vs Ultra. **A.** Representative gel of Non-template Strand (NTS) cleavage by Ultra and WT AsCas12a. Top two bands represent undenatured uncleaved and cleaved duplex and were not used to calculate fraction cleaved NTS strand. **B.** Quantification of NTS cleavage from data in A for Ultra (blue) and WT (orange). **C.** Representative gel of Template Strand (TS) cleavage by Ultra and WT AsCas12a. Top band represent undenatured uncleaved duplex and was not used to calculate fraction cleaved TS strand. **D**. Quantification of TS cleavage from data in C for Ultra (blue) and WT (orange).

- Ultra mutations have no effect on NTS and TS cleavage kinetics when comparing WT and Ultra.
- As expected from previous studies, NTS cleavage is much faster (Fig 3A,B) compared to TS cleavage (Fig 3C,D).[5]
- NTS and TS cleavage are not rate-limiting steps, so not unsurprising that kinetics at these stages are the same between WT and Ultra.
- $Pro + DNA \overset{K_D}{\rightleftharpoons} PD \overset{K_{R-Loop}}{\rightleftharpoons} PD_R \xrightarrow{k_{NTS}} PD_{NTS} \xrightarrow{k_{TS}} PD_{TS}$

  where $K_{NTS} >> K_{TS} >>> K_{R\text{-}loop}$.[5]
- <u>How do Ultra mutations affect the rate-limiting R-loop formation step?</u>

Figure 5. AsCas12a Ultra exhibits faster R-loop formation compared to WT.



	Fast Phase (τ1)		Slow Phase (τ2)	
Temp	Ultra	WT	Ultra	WT
25 ºC	1.357 ± 0.061	5.33 ± 0.225	25.88 ± 0.49	27.34 ± 0.85
31 ºC	0.639 ± 0.016	1.60 ± 0.047	12.92 ± 0.17	13.43 ± 0.22
37 ºC	0.281 ± 0.011	0.788 ± 0.021	5.73 ± 0.1	6.17 ± 0.01

Figure 5. AsCas12a Ultra exhibits faster R-loop formation compared to WT. R-loop conformational dynamics captured by stopped-flow spectrophotometry. Data was collected using a cleavage incompetent FAM-labelled TS oligo coupled with a flour-quencher. Any small changes in conformational dynamics around the FAM label are captured as an increase/change in fluorescence (y-axis). **A**. R-loop formation at 37°C with AsCas12a Ultra shown in blue and WT in orange. **B** and **C**. Same experiment at 31°C and 25°C, respectively. RFU – Relative Fluorescence Units. **D**. Quantification of time constants generated from graphs shown in A,B and C. **E**. Equation used to fit data in graphs A-C.

Figure 6. Preliminary R-loop propagation measurements of AscAS12a Ultra

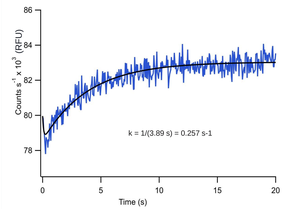


Figure 6. AsCas12a Ultra R-loop propagation. R-loop propagation was measured using stopped-flow spectrophotometry using a 2-amino purine (2-AP) modified nucleotide on the NTS at position 4 of the R-loop at 25°C. Fluorescence is quenched when 2-AP is within a stable base-pair but increases when 2-AP is single-stranded, as indicated by displacement of the NTS during R-loop formation. The fit of the data was performed using the indicated equation under the curve. Experiment based on R-loop propagation measurements for WT AsCas12a.[5] RFU – Relative Fluorescence Units.

- Together, stopped-flow experiments demonstrate efficient R-loop propagation for AsCas12a Ultra.
- Additional effects on R-loop formation/propagation will be measured using mismatched templates for both WT and Ultra.

CONCLUSIONS

- Structurally, AsCas12a WT and Ultra are very similar when complexed with DNA to form a fully extended 20 bp DNA/RNA hybrid. It is possible that the M537R and F870L mutations would have more obvious effects in structures of earlier AsCas12a/DNA complex intermediates.
- M537R results in decreased interactions with the DNA template, which might facilitate quicker DNA/RNA hybrid formation. Additionally, M537R hydrogen bonds with K550 in the WED domain. This interaction could potentially result in a more cleavage competent conformation.
- AsCas12a WT and Ultra do not display different NTS and TS cleavage kinetics, as is expected since neither step is rate limiting.
- R-loop formation is rate limiting in WT AsCas12 and is indeed sped up significantly in AsCas12a Ultra. Previous studies show that AsCas12a specificity is not impacted by the increased efficiency generated by the Ultra mutations.[6]

DISCLOSURES

Linnea Jansson-Fritzberg, Bryant Chica and Steve Wolk are employees and stock-holders at Editas Medicine Inc. Alex Dementiev and Andre White are employees and stock-holders at Schrödinger, Inc.

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References:
1. Deltcheva et al. Nature. 2011. 2. Fonfara et al. NAR. 2014. 3. Zetsche et al. Cell. 2015. 4. Garneau et al. Nature. 2010. 5. Strohkendl et al. Mol. Cell. 2018. 6. Zhang et al. Nat Com. 2021. 7. Strohkendl et al. BioRxiv. 2023. 8. Yamano et al. Cell. 2016.

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