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Type III CRISPR-Cas systems produce cyclic oligoadenylate second messengers
Ole Niewoehner 1*, Carmela Garcia-Doval 1*, Jakob T. Rostl 2, Christian Berk 3, Frank Schwede 4, Laurent Bigler 5, Jonathan Hall 3, Luciano A. Marraffini 2 & Martin Jinek 1
In many prokaryotes, type III clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated (Cas) systems detect and degrade invasive genetic elements by an RNA-guided, RNA-targeting multisubunit interference. The CRISPR-associated protein Csm6 additionally contributes to interference by functioning as a standalone RNase that degrades invader RNA transcripts, but the mechanism linking invader sensing to Csm6 activity is not understood. Here we show that Csm6 proteins are activated through a second messenger generated by the type III interference complex. Upon target RNA binding by the interference complex, its Cas10 subunit converts ATP into a cyclic oligoadenylate product, which allosterically activates Csm6 by binding to its CRISPR-associated Rossmann fold (CARF) domain. CARF domain mutations that abolish allosteric activation inhibit Csm6 activity in vivo, and mutations in the Cas10 Palm domain phenocpy loss of Csm6. Together, these results point to an unprecedented mechanism for regulation of CRISPR interference that bears striking conceptual similarity to oligoadenylate signalling in mammalian innate immunity.
CRISPR loci and Cas proteins constitute an RNA-guided adaptive prokaryotic immune system directed against invasive genetic elements such as bacteriophages and plasmids 1-3. Class 1 CRISPR-Cas systems, comprising type I, III, and IV systems, mediate nucleic acid interference by means of multiprotein-RNA complexes composed of a processed CRISPR RNA (crRNA) and several Cas protein subunits 4, In type III systems, the interference complex (known as Csm complex in type III-A/D systems and Cmr complex in type III-B/C systems) is assembled from the signature multidomain protein Cas10 (Csm1 in type III-A/D or Cmr2 in type III-B/C systems) and additional Cas proteins (Csm2-5 in type III-A/D, or Cmr1 and Cmr3-6 in type III-B/C5. In these systems, interference is transcription-dependent 6-8. The target RNA-bound interference complex functions as a sequence-specific endoribonuclease (RNase) to cleave the target RNA transcript and in addition has a target RNA-stimulated non-specific DNase (DNase) activity that cleaves single-stranded (ss)DNA 6,9-12.
Members of the Csm6 and the related Csx1 protein families are frequently encoded within type III CRISPR-Cas systems 13. These proteins are characterized by the presence of an N-terminal CARF domain and a C-terminal higher ekaryotes and prokaryotes nucleotide-binding (HEPN) RNase domain 13,14. In the Staphylococcus epidermidis type III-A system, Csm6 is required for efficient antiplasmid and antiviral immunity 15,16. Csm6 proteins function as RNases 16-18 that can non-specifically degrade invader-derived RNA transcripts, providing an additional interference mechanism that complements the nuclease activities of the type III interference complex 16. As Csm6 ribonucleases do not appear to physically associate with the Cas10-containing interference complex 19-21, the mechanism linking their nuclease activity to invader recognition is not known.
Allosteric activation of Csm6 ribonucleases
We previously showed that Csm6 is a homodimeric RNase that contains a conserved active site at the dimer interface of the HEPN domains 17. Our structural studies further revealed that Csm6 contains a conserved, positively charged cleft at the dimeric interface of the CARF domains. The CARF domain is a variant of the Rosmann fold and has been proposed to function in nucleotide binding or nucleotide acid sensing 13,22. Moreover, a recent crystal structure of the CARF domain-containing protein Csx3 revealed specific binding

to a linear tetraribonucleotide 13,24. Therefore, we hypothesized that the RNase activity of Csm6 might be allosterically regulated by oligonucleotide ligands (Extended Data Fig. 1). To test this, we first analysed the effect of linear tetraridynlate nucleotides on the RNase activity of Thermus thermophilus (Tt) Csm6 in assays that used either a fluorophore-labeled ssRNA substrate (Fig. 1a) or a fluorogenic RNA substrate whose cleavage results in a quantifiable increase in fluorescence signal (Fig. 1b and Extended Data Fig. 2). TtCsm6 was markedly activated by 3'-hydroxylated tetrarenylate (A4); moreover, the presence of a 2'-3'-cyclic phosphate group further potentiated activation (Fig. 1a, b). Profiling a panel of oligoadenylate (oligoA) nucleotides of varying lengths revealed that the 2',3'-cyclic phosphate-terminated tetraadenylate (A4>P) had the strongest effect on TtCsm6 (Fig. 1c and Extended Data Fig. 2a). To test whether oligoA-mediated activation was a general property of Csm6 enzymes, we additionally examined evolutionarily divergent Csm6 proteins from Methanothermobacter thermautrophicus (MtCsm6) and Enterococcus italicus (EiCsm6) (Extended Data Fig. 2b). Both MtCsm6 and EiCsm6 were efficiently activated by oligoA nucleotides (Extended Data Fig. 2c, d), with MtCsm6 displaying strongest activation by A4>P (Extended Data Fig. 2e) and EiCsm6 by 2',3'-cyclic phosphate-terminated hexaadenylate (A6>P; Fig. 1d and Extended Data Fig. 2f). Together, these results demonstrate that Csm6-family ribonucleases are activated by oligoadenylate nucleotides.

CARF domain mediates allosteric activation of Csm6

To quantify the activation of Csm6 ribonucleases, we analysed the RNase activity of EiCsm6 as a function of A6>P concentration, obtaining a half-maximal effective concentration (EC) of ~60 nM (Extended Data Fig. 3). Since HEPN domain ribonucleases typically

a

b

c

d

Figure 1 | Csm6 is allosterically activated by oligoA nucleotides. a, TtCsm6 RNase activity assay using a Cy5-labeled ssRNA substrate, in the presence of linear tetraadenylates containing 3'-OH or 2',3'-cyclic phosphate. b, Top: schematic representation of fluorogenic RNase activity assay; bottom: TtCsm6 RNase activity in the presence of tetraadenylates containing 3'-OH, 3'-phosphate, or 2',3'-cyclic phosphate groups. c, d, TtCsm6 and EiCsm6 RNase activities in the presence of oligoadenylates of varying lengths containing 3'-OH or 2',3'-cyclic phosphate. All data points represent the mean of three replicates ± s.e.m.

bind their RNA substrates with micromolar affinities 25,26, this strongly suggests that the A6>P activator is recognized by an allosteric binding site in EiCsm6 that is distinct from the RNase active site in the HEPN domain. On the basis of the proposed function of CARF domains in binding nucleotide ligands 13,22, we surmised that the CARF domain functions as the allosteric sensor. To test this, we engineered a glutamine-to-alanine substitution in the conserved CARF domain motif Ser112-Gln116 in EiCsm6 (Extended Data Fig. 4a), which maps to the dimeric CARF domain interface in TtCsm6 and would correspond to the ligand-binding face in a canonical Rossmann fold domain (Extended Data Fig. 4b). In contrast to wild-type EiCsm6, the Q116A EiCsm6 mutant (dEiCsm6CARF) no longer responded to A6>P (Fig. 2a, b), indicating that disruption of the CARF domain motif abrogated allosteric activation and confirming that the CARF domain is critical for the sensing of oligoA ligands.

The Csm complex complex generates a Csm6 activator

Since Csm6 proteins function in the context of type III CRISPR-Cas systems, we speculated that allosteric activation of Csm6 is dependent on the activity of the multisubunit type III CRISPR interference complex. Guided by a processed crRNA, the Cas10-containing complex binds to a target RNA, which leads to sequence-specific target RNA cleavage and concurrently stimulates sequence non-specific DNase activity of the complex 9–12. Given that Csm6 proteins have not been found to be physically associated with the interference complex 19,20,27, we hypothesized that the complex would additionally generate a diffusible

allosteric activator of Csm6 in a target RNA-dependent manner. To investigate this, we heterologically expressed and purified the interference complex, hereafter referred to as EiCsm(1-5), from the type III-A system of E. italicus (Extended Data Fig. 5a). The complex displayed subunit stoichiometry consistent with the general molecular architecture of type III interference complexes (Extended Data Fig. 5b) and was capable of cleaving a cognate RNA target, yielding a characteristic
pattern of cleavage products at six-nucleotide intervals 19,20,28. As expected, target RNA cleavage was dependent on the presence of a conserved aspartate residue in the Csm3 subunits (Asp32), since alanine substitution of the Csm3 aspartate (dCsm 3D32A) resulted in loss of RNase activity (Extended Data Fig. 5c).

To test whether the allosteric activator of Csm6 is produced by the type III interference complex, we incubated the EiCsm(1–5) complex together with a target RNA oligonucleotide in the presence of ATP and magnesium ions at 37° C. We subsequently heat-inactivated the complex, removed precipitated proteins by centrifugation and added a fraction of the deproteinized supernatant to an assay reaction mixture containing EiCsm6 and the fluorogenic RNA substrate. We observed robust activation of EiCsm6, with 0.01% of the added supernatant having slightly stronger effect than 500 nM AG>P, suggesting that the EiCsm(1–5) complex produces a low molecular mass product capable of activating EiCsm6 (Fig. 3a). Activator production was dependent on the presence of ATP, which could not be substituted by any of the three other nucleotide triphosphates, and required Mg 2+ ions, as addition of EDTA was inhibitory (Fig. 3b). Moreover, the activator was only produced in the presence of a cognate target RNA but not when a non-cognate control RNA was used instead (Fig. 3b). Together, these results show that the EiCsm(1–5) complex generates a diffusible Csm6 activator by an ATP-, magnesium-, and target RNA-dependent mechanism.

Since crRNA-guided target RNA cleavage by the type III interference complex yields hexanucleotide products that could potentially act as Csm6 activators 19,20,28, we tested whether the RNase activity of the EiCsm(1–5) complex was required to generate the EiCsm6 activator. Supernatant from a reaction containing EiCsm(1–5) complex in which the crRNA-guided RNase activity was abolished by the D32A mutation in the Csm3 subunits activated EiCsm6 even more strongly than the wild-type complex (Fig. 3c). These results indicate that activator generation is dependent on target RNA binding but the activator is not

a

Figure 2 | The CARF domain mediates allosteric activation of Csm6. a, RNase activity assay using a Cy5-labeled ssRNA and either wild-type (WT) EiCsm6 or dEiCsm6CARF (Q116A) in the presence or

generated by cleavage of target RNA. Moreover, the observed hyper-activity of the RNase-deficient EiCsm(1–5) complex implies that production of the Csm6 activator is itself allosterically activated by target RNA binding by the type III interference complex.

The Cas10 Palm domain generates cyclic oligoA

Having ruled out the crRNA-guided target RNA cleavage activity as the source of the allosteric activator, we examined other enzymatic activities within the type III interference complex. The Cas10 (Csm1) subunit of the complex contains two putative catalytic domains. The histidine-aspartate (HD) domain of Cas10 possesses sequence non-specific DNase activity and mediates target RNA-dependent DNA degradation 9–12. In turn, the Palm domain shares structural similarity with nucleotidyl cyclases and nucleotide polymerases, and it contains

b

absence of A6>P. b, RNase activity assay using WT EiCsm6, dEiCsm6HEPN (R372A/N373A), and dEiCsm6CARF proteins in the presence of A6>P. All data points represent the mean of three replicates ± s.e.m.

a putative, conserved Gly-Gly-Asp-Asp (GGDD) catalytic motif 99. To test whether either of the domains was required for the production of the Csm6 activator, we generated EiCsm(1–5) complexes harbouring inactivating point mutations in the HD domain (H14A/D15N, dCas10HD) or in the Palm domain motif (D584A/D585A, dCas10Palm) of EiCas10. Whereas inactivation of the HD domain had no effect on the production of the Csm6 activator, mutations in the Palm domain motif abrogated Csm6 activation (Fig. 4a),

indicating that the Cas10 Palm domain generates the Csm6 activator.
Previous structural studies of the Cas10 subunit (Cmr2) from the type III-B interference complex of Pyrococcus furiosus revealed that the putative Palm domain active site can accommodate two ATP molecules, while the GGDD motif coordinates divalent metal ions 30. On the basis of this, we tested whether the Palm domain functions as an
a
c
Figure 3 | The Cas10 complex activates Csm6 via a diffusible second messenger. a, Left: schematic representation of the EiCsm(1–5) complex activation assay; right: RNase activity of EiCsm6 in the presence of synthetic A6>P or the product generated by the EiCsm(1–5) complex.
b, Control assay to identify components necessary for activator production by the EiCsm(1–5) complex. c, EiCsm6 RNase activity in the presence of the product generated by WT EiCsm(1–5) and EiCsm(1–5)–dCsm3D32A complexes. All data points represent the mean of three replicates ± s.e.m.
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Figure 4 | The Palm domain of Cas10 generates cyclic hexaadenylate in vitro. a, EiCsm6 RNase activity in the presence of products generated by WT and mutant EiCsm(1–5) complexes. b, ATP oligomerization using [α-32P]ATP. c, LC–MS analysis of the products generated by EiCsm(1–5)–dCsm3D32A, EiCsm(1–5)–dCas10Palm/dCsm3D32A, and a synthetic A6>P standard. d, Treatment of the product generated by the
oligoA synthetase to directly catalyse 5'-3' phosphodiester formation between ATP molecules. To do this, we incubated the EiCsm(1)-5) complex in the presence of target RNA, Mg 2+ ions, and [co32P]ATP, and visualized reaction products by denaturing polycrylamide gel electrophoresis (PAGE) and phosphorimaging. In the presence of cognate target RNA, the EiCsm(1-5) complex converted ATP into a product displaying lower electrophoretic mobility (Fig. 4b and Extended Data Fig. 6a). In contrast, product formation did not occur when a non-cognate RNA was supplied. Moreover, the activity was dependent on the presence of an intact GGDD motif in the Palm domain, whereas inactivating point mutations in the Cas10 HD domain or in Csm3 had no effect (Fig. 4b). Together, these results indicate that, within the Csm(1-5) complex, the Cas10 Palm domain is allosterically activated by target RNA binding to convert ATP into a specific oligoA product.
Liquid chromatography–mass spectrometry (LC–MS) analysis revealed that the EiCsm(1–5) complex generates a single dominant molecular species with a molecular mass of 1,974.75 daltons (Da), consistent with the product being either A6>P or a cyclic hexadenylate (c–A6), both having a theoretical mass of 1,974.32 Da (Fig. 4c and Extended Data Fig. 6b). However, the retention time of the purified product (3.93 min) was markedly different from that of a synthetic A6>P standard (3.74 min), suggesting that the product is cyclic and not linear (Extended Data Fig. 7). Moreover, the product was refractory to treatments with polynucleotide kinase (PNK), alkaline phosphatase, and the pyrophosphatase RppH, indicating that it locked 5'- or 3'-phosphate, 2,3'-cyclic, or 5'-triphosphate moieties. Digestion of the product with S1 nuclease yielded a single terminal species,
EiCsm(1-5)-dCsm3D32A complex by T4 PNK, FastAP, pyrophosphatase RppH, and S1 nuclease. e, The log(dose)-versus-response curve and EC50 derived from assays in Extended Data Fig. 8b. Errors are indicated as 95% confidence interval (CI) and each data point represents the mean of three replicates ± s.e.m.
further confirming that the product is a cyclic, 3'-5' linked hexanucleotide (Fig. 4d and Extended Data Fig. 8a). The purified c-A6 product potentially stimulated the RNase activity of EiCsm6 with an EC to 3.5 nm (Fig. 4e and Extended Data Fig. 8b). Together, these results imply that the Palm domain of the Cas10 subunit is a cyclic oligoadenylate synthetase that uses ATP to generate a cyclic hexaadenely product. Notably, the molecular mass of the product generated in the presence of all ribonucleotide triphosphates (ATP, UTP, CTP, and GTP) remained unchanged (Extended Data Fig. 9), indicating that the EiCsm(1-5) complex only incorporates adenosines into the cyclic product and does not generate mixed-base

species. Overall, our results thus suggest that cyclic oligoadenylates are allosteric activators of Csm6 enzymes in vivo and that the activators are synthesized by the Cas10 subunit of the type III interference complex upon target RNA recognition. In light of our TiCsm6 data, these results also hint that other type III CRISPR-Cas systems might generate cyclic tetranucleotides instead.

Csm6 activity is dependent on Cas10 in vivo.

In the S. epidermidis type III-A CRISPR-Cas system, the RNase activities of Csm6 and Csm3 result in the degradation of phage transcripts, which is required for efficient anti-phage immunity when the target site is located in a gene transcript expressed late during infection 16. To corroborate our biochemical studies, we tested whether the in vivo activity of Csm6 depends on allosteric activation by cyclic oligoadenylates generated by Cas10. We first determined whether EiCsm6 can function together with the S. epidermidis type III system in the absence of its endogenous csm6 gene. To do this, we engineered

a

b

Figure 5 | In vivo activity of Csm6 is dependent on cyclic oligoA and Cas10. a, Growth of S. aureus cells containing S. epidermidis type III-A (dCsm3D32A/ \Delta Csm6) system programmed with a gp43 spacer supplemented with wild-type or mutated S. epidermidis or E. italicus Csm6. Cells were infected at 60 min with \PhiNM16 at a MOI of 0.25. b, Growth curves of S. aureus harbouring the S. epidermidis type III-A system with a gp43 spacer and indicated mutations. Infection with \PhiNM16 is initiated at 60 min at a MOI of 30. Each data points represents the mean of three replicates ± s.e.m.

Staphylococcus aureus strains containing an RNase-deficient version of the S. epidermidis type III-A system that was programmed to target the gp43 gene of phage ΦNM1γ6, contained an inactivating point mutation in the SeCsm3 protein, and locked SeCsm6. The strains co-expressed either wild-type EiCsm6 or the unresponsive EiCsm6 Q116A mutant (dEiCsm6CARF) from a plasmid. Cell cultures were infected with ΦNM1/6/phage at a low multiplicity of infection (MOI, ~0.25) and cell survival was measured over time (Fig. 5a). Staphylococci expressing EiCsm6 were equally, if not more, immune as those expressing catalytically active SeCsm6, demonstrating that the SeCsm(1–5) complex can activate EiCsm6. When the dEiCsm6CARF mutant was expressed instead, CRISPR-mediated immunity was lost and staphylococci sc-cumbled to phage infection, as did control strains expressing the HEPN RNase active site mutants of SeCsm6 or EiCsm6 (Fig. 5a). These results suggest that oligoA sensing by the Csm6 CARF domain is required for Csm6 function in vivo and affirm that Csm6 activation relies on a diffusible ligand rather than a direct physical interaction with the type III interference complex.

We then proceed to test whether the function of the endogenous Csm6 protein is dependent on the enzymatic activity of the Palm domain of Cas10. To do this, we infected staphylococci containing the S. epidermidis system programmed to target the late-expressed gene gp43 with phage ΦNM1~6. Whereas the activity of either Csm3 or Csm6 is sufficient to support immunity at low MOI (~1)16, at high MOI (~30) mutation of the Csm6 HEPN RNase domain (dSeCsm6HEPN) resulted in a significant reduction of cell survival, even in the presence of active Csm3 (Fig. 5b). Mutation of the GGDD motif in the Cas10 subunit (dSeCas10Palm) resulted in a similar reduction in cell survival as that observed for the dSeCsm6HEPN mutant (Fig. 5b). It was previously reported that this mutation in S. epidermidis Cas10 prevents DNA cleavage in vitro 6, and therefore the defect in immunity could be attributed to a functional role of the Palm domain in Csm6 activation, DNA cleavage, or both. To distinguish between these possibilities, we additionally tested a dSeCsm6HEPN/dSeCas10Palm double mutant strain, reasoning that the combined effect of the mutations would not be additive if immunity is a consequence of Cas10-dependent Csm6 activation. Indeed, the double mutant displayed a cell survival profile similar to each of the individual mutants, indicating that Cas10 Palm domain and Csm6 RNase activity are genetically linked. Altogether, these results thus demonstrate that the in vivo activity of Csm6 is dependent on the cyclic oligoadenylate synthetase activity of the type III interference complex.

Discussion

Previous studies demonstrated that Csm6 family ribonucleases provide an additional interference mechanism in type III CRISPR-Cas

Figure 6 | Proposed model for the molecular mechanism of type III CRISPR-Cas systems. The type III interference complex has three enzymatic activities: (1) a crRNA-guided RNase activity harboured by the Csm3 subunits to cleave target RNA, (2) target RNA-stimulated DNase activity harboured by the HD domain of Cas10, and (3) target RNA-stimulated cyclic oligoadenylate synthetase activity harboured by the Palm domain of Cas10 to allosterically activate Csm6 RNase.

systems by targeting invader transcripts 16. Here we show that Csm6 proteins are allostericically activated by cyclic oligoadenylate activators through binding to the CARF domain. We also demonstrate that the activator is synthesized by the Palm domain of the Cas10 subunit of the type III interference complex in response to target RNA recognition. Our findings thus reveal that type III systems use second messengers' signal target RNA sensing to CARF domain ribonucleases, as has been proposed in a previous comparative genomic study 22. Although bacteria utilize numerous nucleotide metabolites such as c-di-AMP, c-di-GMP, or ppGpp for signalling and stress response pathways 31, cyclic oligoA metabolites have not been reported to function as second messengers in prokaryotes before. However, the mechanism brings up striking parallels with the vertebrate innate immune system, where detection of viral double-stranded RNAs by oligoadenylate synthetase enzymes triggers production of linear 2'–5' linked oligoadenylates that allosterically activate RNase L 32.

We conclude that the type III interference complex is not only a crRNA-guided RNase and DNase, but also a cyclic oligoadenylate synthetase (Fig. 6). The likely mechanism of cyclic oligoA synthesis by Cas10 would involve linear oligomerization, followed by final cyclization of the linear intermediate through its 5'-triphosphate group (Extended Data Fig. 10). As the cyclic oligoadenylate synthetase activity is stimulated by target RNA binding, this would provide a fail-safe interference mechanism in case the intrinsic DNase and RNase activities of the type III interference complex are insufficient to clear the invader DNA and/or its transcripts, such as when the target gene is expressed late in the phage lytic cycle or contains mismatches to the crRNA guide 16. Although the interference in type III CRISPR-Cas systems might in part be due to non-specific RNA degradation by Csm6 enzymes, in analogy with the proposed mechanism of type VI systems 33, we note that the Csm6 activity appears to be confined to phage-derived transcripts in the S. epidermidist type III system 16. As CARF domain ribonucleases of the Csm6 and Csx1 families are only associated with type III CRISPR-Cas systems containing a Cas10 subunit with a catalytically competent Palm domain, this nevertheless hints that the second messenger-mediated activation mechanism is nearly universally conserved in type III-A and III-B CRISPR-Cas systems. Moreover, other CRISPR-associated CARF domain proteins, notably Csa3 (refs 13, 34, 35), appear to be transcription factors rather than nucleases. This raises the intriguing possibility that CRISPR-Cas systems also use cyclic oligoA signalling for transcriptional regulation across different CRISPR-Cas loci within the same organism, or to induce other genome defence or stress response pathways upon invader detection.

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Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.
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Author Contributions O.N., C.G.-D., and M.J. conceived the study. O.N., C.G.-D., J.T.R., L.M., and M.J. designed experiments. O.N. expressed and purified recombinant Csm6 proteins, performed oligoA activation and ATPase assays, and performed enzymatic probing of the cyclic oligoA product. C.G.-D., expressed and purified recombinant EiSm(1–5) complexes, performed oligoA activation assays, and assisted with LC-MS analysis. J.T.R. performed phage infection assays under supervision of L.M. C.B. synthesized 2,3'-cyclic phosphate-terminated nucleotides and performed LC-MS analysis under supervision of J.H. F.S. synthesized 2,3'-cyclic phosphate-terminated A4 nucleotide and advised on nucleotide chemistry. L.B. performed additional LC-MS analyses of Csm6 activators. O.N., C.G.-D., and M.J. wrote the manuscript, with input from the remaining authors.
Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
Correspondence and requests for materials should be addressed to M.J. (jinek@bioc.uzh.ch).
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No statistical methods were used to predeterminme sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Plasmid DNA constructs. DNA sequences encoding to TrCsm6 (T. thermophilus DSM-579), EiCsm6 (E. italicus DSM-15952), and MtCsm6 (M. thermautotrophicus DSM-1053) were amplified from their respective genomic DNAs by a standard PCR protocol and were subsequently cloned into a 2HR-T vector (Addgene 29718) using ligation-independent cloning, resulting in constructs carrying an N-terminal hexa-histidine tag followed by a StrepII tag, a tobacco etch virus protease cleavage site, and the Csm6 typeptide sequence. To obtain a plasmid vector for heterologous expression, a functional EiCsm-1(5-) interference complex from the E. italicus DSM-15952 type III-A CRISPR locus, a DNA fragment spanning from the cas10 gene to the end of the second spacer (GenBank GL622241.1; complement 1999065-1991480), was amplified from E. italicus genomic DNA using primers of CGD208 and oCGD209 and cloned into a p7XNH3 vector (Addgene 47064) using FX cloning 36, resulting in the pCGD253 plasmid. In this plasmid, Cas10 (Csm1) was N-terminally fused downstream of a deca-histidine tag and a human rhinovirus 3C protease cleavage site. Point mutations in all constructs were inserted by inverse PCR using the primers and templates listed in Supplementary Table 1, and resulting plasmids were verified by DNA sequencing. Synthetic DNA and RNA oligonucleotides were obtained from Sigma-Aldrich or IDT and are listed in Supplementary Table 2.

The plasmids containing the wild-type S. epidermidis RP62a CRISPR system with an anti-gp43 spacer and wild-type SeCsm6 (pWJ191) or inactive dCsm6HEPN (R364A, H369A) mutant (pWJ241), as well as the no-space control plasmid (pgG-BsaI-R), were previously described7,16 To construct the Cas10 Palm GGAA (D586A, D587A) mutation (dCas10Palm), the wild-type plasmid was amplified by PCR with primer pairs W852/W1169 and W614/W1170, and the resulting fragments were ligated by Gibson assembly. The plasmid carrying the dCsm6HEPN (R364A, H369A) and dCas10Palm double mutation (pJTR147) was generated by amplifying PCR products from pWJ241 with primers W614/PS93 and from pWJ299 with primers W852/PS94, and joining them by Gibson assembly. pWJ235 was generated by amplifying PCR products from the plasmid pWJ192 with primer pairs PS465/W1022 and W494/PS466, and joining them by Gibson assembly. pWJ156, containing SeCsm6, was constructed by a one-piece Gibson reaction using the PCR product from the amplification of pAS9 with primers W795 and W796. The plasmid carrying dCsm6HEPN was generated by amplifying pWJ156 with primers NP38/PS247 and NP39/PS248, and joining the products by Gibson ligation. The plasmids containing wild-type Csm6 (pJTR179), deiCsm6HEPN (pJTR180), and deiCsm6CARF (pJTR181) were generated by amplifying DNAs from plasmids popP214, popP215, or popP218 respectively with primers TJR504/JTR505. Each resulting fragment was combined by Gibson ligation with the PCR product amplified from pWJ156 with primers JTR502/JTR503.

Protein expression and purification. For expression of TtCsm6, MtCsm6, EiCsm6, and EiCsm(1-5) complex, the corresponding plasmid was transformed into Escherichia coli BL21 Rosetta2 (DE3) cells. Cells were grown in LB medium supplemented with appropriate antibiotics until they reached an absorbance, $A_{600\text{nm}}$ of 0.6 and expression was induced by addition of 0.2 mM IPTG (isopropyl-β-d-thiogalactopyranoside). Proteins were expressed at 18° C for 16 h. For TtCsm6, MtCsm6, and EiCsm6, the cells were harvested and resuspended in lysis buffer containing 20 mM HEPES pH 8.0, 500 mM KCl, and 5 mM imidazole pH 8.0. The cell suspension was lysed by ultrasonication and lysate was cleared by centrifugation at 20,000g for 40 min. Cleared lysate was applied to a 5 mL Ni-NTA cartridge (Qiagen), the column was washed with ten column volumes of lysis buffer, and bound proteins were eluted with five column volumes of the same buffer supplemented with 250 mM imidazole pH 8.0. Eluted proteins were dialysed overnight against 20 mM HEPES pH 7.5 and 500 mM KCl in the presence of tobacco etch virus protease. Dialysed proteins were passed through a 5 mL Ni-NTA cartridge. The flow-through fraction was concentrated and further purified by size-exclusion chromatography using an S200 (16/600) column (GE Healthcare) in 20 mM HEPES pH 7.5 and 500 mM KCl, yielding pure, monodisperse proteins. Purified proteins were concentrated to 5–75 mg ml −1 using 30,000 molecular weight cut-off centrifugal filters (Merck Millipore) and flash-frozen in liquid nitrogen.

For EiCsm(1-5) complex, cells were harvested and resuspended in lysis buffer containing 20 mM HEPES pH 8.0, 400 mM KCl, and 5 mM imidazole supplemented with EDTA-free protease inhibitor (Roche) and lysed using an HPL6 cell homogenizer (Maximator Schweiz). Lysate was clarified by centrifugation at 20,000 g for 40

min. The lysate was applied to a 5 mL Ni-NTA cartridge (Qiagen) and the column was washed in two steps with lysis buffer supplemented 10 and 50 mM imidazole. The complex was eluted with elution buffer containing 20 mM HEPES
pH 7.5, 400 mM KCl, and 250 mM imidazole. The complex was concentrated and further directly purified by size-exclusion chromatography using two Superose-6 (10/300) columns connected in tandem, or a Sephacryl S-300 (26/600) column (GE Healthcare), in buffer containing 20 mM HEPES pH 7.5, 300 mM KCl, and 5 mM MgCl. Appropriate fractions were pooled and concentrated using 100,000 molecular weight cut-off centrifugal filters (Merck Millipore), and samples were analysed by SDS-PAGE using a 4–15% gradient polacyramamide gel (Bio-Rad). All mutant complexes were purified using the same protocol as for the wild-type complex. The concentration of EiCsm(1-5) complex was calculated according to a formula taking nucleic acid contamination (that is, crRNA) into consideration: (1.55 × Anm) − (0.75 × Anm).
Synthetic RNA oligonucleotides. A synthetic target oligonucleotide for the EiCsm(1–5) was obtained from IDT with the sequence 5'-GGGUAAGGAUAGUG AAAUAGAUGCAGUGGAAGCAUACCAAGAGCA-3'. A non-cognate control RNA contained the sequence 5'-GGUCUAUUGCCCUCUAUAUCGGGGCUGUU CUCCAAAACCGGUCUGGGUACCUUCGUACGGACGACCUUC-3'. Linear 3'-hydroxylated oligo A nucleotides were obtained from Dharmacon. Linear tetraadenylate carrying a 3'-phosphate group (ApApApAp) was obtained from Biospring, and subsequently converted to A4>Pas follows. Two and a half militites of 2-(N-morpholino)ethanesulfonic acid buffer (MES, 20 mM, pH 6) and 1-ethyl-3-3-dimethylaminopropyl)carbodiimide (EDC, 5,9 mg, 38 mol, 3,1 eg.) were added to ApApApAp (12,3 mol in 486 l water) and the resulting solution was stirred at room temperature. Further portions of 1 mL MES-buffer and 1.91 mg (1 eq.) EDC were added to the reaction mixture after 24 h and 48 h. High-performance liquid chromatography (HPLC) analysis after 55h indicated ~90% product formation. The reaction mixture was diluted with 10 ml water and extracted with chloroform (4 × 40 ml). The combined organic phases were back-extracted with 10 ml water and the combined product-containing aqueous phase was filtered (regenerated cellulose membrane, 5 m) to remove particulate components. The product solution was diluted with water to ~90 ml and applied to a Q Sepharose Fast Flow anion exchange column (40 – 165 m; 100 mm × 10 mm), previously generated with 2 M sodium chloride and washed with water. The column was washed with water, followed by a gradient of 0 – 2 M NaCl. A4>P was eluted with approximately 0.2 – 0.3 M NaCl. Product-containing fractions were carefully concentrated to a final volume of approximately 10 mL. Desalting of A4>P was accomplished by semi-preative reverse-phase HPLC. The product solution was applied to a YMCGEL ODS-A 12 nm column (10 m; 250 mm × 16 mm) pre-equibrated with 100% water. The column was washed with water to remove excess NaCl. Methanol in water (50%) was used to elute residual title compound. Product-containing fractions of appropriate purity were pooled, concentrated with a rotary evaporator, and subsequently lyophilized in high vacuum for 16 h to yield 5.8 mol A4>P, sodium salt (theoretical yield: 47.2%; purity: 99.96% by HPLC).
All other linear oligoA nucleotides containing 2,3'-cyclic phosphate groups (A5>P, A6>P, A7>P, A8>P) were synthesized as follows. Phosphoramidites were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA). 2',3'-Cyclic phosphate oligonucleotides were synthesized on an MM12 synthesizer from Bio Automation (BioAutomation, Irving, Texas, USA) using 5 mg 5'-DMT-2', 3'-cyclic phosphate adosenine (N-Bz) 1000 CPG (ChemG Genes, Wilmington, Massachusetts, USA), and 2'-O-tert-butyldimethylsilyl groups (2'-TBDMS) adenosine (N-Bz) CED phosphoramidite with a coupling time of 2×90s. Phosphoramidite was prepared as a 0.08 M solution in dry acetonitrile (ACN); the activator 5-(benzylthio)-1H-tetrazole (Biosolve, Valkenswaard, the Netherlands) was prepared as a 0.24 M solution in dry ACN. Oxidizer was prepared as a 0.02 M 1 solution in THF/pyridine/HO (70:20:10, w/v/v). Capping reagent A was THF/lutidine/acetic anhydride (8:1:1) and capping reagent B was 16% N-methylimidazole/THF. Dichloroacetic acid in dichloromethane (3%) was used as deblock solution. 2',3'-Cyclic phosphate oligonucleotides were synthesized DMT-off. After completion of the automated synthesis, oligonucleotides were cleaved from the solid support, and the protecting groups on the exocyclic amino groups (N6-benzoyl) and the backbone (2-cyanoethyl) were removed using a 1-1 mixture of 40% aqueous methylamine and 25% aqueous ammonia for 1 h at 65° C. 2'-O-tert-butyldimethylsilyl groups (TBDMS) were removed and simultaneously the 2'-3'-cyclic

phosphate terms in formed using a fresh mixture of N-methyl-2-pyrolidone (60 iul), triethylamine (30 iul), and triethylamine trihydrofluoride (40 l), at 40° C for 6 h. Desilylation was quenched with trimethylethoxysilane (200 l), and disopropyl ether (200 l) was subsequently added to precipitate the oligonucleotide. The precipitate was dissolved in HO and purified on an Agilent 1200 series preparative HPLC fitted with a Waters XBridge Oligonucleotide BEH C18 column (10 mm x 50 mm, 2.5 m) at 65° C using a gradient of 5-20% buffer B over 8 min and flow rate of 5 ml min -1. Buffer A was 0.1 M triethylammonium acetate pH 8.0; buffer B was methanol. Fractions were pooled, dried under vacuum.

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and dissolved in HO. The c-A6 product generated by EiCsm(1-5) was purified using the same protocol.

Gel-based Csm6 RNase activity assay. Gel-based cleavage assays were performed as described previously 17. Briefly, 24-nucleotide synthetic RNA (250 nM), 5'-end labelled with Cy5, was incubated with Csm6 in the presence of indicated concentrations of oligonucleotide activators at 37° C. At the indicated time points, the reaction was quenched by addition of an equal volume of quencher solution (95% formamide, 5% glycerol, and 0.005% bromophenol blue) and products were resolved on a 16% denaturing (7M urea) polyacrylamide gel and visualized using a fluorescence gel scanner (Typhoon FLA 9500, GE Healthcare). Protein concentrations are indicated in the respective figure panels.

Fluorescence-based Csm6 RNase activation assay. To obtain kinetics of Csm6 RNase activity, 2 l of 2 l mM RNase Alert substrate (IDT) and 1 l of synthetic oligoA or supernatant from a EiCsm(1 - 5) cyclic oligoadenylate synthetase reaction were added to a well in a 96-well plate. The reaction was started by addition of 97 l Csm6 protein in 20 mM HEPES pH 7.5 and 50 mM KCl. Fluorescence signal (excitation at 490 nm, emission at 520 nm) was measured over time in TECAN Infinite M1000 or PHERAstar FSX (BMG Labtech) multimodal plate readers. Final protein and ligand concentrations used in the assay reactions are indicated in corresponding figure panels.

Calculation of EC. Cleavage kinetics for reactions containing EiCsm6 supplemented with indicated concentrations of A6>P or c-A6 were measured and initial velocities were calculated over the first 10 s of the reaction. Initial velocities were then plotted against ligand concentrations in GraphPad and fitted by using a nonlinear regression analysis using the log(dose)-versus-response relationship, assuming a variable Hill coefficient.

In vitro cyclic oligoadenylate synthetase assays using EiCsm(1 - 5). To utilize the EiCsm(1 - 5) complex and mutants for in vitro synthesis of cyclic oligoadenylates, 0.15 mg ml -1 EiCsm(1 - 5) complex was mixed with 10 l M target RNA, 1 mM MgCl and 0.5 mM ATP in a buffer containing 20 mM HEPES pH 7.5 and 50 mM KCl in a total volume of 30 l. The reaction was incubated at 37° C for ~16 h and the proteins were subsequently denatured at 95° C for 10 min. Denatured protein was pelleted by centrifugation and the deproteinized supernatant was transferred to a fresh tube. The supernatant was diluted as indicated and added to the Csm6 RNase activity assay as described above.

To directly visualize the synthesis of cyclic oligoadenylates, the same reaction was supplemented with 1 Ci of [\alpha_0^{-32}\text{P]ATP (Hartmann Analytic) and the reaction time was shortened to 30 min. The reaction was stopped by heat inactivation at 95° C for 10 min. The products were mixed with equal volumes of quencher solution (95% formamide, 5% glycerol, and 0.005% bromophenol blue) and subsequently resolved on a 20% denaturing (7M urea) polyacrylamide gel run at 60W for ~5h. The gel was dried and products were detected by phosphorimaging using a Typhoon FLA 9500 imager (GE).

For time-course experiments, 0.05 mg ml -1 EiCsm(1 - 5) complex was mixed with 10 M target RNA, 1 mM MgCl, and 0.5 mM ATP in a buffer containing 20 mM HEPES pH 7.5 and 50 mM KCl in a total volume of 30 l. Aliquots (3l) were taken at indicated time points and reactions were quenched by addition into 7 l quencher solution. Samples were resolved and imaged as above.

Nuclease S1 digest or EI(Csm1-5) product. The radiolabelled product from the EiCsm(1-5) assay was digested with Nuclease S1 (Thermo Fisher Scientific). Cleavage reactions contained 4 l 5 × nuclease S1 buffer, 15 l of [α-32P] ATP-labeled oligoadenylates, and 1 l of nuclease S1 diluted 1:50 in its commercial protein storage buffer (final concentration 0.1 U l -1) in a total volume of 20 l. The reaction was

incubated at 37° C and 2 l aliquots were taken at the indicated time points. Reactions were quenched by addition into ten volumes of quencher solution and subsequently resolved and imaged as described above.
Enzymatic probing of Ei(Csm1–5) product. Aliquots of the radiolabelled product from the EiCsm(1–5) assay were treated with PNK (NEB) in the absence and presence of 1 mM ATP with Fast Alkaline Phosphatase (Thermo Fisher Scientific) and E. coli RNA 5' pyrophosphohydrolase (RppH, purified in-house). All reactions were performed in reaction buffers optimized for the respective enzymes as recommended by the supplier. All reactions were performed in total volumes of 10l containing 1l enzyme, 1.5l (α·32P) ATP-labeled oligoadenylates, 1l 10× buffer, and 6.5l HO. Reactions were incubated at 37° C for 90 min and products were resolved and imaged as above.
LC-MS analysis. To analyse the product generated by EiCsm(1-5) complex, the EiCsm(1-5)-dCsm 332A complex was used to prevent cleavage of the target RNA by the Csm3 subunits and ensure persistent activation of the complex. A reaction containing 0.15 mg ml −1 of EiCsm(1-5) complex (500 nM), 10 M target RNA, 1 mM MgCl and 0.5 mM ATP in buffer containing 20 mM HEPES pH 7.5 and 50 mM KCl was incubated for 30 min at 37° C. To test the incorporation of other ribonucleotides, the reaction was additionally supplemented with 0.5 mM of GTP, CTP and UTP Reactions were stopped by heating to 95° C for 10 min, the denatured protein was removed by centrifugation, and 1 l of the deproteinized supernatant was diluted in 30 l of HO and subjected to LC-MS. Analysis of purified oligonucleotides and enzymatic assays was conducted on an Agilent 1200/6130 LC-MS system fitted with a Waters Acquity UPLC OST C18 column (2.1 mm × 50 mm, 1.7 m), at 65° C, with a gradient of 5–20% buffer B in 10 min with a flow rate of 0.3 ml min −1. Buffer A was aqueous hexafluoroisopropanol (0.4 mM) supplemented with triethylamine (15 mM) Buffer B was methanol. The supernatants obtained from the EiCsm(1-5)-dCsm 332A complex or the EiCsm (1-5)-dCsm 332A/dCas10 20m complex were analysed using the same protocol. To analyse the SI nucase digest of the EiCsm(1-5) product, 15 l of the supernatant were treated with 0.5 U l −1 of nucase S1 enzyme for 30 min at 37° C. The sample was mixed with 20 l chloroform, vortexed, and centrifuged for 5 min at 5,000 r.p.m. The upper (aqueous) phase was transferred to a fresh tube without disturbing the interphase and analysed on the same system as above with a gradient of 1–10% buffer B in 10 min.
RNA cleavage assay by EiCsm(1–5) complexes. The reaction was initiated by adding 250 nM target RNA substrate (3'-labelled with Alexa 532) to 250 nM EiCsm(1–5) complex (wild type or inactive mutants) in a buffer containing 20 mM HEPES pH 7.5 and 50 mM KCl. After 1 min at 37 ° C, the reaction was stopped by addition of an equal volume of quencher solution (90% formamide, 5% glycerol, 25 mM EDTA) and heated at 95 ° C for 5 min. Ten per cent of the final reaction was resolved on a 16% denaturing (7M urea) polyacrylamide gel. The products were visualized using a Typhoon FLA 9500 fluorescence gel scanner.
Phage infection assays. S. aureus RNA220 (ref. 37) was grown in tryptic soy broth (TSB) medium at 37° C, and supplied with 10 g ml −1 chloramphenicol or 10 g ml −1 erythromycin for plasmid maintenance. During bacteriophage infection, 5 mM CaCl was added to the media. For both EiCsm6 complementation and Csm6-Cas10 phenocopy experiments, overnight cultures initiated from single colonies were diluted 1:20 in TSB broth. After growth for 1 h, cell densities were normalized and cultures were added to a 96-microwell plate (Cellstar, 655180) in the presence of appropriate antibiotics and 5 mM CaCl. The plate was shaken in a microplate reader (TECAN Infinite 200 PRO) at 37° C, with optical measurements taken at 600 nm every 10 min. Bacteriophage \Phi NMI-6 (ref. 7) was added at an MOI of approximately 0.25 or 30 after 60 min of shaking. For the implementation experiment, cells containing plasmid pWJ235 (gp43 spacer, dCsm3, \Delta Csm6) in combination with one of the plasmids pWJ156, pJTR677, pJTR179, pJTR180, or pJTR181 were inoculated from single colonies and grown in TSB supplemented with 10 g ml −1 chloramphenicol and 10 g ml −1 erythromycin. For the phenocopy experiment, overnight cultures of S. aureus RNA4220 containing pWJ191, pWJ241, pWJ299, pJTR147, or pGG-Basal-R were inoculated from single colonies and grown in the TSB supplemented with 10 g ml −1 chloramphenicol.
Data availability. Raw images of uncropped gels shown in Figs 1a, 2a, 4b, d and Extended Data Figs 1, 2 and 5 are provided in Supplementary Fig. 1. Other data are available from the corresponding author upon reasonable request.

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Extended Data Figure 1 | AfCsx3 resembles the CARF domain of TtCsm6. Left: structure of AfCsx3 (Protein Data Bank (PDB) accession number 3WZI) bound to a tetraribonucleotide. Centre: structure of the CARF domain dimer of TtCsm6 (PDB 5FSH). Right: superimposition of AfCsx3 (grey) and TtCsm6 (pink and blue). The structures were aligned using PDBeFold 38.

a
b
c
d
e
f
Extended Data Figure 2 | Activation of Csm6 ribonucleases by oligoadenylates. a, TtCsm6 RNase activity assay in the presence of 3′-hydroxylated oligoadenylates ranging from A3 to A10. b, SDS-PAGE analysis of purified WT and mutant Csm6 proteins from T. thermophilus (TtCsm6), M. thermautotrophicus (MtCsm), and E. italicus (EiCsm6). c, d, MtCsm6 and EiCsm6 RNase activity assays in the presence of 3′-hydroxylated oligoadenylates ranging from A3 to A10. e, MtCsm6 RNase activity assay in the presence of oligoadenylates carrying 3′-hydroxyl (–OH) or 2′,3′-cyclic phosphate (>P) groups. f, TtCsm6 and EiCsm6 RNase activity assay in the presence of A4>P and A6>P. All data points represent the mean of three replicates ± s.e.m.

a
b
Extended Data Figure 3 | Activation of EiCsm6 by A6>P. a, EiCsm6 RNase assay in the presence of varying concentrations of A6>P. b, The log(dose)-versus-response curve and EC50 derived from assays in a. Errors are indicated as 95% confidence interval. All data points represent the mean of three replicates ± s.e.m.

a
E.italicus Csm6 \rightarrow WP_0072089533 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow WP_004333841 \rightarrow W
b
Extended Data Figure 4 | Conservation and structure of the CARF domain motif. a, Multiple sequence alignment of the CARF domain motifs in Csm6. The polypeptide sequence of EiCsm6 (denoted with an arrow) was used for a BLAST search against the National Center for Biotechnology Information (NCBI) non-redundant

protein sequences database and 19 best scoring hits with >95% query coverage were selected. The multiple sequence alignment was performed using Clustal Omega 39 and visualized using ESPript 3 (ref. 40). Green asterisk denotes the conserved glutamine residue mutated to abrogate allosteric activation of EiCsm6. b, Zoom-in view of a structural model of the dimeric interface of the CARF domains in the EiCsm6 dimer. The model was generated using Phyre2 (ref. 41) on the basis of the TtCsm6 crystal structure (PDB 5FSH).

a

E. italicus DSM 15952

b

Extended Data Figure 5 | Purification and crRNA-guided RNA cleavage by the EiCsm(1–5) complex. a, E. italicus DSM15952 contains a CRISPR-Cas locus with nine cas genes followed by a repeat-spacer array. For heterologous expression of the EiCsm(1–5) complex, the genomic DNA fragment spanning genes csm1–csm6 and cas6 and the first two

c

repeat-spacer units in the CRISPR array was cloned into the expression plasmid. b, SDS-PAGE analysis of purified wild-type and mutant EiCsm(1–5) complexes. c, Denaturing gel of the products of a target RNase cleavage assay performed using a fluorophore-labeled target ssRNA substrate in the presence of WT or mutant EiCsm(1–5) complexes.

a

b

Extended Data Figure 6 | The EiCsm(1-5) complex generates cyclic hexaadenylate. a, Denaturing 20% polyacrylamide gel indicating time-resolved [α-32P] ATP oligomerization by the EiCsm(1-5) complex. b, Mass spectra of LC-MS peaks from the analysis of the products generated by the EiCsm(1–5)–dCsm3D32A and EiCsm(1–5)–dCas10Palm/dCsm3D32A complexes shown in Fig. 4c. The mass spectrum of synthetic A6>P standard is shown for comparison.

a

b

C

d

e

f

Extended Data Figure 7 | LC-MS analysis of purified EiCsm(1-5) product. a, c, e, LC-MS analysis of the HPLC-purified activator product generated by the EiCsm(1-5) complex as well as an A6>P standard and purified product spiked with the A6>P standard. b, d, f, Mass spectra of the peaks indicated in a, c, e.

a

b

Extended Data Figure 8 | The EiCsm(1–5) complex generates cyclic hexaadenylate. a, Left: LC–MS analysis of the nuclease S1 digested activator. Right: mass spectrum profile of the peak obtained after S1 digest. b, EiCsm6 RNase assay in the presence of varying concentrations

c

of c-A6. c, Overlay of EC values obtained for A6>P and c-A6. Errors are indicated as 95% confidence interval and data points represent mean of three replicates ± s.e.m.

a
b
Extended Data Figure 9 | The EiCsm(1–5) complex selectively incorporates adenosines into the activator. a, Stimulation of EiCsm6 RNase activity by products generated by the EiCsm(1–5) complex in the presence of various ribonucleotide triphosphate combinations. b, LC-MS analysis of indicated samples from a. The elution profile (left) and mass spectrum (right) of the product remain unaltered upon addition of all four ribonucleotide triphosphates, indicating ATP selectivity of the EiCsm(1–5) complex.
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Extended Data Figure 10 | Proposed mechanism of cyclic oligoadenylate formation. The Cas10 Palm domain initially catalyses 5'-3' phosphodiester bond formation by a nucleophilic attack of the 3'-hydroxyl group of one ATP substrate molecule onto the α-phosphate group of a second ATP molecule, yielding a 5'-triphosphorylated dinucleotide and inorganic pyrophosphate (PPi). The dinucleotide is extended by addition of successive AMP moieties to the 3'- end to generate a linear oligonucleotide. Final cyclization involves the 3'-hydroxyl group attacking the α-phosphate group of the 5'-triphosphate moiety in the linear oligoadenylate intermediate. Notably, all reaction steps use the same catalytic mechanism and thus could be performed by a single catalytic active site in the Palm domain of Cas10.
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Corresponding author(s): Martin Jinek
☐ Initial submission ☐ Revised version Final submission
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 Experimental design
1. Sample size.
Describe how sample size was determined.	not applicable
2. Data exclusions	
Describe any data exclusions.	no applicable
3. Replication
Describe whether the experimental findings were reliably reproduced.	Experimental findings in all figures were reliably reproduced.
4. Randomization
Describe how samples/organisms/participants were allocated into experimental groups.	not applicable
5. Blinding
Describe whether the investigators were blinded to group allocation during data collection and/or analysis.	not applicable
Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters
For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).
n/a	Confirmed
	The exact sample size (n) for each experimental group/condition, given as a discrete number and unit

of measurement (animals, litters, cultures, etc.)|
	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
	A statement indicating how many times each experiment was replicated
	The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
	The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
	A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
	Clearly defined error bars
See the web collection on statistics for biologists for further resources and guidance.
Software
Policy information about availability of computer code
7. Software
Describe the software used to analyze the data in this study.
GraphPad and Microsoft Excel
For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.
Materials and reagents
Policy information about availability of materials
8. Materials availability
Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.
A4>P was custom-made by BIOLOG GmbH. As we only have limited quantities, we are unable to distribute large quantities of the material - this would require another custom synthesis, either by BIOLOG or other CMO.
9. Antibodies
Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).
not applicable
10. Eukaryotic cell lines
a. State the source of each eukaryotic cell line used.
not applicable
no applicable
not applicable
not applicable
b. Describe the method of cell line authentication used.
c. Report whether the cell lines were tested for mycoplasma contamination.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.
Animals and human research participants
Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines
11. Description of research animals
Provide details on animals and/or animal-derived materials used in the study.
not applicable

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

|not applicable|