Antiviral signaling by a cyclic nucleotide activated CRISPR protease

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CRISPR defense systems such as the well-known DNA-targeting Cas9 and the RNA-targeting type III systems are widespread in prokaryotes[1,2]. The latter can orchestrate a complex antiviral response that is initiated by the synthesis of cyclic oligoadenylates (cOAs) upon foreign RNA recognition[3–5]. Among a large set of proteins that were linked to type III systems and predicted to bind cOAs[6,7], a CRISPR associated Lon protease (CalpL) stood out to us. The protein contains a sensor domain of the SAVED (SMODS-associated and fused to various effector domains) family[7], fused to a Lon protease effector domain. However, the mode of action of this effector was unknown. Here, we report the structure and function of CalpL and show that the soluble protein forms a stable tripartite complex with two further proteins, CalpT and CalpS, that are encoded in the same operon. Upon activation by cA_4, CalpL oligomerizes and specifically cleaves the MazF-homolog CalpT, releasing the extracytoplasmic function (ECF) sigma factor CalpS from the complex. This provides a direct connection between CRISPR-based foreign nucleic acid detection and transcriptional regulation. Furthermore, the presence of a cA_4-binding SAVED domain in a CRISPR effector reveals an unexpected link to the cyclic oligonucleotide-based antiphage signaling system (CBASS).

Main

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a prokaryotic adaptative immune system that enables microorganisms to fend off attacks from mobile genetic elements such as phages, viruses, or plasmids[8]. The protein complex Cas1-Cas2 captures short DNAs from invaders and integrates them as "memories" into a CRISPR locus[9]. Transcripts of these "memories" are processed into small CRISPR RNAs (crRNAs) and integrated into large ribonucleoprotein (RNP) complexes, which can sense the presence of a matching foreign nucleic acid in the cell[10]. Once an invading sequence is detected, an antiviral response is triggered. Depending on the type of CRISPR system[1], this response can be markedly different, ranging from cleavage of the invading nucleic acid by the RNP as in the case of Cas9[11], to a complex multipronged defense strategy as found in type III CRISPR systems[12]. For the latter, the Cas10 subunit of the RNP has a cyclase activity that converts ATP into a recently discovered class of cyclic oligoadenylates (cOAs) upon viral RNA recognition[3–5]. The cOAs are constructed from 3 to 6, 3'-5' linked AMP units[13] and act as second messengers, typically by binding to proteins harboring a CARF (CRISPR-associated Rossmann-fold) domain[14]. There is a wide variety of CARF proteins linked to effector domains with functions ranging from RNA cleavage, supercoiled DNA nicking, dsDNA cleavage to transcription modulation[12,15–20]. The downstream effects of those cOA-activated proteins can lead to viral clearance, an abortive infection or a dormant state of the cell, enabling it to weather the phage attack[18,21].

Recently, two bioinformatic teams cataloged CARF-domain encoding genes that are likely linked to a functional type III system in bacterial and archaeal genomes[6,7]. Together, the studies revealed more than 100 such genes, including several membrane proteins and many proteins with currently unknown functions. Another study proposed that some of those type III-associated proteins contain a SAVED domain ('SMODS-associated and fused to various effectors domains'; SMODS being the acronym for 'second messenger oligonucleotide or dinucleotide synthetase'[22]) instead of CARF, reminiscent of the recently discovered CBASS system ('cyclic-oligonucleotide-based antiphage signaling systems'[23]). One of these proteins is CalpL (CRISPR associated Lon protease; initially termed Lon-CARF[6]), a 60 kDa protein with two predicted transmembrane helices, a Lon-protease domain and a SAVED4 domain[24].

Here we report the structure and function of the CalpL protein from the thermophilic bacterium *Sulfurihydrogenibium sp.* YO3AOP1. We find that CalpL is a soluble monomer and forms a 1:1:1 complex with CalpT and CalpS, encoded by adjacent genes in the locus. Once activated by cA_4, CalpL oligomerizes and proteolytically cleaves CalpT, releasing the $CalpT_{23}$/S complex, which has striking similarities to bacterial σ-factor/anti-σ-factor pairs.

3

Structure of CalpL

A synthetic, codon-optimized variant of the CalpL gene from *Sulfurihydrogenibium sp.* YO3AOP1 (UniProt ID B2V8L9) was expressed in *E. coli.* Although predicted to be a trans-membrane protein[6,7], the protein was found in the soluble fraction of the cell lysate and behaved as a monomer during size exclusion chromatography (Extended Data Fig. 1a,b and below). CalpL was crystallised and the structure was solved at a resolution of 2.1 Å by single-wavelength anomalous dispersion phasing and refined to R/R$_{free}$ values of 19.3/22.5 (Fig. 1a, Extended Data Fig. 1c,d, Extended Data Table 1)[25,26].

The Lon protease domain consists of a four-stranded mixed β-sheet (β1-4), sandwiched between αD of the N-terminal domain and αG, H, J on the other side (Fig. 1a, Extended Data Fig. 1d). Various close structural homologs were identified and are listed in Extended Data Fig. 2. In CalpL, the catalytic Ser-Lys dyad, a hallmark of Lon proteases, is formed by S152 (loop β4-αH) and K193 (αJ) and lies at the end of a narrow channel that presumably binds the substrate peptide (Extended Data Fig. 2c). A superposition of the Lon-protease domain of CalpL with the acyl-enzyme intermediate state of the yellowfin ascites virus ATP-independent Lon protease[27] hints at the location of the P1 site in CalpL (Extended Data Fig. 2d). Structural modelling indicated that only amino acids with small hydrophobic side chains such as Ala or Gly can be accommodated in this site.

The C-terminal part of CalpL folds into a SAVED4 domain[24]. It consists of two pseudo-symmetric CARF-like domains with a pseudo-two-fold axis running between helices αP and αS (Fig. 1a). Interestingly, the TMHMM 2.0 server predicted that those helices and the directly preceding β-strands form transmembrane helices or at least membrane associated helices[6,28] (Extended Data Fig. 1b), which is clearly not the case. The SAVED domain has an extensive, positively charged cavity on its molecular surface, suited to bind a cOA ligand. The CARF- or SAVED-domains of the cOA-activated effector proteins Cap4, Cap5, and Can1 are structural homologs found in CBASS and CRISPR systems [17,23,29] (Extended Data Fig. 2b). Despite the low sequence identities (9-14% identical amino acids) the fold of the CARF-like domains is conserved. The position of the effector domain relative to the SAVED or CARF domains is, however, entirely different between the four structures (Extended Data Fig. 2e-g).

Surface plasmon resonance experiments showed that of the four tested cOAs CalpL selectively binds cA$_4$ with a dissociation constant of ~1 nM (Fig. 1b). We determined a 2.2 Å crystal structure of the CalpL/cA$_4$ complex and found the ligand bound to the SAVED domain at the expected position (Fig. 1b,c). As for the SAVED domain itself, the bound cA$_4$ molecule adopts a pseudo two-fold symmetry (Extended Data Fig. 3a). The cyclic tetra adenylate is

109 involved in a large number of polar and hydrophobic interactions with the SAVED domain,

110 such that three of the four phosphate groups of the ligand are solvent exposed (Extended Data

111 Fig. 3a). A comparison between the apo- and the cA_4-complex structure reveals no major

112 conformational changes, apart from small shifts in the loops surrounding the cA_4 ligand

113 (Extended Data Fig. 3b). A comparison with the cA_3 bound Cap4 structure (Extended Data Fig.

114 3c) illustrates how the loops on top of the SAVED domains shape the binding site for the

115 cognate ligand in each structure.

116 The N-terminal domain of CalpL forms a bundle of six α-helices (αA-F, Fig. 1a, Extended

117 Data Fig. 1d) with weak structural similarity to the N-terminal protein-protein interaction

118 domain of Katanin p60-N in the p60p80-CAMSAP complex (Extended Data Fig. 2b)[30].

119

CalpL specifically cleaves CalpT

121 We used the WebFLAGs server[31] to study the gene neighborhood of CalpL homologs and

122 noticed a small 812 bp open reading frame (271 amino acids, 31.8 kDa, UniProt ID B2V8L8)

123 with no annotated function upstream of the *calpL* gene (Fig. 2a). We analyzed its sequence with

124 HHPRED[32] and found homologies to the MazF toxin in the N-terminal half of the protein and

125 weak homologies to DUF2080, a "domain of unknown function", in the C-terminal half (Fig.

126 2a). We predicted the structure with AlphaFold2[33]. The software produced a model of a two-

127 domain protein with a ~23 kDa and a ~10 kDa domain connected by an apparently flexible

128 linker (Fig. 2b). The structural model was submitted to the DALI server[34], revealing structural

129 similarities to MazF-like toxins (N-terminal fragment) and various immunoglobulin fold

130 containing proteins (C-terminal fragment). Interestingly, the predicted structure appears as a

131 structural mimic of the MazEF complex with helices αA, αD, and αE blocking the region that

132 binds to the ssRNA target of MazF in a similar fashion to MazE (Extended Data Fig. 4a,b)[35].

133 We investigated whether this protein (named "CalpT" for "target") is cleaved by the CalpL

134 protease. The gene was expressed in *E. coli* and the protein purified to near homogeneity

135 (Extended Data Fig. 4c). CalpL, CalpT, and different cOAs (3, 4, 5, 6) were mixed at 1:1:1.5

136 molar ratios and incubated at 60 °C for one hour. Strikingly, we found that in the presence of

137 cA_4, CalpT was cleaved by CalpL. SDS-PAGE analysis revealed two distinct cleavage products

138 with molecular weights of 23 kDa and 10 kDa, respectively, suggesting a single cleavage site

139 (Fig. 2c). The activity for the other cOAs was significantly lower. We repeated the experiment

140 with an S152A variant of CalpL, which lacks the nucleophilic serine needed for its peptidase

141 activity. Since this variant showed no protease activity, the CalpL protease active site is

142 responsible for the observed proteolytic activity (Fig. 2c).

143   The peptide sequences of the two cleavage fragments were determined with peptide mass

144   fingerprinting (Extended Data Fig. 4d). This analysis confirmed that the 23 kDa (CalpT23)

145   fragment corresponds to the N-terminal two-thirds of the CalpT protein and the 10 kDa

146   fragment (CalpT10) to the C-terminal one-third. Based on this result and considering the

147   predicted structure (Fig. 2b), we mapped the location of the cleavage site to the stretch of

148   residues between amino acids ~170-200 of CalpT. As mentioned above, our CalpL structure

149   suggested that only peptides containing an alanine or glycine as the P1 residue will fit into the

150   active site of CalpL. We therefore created glutamic acid mutants of all four alanine residues in

151   the cleavage region: A172, A182, A195, and A201 (Fig. 2b, magenta spheres; the stretch of

152   residues does not contain any glycine). Peptidase assays with all four CalpT variants were

153   conducted and only the A195E mutation abolished the cleavage completely (Extended Data

154   Fig. 4e). At this position, the amino acid sequence reads V_{190}LRHVA|ST, where A195 is most

155   likely the P1 residue. Notably, A195 is conserved amongst CalpT homologs (Fig. 2d). The

156   peptide fingerprint data in Extended Data Fig. 4d also supports this conclusion, as for CalpT23,

157   the peptide coverage extended almost exactly to the identified cleavage site. We did not observe

158   any non-tryptic peptides that corresponded to the identified cleavage site. Thus, the exact

159   molecular weights of the CalpT cleavage products are 23.0 kDa (CalpT23) and 8.7 kDa

160   (CalpT10), fitting to the sizes observed in SDS PAGE analysis (Fig. 2c).

161

162   **CalpL and CalpT form a 1:1 complex**

163   To test whether CalpL and CalpT form a stable complex, we analysed the individual proteins

164   and their equimolar mixtures by SEC-MALS (Fig. 3a). CalpL alone eluted in a single peak at

165   17.1 ml and the CalpT protein eluted at 17.9 ml, both at the expected molecular weights for the

166   monomeric proteins. The 1:1 mixture of the two proteins resulted in a single elution peak at

167   16.2 ml. The MW_{MALS} of the complex was 82.4 kDa, suggestive of a 1:1 complex of the CalpL

168   and CalpT proteins (52 + 30.5 kDa). We used surface plasmon resonance (SPR) to quantify the

169   interaction strength between CalpL and -T and found that the two proteins form a very strong

170   complex with a sub-nanomolar K_D (Extended Data Fig. 5a). Interestingly, a similar affinity was

171   observed for a construct where CalpT10 (including the cleavage site) was fused to a VHH

172   domain targeting an unrelated protein (Extended Data Fig. 5b,c). The artificial construct was

173   readily cleaved by the protease upon activation by cA_4 (Extended Data Fig. 5d). Thus, the

174   CalpT23 fragment plays no important role either in the formation of the CalpL/T complex or in

175   the cleavage process. A second artificial construct, where the CalpT10 moiety was also replaced

176   by an unrelated VHH was not cleaved (Extended Data Fig. 5d). Hence, the CalpT$_{10}$ subunit is

177   required for cleavage.

178   To follow the fate of the complex after cleavage, we repeated the experiment in presence of

179   an excess (1:1.1) of cA$_4$ (Fig. 3a, violet). Here, we observed three peaks corresponding to

180   CalpL/T$_{10}$, CalpT$_{23}$ and cA$_4$. Correspondingly, for the inactive CalpL S152A variant, the

181   CalpL/T complex was observed but the addition of cA$_4$ did not lead to the observed split into

182   three peaks (Extended Data Fig. 5e). We also checked whether the four cleavage-site variants

183   of CalpT could still form a complex with CalpL (Extended Data Fig. 5f). Whereas A172E,

184   A182E, and the P1 site variant A195E did, the A201E variant did not. The glutamate at this

185   position apparently weakened the interaction, explaining the reduced cleavage efficiency of this

186   mutant (Extended Data Fig. 4e).

187   We isolated the CalpL/T$_{10}$ complex for crystallization and determined its structure at 3.3 Å

188   resolution by molecular replacement, using the CalpL crystal structure and the AlphaFold

189   model of CalpT$_{10}$ as search models (Fig. 3a, Extended Data Fig. 5g, Extended Data Table 1).

190   Indeed, the CalpT$_{10}$ fragment binds to the N-terminal domain of CalpL and, as indicated by the

191   mutation analysis, A201 of CalpT is part of the interface in addition to the CalpL hydrophobic

192   residues W28, L6, V14, L18, E20, E13, K8, H2 and CalpT residues K200, Y210, Y203, E222

193   (Extended Data Fig. 5g). A small-angle X-ray scattering (SAXS) experiment was performed to

194   exclude a crystal packing artefact, by measuring the SAXS profiles of CalpL and CalpL/T$_{10}$

195   complex by SEC-SAXS (Extended Data Table 2, experimental session I). The two profiles were

196   fitted simultaneously using the multi-phase *ab initio* shape reconstruction program MONSA.

197   The *ab initio* model was in excellent agreement with the SAXS data and compares well to our

198   crystal structure (Extended Data Fig. 5h), thus confirming the arrangement of the two subunits

199   in solution.

200

201   **cA$_4$ induced oligomerization of CalpL/T**

202   Intriguingly, while the C-terminal part of the CalpL cleavage site (T197 of CalpT) is visible in

203   the complex crystal structure, it is more than 35 Å away from the protease active site (Fig. 3a),

204   indicating that a cA$_4$ induced structural rearrangement of CalpL must occur to allow cleavage

205   of CalpT. Recent studies on other SAVED-domain containing CBASS effectors demonstrated

206   that cOA binding induces an oligomerization, which then activates the effector[23,29,36]. Dynamic

207   light scattering (DLS) and SAXS experiments showed such a cA$_4$- and protein concentration-

208   dependent oligomerization of CalpL (Fig. 3b, Extended Data Fig. 6a,b). A representative *ab*

209   *initio* model for monomeric CalpL in the presence of cA$_4$ was obtained by SEC-SAXS

210 (Extended Data Table 2), resulting in an elongated and slightly bent model featuring two main

211 lobes connected by a slightly thinner region at SAXS resolution. Using this monomeric unit,

212 the concentration series in the range 2-5 mg/mL was modeled as a dimerizing mixture by a

213 global SASREFMX fitting without imposing symmetry elements (as non-identical binding

214 interfaces are to be expected in the presence of the cA_4 ligand). SAXS modelling of the

215 dimerizing mixture produced stable solutions (normalized spatial discrepancy (NSD) ~0.95)

216 featuring elongated shapes that were large enough to accommodate two CalpL molecules

217 (Extended Data Fig. 6c).

218   We noticed a distinct positively charged patch on the face opposite of the cA_4 binding site,

219 where R361, R338 and K364 coordinate a sulfate ion in the cA_4 complex structure (Fig. 1c).

220 Due to this structural feature, binding of the negatively charged cA_4 molecule would result in a

221 charge complementarity between the top and bottom sides of the SAVED domain. This supports

222 an arrangement where two or more CalpL molecules would form stacks, with cA_4 sandwiched

223 in between, similar to the architecture observed for other SAVED domain oligomers[23,29,36,37].

224 The SAXS *ab initio* model in Extended Data Fig. 6c would best agree with a staggered

225 arrangement of the CalpL monomers. An attractive model for the activation of CalpL by cA_4

226 would thus be an in-trans cleavage reaction in the observed oligomers. To test this, we

227 performed a cleavage assay, were mixtures of preformed CalpL/T complexes, for instance

228 CalpL/T (wt/wt), CalpL/T (S152A/wt), CalpL/T (wt/A195E), were tested for cA_4 induced

229 cleavage. A 1:1 mixture of CalpL/T (S152A/wt) with CalpL/T (wt/A195E), *i.e.*, two complexes

230 that are not capable of in-cis cleavage, led to 50% cleaved CalpT (Fig. 3c). The remaining 50%

231 could not be cleaved due to the A195E mutation, but this could be titrated by changing the ratio

232 of the two complexes (Fig. 3c). Further, a mixture of CalpL/T (S152A/wt) with uncomplexed

233 CalpL led to complete cleavage of CalpT. To support the idea of oligomerization induced

234 cleavage, we introduced mutations to the backside of the SAVED domain, aiming to disturb

235 the presumed oligomerization interface (Fig. 3d and 1c). While mutant R361E had a ~50%

236 reduced activity, R338E had no cleavage activity. We also found that the R493C mutant used

237 for the SPR experiments had a ~50% reduced cleavage activity.

238   While our data show that in-trans cleavage occurs, we can currently not distinguish, whether

239 cleavage occurs inside one particular CalpL/T oligomer or between two oligomers. For the

240 latter, three CalpL/T units would have to assemble for the VA/ST sequence in CalpT to be able

241 to reach the protease active site of a CalpL molecule in the oligomer. A high-resolution structure

242 of such a CalpL/T stack will be necessary to unravel the molecular details of the activation.

243

CalpL/T bind the ECF σ factor CalpS

Our initial assumption that CalpT will have a MazF-like nuclease activity could not be confirmed experimentally. We could neither identify any signs of an RNase activity biochemically (Extended Data Fig. 7a-c), nor with RNase-seq of random libraries (Extended Data Fig. 7d,e). Moreover, expression of CalpT$_{23}$ constructs in *E. coli* were tolerated by the cells (not shown). We also looked at a dimerization of the CalpT$_{23}$ fragment, similar to the active dimeric MazF enzyme. To test this, we spin labelled the CalpL/CalpT complex at position 119 of CalpT (Extended Data Fig. 7f,g) and measured the interspin distance in the presence and absence of cA$_4$. According to the EPR data, the cA$_4$ induced cleavage did not lead to changes of the conformational state of the CalpT$_{23}$ (Extended Data Fig. 7h-j).

All this turned our attention towards CalpS, a third conserved protein encoded by the operon (224 amino acids, 26.5 kDa, UniProt ID B2V8L7). The protein has strong sequence similarities to ECF family σ factors, which tailor transcription in diverse stress conditions[32,38,39] (Extended Data Fig. 8a). Interestingly, AlphaFold2 supported a heterotrimeric complex between CalpL, -T and -S, which was consistent with our finding that the CalpT$_{23}$ domain is not involved in CalpL/T complex formation (Fig. 4a). The predicted CalpT/S interface has a combined buried surface area of ~4000 Å^2 [40], high confidence scores in the interface area and convincing sidechain interactions. In the prediction, CalpT binds to both the σ$_2$- and σ$_4$-domains of the σ-factor and blocks most of the -10-region interface (Extended Data Fig. 8bc). The same interface is targeted by so called anti-σ-factors, preventing the interaction of the σ-factor/RNA-polymerase (RNAP) complex with its cognate promotor (Extended Data Fig. 8d)[38,39]. Note that in the predicted CalpT/S complex, the σ$_2$- and σ$_4$ domains are tied together in a way that would not allow the σ-factor to bind to the RNAP (Extended Data Fig. 8e).

To put the existence of this complex to the test, we cloned the *calpS* gene and co-expressed the His-CalpT/S proteins in *E. coli*. As predicted, the two proteins formed a stable complex that could be isolated by gel filtration (Fig. 4b). Furthermore, addition of CalpL to the CalpT/S complex resulted in a ternary complex, that disintegrated into CalpL/T$_{10}$ and CalpT$_{23}$/S upon addition of cA$_4$ (Fig. 4bcd).

We noticed that expression of CalpS alone (instead of coexpression with CalpT) led to a copurification of the protein with the α- and β subunits of the DNA-directed RNA polymerase of *E. coli* (44% sequence identity between β subunits of *Sulfurihydrogenibium sp.* and *E. coli* RNAP). This corroborates the prediction that CalpS is a σ-factor and that CalpT inhibits its interaction with the RNAP (Fig. 4e). Thus, CalpT has striking functional similarities to anti-σ-

277 factor proteins and literally links the cA_4 sensor CalpL to the transcription machinery of the
278 cell.

279

280 **Discussion**

281 Protease signaling cascades are a common scheme in evolution that are often employed in
282 emergency situations. Prokaryotic type-II toxin-antitoxin (TA) systems, for instance, are
283 activated by degradation of the antitoxin by ATP-dependent Lon proteases[41]. The innate
284 immune system of higher organisms also employs proteases such as caspases to initiate and
285 amplify fast responses to external threats. Another well-known example are the cascades of
286 proteases that control the clotting of blood[42]. Our work shows that the CalpL/T/S cascade
287 amalgamates aspects of different defense systems such as CRISPR, CBASS, toxin/antitoxin
288 systems, and σ/anti-σ-factors into a cA_4-controlled "fast response" signaling cascade.

289   Our data are summarized in the model sketched in Fig. 5, where in its inactive state, CalpL
290 will be present in a 1:1:1 complex with CalpT and CalpS. Upon detection of a foreign RNA by
291 the type III effector complex, cA_4 will be synthesized by its Cas10 subunit and the second
292 messenger will bind to the SAVED domain of CalpL with nanomolar affinity. This drastically
293 changes the surface electrostatics of the SAVED domain, enabling oligomerization of CalpL,
294 as observed by SAXS and DLS (Fig. 3b and Extended Data Fig. 6). This activation mechanism
295 has now emerged as a common theme in the SAVED-domain based effectors of CBASS
296 defense systems[23,29,36] but has not been observed in CRISPR systems. The CalpL
297 oligomerization triggers an in-trans cleavage of CalpT (Fig. 3c,d), releasing the $CalpT_{23}$/S
298 subcomplex in a strictly cA_4 dependent manner (Fig. 4bcd). Some CARF domain proteins are
299 known to auto deactivate by degrading cOA species [12], but this has not been observed for
300 SAVED domains and has not yet been investigated for CalpL.

301   CalpS is member of the ECF family of ECF σ factors, which play a role in the sensing of
302 extracellular stress events, such as cell envelope- or oxidative stress[38,39]. In striking resemblance
303 to the Calp cascade, the release of such anti-σ factors is orchestrated by a sequence of
304 proteolytic events called regulated intermembrane proteolysis (RIP)[43]. The activation of σ^E
305 from *E. coli*, for instance, proceeds via proteolytic cleavage of the membrane bound anti sigma
306 factor RseA, releasing a soluble σ-factor/anti-σ-factor complex. The anti-σ-factor is
307 subsequently degraded by ATP-dependent ClpXP proteases. [38,44]. Following the established
308 paradigms, one might speculate that further proteolysis of $CalpT_{23}$ releases the sigma factor
309 CalpS to allow transcriptional response.

10

310   Recently, two studies have revealed that type III-E CRISPR systems also function by

311   activating the protease Csx29 (also known as TRP-CHAT) [45,46]. There are notable differences,

312   as the protease (from the Caspase family) is completely unrelated to CalpL and is not activated

313   by cOA. Csx29, part of the Type III-E effector complex, is activated by conformational changes

314   upon foreign RNA detection and cleaves an uncharacterized protein (Csx30) encoded in the

315   operon [47]. Interestingly, Csx30 binds a σ-factor also homologous to the ECF family (termed

316   CASP-σ). Furthermore, it was shown that the Csx30 protein inhibits CASP-σ and this inhibition

317   is relieved by Csx29 mediated proteolytic cleavage. CASP-σ has a high affinity for a DNA

318   sequence that is found in the promotor of Cas1-2, proteins of CRISPR adaptation for the

319   acquisition of new viral memories [48]. Since the CRISPR effectors and the two proteases are

320   completely unrelated, this appears to be a striking example of convergent evolution.

321   Here, we have uncovered a cOA-mediated signaling cascade from viral RNA detection to the

322   proteolytic release of a σ-factor that binds RNA polymerase. Notably, the Cas10 proteins

323   associated with Calp operons lack an HD nuclease domain and auxiliary cOA activated

324   nucleases such as Csx1 are rarely found, suggesting transcriptional changes sufficient for

325   CRISPR antiviral immunity in organisms such as *Sulfurihydrogenibium*. It will be exciting to

326   find out the DNA targets of CalpS to understand better how the Calp cascade shapes the

327   antiviral response, buying the organism enough time to survive a viral attack[18,21].

328

Main text references

1. Makarova, K. S., Wolf, Y. I. & Koonin, E. V. Classification and nomenclature of CRISPR-Cas systems: where from here. *The CRISPR Journal* 1, 325-336 (2018).
2. Zhu, Y., Klompe, S. E., Vlot, M., van der Oost, J. & Staals, R. H. J. Shooting the messenger: RNA-targetting CRISPR-Cas systems. *Bioscience reports* 38, BSR20170788 (2018).
3. Kazlauskiene, M., Kostiuk, G., Venclovas, Č., Tamulaitis, G. & Siksnys, V. A cyclic oligonucleotide signaling pathway in type III CRISPR-Cas systems. *Science* 357, 605-609 (2017).
4. Niewoehner, O., Garcia-Doval, C., Rostøl, J. T., Berk, C., Schwede, F., Bigler, L., Hall, J., Marraffini, L. A. & Jinek, M. Type III CRISPR-Cas systems produce cyclic oligoadenylate second messengers. *Nature* 548, 543-548 (2017).
5. Rouillon, C., Athukoralage, J. S., Graham, S., Grüschow, S. & White, M. F. Control of cyclic oligoadenylate synthesis in a type III CRISPR system. *Elife* 7, e36734 (2018).
6. Shmakov, S. A., Makarova, K. S., Wolf, Y. I., Severinov, K. V. & Koonin, E. V. Systematic prediction of genes functionally linked to CRISPR-Cas systems by gene neighborhood analysis. *Proc Natl Acad Sci U S A* 115, E5307-E5316 (2018).
7. Shah, S. A., Alkhnbashi, O. S., Behler, J., Han, W., She, Q., Hess, W. R., Garrett, R. A. & Backofen, R. Comprehensive search for accessory proteins encoded with archaeal and bacterial type III CRISPR-cas gene cassettes reveals 39 new cas gene families. *RNA Biol* 16, 530-542 (2019).
8. Gasiunas, G., Sinkunas, T. & Siksnys, V. Molecular mechanisms of CRISPR-mediated microbial immunity. *Cell Mol Life Sci* 71, 449-465 (2014).
9. Sasnauskas, G. & Siksnys, V. CRISPR adaptation from a structural perspective. *Curr Opin Struct Biol* 65, 17-25 (2020).
10. Jiang, F. & Doudna, J. A. CRISPR–Cas9 Structures and Mechanisms. *Annual Review of Biophysics* 46, 505-529 (2017).
11. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A. & Charpentier, E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816-821 (2012).
12. Athukoralage, J. S. & White, M. F. Cyclic oligoadenylate signalling and regulation by ring nucleases during type III CRISPR defence. *RNA* rna.078739.121 (2021).
13. Jia, N., Jones, R., Sukenick, G. & Patel, D. J. Second messenger cA4 formation within the composite Csm1 Palm pocket of type III-A CRISPR-Cas Csm complex and its release path. *Molecular cell* 75, 933-943. e6 (2019).
14. Makarova, K. S., Anantharaman, V., Grishin, N. V., Koonin, E. V. & Aravind, L. CARF and WYL domains: ligand-binding regulators of prokaryotic defense systems. *Frontiers in genetics* 5, 102 (2014).
15. Lau, R. K., Ye, Q., Birkholz, E. A., Berg, K. R., Patel, L., Mathews, I. T., Watrous, J. D., Ego, K., Whiteley, A. T. & Lowey, B. Structure and mechanism of a cyclic trinucleotide-activated bacterial endonuclease mediating bacteriophage immunity. *Molecular cell* 77, 723-733. e6 (2020).
16. Lintner, N. G., Frankel, K. A., Tsutakawa, S. E., Alsbury, D. L., Copié, V., Young, M. J., Tainer, J. A. & Lawrence, C. M. The structure of the CRISPR-associated protein Csa3 provides insight into the regulation of the CRISPR/Cas system. *Journal of molecular biology* 405, 939-955 (2011).
17. McMahon, S. A., Zhu, W., Graham, S., Rambo, R., White, M. F. & Gloster, T. M. Structure and mechanism of a Type III CRISPR defence DNA nuclease activated by cyclic oligoadenylate. *Nat Commun* 11, 500 (2020).

379   18.   Rostøl, J. T., Xie, W., Kuryavyi, V., Maguin, P., Kao, K., Froom, R., Patel, D. J. &
380   Marraffini, L. A. The Card1 nuclease provides defence during type III CRISPR
381   immunity. *Nature* 590, 624-629 (2021).
382   19.   Garcia-Doval, C., Schwede, F., Berk, C., Rostøl, J. T., Niewoehner, O., Tejero, O., Hall,
383   J., Marraffini, L. A. & Jinek, M. Activation and self-inactivation mechanisms of the
384   cyclic oligoadenylate-dependent CRISPR ribonuclease Csm6. *Nature communications*
385   11, 1-9 (2020).
386   20.   Lawrence, C. M., Charbonneau, A. & Gauvin, C. Cyclic Tetra-Adenylate (cA4)
387   Activates CRISPR Associated Transcription Factor Csa3, Providing Feedback
388   Activation of Protospacer Acquisition and crRNA Expression. *The FASEB Journal* 34,
389   1-1 (2020).
390   21.   Meeske, A. J., Nakandakari-Higa, S. & Marraffini, L. A. Cas13-induced cellular
391   dormancy prevents the rise of CRISPR-resistant bacteriophage. *Nature* 570, 241-245
392   (2019).
393   22.   Burroughs, A. M., Zhang, D., Schäffer, D. E., Iyer, L. M. & Aravind, L. Comparative
394   genomic analyses reveal a vast, novel network of nucleotide-centric systems in
395   biological conflicts, immunity and signaling. *Nucleic Acids Res* 43, 10633-10654
396   (2015).
397   23.   Lowey, B., Whiteley, A. T., Keszei, A. F. A., Morehouse, B. R., Mathews, I. T., Antine,
398   S. P., Cabrera, V. J., Kashin, D., Niemann, P., Jain, M., Schwede, F., Mekalanos, J. J.,
399   Shao, S., Lee, A. S. Y. & Kranzusch, P. J. CBASS Immunity Uses CARF-Related
400   Effectors to Sense 3'-5'- and 2'-5'-Linked Cyclic Oligonucleotide Signals and Protect
401   Bacteria from Phage Infection. *Cell* 182, 38-49.e17 (2020).
402   24.   Makarova, K. S., Timinskas, A., Wolf, Y. I., Gussow, A. B., Siksnys, V., Venclovas, Č.
403   & Koonin, E. V. Evolutionary and functional classification of the CARF domain
404   superfamily, key sensors in prokaryotic antivirus defense. *Nucleic acids research* 48,
405   8828-8847 (2020).
406   25.   Zwart, P. H., Afonine, P. V., Grosse-Kunstleve, R. W., Hung, L.-W., Ioerger, T. R.,
407   McCoy, A. J., McKee, E., Moriarty, N. W., Read, R. J., Sacchettini, J. C., Sauter, N. K.,
408   Storoni, L. C., Terwilliger, T. C. & Adams, P. D. Automated structure solution with the
409   PHENIX suite. *Methods in Molecular Biology (Clifton, N.J.)* 426, 419-435 (2008).
410   26.   Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G.
411   J., Murray, L. W., Richardson, J. S. & Richardson, D. C. MolProbity: all-atom structure
412   validation for macromolecular crystallography. *Acta Crystallographica Section D* 66,
413   12-21 (2010).
414   27.   Chung, I. Y. & Paetzel, M. Crystal structures of yellowtail ascites virus VP4 protease:
415   trapping an internal cleavage site trans acyl-enzyme complex in a native Ser/Lys dyad
416   active site. *J Biol Chem* 288, 13068-13081 (2013).
417   28.   Krogh, A., Larsson, B., von Heijne, G. & Sonnhammer, E. L. L. Predicting
418   transmembrane protein topology with a hidden markov model: application to complete
419   genomes. *Journal of Molecular Biology* 305, 567-580 (2001).
420   29.   Fatma, S., Chakravarti, A., Zeng, X. & Huang, R. H. Molecular mechanisms of the
421   CdnG-Cap5 antiphage defense system employing 3′, 2′-cGAMP as the second
422   messenger. *Nature Communications* (2021).
423   30.   Jiang, K., Faltova, L., Hua, S., Capitani, G., Prota, A. E., Landgraf, C., Volkmer, R.,
424   Kammerer, R. A., Steinmetz, M. O. & Akhmanova, A. Structural Basis of Formation of
425   the Microtubule Minus-End-Regulating CAMSAP-Katanin Complex. *Structure* 26, 375-
426   382.e4 (2018).
427   31.   Saha, C. K., Sanches Pires, R., Brolin, H., Delannoy, M. & Atkinson, G. C. FlaGs and
428   webFlaGs: discovering novel biology through the analysis of gene neighbourhood
429   conservation. *Bioinformatics* 37, 1312-1314 (2021).

430   32.   Zimmermann, L., Stephens, A., Nam, S.-Z., Rau, D., Kübler, J., Lozajic, M., Gabler, F.,
431   Söding, J., Lupas, A. N. & Alva, V. A completely reimplemented MPI bioinformatics
432   toolkit with a new HHpred server at its core. *Journal of molecular biology* 430, 2237-
433   2243 (2018).
434   33.   Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O.,
435   Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C.,
436   Kohl, S. A. A., Ballard, A. J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R.,
437   Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M.,
438   Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A. W.,
439   Kavukcuoglu, K., Kohli, P. & Hassabis, D. Highly accurate protein structure prediction
440   with AlphaFold. *Nature* 596, 583-589 (2021).
441   34.   Holm, L. & Rosenström, P. Dali server: conservation mapping in 3D. 38, W545-9
442   (2010).
443   35.   Simanshu, D. K., Yamaguchi, Y., Park, J.-H., Inouye, M. & Patel, D. J. Structural basis
444   of mRNA recognition and cleavage by toxin MazF and its regulation by antitoxin MazE
445   in Bacillus subtilis. *Molecular cell* 52, 447-458 (2013).
446   36.   Hogrel, G., Guild, A., Graham, S., Rickman, H., Grushow, S., Bertrand, Q., Spagnolo,
447   L. & White, M. F. Cyclic nucleotide-induced superhelical structure activates a bacterial
448   TIR immune effector. *bioRxiv* (2022).
449   37.   Hogrel, G., Guild, A., Graham, S., Rickman, H., Grüschow, S., Bertrand, Q., Spagnolo,
450   L. & White, M. F. Cyclic nucleotide-induced helical structure activates a TIR immune
451   effector. *Nature* 1-5 (2022).
452   38.   Paget, M. S. Bacterial sigma factors and anti-sigma factors: structure, function and
453   distribution. *Biomolecules* 5, 1245-1265 (2015).
454   39.   Sineva, E., Savkina, M. & Ades, S. E. Themes and variations in gene regulation by
455   extracytoplasmic function (ECF) sigma factors. *Current opinion in microbiology* 36,
456   128-137 (2017).
457   40.   Krissinel, E. & Henrick, K. Inference of macromolecular assemblies from crystalline
458   state. *Journal of Molecular Biology* 372, 774-797 (2007).
459   41.   Schuster, C. F. & Bertram, R. Toxin–antitoxin systems are ubiquitous and versatile
460   modulators of prokaryotic cell fate. *FEMS microbiology letters* 340, 73-85 (2013).
461   42.   Walsh, P. N. & Ahmad, S. S. Proteases in blood clotting. *Essays in biochemistry* 38, 95-
462   112 (2002).
463   43.   Brown, M. S., Ye, J., Rawson, R. B. & Goldstein, J. L. Regulated intramembrane
464   proteolysis: a control mechanism conserved from bacteria to humans. *Cell* 100, 391-398
465   (2000).
466   44.   Fei, X., Bell, T. A., Barkow, S. R., Baker, T. A. & Sauer, R. T. Structural basis of
467   ClpXP recognition and unfolding of ssrA-tagged substrates. *Elife* 9, e61496 (2020).
468   45.   Hu, C., van Beljouw, S. P. B., Nam, K. H., Schuler, G., Ding, F., Cui, Y., Rodríguez-
469   Molina, A., Haagsma, A. C., Valk, M. & Pabst, M. Craspase is a CRISPR RNA-guided,
470   RNA-activated protease. *Science* eadd5064 (2022).
471   46.   van Beljouw, S. P. B., Haagsma, A. C., Rodríguez-Molina, A., van den Berg, D. F.,
472   Vink, J. N. A. & Brouns, S. J. J. The gRAMP CRISPR-Cas effector is an RNA
473   endonuclease complexed with a caspase-like peptidase. *Science* 373, 1349-1353 (2021).
474   47.   Kato, K., Okazaki, S., Schmitt-Ulms, C., Jiang, K., Zhou, W., Ishikawa, J., Isayama, Y.,
475   Adachi, S., Nishizawa, T. & Makarova, K. S. RNA-triggered protein cleavage and cell
476   growth arrest by the type III-E CRISPR nuclease-protease. *Science* eadd7347 (2022).
477   48.   Strecker, J., Demircioglu, F. E., Li, D., Faure, G., Wilkinson, M. E., Gootenberg, J. S.,
478   Abudayyeh, O. O., Nishimasu, H., Macrae, R. K. & Zhang, F. RNA-activated protein
479   cleavage with a CRISPR-associated endopeptidase. *Science* eadd7450 (2022).
480

Figure legends

Fig. 1 | Structure of apo and cA$_4$ bound CalpL. a, Overall structure of CalpL in the apo state. The structure is shown as a cartoon model and the individual domains are labelled and color-coded. The N- and C-termini, as well as the protease active site residues, are marked by spheres. The positions of key structural elements are indicated. **b**, Single cycle kinetics SPR measurements of different cOAs binding to immobilized CalpL. The experiment was performed multiple times (n=3 technical replicates) for cA$_4$ and once for the other cOAs. **c**, The CalpL/cA$_4$ complex structure. CalpL is shown as a surface model and the electrostatic potential is mapped onto the structure (blue - positive, red – negative). The bound cA$_4$ molecule is shown as spheres. Turning the structure by 180° reveals a positively charged patch opposite of the SAVED domain.

Fig. 2 | CalpL is activated by cA$_4$ and cleaves CalpT. a, The WebFLAGs server [31] was used to investigate the genomic neighborhood of CalpL (green). The primary structure of CalpT (red) is shown on top. Regions with homologies found by HHPRED [32] are marked. **b**, A structural prediction (AlphaFold2, [33]) of CalpT. The protein is shown as cartoon and colored according to the prediction confidence (pLDDT [49], predicted local distance difference test). **c**, SDS-PAGE analysis of CalpL induced cleavage of CalpT. The experiment was repeated multiple times (n =2 biological replicates and n>>3 technical replicates) **d**, Sequence alignment [50] showing that the identified P1 site at A195 is conserved among CalpT homologs. For gel source data, see Supplementary Figure 1.

Fig. 3 | CalpL and -T form a stable complex and cA$_4$-induced oligomerization of CalpL. a, SEC-MALS traces (solid lines: UV$_{280}$, dashed lines: MW$_{MALS}$) of proteolysis reactions with different combinations of CalpL wt, CalpT wt, and cOA. The SEC-MALS experiment was performed multiple times with slight variations of buffer and concentrations (n = 3 technical replicates). The schematic indicates the molecular species behind the individual peaks. Inset: Crystal structure of the CalpL/T$_{10}$ complex. The distance of 36 Å between the P-1 position (S196) and the protease active site is indicated. **b**, Top: Concentration-normalized small angle X-ray scattering curves recorded at four different concentrations of CalpL. For each experiment, thirty sample intensity frames and sixty buffer intensity frames were collected and averaged. For each data set and angular point the errors were computed following the Poisson statistics. The data points represent the average intensity difference (sample-buffer) and the error bars represent the standard deviation. The experiment was performed once for each concentration. Bottom: Molecular weights from forward scattering I(0) calculated from the SAXS curves plotted vs the concentration. The apparent molecular weight of the protein in the presence of cA$_4$ increases with the concentration. **c**, Protease assays with preformed CalpL/T complexes as indicated in the figure. The experiment was performed three times (n = 3 technical replicates). **d**, Protease assays with CalpL mutants in the positive patch on the backside of the SAVED domain (Fig. 1). The experiment was performed twice (n = 2 technical replicates). For gel source data, see Supplementary Figure 1.

Fig. 4 | CalpT links cA$_4$ detection to the transcription machinery of the cell. a, Structural information about the CalpL/T/S complex as obtained by crystallography, SAXS and AlphaFold2. **b-d**, CalpT and S form a complex that is stable during gelfiltration and disintegrates into CalpL/T$_{10}$ and CalpT$_{23}$/S. The experiments were performed twice (n=2 technical replicates). **e**, His-CalpS and CalpT can be copurified from *E. coli*. Overexpression of CalpS alone leads to copurification of the α- and β-subunits of the *E. coli* RNAP. The complex formations were replicated multiple times in two independent laboratories (n>3 biological replicates). For gel source data, see Supplementary Figure 1.

532

Fig. 5 | Model for CalpL/T/S mediated antiviral defense. a, The *calpL*, *calpT* and *calpS*
genes are located in close proximity to the type III-B CRISPR genes of *Sulfurihydrogenibium*
sp. YO3AOP1 (modified from [6]). **b**, Once activated, the Cas10 subunit of the RNP synthesizes
cA_4 from ATP. The second messenger binds to preformed CalpL/T/S complexes.
Oligomerization leads to proteolytic cleavage of CalpT, releasing the $CalpT_{23}$/S fragment.
$CalpT_{23}$ is likely degraded by proteases, allowing CalpS to bind to the RNA polymerase.

539

Methods

Expression and purification of CalpL

The codon-optimized gene for CRISPR–Lon was cloned into a pET11a vector with an N-terminal 10xHis-TEV tag. Site-directed mutagenesis was performed according to a protocol by Liu et al.[51]. All CalpL constructs were expressed in lysogeny broth (LB) medium. *E. coli* BL21(DE3) cells were grown at 37 °C until an OD_{600} of 0.6-0.8 was reached. Then, protein expression was started by induction with 0.4 mM IPTG, and the cell suspension was incubated at 30 °C for 4.5 h with shaking. Cells were harvested by centrifugation at 4,000*rcf for 25 min. at 20 °C and resuspended in lysis buffer (20 mM Tris, 50 mM NaCl, pH 8.0). The cells were lysed with a sonicator and cell debris was removed by centrifugation at 48,000*rcf for 45 min. at 4 °C. For protein purification, Ni^{2+}-affinity chromatography (20 mM Tris, 50 mM NaCl, pH 8.0; 500 mM imidazole was included for elution) was followed by size-exclusion chromatography (20 mM Tris, 50 mM NaCl, pH 8.0) using a Superdex 200 16/600 column. After that, the His-tag was cleaved off by overnight incubation at 4 °C with a 1:50 molar ratio of protein to TEV protease (20 mM Tris, 50 mM NaCl, pH 8.0). A second Ni^{2+}-affinity chromatography was used to remove the TEV protease and uncleaved protein. The purity of the protein was checked by SDS-PAGE after each purification step. After successful purification, the proteins were concentrated, flash-frozen in liquid nitrogen, and stored at -80 °C in 20 mM Tris, 50 mM NaCl, pH 8.0. The selenomethionine derivative of CalpL was prepared using *E. coli* B834 cells and the "SelenoMethionine Medium Complete" kit from Molecular Dimensions according to the instructions. Protein expression and purification were done in the same way as for the native protein.

Expression and purification of CalpT

The codon-optimized synthetic gene (BioCat) for CalpT (UNIPROT-ID: B2V8L8), including an N-terminal 10x His-TEV tag was cloned into a pET11a vector. Protein expression was done using the same expression strain and the same conditions as for CalpL. Cells were harvested by centrifugation at 4,000*rcf for 25 min. at 20 °C and resuspended in lysis buffer (25 mM Tris, 500 mM NaCl, 10% glycerol, 1 mM DTT, pH 8.0). The cells were lysed with a sonicator and cell debris was removed by centrifugation at 48,000*rcf for 45 min. at 20 °C. For protein purification, Ni^{2+}-affinity chromatography (25 mM Tris, 500 mM NaCl, 1 mM DTT, 10% glycerol, pH 8.0; 1 M imidazole was included for elution) was followed by size-exclusion chromatography (25 mM Tris, 500 mM NaCl, 1 mM DTT, 10% glycerol, pH 8.0) using a Superdex 75 16/600 column. After that, the His-tag was cleaved off by overnight incubation at

574 4 °C with a 20:1 ratio (m/m) of protein to TEV protease (25 mM Tris, 500 mM NaCl, 1 mM
575 DTT, 10% glycerol, pH 8.0). A second Ni^{2+}-affinity chromatography was used to separate the
576 TEV protease and uncleaved protein. The purity of the protein was checked by SDS-PAGE
577 after each purification step. After successful purification, the proteins were concentrated, flash-
578 frozen in liquid nitrogen, and stored at -80 °C in 25 mM Tris, 500 mM NaCl, 1 mM DTT, 10%
579 glycerol, pH 8.0.

580

581 **Expression and purification of CalpS**

582 The codon-optimized gene of CalpS was purchased from Integrated DNA Technologies (IDT,

583 Coralville, Iowa, USA) as a G-Bock with flanking restriction sites for cloning. *SF* was cloned

584 into NcoI and BamHI restriction sites of vector pEV5HisTEV[52], allowing expressed proteins

585 with an N-terminal 8x His-TEV tag. For expression, *E. coli* C43(DE3) cells with sequencing-

586 verified construct were incubated at 37 °C with shaking at 180 rpm until OD_{600} of the cells was

587 between 0.6 and 0.8. Then, the cell culture was grown at 16 °C overnight after inducing with

588 0.2 mM IPTG. The cell pellet was collected by centrifugation at 4000 rpm (Beckman Coulter

589 Avanti JXN-26; JLA8.1 rotor) at 4 °C for 15 min. For purification, cell pellet was resuspended

590 into buffer A (50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 20 mM imidazole, and 10% glycerol) and

591 lysed by sonication. The cleared cell lysate was loaded onto a 5 mL HisTrap FF column (GE

592 Healthcare) equilibrated with buffer A. The His-tagged SF was eluted in a linear gradient with

593 buffer B (50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.5 M imidazole, and 10% glycerol). The his-

594 tag was then removed by incubating with TVE protease at room temperature overnight before

595 recovering TEV-cleaved SF through a HisTrap column again. Size-exclusion chromatography

596 was finally used to purify the SF in SEC buffer (20 mM Tris–HCl, 0.25 M NaCl, 1 mM DTT,

597 10% glycerol, pH 7.5). The purity of SF was evaluated on the SDS-PAGE at each purification

598 step. Concentrated SF was flash-frozen in liquid nitrogen and stored at -70 °C.

599

600 **Co-expression and co-purification of sigma factor (SF) and CalpT**

601 For expression of His-tagged CalpS with CalpT, the fragment of CalpT flanking NdeI and XhoI

602 sites was cloned into MCS-2 of vector pCDFDuet™-1 (Novagen, Merck Millipore). The

603 constructs pEV5HisTEV-SF and pCDFDuet-CalpT were co-transformed into *E. coli* C43(DE3)

604 cells. The cell was induced by 0.2 mM IPTG after reaching OD600 of 0.6-0.8 and grown

605 overnight at 16 °C with shaking at 180 rpm.

606 For expression of His-tagged CalpT with CalpS, a G-Block of SF was constructed into MCS-1

607 (NcoI and BamHI) of vector pCDFDuet™-1. *E. coli* C43(DE3) cells were transformed with

608   constructs pET11a-CalpT and pCDFDuet-SF and grown at 37°C with shaking at 180 rpm. Cell

609   was induced by 0.2 mM IPTG once OD600 of 0.6-0.8 and cultivated at 16 °C overnight, then

610   purified as described above.

611

612   **Protease assay**

613   For protease activity assays CalpL and CalpT were used at a final concentration of c = 4.64 µM

614   each. The different cOAs were used at a final concentration of c = 5.11 µM. The protein

615   solutions were prepared in 20 mM Tris, 50 mM NaCl, pH 8.0 and incubated for 1 hr at 60 °C.

616   Subsequently, the cOA was added and the mixture was incubated for another 1 hr at 60 °C. For

617   SDS-PAGE 3 µl of 4x SDS-loading buffer was added to 9 µl of the sample, the mixture was

618   heated for 5 min at 94 °C and 10 µl were loaded to a 15% polyacrylamide gel, which was run

619   at 250 V for 40 min.

620

621   **Size exclusion chromatography analysis (SEC)**

622   To determine the interaction of the complex of CalpS and CalpT with CalpL, the SEC runs

623   were carried out on a Superose6 increase 10/300 chromatography column (GE Healthcare)

624   equilibrated with SEC buffer (20 mM Tris, 0.25 M NaCl, 1 mM DTT, 10% glycerol, pH 8.0).

625   The injected volume of tested sample solution was 200 ul at flow rate of 0.5 ml min^{-1}. The final

626   concentrations were set to c(CalpL) = 63.3 µmol*l^{-1}, c(CalpT/S) = 115.8 µmol*l^{-1}, and c(cA$_4$)

627   = 60 µmol*l^{-1} diluted by using SEC buffer. All samples were incubated at 60 °C for 60 min

628   before cooling down to room temperature and loading onto column.

629

630   **Pull-down assay**

631   The magnetic nickel beads-based immobilized metal affinity chromatography (IMAC) was

632   performed to detect releasing of CalpS from CalpL/T/S complex. The complex of His-tagged

633   CalpS and CalpT was incubated with CalpL in binding buffer (20 mM Tris-HCl, pH 7.5, 60

634   mM NaCl, 0.01% Tween^{TM}-20) at 60 °C for 1 hr in presence or absence of cA$_4$. After cooling

635   down to room temperature, the sample solution was mixed with pre-equilibrated beads (Magne,

636   His Ni particle, Promega) with binding buffer on a roller for 20 min at 4 °C. The beads were

637   washed tree times with 300 ul wash buffer (20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 10 mM

638   imidazole, 0.01% Tween^{TM}-20) before eluted twice using 25 ul elution buffer (20 mM Tris-

639   HCl, pH 8.0, 120 mM NaCl, 300 mM imidazole, 0.01% Tween^{TM}-20). The samples from first

640   elution and 20% input were analyzed on the SDS-PAGE. The final concentrations were set to

641   c(CalpT/S) = 0.208mg/ml, c(CalpL) = 0.127 mg/ml, and c(cA$_4$) = 2.5 µmol*l^{-1} diluted by

642   binding buffer.

643

644   **Analytical gel filtration and SEC-MALS analysis**

645   To investigate the complex formation of CalpS with CalpT and CalpL, analytical gel filtration

646   was carried out on a SD 200 increase 10/300 column. When purifying CalpS, the complex

647   consisting of CalpS and DNA-directed RNA polymerase subunits alpha and beta, eluted in a

648   defined 50 mAU peak on a SD 200 16/600 gel filtration column. This peak was pooled and

649   concentrated to V ≈ 1.5 ml. Thereafter, 440 µl of the complex were incubated with 60 µl of

650   CalpT (350 µM), 60 µl of CalpT-CalpL S152A complex (195 µM) and 60 µl of CalpS buffer

651   (20 mM Tris–HCl, 0.25 M NaCl, 1 mM DTT, 10% glycerol, pH 7.5), respectively. After

652   centrifugation at 15000 rcf and 10 °C for 10 min, each sample was loaded onto a SD 200

653   increase 10/300 column for size-exclusion chromatography in CalpS buffer.

654   For determination of interactions between CalpL and CalpT, SEC-MALS runs were performed

655   at room temperature on an Agilent 1260 Infinity II Prime Bio LC System coupled with a Wyatt

656   miniDAWN® MALS detector, a Optilab rEX refractive index detector and a Superose6 increase

657   10/300 chromatography column (GE Healthcare) equilibrated with 25 mM Tris, 500 mM NaCl,

658   1 mM DTT, 10% glycerol, pH 8.0. Data acquisition and evaluation were carried out using

659   ASTRA 8 software (Wyatt Technologies). The flow rate was set to 0.5 ml min^{-1} and an injection

660   volume of 50 µl was used for the experiments. Final concentrations were set to

661   c(CalpL) = 51 µmol l^{-1}, c(CalpT) = 51 µmol l$^{-1,}$ and c(cA4) = 60 µmol l^{-1} by dilution with

662   25 mM Tris, 500 mM NaCl, 1 mM DTT, 10% glycerol, pH 8.0. The proteins were incubated

663   for 40 min at 60 °C, cA$_4$ was added followed by an additional 20 min incubation at 60 °C. The

664   samples were centrifuged at 15.000*g for 10 min. before injection.

665

666   **Mass spectrometry**

667   The gel bands were excised and cut into 1 mm^3 cubes. The samples were destained with 2x

668   rinses each of ethanol, acetonitrile and 25mM ammonium bicarbonate, then subjected to

669   reduction with 10 mM dithiorethritol, followed by alkylation with 20 mM iodoacetamide. The

670   gel pieces with shrunk with acetonitrile and then soaked in 25 mM AmBic with 2 ng/µl trypsin

671   and left to digest overnight at 37 °C. The peptides were soaked from the gel with 1% formic

672   acid and concentrated to 20 µl in a speedvac. Between 1-7 µl of the sample, dependent on

673   original gel coomassie staining, was loaded onto a Eksigent 2D ultra nano HPLC with Sceix

674   5600+ mass spectrometer. The Thermoscientific Acclaim Pepmap 100 trap (20 mm x 75 µm)

675 and column (150 mm x 75 µm) were in trap elute configuration with a flow of 5 µl/min and 300

676 nl/min respectively. The peptides were loaded onto the trap and washed for 5 minutes at 100%

677 loading buffer (100% water, 0.05% TFA) before the trap was switched in line with the column

678 and the peptides eluted with a linear gradient over 20 minutes of 98% A to 98% B where A is

679 100% water with 0.1% formic acid and B is 80% acetonitrile, 20% water, 0.1% formic

680 acid. The eluent was sprayed directly into the nanosource of the mass spectrometer. MS data

681 was collected from 400-1250 m/z in positive ionisation for 150 msec. Data dependant

682 acquisition mode was utilized to collect MSMS data from 100-2000 m/z on the 20 strongest

683 peptides with 2-5+ charge states. The peak list was extracted from the .wiff file using

684 MSconvert and the .mgf file searched against an inhouse database of 7000 protein sequences to

685 which the sequences of the proteins of interest were added. The following settings were used

686 in the mascot search, trypsin, and semi trypsin as digest enzymes, fixed modification of

687 carbamidomethyl (c) and variable modifcation of oxidation (M). MS tolerance was set at

688 20 ppm and MSMS at 0.1 Da.

689

690 **Surface plasmon resonance of cOA and CalpT binding to CalpL**

691 All surface plasmon resonance experiments were run on a Biacore^{TM} 8K instrument (GE

692 healthcare life sciences), using a streptavidin-functionalized sensor chip (Serie S Sensor Chip

693 SA, GE healthcare life sciences). Data was recorded at a rate of 10 Hz and 25°C flow cell

694 temperature. The running buffer contained 25 mM Tris-HCl pH 8.0, 250 mM NaCl, 5%

695 Glycerol, 0.05% TWEEN20. After three initial injections of 1 M NaCl in 50 mM NaOH (10

696 µL/min, 60 s), the biotinylated CalpL construct, R493C-biotin, was immobilized on the chip

697 (86 nM, 5 µL/min, 180 s). Binding of CoA and CalpT was measured as single cycle kinetics.

698 For the cOAs, a series of seven different concentrations (0.086, 0.26, 0.78, 2.33, 7, 21, 63 nM)

699 were injected at a flow rate of 30 µL/min (contact time: 120s, dissociation time: 600 s). For

700 CalpT and NIS038, a series of seven different concentrations (0.0625, 0.25, 1, 4, 16, 64, 256

701 nM) were injected applying the same parameters as above. The recorded data were double

702 referenced by reference flow cell and blank cycle subtraction and data was analysed and fitted

703 using the Biacore Insight Evaluation Software.

704

705 **X-ray crystallography**

706 Pure CalpL protein was concentrated to 20 mg/ml and crystallized at 20 °C using a Gryphon

707 pipetting robot (Art Robbins) and commercial crystallization screens (Molecular Dimensions)

708 using sitting drop plates. Hexagonal crystals appeared after one day in condition D7 of the

709   JCSG+ screen. Several rounds of optimization in sitting- and hanging drop plates were

710   performed to achieve well-diffracting crystals. The final crystallization condition was 0.1 M

711   Tris-Cl pH 8.0, 38.8% PEG400, 0.29 M Li_2SO_4. The SeMet derivative (see above) was

712   crystallized under similar conditions and yielded identical crystals. The crystals were harvested

713   without further cryo-protection and a diffraction dataset was recorded at beamline P13

714   ($\lambda = 0.9795$) operated by EMBL Hamburg at the PETRA III storage ring (DESY, Hamburg,

715   Germany)[53]. The diffraction data were automatically processed with XDS[54]. The structure was

716   solved using phenix.autosol and refined with phenix.refine[55]. Further model building was

717   performed in Coot[56] and figures were prepared with PyMOL (www.pymol.org). The geometry

718   of the model was checked with MolProbity[57]. The molprobity score was 1.43, the clashscore

719   4.53 and the Ramachandran statistics (outliers/favored) were 0.0/97.0%.

720   The same crystallization condition was used to obtain CalpL crystals for soaking with cA_4.

721   After harvesting the crystals, they were incubated for approximately 3 minutes in a solution of

722   mother liquor supplemented with 5% PEG 400 and 5 mM cA_4. Diffraction experiments were

723   done at beamline P13 ($\lambda = 0.$ 0.97626) at the PETRA III storage ring (DESY, Hamburg,

724   Germany)[53]. A full dataset was recorded and automatically processed with XDS[54]. Molecular

725   replacement was run with PHASER[58] using CalpL as search model. Further refinement of the

726   structure was done with phenix.refine[55]. Using Coot[56], cyclic tetraadenylate could be fitted

727   perfectly into a defined difference electron density inside the cA_4 binding pocket. The

728   molprobity score was 1.32, the clashscore 2.85 and the Ramachandran statistics

729   (outliers/favored) were 0.4/96.6%.

730   To obtain the crystal structure of the CalpL/T_{10} complex, CalpL and CalpT protein solutions

731   were mixed at 1:1 molar ratio (155 µM each) and incubated at 50 °C for 40 min. After

732   incubation, cA_4 was added to a final concentration of 175 µM followed by 20 min of incubation

733   at 50 °C. A total volume of 400 µl was loaded to a SD 200 increase 10/300 column and size-

734   exclusion chromatography was conducted using CalpL buffer. The complex eluted in one single

735   peak which was pooled and concentrated to approximately 30 mg/ml. Sitting drop

736   crystallization plates were set up as described above. Crystals were obtained after several days

737   in condition E2 of the JCSG+ screen (2 M ammonium sulfate, 0.1 M ammonium cacodylate,

738   0.2 M NaCl at pH 6.5). The crystals were harvested with 35% glycerol for cryo-protection and

739   a diffraction dataset was recorded at beamline P13 ($\lambda = 0.$ 0.9762) operated by EMBL Hamburg

740   at the PETRA III storage ring (DESY, Hamburg, Germany)[53]. Automatic data processing was

741   achieved using XDS[54]. CalpL was used as search model for molecular replacement with

742   PHASER [58]. Structure refinement and model building was done with phenix.refine[55] and

743   Coot[56], respectively. The molprobity score was 2.36, the clashscore 11.93 and the

744   Ramachandran statistics (outliers/favored) were 0.9/92.3%.

745   Geometric parameters of all described structures were checked with MolProbity[57]. All figures

746   were prepared with PyMOL (www.pymol.org).

747

748   **Small-angle X-ray scattering**

749   Small-angle X-ray scattering (SAXS) experiments were conducted at the P12[59] beamline of the

750   Petra III synchrotron (Hamburg, Germany), in two separate experimental sessions. The

751   parameters for the data collections are reported in Extended Data Table 2. The scattering

752   intensity, I(s) was collected as a function of the momentum transfer defined as $s=(4\pi\sin\theta)/\lambda$,

753   where 2θ is the scattering angle and λ the X-ray wavelength employed.

754   The buffer employed for all SAXS experiments, also employed for SEC and for background

755   subtraction, contained 20 mM Tris pH=8.0, 50 mM NaCl. CalpL, CalpL/T_{10} and CalpL

756   monomer SAXS curves were collected by SEC-SAXS at room temperature[60], employing a

757   SD200 5/150 increase SEC column (GE Healthcare) online to the SAXS flow capillary. For

758   each SEC-SAXS run, 15 min elutions at 0.3 mL/min flow rate were performed, collecting 900x

759   1 s exposures on the eluate.

760   Concentration series of CalpL in the range 1-5 mg/mL with and without a 1.2-fold molar excess

761   of cA_4 were collected in batch mode after centrifugation 30 min at 30000 xg (5 °C), and 30

762   exposures of 0.1 s were collected while flowing 35 µL of solution through the 1 mm quartz

763   capillary. The apparent molecular weights for these measurements are obtained from the

764   forward scattering, I(0), using the I(0) of a SAXS curve from bovine serum albumin at 1.9

765   mg/mL in a HEPES buffer as secondary standard.

766   The primary data reduction was performed with the program SASFLOW[61], including automatic

767   selection of the exposures to monitor for radiation damage prior to data averaging. The reduced

768   data inspected and processed to obtain the overall protein parameters using PRIMUS[62] and the

769   programs of the ATSAS suite[63]. Comparison of experimental SAXS curves with the crystal

770   structures was performed with CRYSOL[64] *Ab initio* modeling of low-resolution protein

771   structures was performed with DAMMIF[65], and MONSA[66] for multi-phase modeling, repeating

772   10 modeling runs. The resulting models were compared and averaged using DAMAVER[67],

773   providing a normalized spatial discrepancy (NSD) value[68] that reflects the stability of the

774   structural reconstructions (stable reconstructions have NSD<1). SAXS curves of CalpL at 2, 3,

775   5 mg/mL in the presence of cA4 were globally fitted as a mixture of monomer and dimer, using

776   the program SASREFMX[69]. The mixture results in an overall scattering intensity which is the

777   linear combination of the scattering intensity of monomer and dimer species at varying volume

778   fractions along the concentration series.

779

780   **Pulsed EPR experiments**

781   For site-specific spin labelling, a single cysteine mutant E119C of CalpT of was expressed and

782   purified as described for wild-type CalpT. After purification, 250 µl of a 315 µM CalpT E119C

783   solution was bound to Ni^{2+}-NTA beads. These were washed with 10 ml reducing buffer (25 mM

784   Tris, pH = 8, 250 mM NaCl, 1 mM TCEP, 10% glycerol) and 20 ml wash buffer (25 mM Tris,

785   pH = 8, 250 mM NaCl, 10% glycerol). Thereafter, the protein was eluted in buffer containing

786   MTSSL (25 mM Tris, pH = 8, 250 mM NaCl, 1 M imidazole, 10% glycerol, 0.6 mM MTSSL).

787   A PD10 desalting column was used to remove imidazole and free spin label, as well as for

788   buffer exchange of CalpT E119R1, wild-type CalpL and CalpL S152A. All final buffers

789   contained D_2O instead of H_2O and no reducing agent. An activity assay was done as described

790   using the prepared samples with a final protein concentration of 55 µM and deuterated buffer

791   (25 mM Tris, pH = 8, 250 mM NaCl, 5% glycerol). The success was checked on SDS-PAGE.

792   All samples were flash frozen to $N_{2\,(l)}$ and sent on dry ice for the measurement. The labelling

793   efficiency was determined to be 103% by cw-EPR spectroscopy (average of two

794   measurements).

795   For pulse EPR measurements, samples of spin-labeled CalpL/T E119R1 in presence or absence

796   of 1 molar equivalent cA_4 were mixed with 45% (v/v) deuterated ethylene glycol to yield

797   27.5 µM CalpL/T E119R1 in 65 µl final volume. Samples were transferred to 3 mm EPR quartz

798   tubes, flash-frozen and stored in liquid nitrogen until use.

799   Pulsed electron-electron double resonance (PELDOR)[70] distance measurements were obtained

800   at Q-band frequency (34 GHz) on a Bruker ELEXSYS E580 spectrometer with 3 mm

801   cylindrical resonator (ER 5106QT2-2w, TE012 mode) using a pulse travelling wave tube

802   (TWT) amplifier (Applied Systems Engineering) with nominal output of 150 W and an arbitrary

803   waveform generator for rectangular pulses.

804   PELDOR experiments were performed with the 4-pulse DEER[71,72] pulse sequence ($\pi/2(\nu_A)$ –

805     $\tau_1 - \pi(\nu_A) - (\tau_1 + t) - \pi(\nu_B) - (\tau_2 - t) - \pi(\nu_A) - \tau_2$ – echo) at 50 K, with a frequency offset (pump

806   – detection frequency) of +80 MHz (~3 mT). Shot repetition time (SRT) was set to 2.5 ms; τ_1

807   was set to 380 ns, and τ_2 was set to 5000 ns. Pulse lengths were 16 and 32 ns for $\pi/2$ and π

808   detection, and 12 ns for the inversion π pump pulse. Unwanted echoes were suppressed with a

809   16-step phase cycle and nuclear modulation was averaged by adding 16 traces with τ_1

810   incremented by 8 ns. The pump pulse was placed on the resonance frequency of the resonator

811   and applied to the maximum of the nitroxide field-swept spectrum.

812   PELDOR data were analyzed using the ComparativeDeerAnalyzer version 2.0[73,74] within

813   DeerAnalysis2022[75]; shown are the respective consensus fits and distance distributions.

814

815   **Ribonuclease assay**

816   Ribonuclease activity of cleaved CalpT (23-kD fragment) was assayed by incubating full-length

817   CalpT with CalpL and five different fluorescent-labelled RNA substrates, which were

818   synthesised with the fluorescent dye (6-FAM) attached at 5' end or at 3' end (purchased from

819   Integrated DNA Technologies (IDT), Extended Data Fig. 7c). The mixture of CalpL (5.5 µM)

820   and CalpT (5.5 µM) was incubated at 60 °C in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl and 1

821   mM EDTA for 15 min, cA_4 (10 µM) was then added and the mixture was incubated for another

822   15 min at 60 °C, followed by adding one of the above RNA substrates into the mixture,

823   incubating for an additional 30 min at 60°C. Finally, 6 µl of the sample was analyzed on SDS-

824   PAGE (NuPAGE Bis-Tris Gel, Thermo Fisher Scientific) by heating at 95 °C for 5 min with 2

825   µL of SDS-PAGE loading dye (Thermo Fisher Scientific; NuPAGE Sample Reducing Agent

826   and LDS Sample Buffer). The remaining 14 µl of the sample were loaded to 20% acrylamide,

827   7 M urea, 1×TBE denaturing gel, which was run at 30W, 45 °C for 2 hr. The gel was finally

828   imaged by Typhoon FLA 7000 imager (GE Healthcare) at a wavelength of 532 nm (pmt

829   600~700).

830

831   **Ribonuclease target motif profiling (RNase-Seq)**

832   To investigate MazF activity, 20 U mRNA Interferase™ -MazF (TaKaRa, Cat# 2415A) were

833   incubated with 400 ng of a single-stranded RNA library containing 10 random bases in 1X

834   MazF Buffer (200 mM sodium phosphate, pH 7.5, and 0.05% Tween-20) at 37 °C. After 10

835   min or 2 hr, reactions were stopped by placing the samples on ice. Ribonuclease activity of

836   CalpT (23 kDa fragment) was investigated by incubating a solution containing 4.64 µM CalpL

837   and 5.57 µM CalpT in 20 mM Tris pH 8.0, 50 mM NaCl at 37 °C. After 30 min, 400 ng of a

838   ssRNA library was added together with cOA_4 to a final concentration of 5.57 µM. The reaction

839   was incubated for 10 min or 2 hr at 37 °C. To prepare next generation sequencing libraries, RT-

840   PCR was performed on 1 µl of each sample in a 10 µl reaction containing 1x KAPA HiFi

841   HotStart ReadyMix (pre-heated for 5 min at 98 °C; Roche, Cat# KK2602), 15 U WarmStart

842   RTx Reverse Transcriptase (NEB, Cat# M0380) and 0.5 µM staggered MiSeq gRNA primer

843   mix using the following temperature conditions: 15 min 65 °C, 3 min 72 °C, 30 sec 98 °C, 20

844   cycles of: Denaturation (10 sec at 98 °C), annealing (20 sec at 65 °C) and extension (1 min at

845   72 °C); Final extension: 5 min at 72 °C. Barcodes and Illumina-compatible constant handles

846   were added using a secondary NEBNext (NEB) PCR. Samples were pooled and column-

847   purified using QIAprep Spin columns. The final library was quantified using a NanoDrop

848   photospectrometer and sequenced on an Illumina MiSeq using the v2 chemistry.

849   The primers used for ribonuclease target motif profiling are listed in Extended Data Fig. 7e.

850

851   **Structural predictions with AlphaFold2**

852   The source code of the AlphaFold2 algorithm was downloaded from

853   https://github.com/AlphaFold and installed as described https://github.com/AlphaFold. The

854   algorithm was run locally using the CASP14 preset or via ColabFold[76].

855

856   **Statistics and reproducibility**

857   Information concerning statistics and reproducibility for the experiments shown in this study

858   are given in the figure legends of the corresponding experiments. The key findings of this study

859   (enzymatic activities and formation of macromolecular complexes) have been reproduced in

860   two laboratories (G.H. at the Institute of Structural Biology of the University of Bonn, Germany

861   and M.F.W. at the Biomedical Sciences Research Complex of the University of St Andrews,

862   Scotland, UK).

863

864

Additional references

866

867   49. Tunyasuvunakool, K., Adler, J., Wu, Z., Green, T., Zielinski, M., Žídek, A., Bridgland,
868   A., Cowie, A., Meyer, C. & Laydon, A. Highly accurate protein structure prediction for
869   the human proteome. *Nature* 596, 590-596 (2021).
870   50. Robert, X. & Gouet, P. Deciphering key features in protein structures with the new
871   ENDscript server. *Nucleic acids research* 42, W320-W324 (2014).
872   51. Liu, H. & Naismith, J. H. An efficient one-step site-directed deletion, insertion, single
873   and multiple-site plasmid mutagenesis protocol. *BMC Biotechnology* 8, 91 (2008).
874   52. Rouillon, C., Athukoralage, J. S., Graham, S., Grüschow, S. & White, M. F.
875   Investigation of the cyclic oligoadenylate signaling pathway of type III CRISPR
876   systems. *Methods Enzymol* 616, 191-218 (2019).
877   53. Cianci, M., Bourenkov, G., Pompidor, G., Karpics, I., Kallio, J., Bento, I., Roessle, M.,
878   Cipriani, F., Fiedler, S. & Schneider, T. R. P13, the EMBL macromolecular
879   crystallography beamline at the low-emittance PETRA III ring for high-and low-energy
880   phasing with variable beam focusing. *Journal of synchrotron radiation* 24, 323-332
881   (2017).
882   54. Kabsch, W. Automatic-Indexing of Rotation Diffraction Patterns. 21, 67-71 (1988).
883   55. Liebschner, D., Afonine, P. V., Baker, M. L., Bunkóczi, G., Chen, V. B., Croll, T. I.,
884   Hintze, B., Hung, L.-W., Jain, S. & McCoy, A. J. Macromolecular structure
885   determination using X-rays, neutrons and electrons: recent developments in Phenix.
886   *Acta Crystallographica Section D: Structural Biology* 75, 861-877 (2019).
887   56. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta
888   Crystallographica Section D* 60, 2126-2132 (2004).
889   57. Williams, C. J., Headd, J. J., Moriarty, N. W., Prisant, M. G., Videau, L. L., Deis, L. N.,
890   Verma, V., Keedy, D. A., Hintze, B. J. & Chen, V. B. MolProbity: More and better
891   reference data for improved all-atom structure validation. *Protein Science* 27, 293-315
892   (2018).
893   58. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. &
894   Read, R. J. Phasercrystallographic software. *Journal of Applied Crystallography* 40,
895   658-674 (2007).
896   59. Blanchet, C. E., Spilotros, A., Schwemmer, F., Graewert, M. A., Kikhney, A., Jeffries,
897   C. M., Franke, D., Mark, D., Zengerle, R., Cipriani, F., Fiedler, S., Roessle, M.,
898   Svergun, D. I. & IUCr. Versatile sample environments and automation for biological
899   solution X-ray scattering experiments at the P12 beamline (PETRA III, DESY). *Journal
900   of Applied Crystallography* 48, 431-443 (2015).
901   60. Graewert, M. A., Da Vela, S., Gräwert, T. W., Molodenskiy, D. S., Blanchet, C. E.,
902   Svergun, D. I. & Jeffries, C. M. Adding size exclusion chromatography (SEC) and light
903   scattering (LS) devices to obtain high-quality small angle X-ray scattering (SAXS) data.
904   *Crystals* 10, 975 (2020).
905   61. Franke, D., Kikhney, A. G. & Svergun, D. I. Automated acquisition and analysis of
906   small angle X-ray scattering data. *Nuclear Instruments and Methods in Physics
907   Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment*
908   689, 52-59 (2012).
909   62. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H. J. & Svergun, D. I.
910   PRIMUS: a Windows PC-based system for small-angle scattering data analysis. 36,
911   1277-1282 (2003).
912   63. Manalastas-Cantos, K., Konarev, P. V., Hajizadeh, N. R., Kikhney, A. G., Petoukhov,
913   M. V., Molodenskiy, D. S., Panjkovich, A., Mertens, H. D. T., Gruzinov, A. & Borges,

914   C. ATSAS 3.0: expanded functionality and new tools for small-angle scattering data
915   analysis. *Journal of Applied Crystallography* 54, 343-355 (2021).

916   64.   Svergun, D., Barberato, C. & Koch, M. H. J. CRYSOL– a Program to Evaluate X-ray
917   Solution Scattering of Biological Macromolecules from Atomic Coordinates. *Journal of*
918   *Applied Crystallography* 28, 768-773 (1995).

919   65.   Franke, D. & Svergun, D. I. DAMMIF, a program for rapid ab-initio shape
920   determination in small-angle scattering. 42, 342-346 (2009).

921   66.   Svergun, D. I. Restoring low resolution structure of biological macromolecules from
922   solution scattering using simulated annealing. *Biophysj* 76, 2879-2886 (1999).

923   67.   Volkov, V. V. & Svergun, D. I. Uniqueness of ab initio shape determination in small-
924   angle scattering. 36, 860-864 (2003).

925   68.   Kozin, M. B. & Svergun, D. I. Automated matching of high- and low-resolution
926   structural models. 34, 33-41 (2001).

927   69.   Petoukhov, M. V., Franke, D., Shkumatov, A. V., Tria, G., Kikhney, A. G., Gajda, M.,
928   Gorba, C., Mertens, H. D. T., Konarev, P. V. & Svergun, D. I. New developments in the
929   ATSAS program package for small-angle scattering data analysis. 45, 342-350 (2012).

930   70.   Milov, A., Salikohov, K. & Shirov, M. Application of Endor in Electron-Spin Echo for
931   Paramagnetic Center Space Distribution in Solids. *Fizika Tverdogo Tela* 23, 975-982
932   (1981).

933   71.   Pannier, M., Veit, S., Godt, A., Jeschke, G. & Spiess, H. W. Dead-time free
934   measurement of dipole-dipole interactions between electron spins. *Journal of Magnetic*
935   *Resonance (San Diego, Calif.: 1997)* 142, 331-340 (2000).

936   72.   Larsen, R. G. & Singel, D. J. Double electron–electron resonance spin–echo
937   modulation: Spectroscopic measurement of electron spin pair separations in
938   orientationally disordered solids. *The Journal of chemical physics* 98, 5134-5146
939   (1993).

940   73.   Worswick, S. G., Spencer, J. A., Jeschke, G. & Kuprov, I. Deep neural network
941   processing of DEER data. *Science advances* 4, eaat5218 (2018).

942   74.   Fábregas Ibáñez, L., Jeschke, G. & Stoll, S. DeerLab: a comprehensive software
943   package for analyzing dipolar electron paramagnetic resonance spectroscopy data.
944   *Magnetic Resonance* 1, 209-224 (2020).

945   75.   Jeschke, G., Chechik, V., Ionita, P. & Godt, A. DeerAnalysis2006—a comprehensive
946   software package for analyzing pulsed ELDOR data. *Applied Magnetic Resonance* 30,
947   473-498 (2006).

948   76.   Mirdita, M., Schütze, K., Moriwaki, Y., Heo, L., Ovchinnikov, S. & Steinegger, M.
949   ColabFold: making protein folding accessible to all. *Nature Methods* 1-4 (2022).

950   77.   Cha, S. S., An, Y. J., Lee, C. R., Lee, H. S., Kim, Y. G., Kim, S. J., Kwon, K. K., De
951   Donatis, G. M., Lee, J. H., Maurizi, M. R. & Kang, S. G. Crystal structure of Lon
952   protease: molecular architecture of gated entry to a sequestered degradation chamber.
953   *EMBO J* 29, 3520-3530 (2010).

954   78.   Zorzini, V., Mernik, A., Lah, J., Sterckx, Y. G. J., De Jonge, N., Garcia-Pino, A., De
955   Greve, H., Versées, W. & Loris, R. Substrate Recognition and Activity Regulation of
956   the Escherichia coli mRNA Endonuclease MazF. *Journal of Biological Chemistry* 291,
957   10950-10960 (2016).

958   79.   Simanshu, D. K., Yamaguchi, Y., Park, J.-H., Inouye, M. & Patel, D. J. Structural basis
959   of mRNA recognition and cleavage by toxin MazF and its regulation by antitoxin MazE
960   in Bacillus subtilis. *Molecular cell* 52, 447-458 (2013).

961   80.   Hagelueken, G., Ward, R., Naismith, J. H. & Schiemann, O. MtsslWizard: In Silico
962   Spin-Labeling and Generation of Distance Distributions in PyMOL. *Applied Magnetic*
963   *Resonance* 42, 377-391 (2012).

81. Campagne, S., Marsh, M. E., Capitani, G., Vorholt, J. A. & Allain, F. H. T. Structural basis for− 10 promoter element melting by environmentally induced sigma factors. *Nature structural & molecular biology* 21, 269-276 (2014).
82. Lane, W. J. & Darst, S. A. The structural basis for promoter− 35 element recognition by the group IV σ factors. *PLoS biology* 4, e269 (2006).
83. Li, L., Fang, C., Zhuang, N., Wang, T. & Zhang, Y. Structural basis for transcription initiation by bacterial ECF σ factors. *Nat Commun* 10, 1153 (2019).

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Author contributions

C.R. and G.H. conceived and supervised the study and performed initial protein expression and crystallization experiments on CalpL. R.S. and W.B. cloned the initial CalpL construct. N.S., C.R., M.F.W. and G.H. designed experiments. N.S. optimized the purification of CalpL and CalpT, crystallized CalpL, CalpL/cA$_4$ and CalpL/T$_{10}$ and established and performed the cleavage assays, SEC-MALS and DLS experiments. N.S. and M.F.P. cloned all mutants. G.H. and N.S. solved and refined the CalpL, CalpL/cA$_4$ and CalpL/T$_{10}$ structures. J.M. and M.G. designed and performed the SPR experiments. H.C. and M.F.W. planned and performed the ribonuclease assay. H.C., N.S. and M.F.W. cloned and purified CalpS and performed binding and coexpression experiments involving CalpS. S.D.V. performed SAXS experiments. S.D.V. and D.S. performed the SAXS data analysis and interpretation and wrote the corresponding sections. B.E.B. and K.A. performed the pulsed EPR experiments, analyzed the data, prepared figures and wrote the corresponding sections. K.B. and J.L.S.B. designed and performed the RNase-Seq assay. C.R., M.F.W., H.C., N.S., and G.H. analyzed the data and wrote the paper. All authors discussed the results and commented on the manuscript at all stages.

Competing interest statement

The authors declare no competing interests.

Additional information statement

A previous version of this work has been published as preprint: https://doi.org/10.1101/2021.12.06.471393. Requests for materials should be addressed to G.H. (hagelueken@uni-bonn.de).

Data availability statement

The crystal structures have been deposited in the PDB with the accession codes 7QDA [https://doi.org/10.2210/pdb7QDA/pdb], 8B0R [https://doi.org/10.2210/pdb8B0R/pdb], 8B0U [https://doi.org/10.2210/pdb8B0U/pdb]. The SAXS data and models have been deposited in the SASBDB with the accession codes: SASDQM4 [https://www.sasbdb.org/data/SASDQM4], SASDQN4 [https://www.sasbdb.org/data/SASDQN4], SASDQP4 [https://www.sasbdb.org/data/SASDQP4], SASDQQ4 [https://www.sasbdb.org/data/SASDQQ4]. The following PDB entries have been used in this study: 2H27 [https://doi.org/10.2210/pdb2H27/pdb], 3K1J [https://doi.org/10.2210/pdb3K1J/pdb], 4ME7 [https://doi.org/10.2210/pdb4ME7/pdb], 4IZJ [https://doi.org/10.2210/pdb4IZJ/pdb], 4LUP [https://10.2210/pdb4LUP/pdb], 5ZX2 [https://doi.org/10.2210/pdb5ZX2/pdb], 5CR2 [https://doi.org/10.2210/pdb5CR2/pdb], 6VM6 [https://doi.org/10.2210/pdb6VM6/pdb], 6SCE [https://doi.org/10.2210/pdb6SCE/pdb], 7RWK [https://doi.org/10.2210/pdb7RWK/pdb]

Code availability statement

No custom code was used in this work.

Extended Data Figure legends

Extended Data Fig. 1 | Purification and Structure of CalpL. a, Gelfiltration chromatography (Superdex 200 16/60) of CalpL. Inset: SDS-PAGE analysis of the fractions indicated by the black bar in the chromatogram. The experiment was performed multiple times (n > 3 biological replicates). **b,** TM-prediction by the TMHMM 2.0 server[28] vs experimental structure. **c,** Representative electron density of the SeMet CalpL crystal structure. The structural model is drawn in ball-and-stick representation. Selected residues are labeled. The black mesh is a $2mF_o$-DF_c electron density map contoured at 1.0 σ. **d,** Topology diagram of CalpL. For gel source data, see Supplementary Figure 1.

Extended Data Fig. 2 | CalpL in comparison to structurally related proteins. **a,** CalpL is drawn as a cartoon model color-coded as in Fig. 1. The Lon protease from *T. onnorineus* (PDB-ID: 3K1J, DALI Z-score: 12.8[77]) is shown as a white cartoon model. **b,** Table listing proteins with similar domain structures. **c,** Surface electrostatics of the Lon protease active site region. The catalytic dyad is marked. The grey line marks the likely substrate binding site. **d,** Superposition of CalpL active site with the acyl-enzyme intermediate of yellowfin asciitis virus protease. CalpL is in sticks representation and color-coded as in Fig. 1. Chain D of structure 4IZJ[27] (residues 630-640) was superimposed on the corresponding residues of CalpL (150-160) leading to an r. m. s. d. of 0.314 Å. Of 4IZJ, only the acyl-enzyme intermediate is shown in sticks mode. Selected residues and the positions of the P1-P3 sites are indicated. **e,** Superposition of CalpL (color scheme as in Fig. 1) with the Cap4 protein (white, PDB-ID: 6VM6[23]). **f,** Superposition of the CalpL SAVED domain (color scheme as in Fig. 1) with the Cap5 protein (white, PDB-ID: 7RWK[29]). **g,** Superposition of the CalpL SAVED domain (color scheme as in Fig. 1) with the CARF domains of the Can1 protein (white, PDB-ID: 6SCE[17]).

Extended Data Fig. 3 | The CalpL/cA$_4$ complex a, Close-up of cA$_4$ (green) bound to the SAVED domain of CalpL. The blue mesh is a $2mF_o$-DF_c electron density map contoured at 1.0 σ. **b,** Superposition of CalpL apo (white) onto the cA4 complex structure (color coded as in Fig. 1). **c,** Structural alignment of the SAVED domains of CALP/cA$_4$ and Cap4/cA$_3$ (white).

Extended Data Fig. 4 | CalpT is a MazF homolog and the target of the CalpL protease. a, b, A superposition of the predicted CalpT structure (compare Fig. 2B) with one monomer of the MazF/ssRNA complex (purple/orange) (PDB-IDs: 5CR2[78]). The AlphaFold2[33] prediction confidence is mapped onto the CalpT structure (pLDDT[49], predicted local distance difference test). **b,** A superposition of the predicted CalpT structure (compare Fig. 2b) with one monomer of the MazE/F complex (PDB-IDs: 4ME7[79]). The AlphaFold2[33] prediction confidence is mapped onto the CalpT structure (pLDDT[49], predicted local distance difference test). **c,** Gel filtration chromatography (Superdex 75 16/60) of CalpT The experiment was performed multiple times (n > 3 biological replicates). According to the MALS data in Fig. 3, isolated CalpT behaves as a monomer. **d,** Peptide fingerprints of cleavage bands. The indicated gel-bands were cut from the gel and submitted for identification at the Mass spectrometry and proteomics facility at the University of St Andrews (Fife, UK, https://mass-spec.wp.st-andrews.ac.uk). Red letters indicate peptides that were identified in the respective sample. The experiment was performed once. **e,** Mutational analysis of potential CalpL cleavage sites in CalpT. The positions of the mutants are indicated as magenta spheres on the right. (pLDDT[49], predicted local distance difference test) The experiment was performed twice (n = 2 technical replicates). **e)** SDS-PAGE analysis of the fractions indicated by the black bar in a) The experiment was performed multiple times (n > 3 technical replicates). For gel source data, see Supplementary Figure 1.

Extended Data Fig. 5 | Characterization of the CalpL/T complex. a, Single cycle kinetics SPR data of the CalpL/T interaction. The interaction is very strong but cannot be satisfyingly fitted with a 1:1 binding model. The experiment was performed twice (n = 2 technical replicates). **b,** As **a**), but an artificial construct of an unspecific VHH fused to CalpT was used as analyte in this experiment. The interaction is very similar to the CalpL/T interaction. The experiment was performed twice (n = 2 technical replicates). **c,** Schematics of two artificial constructs containing the CalpL cleavage site. **d,** CalpL cleaves an artificial construct of an unspecific VHH fused to $CalpT_{10}$ but not a construct of two VHHs fused by the CalpL cleavage site. The experiment was performed once. **e,** SEC-MALS traces (solid lines: UV_{280}, dashed lines: MW_{MALS}) of proteolysis reactions with different combinations of CalpL S152A, CalpT, and cOA. The schematic indicates the molecular species behind the individual peaks. The experiments were performed twice with slightly different buffer conditions (n=2 technical replicates). **f,** Binding of CalpL wt to the indicated CalpT mutants in the absence of cA_4. The schematic indicates the position of the mutant in the CalpL/T complex. The experiments were performed once. **g,** Representative electron density of the $CalpL_{/T10}$ crystal structure. Selected residues are labeled. The black mesh is a $2mF_o-DF_c$ electron density map contoured at 1.0 σ. **h,** SEC-SAXS experiment of the $CalpL/T_{10}$ complex. The experiment was performed once. Thirty sample intensity frames and sixty buffer intensity frames were collected and averaged. For each data set and angular point the errors were computed following the Poisson statistics. The data points represent the average intensity difference (sample-buffer) and the error bars represent the standard deviation. For gel source data, see Supplementary Figure 1.

Extended Data Fig. 6 | cA_4 induced oligomerization of CalpL studied by DLS and SAXS. a, Dynamic light scattering experiments (six timeseries, each series marked by a dashed circle, single data points are shown) at different protein concentrations and in the absence (t=0: light grey to t= 60 min: dark grey) and presence (t=0: cyan to t= 60 min: violet) of cA_4 reveal a cA_4-dependent oligomerization of CalpL. The experiment was performed twice (n=2 technical replicates) **b,** SAXS experiments at different concentrations. The experiments were performed once. For each experiment, thirty sample intensity frames and sixty buffer intensity frames were collected and averaged. For each data set and angular point the errors were computed following the Poisson statistics. The data points represent the average intensity difference (sample-buffer) and the error bars represent the standard deviation. **c,** *Ab initio/rigid-body* model of a CalpL dimer created with DAMMIF and SASREFMX by a global fit of a monomer-dimer mixture to the different concentrations (red lines). The crystal structure of the CalpL monomer is shown on the same scale.

Extended Data Fig. 7 | Probing the RNase activity of the activated toxin and checking for cA4 induced dimerization of CalpT with pulsed EPR. a, Fluorescence image of the denaturing PAGE to determine ribonuclease activity of the reactions in **b**) against six fluorescently labelled RNA substrates (listed in **c**)). No cleavage was observed after 30 min incubation with RNAs at 60 °C. The experiment was performed three times (n=3 biological replicates) **b,** SDS-PAGE analysis of cA_4-induced cleavage of CalpT (33 kDa) by CalpL. Cleavage is complete after 60 min at 60 °C. The experiment was performed three times (n=3 biological replicates) **c,** Sequences of the RNA substrates **d,** left: MazF was incubated with a single stranded RNA library containing 10 random bases. Illumina sequencing was used to check for sequences that were cleaved by MazF. Compared to a control reaction without MazF, sequences containing the known MazF target site (ACA) were depleted. right: same experiment but with CalpL/T ± cA_4 instead of MazF. No off-diagonal sequences and hence no ssRNase activity were observed. The experiment was performed two times (n=2 biological replicates). **e,** Oligonucleotides for the experiments in **d**) **f,** AlphaFold2 dimer models of $CalpT_{23}$. **g,** Best model (pLDDT[49], predicted local distance difference test) including MTSSL spin label[80]. **h,** X-

band cw-EPR spectrum of of CalpL/T E119R1. The amount of free label (sharp spikes) is ~10%. The labelling efficiency determined as ~100%. **i,** PELDOR time traces of CalpL/T E119R1 in the presence (red) and absence (black) of cA[4]. **j,** Consensus distributions and corresponding uncertainty bands. Colored bars indicate reliability ranges (green: shape reliable; yellow: mean and width reliable; orange: mean reliable; red: no quantification possible). Predicted distance calculated with mtsslWizard[80]. The EPR experiment was performed twice (n=2 technical replicates). For gel source data, see Supplementary Figure 1.

Extended Data Fig. 8 | AlphaFold2 predictions of CalpS. a, Prediction of CalpS alone. The protein is shown as cartoon and colored according to the prediction confidence (pLDDT[49], predicted local distance difference test) **b,** Prediction of the CalpT/S complex. **c,** Superposition of CalpS with 4LUP[81] and 2H27[82] identify the DNA binding regions of CalpS. **d,** Model of CalpS in the context of a RNAP/ECF σ-factor/promotor complex (PDB: 5ZX2[83], grey, yellow, green) from *M. tuberculosis*. Note that the linker region between the σ2 and σ4 subunits of CalpS has been cut to allow the superposition of the σ_2 and σ_4 domains onto those of 5ZX2. The linker is long enough to bind to the RNAP in a similar way as the σ-factor in the 5ZX2 structure (yellow).

Extended Data Table 1 | Data collection and refinement statistics for crystallographic structures.
*Values in parenthesis are for the highest-resolution shell.
One crystal was used for each data collection.

Extended Data Table 2 | SAXS data collection and parameters.
[*]Rg (radius of gyration) from Guinier approximation, [†]Rg from real-space pair distance distribution function, [‡]Largest intramolecular distance, Dmax, [§]Bayesian molecular mass (Mr) estimate and credibility interval (>90% probability), [‖]Porod volume from regularized curve, [¶]Mr from VP, [#,☆]Volume and Mr of the *ab initio* models, [**]Normalized Spatial Discrepancy of the *ab initio* reconstructions, [††]The χ^2 value is given for the most representative DAMMIF *ab initio* reconstruction.

a

S152 (magenta)
protease active site
K193 (magenta)

αJ

αG

"hinge"
αK   αM   αL   SAVED domain

cOA binding site

αD   β2   β1

N-terminal domain

αA

N

αC

Lon protease domain

αP

αO   αS

C   β19

β17   αQ

b

response units (RU)

$K_D \sim 1nM$

cA₃   cA₄   cA₅   cA₆

time (s)

c

protease active site

cA4
K_D 1 nM

"top side"

~180°

C-term   R493

R338

K366

"bottom side"

sulfate ion   R361

K364

a

MazF-like   DUF 2080

1 30   129   195 219 244 272

CalpS — CalpT — CalpL

Sulfurihydrogenibium sp. YO3AOP1

b

CalpT model

pLDDT

<50 | >90
low confidence | high confidence

DUF2080-like

A201

A195

αG

αF

C

A172

A182

αB

αE

αC

MazF-like

αD

αA

N

RNA binding site in MazF

~23 kDa   ~10 kDa

c

CalpL wt	+	−	+	+	+	+	+	−	−	−
CalpL S152A	−	−	−	−	−	−	−	+	+	+
CalpT	−	+	+	+	+	+	+	−	+	+
cOA	−	−	−	3	4	5	6	−	−	4

kDa

M

70

55 — CalpL

35 — CalpT

25

— CalpT₂₃

15

10 — CalpT₁₀

15% SDS-PAGE, Coomassie

d

cleavage site

```
        170              180              190              200
RIFEILAEPEE...QVIEIAPE.RISEFVLR..HVASTSQKATY  CalpT
RIFNIIDELEKEEYNIIPLSPE.REENYRYT..AVASTEKNTAL  C1DV82
RIFDILEEED....FMNSIYNSLVEREIPLPEVAAAGEKDTFAE  WP_097000650.1
RVFDLIEEAD....NIIYLAPT.KL..EKQP.LSAGKENTVYT.  NPA52460.1
FIFEEDENWD....NVISVDTT.KHDDIKFA..LAASVEKTTAR  HEK25337.1
RVFEE...EE...NVIQISPE.REKEILIP..LVAGREKTVFS.  WP_200674064.1
RIFEELLE.E...NVIQIPPE.REEQIKLK..LVAGKEKLTSR  A0A432RDI1
LPFEQLPELE....NTIKLPPK.RE..ILLE..LVAGKEKTTAF  HHG75456.1
```

a

CalpL/T
CalpL/T+cA₄
CalpL
CalpT
cA₄

CalpT 23 kDa
10 kDa
CalpL 58 kDa

CalpL
K193 S196 A201
36 Å
S152
C-term
CalpT₁₀

b

CalpL w/o cA₄
— 1 — 2 — 3 — 5 mg/ml
CalpL +cA₄
— 1 — 2 — 3 — 5 mg/ml

CalpL+cA₄
CalpL w/o cA₄
monomer 57.7 kDa

c

-	-	-	-	-	-	-	-	-	-	+	+	CalpL_wt
+	+	-	-	-	-	-	-	-	-	-	-	CalpL_wt/T_wt
-	-	+	+	-	-	+	+	+	+	+	+	CalpL_S152A/T_wt
-	-	-	-	+	+	+	+	+	+	-	-	CalpL_wt/T_A195E
-	+	-	+	-	+	-	+	-	+	-	+	cA₄

M
kDa
70
55 —CalpL
35
25 —CalpT
—CalpT₂₃

15% SDS-PAGE, coomassie

d

M   wt   R338E   R361E   K364E   K366E   R493C
     - +   - +   - +   - +   - +   - +   cA₄
kDa
70
55 —CalpL
35 —CalpT
25 —CalpT₂₃

15% SDS-PAGE, coomassie

a

AlphaFold2 | crystallography/SAXS

CalpS

σ₂

-35 interface

RNAP interface

linker

σ₄

CalpT₂₃

-10 interface

K193

S152

CalpL

A195

CalpT₁₀

b

CalpS — CalpT — CalpL

① ②

CalpL/T₁₀

+cA₄

CalpS/T₂₃

③ ④

cA₄

absorbance (mAU)

elution volume (ml)

16   18   20   22

e

CalpS/T

⑤

RNAP — CalpS

⑥

absorbance (mAU)

elution volume (ml)

50   100

M ⑤ M ⑥

kDa

RNAP β

60

50

RNAP α

40

30

CalpT

25

CalpS

20

15

10

c

M ② ①   M ③   M ④

kDa

60

50 — CalpL

40

30 — CalpT

25 — CalpS

20 — CalpT₂₃

15

10 — CalpT₁₀

d

+ − + + +   His-CalpS/CalpT

− + − + +   CalpL

− − − − +   cA₄

M

kDa

60

50 — CalpL

40

30 — CalpT

25 — His-CalpS

20 — CalpT₂₃

15

10

input   1st elution

a *Sulfurihydrogenibium sp.* YO3AOP1

calpS calpT calpL cmr1gr7 cas10 cmr3gr5 cmr4gr7 cmr5gr11 cmr6gr7

b

type-III complex

DNA

transcription

RNA

Cas10

ATP

cA_4 synthesis

CalpS

CalpT CalpL

cA_4

in-trans cleavage.
conformational change?
cleavage between oligomers?

RNAP

$CalpT_{10}$

+

$CalpT_{23}$/S

adaption
to phage attack

degradation of $CalpT_{23}$