

Tetramerisation of the CRISPR ring nuclease Csx3 facilitates cyclic oligoadenylate cleavage

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Abstract

Type III CRISPR systems detect foreign RNA and activate the cyclase domain of the Cas10 subunit, generating cyclic oligoadenylate (cOA) molecules that act as a second messenger to signal infection, activating nucleases that degrade the nucleic acid of both invader and host. This can lead to dormancy or cell death; to avoid this, cells need a way to remove cOA from the cell once a viral infection has been defeated. Enzymes specialised for this task are known as ring nucleases, but are limited in their distribution. Here, we demonstrate that the widespread CRISPR associated protein Csx3, previously described as an RNA deadenylase, is a ring nuclease that rapidly degrades cyclic tetra-adenylate (cA₄). The enzyme has an unusual cooperative reaction mechanism involving an active site that spans the interface between two dimers, sandwiching the cA₄ substrate. We propose the name Crn3 (CRISPR associated ring nuclease 3) for the Csx3 family.

Keywords:

CRISPR, Csx3, ring nuclease, cyclic tetra-adenylate, CARF, cooperative enzyme

Introduction

The CRISPR system provides adaptive immunity against viruses and other Mobile Genetic Elements (MGE) in bacteria and archaea (reviewed in (Wright et al., 2016, Koonin and Makarova, 2017)). Type III CRISPR systems are widespread in archaea and found in many bacteria (Makarova et al., 2011), including the human pathogen *Mycobacterium tuberculosis* (Gröschow et al., 2019). Type III effectors utilise a Cas7 backbone subunit to bind CRISPR RNA (crRNA) (Rouillon et al., 2013). This allows detection of the RNA encoded by invading MGE via base-pairing to the crRNA. Binding of this “target RNA” results in subtle conformational changes in the effector complex, activating the catalytic Cas10 subunit which uses its HD-nuclease domain to degrade DNA (Elmore et al., 2016, Estrella et al., 2016, Kazlauskienė et al., 2016, Jung et al., 2015, Han et al., 2017a) and its cyclase domain to synthesise cyclic oligoadenylate (cOA) molecules by polymerisation of ATP (Kazlauskienė et al., 2017, Niewoehner et al., 2017, Rouillon et al., 2018). These cyclic molecules, which range from 3-6 AMP monomers in size (cA₃ to cA₆), act as second messengers in the cell, signalling viral infection and activating cellular defences.

cA₄ and cA₆ bind specifically to two subtypes of CRISPR Associated Rossmann Fold (CARF) domain (Makarova et al., 2014). These domains are fused to a range of effector domains that carry out defensive duties. The best characterised is the Csx1/Csm6 family that utilises a C-terminal HEPN (Higher Eukaryotes and Prokaryotes, Nucleotide binding) domain to cleave RNA non-

specifically (Niewoehner and Jinek, 2016, Sheppard et al., 2016). The Csx1/Csm6 ancillary ribonucleases are crucial for CRISPR-based immunity *in vivo* in several different organisms, emphasizing the importance of cOA signalling for type III CRISPR defence (Jiang et al., 2016, Gröschow et al., 2019, Foster et al., 2019, Deng et al., 2013). A wide variety of alternative CARF-domain proteins associated with type III CRISPR loci have been identified but not yet described (Shmakov et al., 2018, Shah et al., 2018), and recent work has characterized the cA₄-activated DNA nickase Can1, which is present in *Thermus thermophilus* (McMahon et al., 2020).

Thus, type III CRISPR systems are capable of directing a multi-faceted antiviral defence on detection of foreign RNA in the cell. However, activation of this anti-viral state is known to generate collateral damage to host nucleic acids (Rostol and Marraffini, 2019) that could lead to dormancy or cell death. While this could be an acceptable outcome for an infected cell, if a viral infection can be cleared then the cOA-signalling pathway needs a mechanism to remove the cOA molecules and return the cell to a basal state. Some archaea encode a dedicated ring nuclease (Athukoralage et al., 2018), recently named as the Crn1 family (CRISPR-associated ring nuclease 1) (Athukoralage et al., 2020b). Crn1 is a specialised CARF domain protein that splits the cA₄ ring into two linear A₂ products to switch off the antiviral response (Athukoralage et al., 2018). In other systems, the CARF domains of Csx1/Csm6 family nucleases slowly degrade cOA, thus acting as bi-functional, self-limiting nucleases (Athukoralage et al., 2019, Jia et al., 2019, Garcia-Doval et al., 2020). Many archaeal viruses and some bacteriophage also encode a specialised ring nuclease as an anti-CRISPR (Acr). This enzyme, known as AcrIII-1, binds cA₄ by means of a distinct protein fold (DUF1874) unrelated to the CARF domain, and degrades cA₄ rapidly using conserved active site residues (Athukoralage et al., 2020b). These viral ring nucleases can abrogate type III CRISPR immunity by rapidly destroying the cA₄ infection signal. This enzyme has been co-opted into some bacterial type III CRISPR systems, where it likely acts to degrade cA₄ following clearance of phage infection. In this context, it has been named as CRISPR-associated ring nuclease 2 (Crn2) (Athukoralage et al., 2020b, Samolygo et al., 2020).

Ring nucleases thus appear to be an important constituent of virus:host conflict in the expanding arena of cyclic nucleotide signalling. Here, we focus on the Csx3 family of proteins, which is found associated with many type III CRISPR systems (Shah et al., 2018, Shmakov et al., 2018). Csx3 from *Archaeoglobus fulgidus* has been described as an RNA deadenylation enzyme and crystallised in complex with a pseudo-symmetric RNA tetraloop (Yan et al., 2015). Subsequent structural analysis revealed that the Csx3 protein is a distantly related member of the CARF family of proteins (Topuzlu and Lawrence, 2016). Spurred by these observations, we undertook a detailed study of the Csx3 protein. We demonstrate that Csx3 is, in fact, a cA₄-specific ring nuclease, for which we propose the name Crn3 (CRISPR-associated ring nuclease 3). The enzyme has an unusual cooperative reaction mechanism involving the association of two dimers, bridged by the cA₄ substrate, to complete the active site. This mechanism may allow the cell to respond appropriately to changing cA₄ and Csx3 levels during viral infection and preserve type III CRISPR immunity.

Results

Csx3 is a ring nuclease specific for binding and cleavage of cA₄

The Csx3 protein from *A. fulgidus* was originally crystallised in the absence and presence of a pseudo-symmetric RNA tetranucleotide, and shown to have RNA deadenylase activity *in vitro* (Yan et al., 2015). Given what is now known about cA₄ ring nucleases, we re-assessed the structure and potential function of the Csx3 protein. It has previously been suggested that the binding site for the RNA tetranucleotide could be compatible with binding of a symmetric cyclic oligonucleotide

(Topuzlu and Lawrence, 2016), similar to those observed in other CARF family proteins. Examination of the genomic context of Csx3 in selected archaeal and bacterial species confirmed its close association with type III-B CRISPR systems and with other CARF family proteins such as the ribonuclease Csx1 (Figure 1A), implying a role in cyclic oligoadenylate signalling and providing further impetus to re-examine the function of Csx3. Csx3 is most commonly observed in the euryarchaea and cyanobacteria (Figure 1- figure supplement 1). We therefore cloned, expressed and purified *A. fulgidus* Csx3, allowing biochemical analysis. Initial studies showed that Csx3 binds cyclic tetra-adenylate with an apparent dissociation constant $< 0.1 \mu\text{M}$. In contrast, an RNA oligonucleotide (49-9A) with a 9A poly-adenylate 3' tail was bound 100-fold less tightly, with an apparent dissociation constant around $10 \mu\text{M}$ (Figure 1B).

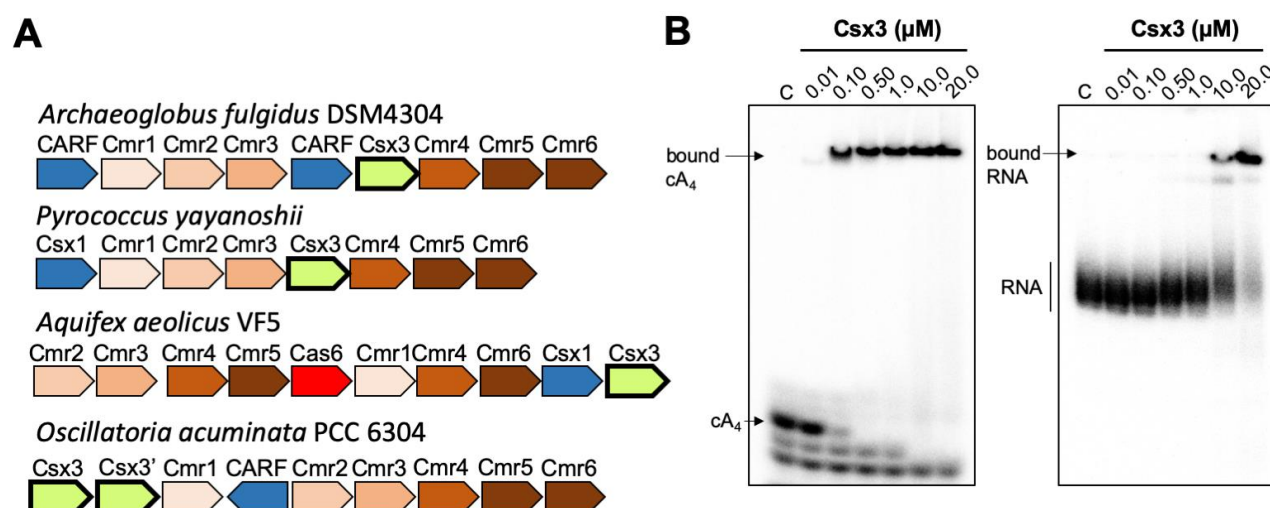


Figure 1. Csx3 is a type III CRISPR accessory protein that binds cA_4 . **A.** Genome context of selected Csx3 orthologues. Csx3 is found next to type III-B CRISPR operons and adjacent to predicted CARF protein ancillary effector proteins. Csx3' is a longer version of Csx3, common in cyanobacteria, consisting of an N-terminal Csx3 domain fused to a C-terminal kinase/transferase domain of unknown function. **B.** Phosphor images of native gel electrophoresis visualising cA_4 (20 nM) or RNA oligonucleotide 49-9A (50 nM) binding by *A. fulgidus* Csx3. Csx3 binds to cA_4 with high affinity (apparent $K_D \sim 50 \text{ nM}$) and binds the RNA 49-9A with significantly lower affinity (apparent $K_D \sim 10 \mu\text{M}$). Images are representative of three technical replicates.



Figure 1 – figure supplement 1. Multiple sequence alignment of Csx3 proteins showing conserved residues. Csx3 proteins are shown from *Archaeoglobus fulgidus*; *Methanosarcinia mazei*; *Methanosarcinia*; *Bathyarchaeota*; *Thermococcus celericrescens*; *Methylobacterium fumariolicum*; *Aquifex aeolicus*; *Dictyoglomus turgidum*; *Spirulina major*; *Oscillatoria nigro-viridis*. Absolutely conserved residues are shaded. Conserved residues H60 and D69 (Afu numbering) are indicated by asterisks.

The enzymatic specificity of Csx3 was tested against an RNA substrate oligonucleotide 49-9A. Csx3 cleaved this substrate in the presence of Mn^{2+} ions, removing nucleotides from the 3' end (Figure 2), in keeping with previous observations (Yan et al., 2015). The rate constant for cleavage was calculated as $0.0063 \pm 0.0013 \text{ min}^{-1}$ at 50°C . By comparison, Csx3 cleaved cA_4 with a single-turnover rate constant of $3.5 \pm 0.16 \text{ min}^{-1}$ at 50°C (Figure 2C). The ~600-fold faster cleavage rate for cA_4 compared to RNA strongly suggests that cA_4 is the physiological substrate, and that RNA with a 3' polyA tail represents a substrate analogue of the cyclic nucleotide, as observed previously for a Csx1/Csm6 family enzyme (Han et al., 2017b). Although the physiological growth temperature of *A. fulgidus* is around 70°C , we conducted these assays at 50°C to enable rate determination.

The products of the cA_4 cleavage reaction were identified by liquid-chromatography high-resolution mass spectrometry (LC-HRMS) as predominantly linear di-adenylate (A_2P), plus a small amount of A_2P with a cyclic phosphate ($A_2>P$) (Figure 2D). This suggests there are two active sites in the dimeric Csx3 structure, as seen for the ring nuclease Crn1 (Athukoralage et al., 2018). Similar results were obtained for Csx3 from the mesophilic archaeon *Methanosarcina mazei* (Figure 2 – figure supplement 1).

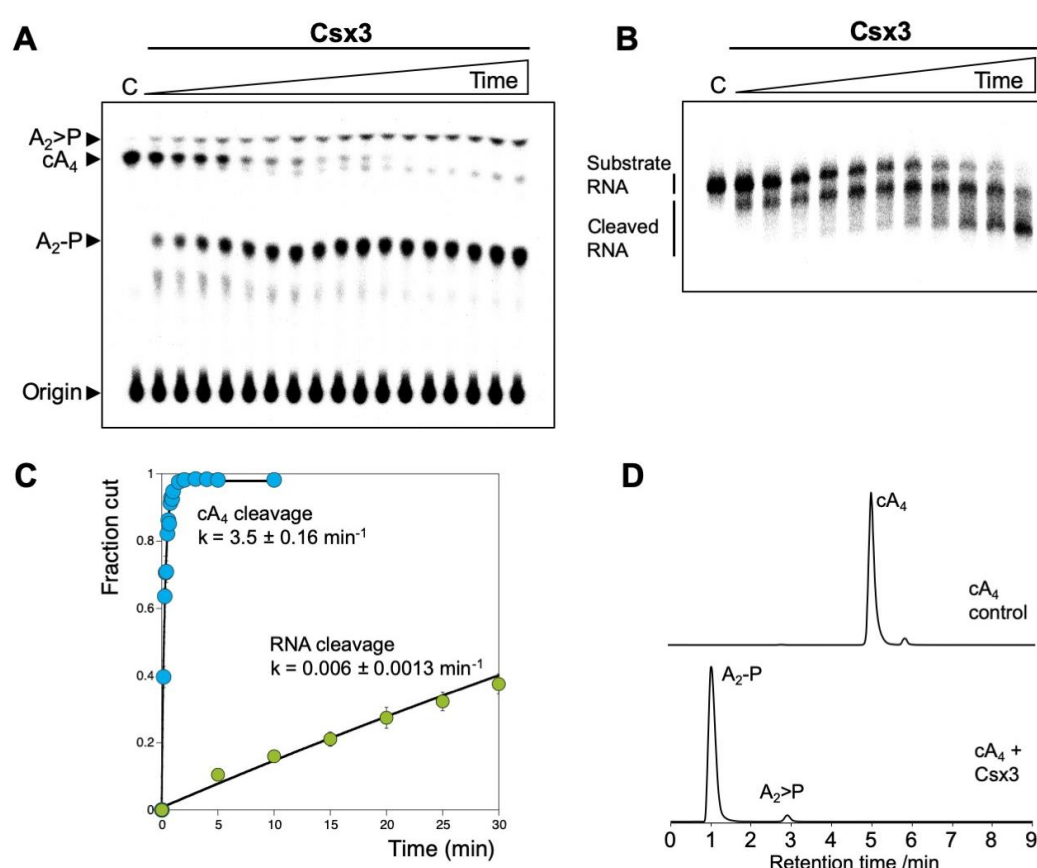


Figure 2. Csx3 is a potent ring nuclease. **A.** TLC analysis of the reaction products of radiolabelled cA_4 (200 nM) incubated with *A. fulgidus* Csx3 (8 μM dimer) in reaction buffer at 50°C (time points every 5 s from 10-60 s then 1.5, 2, 3, 4, 5 and 10 min, $n = 6$ technical replicates). The cA_4 was rapidly converted into A_2P , with a small amount of $A_2>P$ (linear A_2 with a cyclic 2',3' terminal phosphate). **B.** Denaturing polyacrylamide gel electrophoresis visualising cleavage of 5'-end radiolabelled RNA 49-9A (50 nM) by Csx3 (8 μM dimer) at 50°C (time points every 5 min from 5-40 min then 50, 60 and 90 min, $n = 3$ technical replicates). **C.** Plot comparing single-turnover kinetics of cA_4 (blue) and RNA 49-9A (green) cleavage by AfCsx3 at 50°C . Data points are the average of three technical replicates and error bars represent the standard deviation of the mean. **D.** Liquid-chromatography high-resolution mass spectrometry analysis of reaction products when cA_4 (100 μM , top) was incubated with AfCsx3 (10 μM) for 10 min at 50°C (bottom). cA_4 was fully degraded to form A_2P (di-adenylate containing a 3' phosphate) with a minor amount of $A_2>P$ (di-adenylate containing a 2',3' cyclic phosphate).

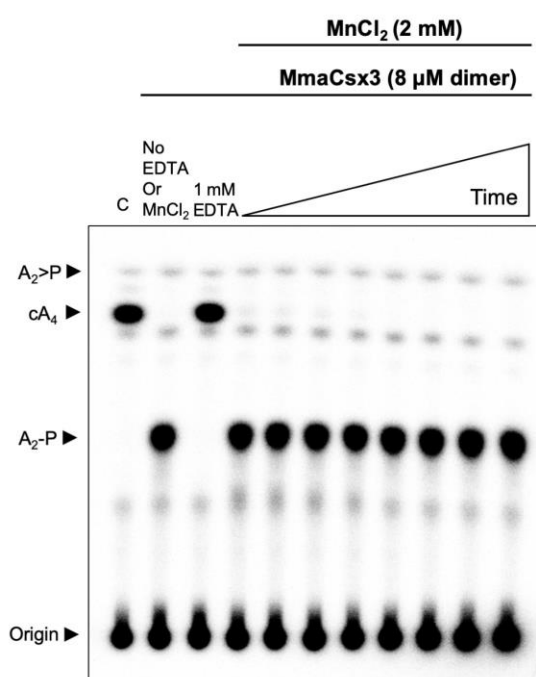


Figure 2 – figure supplement 1. Csx3 from *M. mazei* is a ring nuclease. TLC analysis of cleavage products obtained after incubating radiolabelled cA₄ (~200 nM) with MmaCsx3 (8 μM dimer) in reaction buffer at 50 °C (time points are 10 s, 20 s, 30 s, 1 min, 5 min, 15 min, 30 min and 60 min, n = 3 technical replicates). The cA₄ was rapidly converted into A₂-P, which remained unchanged thereafter.

144

145 Csx3 can decrease cA₄-mediated immunity *in vivo*

146 To determine whether Csx3 displayed properties consistent with a function as a ring nuclease *in*
147 *vivo*, we made use of the synthetic *M. tuberculosis* (Mtb) type III CRISPR system that we recently
148 established in *E. coli* (Grüschow et al., 2019). This system allows expression of the Mtb Csm
149 complex, defined CRISPR RNA and the processing enzyme Cas6 along with a cOA effector
150 protein of choice. In this case, we used the previously characterised Csx1 ribonuclease from
151 *Thioalkalivibrio sulfidophilus*, which is activated by cA₄ (Grüschow et al., 2019). Csx1 provided
152 significant immunity (3 logs) against plasmid transformation when a targeting crRNA specific for
153 the plasmid was provided (Figure 3). As a control, we added the phage ring nuclease AcrIII-1 from
154 bacteriophage THSA-485A, which we previously showed could abolish immunity mediated by cA₄
155 (Athukoralage et al., 2020b), and observed the expected loss of plasmid targeting, reflected in
156 higher transformation efficiencies. When the phage ring nuclease gene was replaced by a gene
157 encoding Csx3 from *M. mazei* (chosen as the organism grows at close to 37 °C, in common with *E.*
158 *coli*) we observed smaller but significant increases in plasmid transformation efficiency, with an
159 effect that increased from 18 to 40 h growth post-transformation. These observations were
160 consistent with a partial deactivation of the Csx1 ribonuclease due to degradation of cA₄ by the
161 Csx3 enzyme. The effect is not as striking as when using a *bona fide* Acr protein, which is
162 consistent with the prediction that Csx3 functions as part of the type III CRISPR defence in cells
163 that express it – rather than an Acr. It should be noted that we expressed the Csx3 enzymes using
164 a strong inducible promoter in these assays and we do not know what the relevant Csx3
165 concentrations are in virally-infected cells.

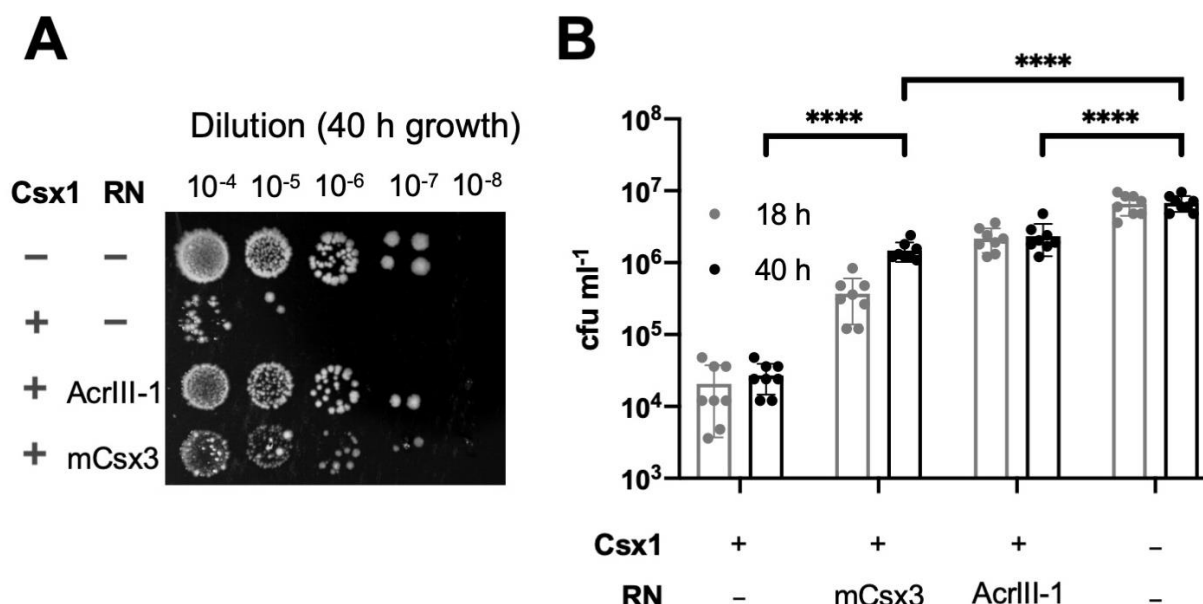


Figure 3. Csx3 functions as a ring nuclease in vivo. **A.** The effector ribonuclease Csx1 prevents an incoming plasmid carrying a CRISPR-target sequence from being established, resulting in a reduction of number of transformants by almost 3 orders of magnitude under selective conditions. Both AcrIII-1 from phage THSA-485 and *M. mazei* Csx3 provide a level of protection against Csx1-mediated plasmid immunity, with *M. mazei* Csx3 requiring extended incubation period to achieve a similar effect as its viral counterpart. **B.** Number of transformants of Csx1-mediated plasmid immunity in the absence and presence of cA₄-degrading ring nucleases. AcrIII-1 almost completely prevented Csx1-mediated cell death or growth arrest, whereas *M. mazei* Csx3 yielded partial but still significant relief from immunity. Transformants were counted after 18 and 40 h of growth. Data are representative of two biological replicates and four technical replicates each, *N* = 8; individual data points are shown and error bars represent the standard deviation. Significance threshold was set at *p* < 0.05, ****: *p* < 0.00001 (unpaired, two-tailed Welch *t* test). Key: RN – ring nuclease; mCsx3 – *M. mazei* Csx3.

Essential catalytic residues are widely separated in Csx3

Previously, the RNA cleavage activity of *A. fulgidus* Csx3 was shown to be dependent on the presence of manganese ions. The H60 residue was shown to be essential for RNA cleavage, as an H60A variant was inactive, while the H57A and H80A variants showed reduced activity (Yan et al., 2015). As these residues are positioned on the opposite surface of the dimer from the RNA binding site (Figure 4A), this led to the prediction that the RNA binding site and RNA cleavage site existed on opposing sides of the dimeric structure, which was plausible for an RNA substrate (Yan et al., 2015). We tested the metal ion dependence of the cA₄ cleavage activity and confirmed that the presence of Mn²⁺ or Co²⁺ ions was required for catalysis (Figure 2 – figure supplement 1 and Figure 4 – figure supplement 1). We recapitulated the H60A variant and found that, in agreement with the previous study, the variant lacked any detectable catalytic activity (Figure 4B). Thus, it appears that H60 and the presumed metal binding site are essential for the ring nuclease activity of Csx3 despite being situated on the opposite face of the Csx3 dimer, over 20 Å away from the cA₄ binding site.

Examination of the multiple sequence alignment of Csx3 (Figure 1 - figure supplement 1) revealed the presence of a conserved aspartate residue, D69, which is positioned adjacent (~4 Å) to the bound RNA in the crystal structure (Figure 4A). The importance of D69 was not explored in previous studies, so we mutated D69 to an alanine to test for a role in catalysis. The D69A variant was completely catalytically inactive as a ring nuclease, confirming the importance of D69 in the catalytic mechanism and suggesting that catalysis requires residues on both faces of the dimer (henceforth denoted as the “D69 face” and “H60 face”). A possible explanation for this was that Csx3 has a shared active site that is formed when two dimers come together, forming a tetramer.

A diagnostic test for this, as first proposed for the shared active site of aspartate transcarbamoylase (Wente and Schachman, 1987), is to mix inactive single variants and look for recovery of activity, as a fraction of intact active sites that can be formed in the quaternary structure. Accordingly, we took the two inactive variants of Csx3, H60A and D69A, and tested their ring nuclease activity when mixed together (Figure 4B). Ring nuclease activity was recovered when the two inactive variants were combined. This result was strongly supportive of the hypothesis that the reaction mechanism involves two half-sites that combine to form a single active site that bridges two dimers of the enzyme.

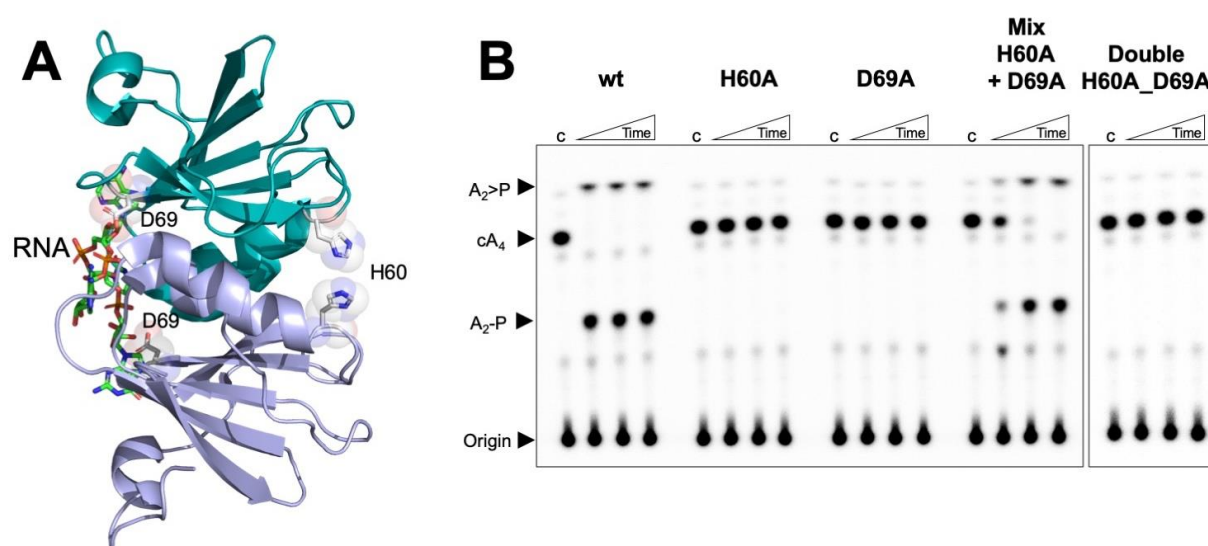


Figure 4. Both faces of the Csx3 dimer are required for ring nuclease activity **A.** Structure of the Csx3 dimer (monomers in mauve and teal) bound to an RNA tetraloop (PDB 3WZI), with residues D69 and H60 indicated. **B.** Phosphorimage of TLC visualising cA_4 (~200 nM) cleavage by *A. fulgidus* Csx3 (8 μ M protein dimer) wild-type and variants at 70 °C. While neither the H60A nor the D69A variant of Csx3 had detectable ring nuclease activity, a mixture of the two inactive variants restored activity. The double mutant was inactive. Control lane c – cA_4 in absence of protein. Time points were 10, 60 & 600 s. This phosphorimage is representative of 3 technical replicates.

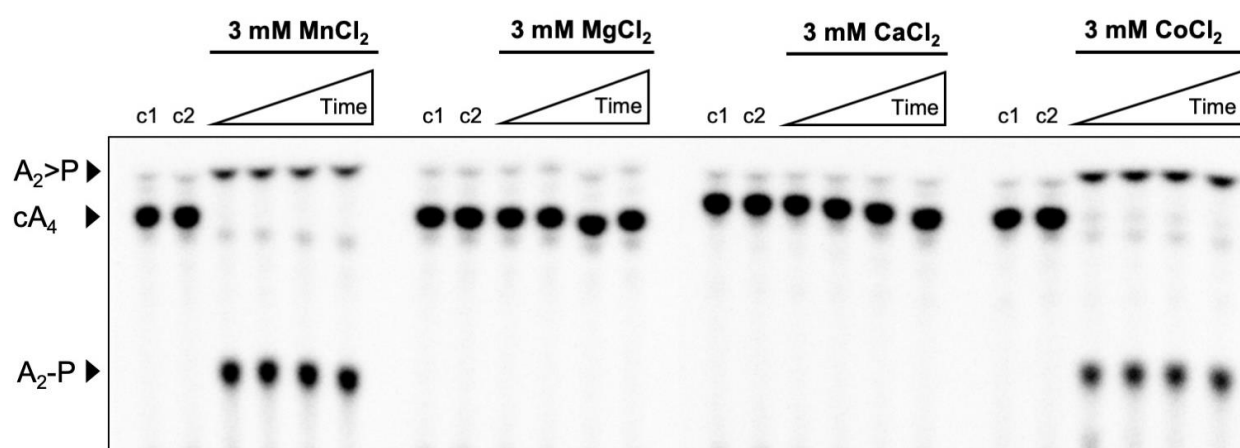


Figure 4 – figure supplement 1. Metal dependence of Csx3. Phosphor image of TLC visualising cA_4 (~200 nM) cleavage by Csx3 (8 μ M protein dimer) at 70 °C when supplemented with 3 mM $MnCl_2$, $MgCl_2$, $CaCl_2$ or $CoCl_2$. Csx3 ring nuclease activity requires the presence of Mn^{2+} or Co^{2+} ions and cannot be substituted with Ca^{2+} or Mg^{2+} . Control lane c1 is cA_4 in absence of protein and the lane c2 is protein incubated in assay buffer containing 1 mM EDTA before adding cA_4 (no metal control). Time points were 10 s, 20 s, 60 s and 10 min. This phosphorimage is representative of 3 technical replicates.

225

226 *The structure of Csx3 reveals a head-to-tail filament stabilised by cA₄*

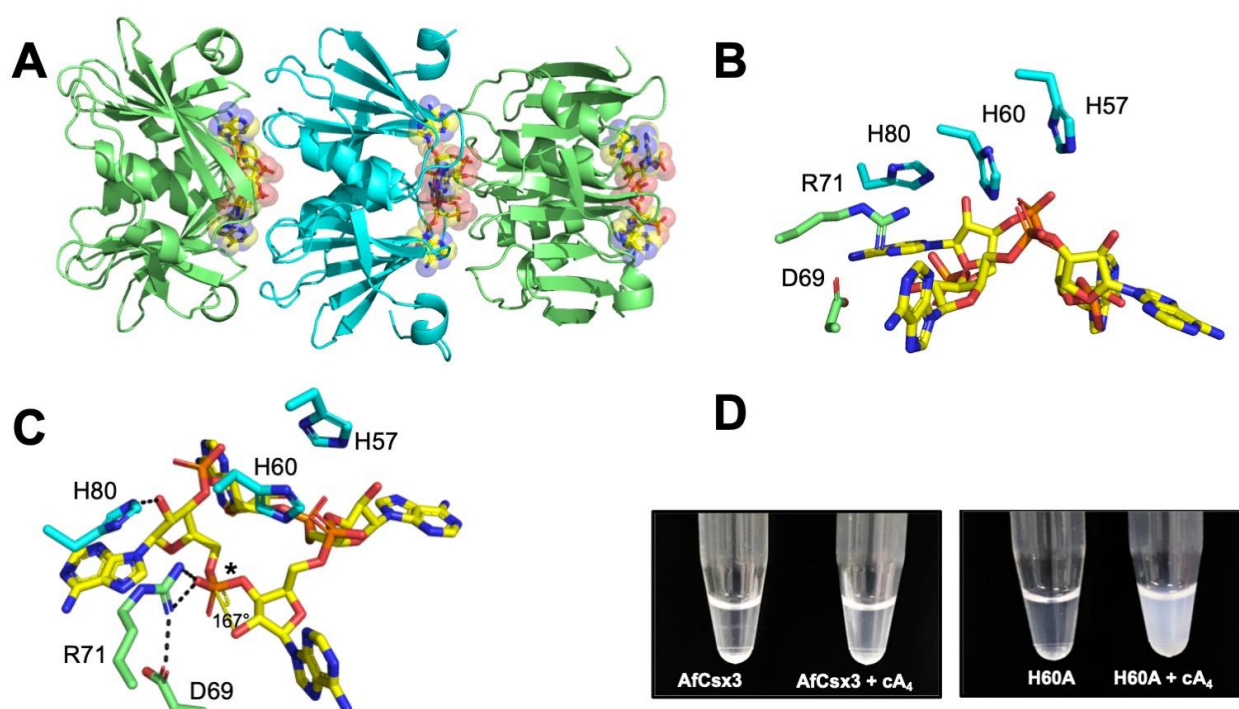
227 In parallel with the biochemical analysis, we crystallised the H60A variant of Csx3 and soaked the
 228 cA₄ substrate into the crystals. The structure was solved using molecular replacement with data to
 229 1.8 Å resolution (Supplementary Table 1). The electron density clearly showed a molecule of cA₄
 230 bound between adjacent dimers of Csx3 (Figure 5A). Notably, the Csx3 structure presented here
 231 crystallised in a different space group to those structures published previously (Yan et al., 2015),
 232 which has allowed the arrangement of Csx3 dimers into a pseudo filament arrangement (Figure 5
 233 – figure supplement 1) with a cA₄ molecule sandwiched between the D69 and H60 faces of the
 234 protein. The rotation between adjacent dimers in the filament is 144 °, coupled with a translation of
 235 28 Å. The calculated buried surface between the complex arrangement of protein and ligand
 236 reflects the number of interactions formed. The interface of the two monomers forming the dimer is
 237 around 1020 Å². The interface area between the D69 face, which forms the majority of the
 238 interactions with cA₄, is around 940 Å² (470 Å² per monomer), and for the H60 face is around 340
 239 Å² (170 Å² per monomer). Interestingly, although not annotated as a tetramer, there is a buried
 240 surface area of around 700 Å² (350 Å² per monomer) between two adjacent dimers.

241 There are surprisingly few residues that interact with cA₄ in the active site of Csx3 given its size.
 242 The orientation of each monomer in the dimer means the interactions with cA₄ are symmetrical.
 243 The majority of interactions are made by the D69 face. The cA₄ ligand hydrogen bonds with the
 244 main chain atoms of I22, S44, G45, and I49, and side chain atoms of S44 and R71 (Figure 5 –
 245 figure supplement 2). Despite the number of main chain interactions, these residues are either
 246 absolutely (G45, I49, and R71) or highly (I22 and Ser44) conserved (Figure 1 – figure supplement
 247 1). The one interaction evident from the H60 face with cA₄ is a strong (2.4-2.5 Å) hydrogen bond
 248 between H80 and the 2'-OH of the ribose ring. H60, H80, and another histidine residue (H57)
 249 nearby on the H60 face are all absolutely conserved in the Csx3 family (Figure 5B, C; Figure 1
 250 figure supplement 1). The ribose of each AMP moiety of cA₄ adopts a relaxed 2'-endo
 251 conformation, with the four adenine bases each filling a pocket. Given the Csx3 structure in
 252 complex with an RNA fragment had two adenine, one uracil and one guanine bases in the same
 253 position as the four adenine bases described here (Yan et al., 2015), there is obviously some
 254 plasticity around these recognition sites.

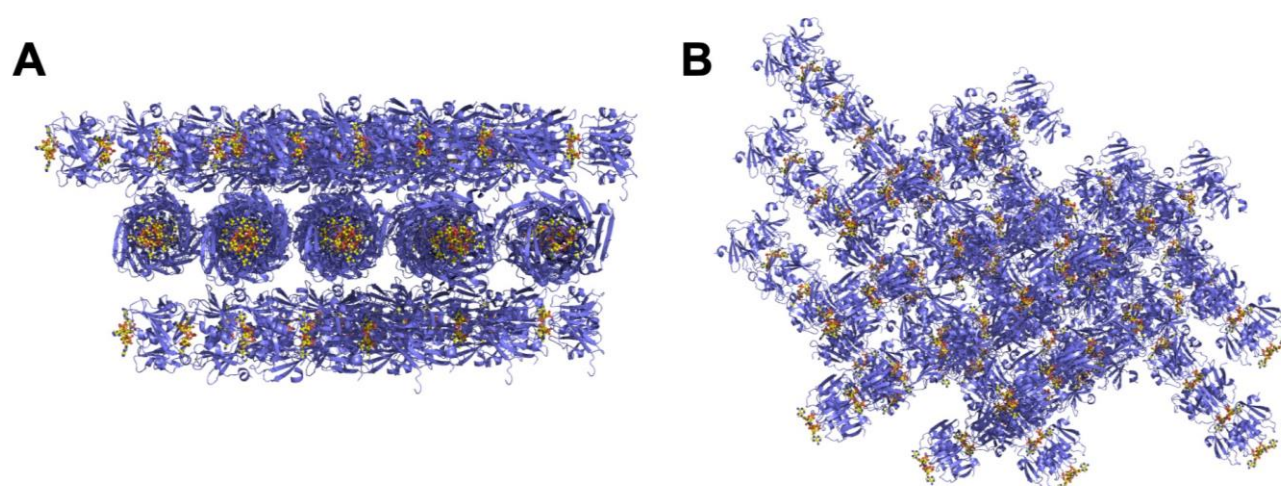
255 We anticipated that the comparison of the structure of Csx3 with cA₄ with the apo structure
 256 published by Yan *et al.* (Yan et al., 2015), in conjunction with activity assays on Csx3 variants,
 257 might provide clues as to the key residues in catalysis. Interestingly, R71 moves around 3.3 Å in
 258 order to form bidentate hydrogen bonds with an oxygen atom of a phosphate group, which we
 259 predict is adjacent to the phosphodiester bond that is cleaved (Figure 5C and text below). This
 260 movement of R71 brings it within hydrogen bonding distance of D69, which has been shown to be
 261 vital for activity. The position of H80 also differs between the two structures; the residue moves
 262 around 3.3 Å in order to hydrogen bond with the cA₄. However, there is the caveat that the packing
 263 arrangement differs in the apo Csx3 structure, meaning H80 does not interact with a ligand or the
 264 face of an adjacent dimer and thus has nothing to 'anchor' it in place.

265 There are a number of histidine residues in or near the active site which could be involved in
 266 coordinating one or more Mn²⁺ ions. H60 and H57 are the most likely candidates; they are both
 267 absolutely conserved, and the position of the alanine residue in the H60A variant structure
 268 suggests this residue does not move significantly on tetramer formation. The position of H57 is
 269 identical in the apo and cA₄-bound structures. H60 and H57 are at the symmetry plane for the
 270 monomers constituting a dimer, meaning there are four histidine residues in close proximity. It is

271 therefore feasible that one or both H60 and/or H57 residues coordinate one or more metal ions,
272 and there is the possibility that just one metal ion bridges the two monomers.



273
274
275 **Figure 5. Structure of the H60A variant of Csx3 in complex with cA₄.** **A** Crystal structure of H60A Csx3
276 co-crystallised with cA₄. The crystal lattice reveals an extended filament with dimers of Csx3 sandwiching
277 cA₄. Three dimers are shown here (green and cyan), with the cA₄ coloured in yellow sticks. **B** and **C** Two
278 views of the Csx3 H60A variant in complex with cA₄ showing the conserved residues implicated in binding
279 and catalysis (colouring as in panel A). Whilst H60 was not present in our structure (as an alanine variant
280 was crystallised), the position has been inferred by superposition with PDB 3WZI. In panel C, hydrogen
281 bonds are indicated as black dashed lines. The geometry of the phosphodiester bond is labelled (*), which is
282 likely positioned by a bidentate hydrogen bond with R71, meaning it is suitable for in-line nucleophilic attack
283 by the adjacent 2'-OH moiety. **D** Photograph comparing wild-type and H60A Csx3 (80 μM dimer) in the
284 absence and presence of equimolar cA₄. The inactive variant forms a milky, colloidal liquid when incubated
285 with cA₄, consistent with the formation of extended fibres.



286
287 **Figure 5 – figure supplement 1. The crystal lattice of the Csx3:cA₄ complex.** **A** and **B** Different views of
288 the crystal lattice of Csx3 (mauve) in complex with cA₄ (yellow). The asymmetric unit contains 5 dimers of
289 Csx3, which with the crystallographic symmetry forms longer filaments.

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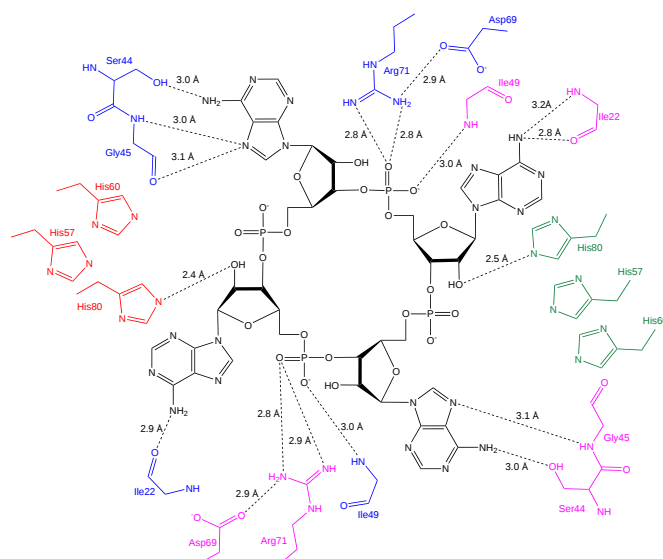


Figure 5 – figure supplement 2. Schematic showing interactions between Csx3 and cA₄. The residues in blue and pink represent the two monomers in the dimer constituting the 'D69 face', and the residues in green and red represent the two monomers in the dimer constituting the 'H60' face. Whilst H60 was not present in our structure, the position has been inferred by superposition with PDB 3WZI.

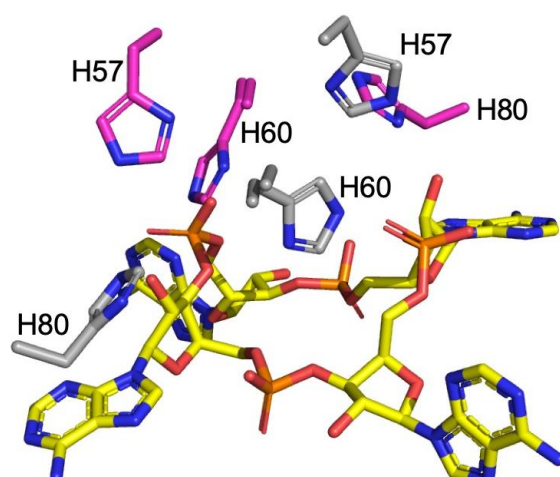


Figure 5 – figure supplement 3. Histidine residues in the active site of Csx3. H57, H60 (H60 was not present in our structure, the position has been inferred by superposition with PDB 3WZI; both the alanine from our structure and superimposed histidine are shown for clarity), and H80 from both monomers (coloured cyan and grey) in a dimer are shown in complex with cA₄ (yellow sticks).

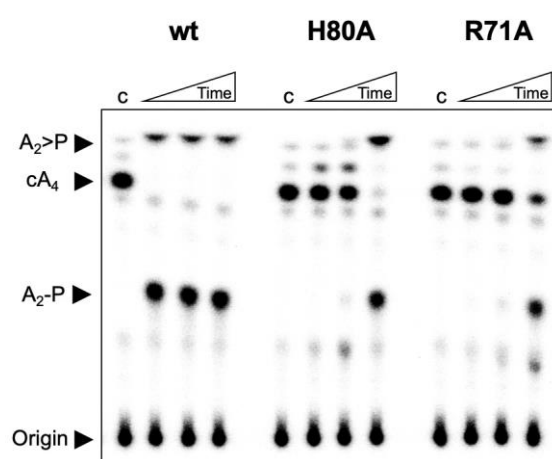


Figure 5 – figure supplement 4. Investigation of H80A and R71A variants of Csx3. TLC comparing ring nuclease activity of wild-type, H80A and R71A variants of Csx3 at 70 °C. Both variants had severely reduced activity. Time points were 10, 60 and 600 s. Control c is reaction in absence of Csx3. This result is representative of 3 technical replicates.

Predicted mechanism of Csx3

The structure of the Csx3:cA₄ complex reveals further details of the catalytic mechanism employed by the enzyme. The other ring nucleases characterised to date are metal independent enzymes

that are predicted to work by catalysing in-line nucleophilic attack by a 2'-hydroxyl group of the cA₄ substrate on an adjacent phosphodiester bond. In this regard, the best candidate for cleavage is the P-O bond of the phosphate (labelled *), as the angle formed between the 2'-OH, P and O moieties is 167° (Figure 5C), identical to that seen for the ring nuclease AcrIII-1 (Athukoralage et al., 2020b). The absolutely conserved residue R71 interacts with this phosphate group via a bidentate hydrogen bond and may participate in stabilisation of the transition state or oxyanion leaving group. The conserved catalytic residue D69 in turn makes polar contacts with R71. These two residues may serve to position each other correctly (and/or perturb their respective pK_a's) to enhance catalysis. The conserved H80 residue forms a hydrogen bond with the cA₄. As it is one of only two residues from the H60 face that interacts with cA₄, H80 may play a 'pinning' role to ensure engagement of the H60 face in catalysis. Csx3 variants R71A and H80A both displayed highly reduced ring nuclease activity (Figure 5 – figure supplement 4), consistent with important roles in cA₄ binding and/or catalysis.

The observations that Mn²⁺ is required for catalysis, and that the primary product of cA₄ cleavage is A₂P rather than A₂>P (Figure 2 – figure supplement 1 and Figure 4 - figure supplement 1) are suggestive (but not diagnostic) of a mechanism whereby a metal-activated hydroxyl ion initiates nucleophilic attack on the phosphodiester bond (Yang, 2011). Uncertainty arises from the observation that the metal independent ring nuclease AcrIII-1 also largely generates A₂P, presumably due to the rapid hydrolysis of the cyclic phosphate following phosphodiester bond cleavage (Athukoralage et al., 2020b). The conserved histidine residues H57 and H60 are in appropriate positions to contribute to catalysis by coordination of the essential catalytic Mn²⁺ ion(s). We see no evidence for the ion in our crystal structure, which may be due to the deletion of the H60 side chain. However, we note that the five Mn²⁺ ions modelled in the Csx3 structure by Yan et al. have ambiguous electron density, hampered by the lower resolution data (Yan et al., 2015). In addition, the coordination distances for a Mn²⁺ ion with a nitrogen atom (of the histidine residues) are longer than expected (Zheng et al., 2017). It remains possible that H80 participates in binding a second metal ion in addition to its observed interaction with cA₄.

The crystal structure of the Csx3:cA₄ complex neatly explains the observation of two distant active site regions, which come together in the complex. During the catalytic cycle, cA₄ binding and dimer:dimer sandwiching would lead to rapid cA₄ degradation that presumably in turn results in dissociation of the tetrameric active form. In support of this model, we observed that the inactive H60A variant of Csx3 had a milky, colloidal property on addition of cA₄, which we interpreted as being due to the formation of long Csx3 fibres bridged by multiple cA₄ molecules (Figure 5D). The wild-type protein did not show this behaviour, presumably because cA₄ was rapidly cleaved. We confirmed the propensity of Csx3 to oligomerise upon cA₄ addition by dynamic light scattering (DLS) measurements in buffer devoid of metal ions. Under these conditions, the addition of cA₄ resulted in mixed oligomeric species with significantly increased particle size and molecular weight, whereas the molecular weight of particles formed in the absence of cA₄ were consistent with homodimers of Csx3 (Supplementary Table 2).

The discovery that two dimers of Csx3 must associate to sandwich the cA₄ substrate to effect cleavage opens the possibility of cooperative kinetic control in the Csx3 reaction cycle. To investigate this, we measured the initial reaction velocity of Csx3 ring nuclease activity under conditions of saturating cA₄ at a range of enzyme concentrations. A plot of V_{max} against [E] revealed a sigmoidal relationship that could be fitted to the Hill equation with a Hill coefficient of 1.8 (Figure 6), consistent with obligate dimerization (of Csx3 dimers) in the reaction cycle. Practically, this cooperativity may be important to modulate the ring nuclease activity of Csx3 appropriately in the cell, particularly if the concentration of Csx3 changes in response to viral infection. The maximal multiple turnover rate constant observed under these conditions was 0.075 min⁻¹ at 70 °C,

about 50-fold lower than the rate for the chemical step of catalysis measured under single turnover conditions at 50 °C. Thus, the association of Csx3 dimers sandwiching cA₄, or the dissociation of the complex following product formation, limits the turnover number of the enzyme significantly.

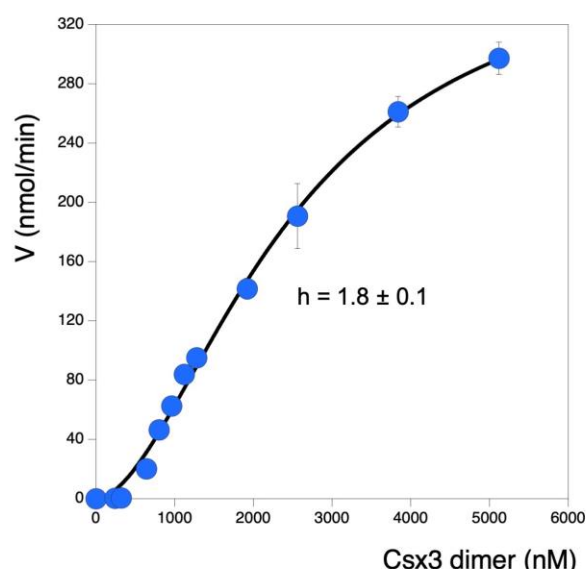


Figure 6. Sigmoidal response of ring nuclease activity as a function of Csx3 concentration. Plot visualising initial reaction rates of cA₄ cleavage across increasing Csx3 concentrations. The cA₄ concentration was 129 μM (25-fold greater than the highest concentration of protein assayed) for all experiments. Instead of a linear relationship between enzyme concentration and activity, a pronounced sigmoidal shape was observed. This could be fitted to a Hill equation to give a good fit with a Hill coefficient of 1.8, consistent with a requirement for two Csx3 dimers to associate with one cA₄ substrate molecule to effect catalysis. The maximum multiple turnover rate constant observed was 0.075 min⁻¹ at 70 °C. Each datapoint is the average of three technical replicates and error bars show the standard deviation of the mean.

Discussion

Csx3 is a cA₄-specific ring nuclease, Crn3

Here we re-examined the specificity of the Csx3 nuclease, which is found in association with type III CRISPR systems in bacteria and archaea. Csx3 was originally described as a manganese dependent RNA deadenylase (Yan et al., 2015). The enzyme was co-crystallised with an RNA tetranucleotide, revealing a pseudo-symmetrical binding mode, and site directed mutagenesis revealed a crucial role for residues including H60 in catalysis – a residue that was situated on the opposite face of the protein from the RNA binding site (Yan et al., 2015). This prompted the authors to make the reasonable speculation that RNA is bound on one side and wraps around the protein to be cleaved on the opposite side. Subsequently, Csx3 was identified as a divergent member of the CARF domain protein family (Topuzlu and Lawrence, 2016) that bind cyclic oligoadenylates generated by the Cas10 subunit of type III CRISPR systems (Niewoehner et al., 2017, Kazlauskienė et al., 2017). Coupled with the lack of an obvious role for a DNA deadenylase in type III CRISPR defence, this led us to speculate on its function *in vivo*. While we confirmed the main experimental observations of the original study (Yan et al., 2015), our analyses have revealed that Csx3 binds much more tightly to cA₄ than to RNA, is considerably more active as a cA₄-specific ring nuclease than an RNA deadenylase, and has properties consistent with a function as a ring nuclease *in vivo*. These data indicate that Csx3 functions as a cA₄-specific ring nuclease in type III CRISPR systems. The default “Csx” nomenclature was designed for proteins of unknown function lacking a clear association with a specific CRISPR subtype (Haft et al., 2005). We therefore propose the family name Crn3 (CRISPR-associated ring nuclease 3) for the Csx3 protein family.

The Crn1 family of ring nucleases, based on the CARF domain fold, was originally identified in the *Sulfolobales* and related crenarchaea (Athukoralage et al., 2018), where their function is thought to be to remove cA₄ from the cell once a viral infection has been cleared. The anti-CRISPR ring nuclease AcrIII-1 appears in many archaeal virus and some bacteriophage genomes (Athukoralage et al., 2020b). Homologues of AcrIII-1 are also found associated with some bacterial type III CRISPR systems, and in this context have been named as CRISPR associated ring

378 nuclease 2 (Crn2) enzymes (Athukoralage et al., 2020b, Samolygo et al., 2020). Ring nuclease
379 activity has also been identified associated with the CARF domains of some Csx1/Csm6 family
380 ribonucleases, which are therefore bifunctional, self-limiting CRISPR ancillary defence enzymes
381 (Jia et al., 2019, Athukoralage et al., 2019, Garcia-Doval et al., 2020). Overall, the emerging
382 picture is becoming clearer for the specific ring nucleases as they are identified in different
383 archaeal and bacterial genomes (Figure 7). It appears to be an emerging paradigm that cells with a
384 type III CRISPR defence require a mechanism to remove the cOA signal, either once viral infection
385 is cleared, or when the system “fires” inappropriately due to self-targeting (Athukoralage et al.,
386 2020a).

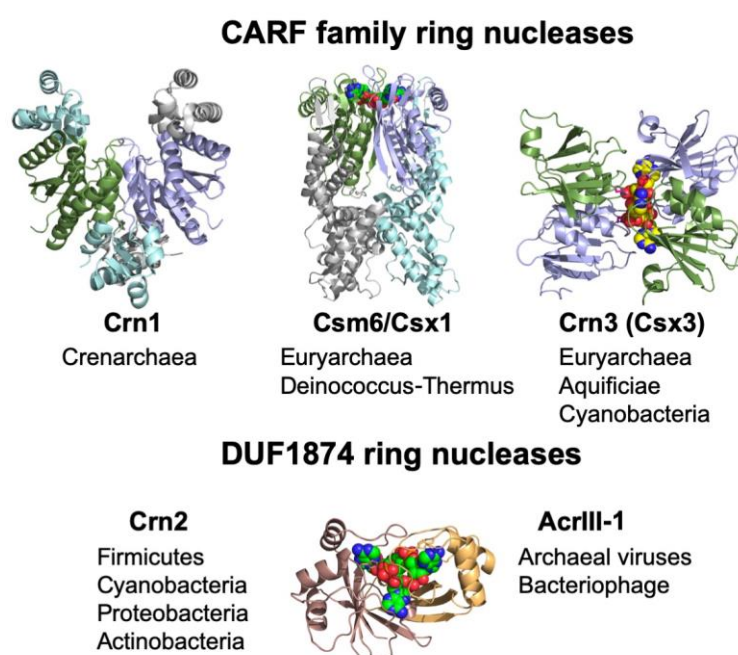


Figure 7. The ring nucleases. The Crn1 family (represented here by Sso1393 PDB code 3QYF) is restricted to the crenarchaea (Athukoralage et al., 2018). The Csm6/Csx1 family (represented here by PDB code 6O6Y) are self-limiting cA₄-dependent ribonucleases (Athukoralage et al., 2019, Jia et al., 2019, Garcia-Doval et al., 2020). The Crn3 family is present in type III CRISPR systems in euryarchaeal and cyanobacteria. In contrast, the DUF1874 family use a different protein fold to bind cA₄. The Crn2 family of ring nucleases is quite widespread in bacterial type III CRISPR systems (Samolygo et al., 2020). The same fold is used by the anti-CRISPR AcrIII-1 in archaeal viruses and bacteriophage (Athukoralage et al., 2020b).

387

388 *Crn3 has a highly unusual cooperative catalytic mechanism*

389 *A. fulgidus* Crn3/Csx3 is a much faster enzyme than Crn1. The single turnover reaction rate for cA₄
390 cleavage of 3.6 min⁻¹ is closer to that of the AcrIII-1 family, which utilise a distinct active site
391 architecture, employing a conserved histidine residue as a general acid to stabilise the oxyanion
392 leaving group (Athukoralage et al., 2020b). This raises an important question: why does Crn3/Csx3
393 degrade cA₄ so quickly when it plays a role in cellular defence rather than viral offense? Clearly,
394 removing a crucial signal of viral infection that mobilises cellular defences is not something that
395 should be undertaken precipitously. A key observation is that Crn3/Csx3 functions via a highly
396 unusual cooperative mechanism where two enzyme dimers associate to sandwich a cA₄ substrate
397 molecule. The active site is thus composed of two half-sites that are present on opposite faces of
398 each dimeric moiety, with enzyme tetramers formed transiently to complete the catalytic cycle.
399 There are many examples of allosteric control of enzyme quaternary structure and activity
400 (Selwood and Jaffe, 2012) and of active sites shared across subunit interfaces. However, the
401 substrate-induced, non-allosteric, dimerization of Csx3 appears unprecedented in the literature – a
402 partial exception being the example of the Arginine Finger of AAA+ ATPases (Nagy et al., 2016),
403 where interdomain interactions with ATP can influence quaternary structure (Zhao et al., 2016).

404 This results in cooperative kinetics where low concentrations of Crn3/Csx3 provide very low levels
405 of ring nuclease activity that rapidly increases with increasing enzyme concentrations.
406 Furthermore, the rate of cA₄ turnover is limited by the formation of protein:cA₄ complexes or
407 dissociation of the products, rather than the catalytic step, which is significantly faster. Together,

these factors may provide a means to control ring nuclease activity in an appropriate manner thus ensuring that cA₄ activation of ancillary ribonucleases is allowed to proceed and provide immunity. Thus, alterations in the gene expression levels of Csx3 would have a large effect on the overall rate of catalysis.

Finally, the observed specificity of Crn3/Csx3 for cA₄ implicates the corresponding type III CRISPR systems as functioning via a cA₄ second messenger. This conforms to the paradigm established from studies of the Crn1 and Crn2/AcrIII-1 family as well as analysis of the specificity of the Csx1/Csm6 ribonucleases, which are most often activated by cA₄ (Grüschow et al., 2019). Although this conclusion may be influenced by a sampling bias, it appears that cA₄ is the default cyclic nucleotide employed to signal infection and activate defences in type III CRISPR systems. It is possible that the pressure applied by cA₄-specific anti-CRISPRs has resulted in the utilization of cA₃ and cA₆ second messengers in some bacterial defence systems. Given the pace of discovery in this area, new cellular and viral enzymes implicated in cyclic nucleotide signalling are anticipated.

Materials and Methods

Cloning

For cloning, synthetic genes (g-blocks) encoding *A. fulgidus* or *M. mazei* Csx3 (Supplementary Figure 1), codon optimised for expression in *Escherichia coli*, were purchased from Integrated DNA Technologies (IDT), Coralville, USA, and cloned into the pEhisV5spacerTev vector between the *NcoI* and *BamHI* restriction sites (Rouillon et al., 2019). Competent DH5α (*E. coli*) cells were transformed with the construct and sequence integrity was confirmed by sequencing (Eurofins Genomics). The plasmid was transformed into *E. coli* C43 (DE3) cells for protein expression. The following constructs were used in plasmid immunity assays: pCsm1-5_ΔCsm6 (*M. tuberculosis* *csm1-5* under T7 and *lac* promoter control) and pCRISPR_TetR (CRISPR array with tetracycline resistance gene targeting spacers and *M. tuberculosis* *cas6* under T7 promoter control) which have been described previously (Grüschow et al., 2019); pRAT-Duet constructs containing *T. sulfidophilus* *csx1* under arabinose-inducible promoter control with and without viral Acr-III which have been previously described (Athukoralage et al., 2020b). *M. mazei* *csx3* was cloned into the pRAT-Duet_tsuCsm1 vector using the *NdeI* and *XhoI* restriction sites. Constructs were verified by sequencing.

Protein Production and Purification

For expression of *A. fulgidus* or *M. mazei* Csx3, the standard protocol described in detail previously was followed (Rouillon et al., 2019). Briefly, 2 L of culture was grown in Luria-Broth at 37 °C to an OD₆₀₀ of 0.8 AU with shaking at 180 rpm. Protein expression was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside and cells were grown at 25 °C overnight before harvesting by centrifugation. For protein purification the cell pellet was resuspended in lysis buffer containing 50 mM Tris-HCl 7.5, 0.5 M NaCl, 10 mM imidazole and 10 % glycerol supplemented with EDTA-free protease inhibitor tablets (Roche; 1 tablet per 100 ml buffer) and lysozyme (1 mg/ml). Cells were lysed by sonication and the lysate was ultracentrifuged at 40,000 rpm (70 Ti rotor) at 4 °C for 35 min. The lysate was loaded onto a 5 ml HisTrap FF Crude column (GE Healthcare) equilibrated with wash buffer containing 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 30 mM imidazole and 10 % glycerol. Unbound protein was eluted with 20 column volumes (CV) of wash buffer prior to elution of His-tagged protein using a step gradient of imidazole (holding at 10 % for 3 CV, and 50 % for 3 CV) of elution buffer containing 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.5 M imidazole and 10 % glycerol. The His-tag was removed by incubating concentrated protein overnight with Tobacco

453 Etch Virus (TEV) protease (1 mg per 10 mg protein) while dialysing in buffer containing 50 mM
454 Tris-HCl pH 7.5, 0.5 M NaCl, 30 mM imidazole and 10% glycerol at room temperature. Cleaved
455 protein was passed through a 5 ml HisTrapFF column and further purified by size-exclusion
456 chromatography (S200 16/60 column; GE Healthcare) in buffer containing 20 mM Tris-HCl pH 7.5,
457 0.125 M NaCl. After SDS-PAGE, pure protein was pooled, concentrated and stored at -80 °C.
458 H60A, D69A, R71 and H80 variants of *A. fulgidus* Csx3 (Supplementary figure 1) were generated
459 using the QuikChange Site-Directed Mutagenesis kit as per manufacturer's instructions (Agilent
460 Technologies) and purified as for the wild-type protein.

461 RNA cleavage assays

462 For RNA cleavage, Csx3 (8 µM protein dimer) was incubated with 50 nM radiolabelled RNA
463 oligonucleotide 49-9A in buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 3
464 units SUPERase•In™ Inhibitor and supplemented with 2 mM MnCl₂ at 50 °C. A control reaction
465 incubating RNA in buffer without protein was also carried out. Reactions were quenched by adding
466 EDTA (17 mM final concentration) and incubated with 20 µg Proteinase K (Invitrogen) for 30 min at
467 37 °C. Subsequently, the reactions were deproteinised by phenol-chloroform extraction and 8 µl
468 reaction product was extracted into 5 µl 100% formamide xylene-cyanol dye. All experiments were
469 carried out in triplicate and RNA cleavage was visualised by phosphor imaging following
470 denaturing polyacrylamide gel electrophoresis (PAGE) as previously described (Rouillon et al.,
471 2019).

472 Oligo 49-9A: 5'-AGGGUACAGUUUGGGUAUUAGCCGUUCUGGUCCUUAUACGAAAAAAAAA

473 Radiolabelled cA₄ cleavage assays

474 Cyclic oligoadenylate (cOA) was generated using *S. solfataricus* Csm complex as detailed in
475 (Rouillon et al., 2019). Single turnover kinetics experiments for *A. fulgidus* or *M. mazei* Csx3 and
476 variants (8 µM protein dimer) were performed by incubating protein with radiolabelled SsoCsm and
477 cOA (~200 nM cA₄) in buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM MnCl₂, 1 mM
478 DTT and 3 units SUPERase•In™ Inhibitor at 50 °C. At desired time points, 10 µl aliquot was
479 removed from the reaction and quenched by adding to phenol-chloroform and vortexing. Products
480 were further isolated by chloroform extraction for thin layer chromatography (TLC). A control
481 reaction incubating cOA in buffer without protein to the endpoint of each experiment was also
482 carried out. All experiments were carried out in triplicate and cA₄ degradation was visualised by
483 phosphor imaging. For experiments examining metal dependence, Csx3 was incubated with 1 mM
484 EDTA in buffer as above before adding cOA and supplementing with 3 mM MgCl₂, CaCl₂ or CoCl₂.

485 Multiple turnover kinetics of Csx3 was carried by varying enzyme (240, 320, 640, 800, 960, 1120,
486 1280, 1920, 2560, 3840, 5120 nM dimer) and incubating with a mix of unlabelled and radiolabelled
487 cA₄ (total concentration 128.5 µM) in buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2
488 mM MnCl₂, 1 mM DTT and 3 units SUPERase•In™ Inhibitor at 70 °C. Reaction products were
489 visualised by phosphor imaging following TLC. All experiments were done in triplicate. Initial rates
490 were calculated and adjusted for labelled:unlabelled cA₄ concentration before fitting to the Hill
491 equation on Kaleidagraph (Synergy Software). For activity rescue of Csx3 variants, D69A (4 µM
492 dimer) variant and H60A variant (4 µM dimer) were mixed together and incubated with cA₄ (~200
493 nM) in a reaction supplemented with 2 mM MnCl₂ at 70 °C. Reactions were quenched at 10, 60
494 and 600 s by adding phenol-chloroform and vortexing. All experiments were done in triplicate.

495 TLC was carried out as previously described (Han et al., 2017a), spotting 1 µl of radiolabelled
496 product 1 cm from the bottom of a 20 x 20 cm silica gel TLC plate (Supelco Sigma-Aldrich). TLC

was carried out at 35 °C in a humidified chamber with running buffer composed of 30% H₂O, 70% ethanol and 0.2 M ammonium bicarbonate, pH 9.2. Sample migration was visualised by phosphor imaging the TLC plate. For kinetic analysis, cA₄ cleavage was quantified using the Bio-Formats plugin (Linkert et al., 2010) of ImageJ as distributed in the Fiji package (Schindelin et al., 2012). The data was fitted to a single exponential curve ($y = m_1 + m_2(1 - \exp(-m_3 \cdot x))$) using Kaleidagraph, as described previously (Sternberg et al., 2012).

Electrophoretic Mobility Shift Assays

Csx3 and variants (0.01, 0.1, 0.5, 1, 10 and 20 µM dimer) were incubated with 20 nM radiolabelled cA₄ in binding buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 mM EDTA supplemented with UltraPure BSA (ThermoFisher) for 10 min at 25 °C. To examine RNA binding by Csx3, 50 nM 5'-end radiolabelled 3' poly-adenylate tailed RNA oligonucleotide 49-9A was incubated with Csx3 as above. A reaction volume equivalent of 20% glycerol was added before native (15% acrylamide, 1X TBE) gel electrophoresis at 30 °C and 250V was performed. Gels were phosphor imaged overnight at -80 °C. in binding buffer as above before native gel electrophoresis. All experiments were done in triplicate.

Protein crystallisation

Crystallisation conditions were tested with JSCG and PACT 96 well commercial screens (Jena Biosciences) with Csx3 H60A at a concentration of 13.5 mg/ml. Following optimisation, crystals were obtained from 25% (v/v) Jeffamine M-600 and 100mM HEPES pH 7.5 using hanging drops in a 24 well plate. 3 µl drops in a 2:1 or 1:1 protein:mother liquor ratio were added to a silanized cover slip over 400 µl mother liquor and sealed with high-vacuum grease (DOW Corning, USA) and left to grow at room temperature. Prior to addition of the cA₄ ligand, crystals were harvested into a fresh 2 µl drop of mother liquor and 1 µl of 16mM cA₄ solution was added and left to soak for 12 hours. Crystals were harvested and cryoprotected with the addition of 2 µl 50% (v/v) Jeffamine M-600 in 0.5 µl increments, mounted on loops and vitrified in liquid nitrogen.

X-ray data processing, structure solution, and refinement

Data on Csx3 H60A crystals soaked with cA₄ were collected at Diamond Light Source (DLS) on beamline I04 at a wavelength of 0.9795 Å to 1.84 Å resolution. Diffraction images were automatically processed through the Xia2 pipeline (Winter, 2010) using DIALS (Gildea et al., 2014) and AIMLESS (Evans, 2006). After checking the likely cell content by the Matthews' coefficient, MOLREP (Vagin and Teplyakov, 1997) was used to solve the structure using molecular replacement with the Csx3 structure (PDB 3WZI) (Yan et al., 2015) as the search model with ligand and water molecules removed. REFMAC5 ((Murshudov et al., 2011) and Coot (Emsley and Cowtan, 2004) were used for automated and manual refinement respectively, which included addition of the ligand and water molecules. cA₄ was drawn using Chemdraw (Perkin Elmer) and restraints generated in JLigand (Lebedev et al., 2012). The model was corrected and validated using tools in PDB-redo (Joosten et al., 2014) and Molprobit (Chen et al., 2010). The Molprobit score is 1.04; centile 100. Ramachandran statistics are 97.34% allowed, 0% disallowed. Data processing and refinement statistics are shown in Supplementary Table 1. The coordinates and data have been deposited in the Protein Data Bank with accession code 6YUD.

Dynamic Light Scattering

Dynamic light scattering experiments were carried out in a 20 µl quartz cuvette using a Zetasizer Nano S90 instrument (Malvern). 80 µM AfCsx3 was either measured alone or when mixed with an

equimolar concentration of cA₄ in phosphate buffered saline, pH 7.5 at 25 °C. Three technical replicates were carried out and by default triplicate measurements were made to produce an average for each technical replicate. For visual inspection of the effect of adding cA₄ to AfC₃ and variants, 80 µM AfC₃ was added to equimolar cA₄ in PBS supplemented with 2 mM MnCl₂ in a 100 µl reaction volume and either heated to 25 °C or 70 °C for 10 min before photographing using a 12-megapixel f/1.8-aperture camera.

Liquid chromatography high-resolution mass spectrometry

Samples were generated by incubating C₃ (10 µM dimer) with 100 µM synthetic cA₄ (BIOLOG Life Science Institute, Bremen) in buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT and 2 mM MnCl₂ for 10 min at 50 °C. Reactions were quenched by adding EDTA to a final concentration of 25 mM, and then deproteinised and desalted using a C18 cartridge (Harvard Apparatus). Liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis was performed on a Thermo Scientific™ Velos Pro instrument equipped with HESI source and Dionex UltiMate 3000 chromatography system as previously described (Grüschow et al., 2019). Data were analysed using Xcalibur™ (Thermo Scientific).

Plasmid immunity assay comparing viral and host ring nucleases

These assays were performed largely as described previously (Athukoralage et al., 2020b). Cells containing *Mycobacterium tuberculosis* (Mtb)Csm1-5, Cas6 and a CRISPR array targeting the tetracycline resistance gene of pRAT-Duet were transformed with the target plasmid containing genes encoding the cA₄-activated ancillary nuclease *T. sulfidophilus* (Tsu) C₃1 and a ring nuclease (*M. mazei* C₃3 or the anti-CRISPR ring nuclease AcrIII-1 from phage THSA-485). Transformants were allowed to recover on selective LB plates in the presence of 0.2 % lactose and 0.02 % arabinose (the former for induction of the Cas genes and the ring nuclease, the latter induces the C₃1 plus ring nuclease). The experiment was run with increasing arabinose concentrations (0, 0.002, 0.02, 0.2 % w/v); there was no difference between 0 and 0.002 %, and a slight difference between 0.02 and 0.2 % arabinose. Four technical replicates on 2 biological replicates were carried out, N = 8. Unpaired Welch t test was performed using GraphPad Prism version 8.3.1, GraphPad Software, La Jolla California USA, www.graphpad.com.

FUNDING

This work was supported by the Biotechnology and Biological Sciences Research Council (REF: BB/S000313/1 to MFW and BB/T004789/1 to MFW and TMG) and by Wellcome Trust Institutional Strategic Support Funding to MFW and TMG (REF: 204821/Z/16/Z).

ACKNOWLEDGEMENTS

We thank Euan Wakefield and Lewis MacDonald for technical assistance with experiments.

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Supplementary Table 1. Data collection and refinement statistics for H60A mutant of Csx3 in complex with cA₄.

	Csx3 H60A mutant + cA ₄
Data collection	
Space group	C 1 2 1
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	194.0, 60.4, 107.1
α , β , λ (°)	90, 116.5, 90
Resolution (Å)	53.56 – 1.84 (1.87 – 1.84)*
<i>R</i> _{sym} or <i>R</i> _{merge}	0.04 (0.72)
<i>I</i> / σ <i>I</i>	11.3 (1.1)
Completeness (%)	96.9 (70.0)
Redundancy	3.2 (2.4)
Refinement	
Resolution (Å)	53.56 – 1.84
No. reflections	88795
<i>R</i> _{work} / <i>R</i> _{free}	0.20 / 0.23
No. atoms	18839
Protein	7629
Ligand/ion	586
Water	440
<i>B</i> -factors	
Protein	37.8
Ligand/ion	43.6
Water	37.9
R.M.S. deviations	
Bond lengths (Å)	0.014
Bond angles (°)	2.02

* Values in parentheses are for the high resolution shell.

Supplementary Table 2. *Dynamic light scattering studies with AfCsx3.* In the absence of cA₄, AfCsx3 (80 μM) forms particles with a molecular weight of 36 ± 9 kDa consistent with protein in a homodimeric state. When an equimolar amount of cA₄ is added, Csx3 forms multiple large molecular weight species (three species detected in each replicate) with significantly greater particles sizes (denoted by increase in the particle Z-average). The mass (%) per volume of each of these species is indicated alongside its molecular weight.

Replicate	AfCsx3			AfCsx3 + cA ₄		
	~MW (kDa)	Mass (%)	Z-Average (nm)	~MW (kDa)	Mass (%)	Z-Average (nm)
1	36 ± 8.8	100	5.5 ± 1.3	453 ± 10	66	16 ± 0.36
				561 ± 12	32	18 ± 0.37
				438 ± 102	0.3	43 ± 0.67
2	38 ± 9.5	100	5.6 ± 1.4	237 ± 15	33	12 ± 0.72
				504 ± 13	33	17 ± 0.35
				10400 ± 706	30	62 ± 4.1
3	36 ± 9.0	100	5.5 ± 1.4	51 ± 2.6	33	6.5 ± 0.33
				1810 ± 124	30	30 ± 1.6
				4080 ± 333	32	55 ± 0.94