A Membrane-Associated DHH-DHHA1 Nuclease Degrades Type III CRISPR Second Messenger

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Graphical Abstract

Type III CRISPR signaling in Sulfolobus

Highlights

- Metal-dependent cOA degradation activity accelerates clearance of high-level cOA
- The metal-dependent activity is associated with the cell membrane
- A membrane-associated DHH-DHHA1 family nuclease (MAD) is found to degrade cOA
- cOA degradation of MAD deactivates the type III CRISPR accessory nuclease

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In Brief
Zhao et al. find that a metal-dependent and membrane-associated nuclease activity accelerates clearance of high-level cOA in Sulfolobus. They further show that a metal-dependent and membrane-associated DHH-DHHA1 family nuclease efficiently degrades cOA and deactivates type III CRISPR accessory nuclease.
A Membrane-Associated DHH-DHHA1 Nuclease Degrades Type III CRISPR Second Messenger

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SUMMARY

Type III CRISPR-Cas systems initiate an intracellular signaling pathway to confer immunity. The signaling pathway includes synthesis of cyclic oligo-adenylate (cOA) and activation of the RNase activity of type III accessory ribonuclease Csm6/Csx1 by cOA. After the immune response, cOA should be cleared on time to avoid constant cellular RNA degradation. In this study, we find a metal-dependent cOA degradation activity in *Sulfolobus islandicus*. The activity is associated with the cell membrane and able to accelerate cOA clearance at a high cOA level. Further, we show that a metal-dependent and membrane-associated DHH-DHHA1 family nuclease (MAD) rapidly cleaves cOA and deactivates Csx1 ribonuclease. The cOA degradation efficiency of MAD is much higher than the cellular ring nuclease. However, the subcellular organization may prevent it from degrading nascent cOA. Together, the data suggest that MAD acts as the second cOA degrader after the ring nuclease to remove diffused redundant cOA.

INTRODUCTION

CRISPR loci and CRISPR-associated (cas) genes constitute an adaptive immune system in bacteria and archaea (Deveau et al., 2010; Koonin et al., 2017; Marraffini and Sontheimer, 2010a). To confer immunity against the invasion of virus and plasmid, CRISPR-Cas systems acquire genetic information from invading genetic elements and use the information to guide Cas protein to destroy nucleic acids and prevent subsequent invasion (Plagens et al., 2015; Sternberg et al., 2016). According to the mechanisms of invading nucleic acid destruction, CRISPR-Cas systems are classified into two classes and six types (Mohanraj et al., 2016). Class 1 systems employ a multi-subunit complex to recognize and destroy invading nucleic acids, while a single multi-domain protein of class 2 systems is able to carry out the same function.

Type III CRISPR-Cas systems belong to the class 1 group (Makarova et al., 2017). Their immunity is dependent on the transcription of the target gene (Deng et al., 2013; Goldberg et al., 2014). Recognition of the target transcript by type III interference effector complex leads to at least three activities: target RNA cleavage (Hale et al., 2009), target RNA-activated DNA degradation (Elmore et al., 2016; Estrella et al., 2016; Han et al., 2017a; Kazlauskienė et al., 2016), and synthesis of cyclic oligo-adenylate (cOA) (Han et al., 2018; Kazlauskienė et al., 2017; Niewoehner et al., 2017; Rouillon et al., 2018). cOA functions as a second messenger to activate the CRISPR-Cas accessory ribonuclease Csm6/Csx1, which uses the CARF (CRISPR-associated Rossman-fold) domain to sense cOA and allosterically activate the RNase activity of the HEPN (higher eukaryotes and prokaryotes nucleotide-binding) domain for unspecific cellular RNA degradation (García-Doval et al., 2020; Jia et al., 2019; Molina et al., 2019). In addition, cOA activates an unusual CARF domain DNase in *Thermus thermophilus* (McMahon et al., 2020). The cOA synthesis and nuclease activation count for the type III CRISPR signaling pathway, which is essential for full protection of the host under many situations, such as late expression of the target viral gene (Bhoobalan-Chitty et al., 2019; Jiang et al., 2016), viral infection at a high dose (Niewoehner et al., 2017), and low expression of the target gene of a plasmid (Rostøl and Marraffini, 2019). The signaling pathway is also essential for the anti-plasmid immunity of *Sulfolobus islandicus* and *Staphylococcus epidermidis* type III systems in their native host (Deng et al., 2013; Hatoum-Aslan et al., 2014) and *Lactococcus lactis*, *Staphylococcus epidermidis*, *Streptococcus thermophilus*, and *Mycobacterium tuberculosis* type III systems when they are expressed in *E. coli* (Foster et al., 2019; Gruschow et al., 2019).

Type III CRISPR signaling has to be stringently controlled to avoid induction of cell death. The control is performed at both the COA synthesis stage (Guo et al., 2019; Kazlauskienė et al., 2017).
Identification of Metal-Dependent cOA Degradation Activity in S. islandicus

We investigated a potential novel cOA degradation mechanism in a model archaeon, Sulfolobus islandicus Rey15A. This organism encodes a type III-Cmr~σ system that generates cA4 (main product) and cyclic tri-adenylate (cA3, byproduct) and a CRISPR accessory ribonuclease Csx1 that is activated by cA4 (Han et al., 2017). The organism also encodes known ring nucleases, such as Csx3 and Csm6 proteins, also categorized as ring nuclease, degrade cA4 or cA6 with their CARF domains, representing an autoinhibitory mechanism (Athukoralage et al., 2018; Garcia-Doval et al., 2020; Jia et al., 2019). Moreover, other type III accessory RNase effectors, such as Csx3 and a Csx1-ring nuclease fusion protein, may also degrade cA4 (Athukoralage et al., 2020; Samolygo et al., 2020). Third, ring nuclease encoded by virus degrades cA4 to suppress type III immunity (Athukoralage et al., 2020b); noticeably, the activity of the virus-encoded ring nuclease is much higher than that of the cellular ring nuclease. Finally, some Csx1/Csm6 proteins can degrade cA4 and cA6 using their HEPN domain (Foster et al., 2020; Garcia-Doval et al., 2020; Jia et al., 2019). All above nucleases degrade cOA in a metal-independent manner and generate products containing 2',3'-cyclic phosphate and 5'-OH groups. Nevertheless, how bacteria and archaea control type III CRISPR immunity by degrading cOA is still unclear. In this study, we reveal that in Sulfolobus islandicus, a model archaeon, metal-dependent and membrane-associated cOA degradation activity contributes to cOA clearance at a high cOA level. We further identify that a membrane-associated DHH-DHHA1 family nuclease (MAD) is hyperactive for cOA degradation and capable of promptly deactivating Csx1 ribonuclease.

RESULTS

Identification of Metal-Dependent cOA Degradation Activity in S. islandicus

To investigate the function of the metal-dependent activity in cOA degradation, we analyzed the cOA degradation rate of CE in the presence of EDTA and MnCl2 at different cOA levels (Figures S1B and 2A). In the presence of EDTA, the main product migrated slower than the substrate, in agreement with the metal-independent ring nuclease activity (Figure S1B). Replacement of EDTA with Mn2+ resulted in both slower and faster products, corresponding to the metal-independent and metal-dependent products, respectively. At 80 nM cOA, the cOA degradation curves of EDTA and Mn2+ were very similar (Figure 2A), indicating that Mn2+ did not accelerate cOA clearance at this cOA level. However, when 1 μM and 5 μM cOA were added into the reaction mixtures, Mn2+ significantly increased cOA-degradation rate (Figure 2A). Specifically, only 5% and 13% cOA were left in the mixtures containing Mn2+ for 1 μM and 5 μM cOA, respectively, while about 35% and 60% cOA were remaining in the presence of EDTA. Further, as the substrate concentrations increased from 80 nM to 5 μM, the final percentage of the metal-independent product decreased from 51% to 6%, whereas the metal-dependent product was the dominant product at the high cOA level at the end of the reaction (Figure 2B). Together, the data indicates that the metal-dependent activity plays an important role in cOA clearance at a high cOA level.
The Metal-Dependent cOA Degradation Activity Is Associated with the Cell Membrane

Further, the effects of detergent on the purification pattern suggest that the metal-dependent activity is associated with the cell membrane (Figure 1). To confirm this, we isolated the membrane-associated fraction and analyzed its cOA degradation activity. For *Sulfolobus*, ultra-centrifugation has been used to isolate the membrane-enriched fraction from the CE (Lower et al., 2000; Roppelt et al., 2010). In our experiments, the pellet after ultra-centrifugation was dissolved with n-Dodecyl-β-D-Maltopyranoside (DDM), corresponding to membrane-enriched fraction (P_DDM), while the supernatant of the CE was the cytoplasm-enriched fraction (Sup). SDS-PAGE analysis of the two fractions and CE show that most proteins were still present in the cytoplasm-enriched fraction (Figure 3A). Then, the activity of these samples was analyzed with 5 μM cOA as substrate in the presence of MnCl₂ and EDTA, respectively. The data show that the metal-dependent product was only detected in the presence of Mn²⁺ (Figure 3B) and that most of the metal-dependent activity existed in the membrane-enriched fraction (Figure 3C). Then, we compared the activity of intact cells and the CE of an equivalent cell mass. The results show that only the CE generated the metal-dependent product (Figure S1C), indicating that the activity does not exist at the outer surface of cells. Together, the data indicate that the metal-dependent cOA degradation activity is located at the inner surface of the cell membrane in *S. islandicus*.
SiRe_0244 Is a Membrane-Associated Nuclease and Degrades cOA

We then analyzed the components of the 7# fraction by mass spectrometry. It contained a mixture of 729 proteins, and 451 of them showed at least two unique peptides (Table S4). From the 451 proteins, we selected four predicted metal-dependent phosphoesterases as cOA-degrading enzyme candidates, including SiRe_0053, SiRe_1902, SiRe_0198, and SiRe_0244 (Table S5). We first attempted to express these proteins in E. coli, and only SiRe_0053 was obtained. Nevertheless, the protein exhibited little cOA degradation activity (Figure S3A). Then, the rest were expressed in the S. islandicus E233S1 strain, yielding purified SiRe_1902 and SiRe_0198, neither of which was capable of cOA degradation (Figures S3B and S3C). Comparatively, purification of SiRe_0244 obtained a small peak in the void volume during gel filtration, which efficiently degraded cOA into the products that migrated faster than cOA (Figure S3D). Both features were observed as we separated the active fraction (Figure 1). Although we failed to detect any band from the small peak by SDS-PAGE, we still assumed that SiRe_0244 may be responsible for the observed cOA degradation activity.

To confirm this, we analyzed whether SiRe_0244 is associated with the cell membrane. The CE was prepared from cells expressing His-tagged SiRe_0244. Sup and pellet were isolated, and the pellet was dissolved with DDM and Triton X-100 (PDDM and Ptriton), respectively. These fractions, together with the CE that did not contain His-tagged SiRe_0244 (negative control, NC) and semi-purified His-tagged SiRe_0244 (positive control, PC), were analyzed with western blotting using an anti-His-tag antibody. Western blot showed that His-tagged SiRe_0244 was present in PDDM and Ptriton but not in the cytoplasm-enriched fraction (Figure 4A). Further, SiRe_0244 was visualized with fluorescence microscopy using an anti-SiRe_0244 polyclonal antibody (Figure 4B). The results show that SiRe_0244 formed a circle surrounding the nucleoid and correlating with the cell shape. These data indicate that SiRe_0244 is a membrane-associated protein.

Thus, we improved the SiRe_0244 purification procedure using DDM as the detergent. With DDM, SiRe_0244 was present in two peaks in gel filtration chromatography: one peaking at ~9 mL, corresponding to the void volume of that in the absence of DDM, and the second one peaking at ~14 mL, indicating that the detergent had solubilized a fraction of membrane-associated SiRe_0244 (Figures 5A and 5B). Moreover, the cOA degradation activity of the gel filtration fractions correlated with SiRe_0244 in the fractions (Figure 5C). The fractions of the peak of ~14 mL were pooled and used for the next experiments. To further confirm whether SiRe_0244 has cOA degradation activity, we constructed and characterized SiRe_0244 mutants carrying alanine substitutions of conserved residues. SiRe_0244 belongs to the DHH-DHHA1 family, which has five conserved motifs that are important for the activity (Aravind and Koonin, 1998). Sequence alignment of SiRe_0244 and well-characterized bacterial DHH-DHHA1 family proteins show that only three of the five motifs are intact in SiRe_0244 (i.e., D10H-D12A, D87H-H88A, and G268GGH271) (Figure S4A). We further constructed the structural model of SiRe_0244 with Mycobacterium tuberculosis Rv2837c (PDB: 5jju) as the template (Figure S4B). In the model, SiRe_0244 adopts a classic two-domain architecture that resembles Rv2837c, containing an N-terminal DHH domain, a C-terminal DHHA1 domain, and a link connecting them (He et al., 2016). In Rv2837c, the DHH domain coordinates two metal ions and acts as the catalytic domain, while the DHHA1 domain plays a role in substrate binding. Moreover, the three conserved motifs adopt a similar conformation to those of Rv2837c (Figures 5D and 5B). Mutation of the motifs yielded three SiRe_0244 variants: M1, D10A-D12A; M2, D87A-H88A-H89A; and M3, H271A. Purification of them revealed that their gel filtration chromatography patterns were similar to that of the wild type (WT) (Figures 5S and 5E). The fractions of the peak of ~14 mL were used for further experiments as well. The bands co-purified with SiRe_0244 were analyzed, and the results indicate that the proteins seem not to be specific partners of SiRe_0244 (Figure 5SD). The cOA degradation assay indicates that mutation of the DxD motif and DHH motif abolished the cOA degradation activity, while the H271A variant was still capable of cOA degradation activity, while the H271A variant was still capable of cOA degradation activity (Figure 5F). Together, the data indicate that SiRe_0244 is a membrane-associated cOA-degrading nuclease and that the DxD and DHH motifs are essential for the activity.
Figure 3. The Metal-Dependent cOA Degradation Activity Is Associated with the Cell Membrane
(A) SDS-PAGE analysis. Sup, the supernatant fraction after ultra-centrifugation; PDDM, the pellet fraction after ultra-centrifugation, which was dissolved in 0.02% DDM.
(B) cOA degradation assay of the fractions from (A) in the presence of EDTA or Mn²⁺. CK, incubation of cOA with buffer containing 0.02% DDM. Three independent repeats were performed, and a representative image is shown. Metal-dependent product is indicated by a red bracket.
(C) Quantification of the metal-dependent product in the presence of Mn²⁺ from (B). Error bars indicate standard deviation of three independent repeats. See also Figure S1.

Figure 4. SiRe_0244 Is Associated with the Cell Membrane
(A) Western blot analysis of the subcellular location of SiRe_0244. NC (negative control), CE without His-tagged SiRe_0244; CE, CE of the cells expressing His-tagged SiRe_0244; Sup, the supernatant fraction of CE; PDDM, the pellet fraction of CE dissolved with Triton X-100 and DDM, respectively; PC (positive control), semi-purified His-tagged SiRe_0244. These samples were analyzed by western blot using antibody against His-tag (upper panel) and SDS-PAGE (lower panel). The red arrow indicates the band of His-tagged SiRe_0244.

(B) Immunofluorescence microscopy analysis of the subcellular location of SiRe_0244. Images show the differential interference contrast (DIC) field, DAPI staining of DNA (blue), Dylight 488 field (green), and merged images (overlay). The black scale bar indicates 500 nm.

and 3'-OH groups (Rao et al., 2010). To gain further insight into the cOA degradation mechanism of SiRe_0244, we analyzed the metal dependency and the products of the SiRe_0244-catalyzed reaction. The activity was highest in the presence of Mn²⁺ but very low with other metals, indicating that the activity of SiRe_0244 is dependent on Mn²⁺ (Figure S2D). In addition, FastAP modified the products, indicating that they carry the free phosphate group (Figure S2E). The data are consistent with the activity of the 7# fraction and characterized DHH-DHHA1 family nucleases.

Depending on different subgroups, the substrates of DHH-DHHA1 family proteins vary from cyclic di-nucleotides, nanoRNA to ssDNA (Srivastav et al., 2019), suggesting that this family of proteins, in contrast to ring nuclease, has a broad substrate spectrum. To preliminarily investigate the substrate spectrum of SiRe_0244, we analyzed whether SiRe_0244 degrades single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), single-stranded DNA (ssDNA), and double-stranded DNA (dsDNA). The data show that all tested substrates are efficiently degraded by SiRe_0244 and that ssRNA was the most preferred substrate (Figures S5 and S6). The efficient degradation of ssRNA suggests that it may interfere with the degradation of cOA as competitors. To test this, cOA was incubated with SiRe_0244 in the presence of 1.4 ng/μl ssRNA, about 10 times the weight/volume concentration of cOA in the reaction mixture (Figures S6E and S6F). The results show that cOA clearance was moderately delayed in the presence of RNA, indicating that RNA could compromise cOA degradation by SiRe_0244 to some extent. Next, we analyzed whether SiRe_0244 degrades ApA> p, the final cleavage product of cA by ring nuclease. In the experiments, cOA was first incubated with ring nuclease SiRe_0455, and then the reaction mixture was incubated with SiRe_0244. SiRe_0244 did not affect the migration of the products generated by SiRe_0455, indicating that SiRe_0244 did not degrade ApA> p (Figure S6G).

It has been reported that a few DHH proteins are associated with the type III CRISPR-Cas system (Shmakov et al., 2018). To investigate whether these proteins degrade cOA, we expressed and characterized a DHH protein (NCBI Reference Sequence: WP_035571781.1) from Halonatronum saccharophilum DSM 13868, which is associated with a type III-B system. The results show that the H. saccharophilum DHH protein did not cleave cOA generated by Cmr-α (Figure S7), indicating that it does not play a role in cOA degradation.

SiRe_0244 Efficiently Degrades cOA and Deactivates Csx1
Next, we analyzed whether cOA degradation by SiRe_0244 could efficiently deactivate the CRISPR accessory ribonuclease Csx1. We set up parallel cOA synthesis reactions with or without 25P-ATP. Both reactions were subjected to the cOA degradation assay of 20 nM SiRe_0244. After quenching the reaction with EDTA at increasing time intervals, radioactive reactions were used to monitor cOA degradation (Figure 6A), while unlabelled reactions were analyzed for their capability to activate Csx1 (Figure 6B). The results show that 5 min incubation of 20 nM SiRe_0244 with 1 μM cOA could almost deactivate Csx1. Further, Csx1 deactivation was correlated with cOA degradation (Figure 6C), and the rate constants of the two
processes were $0.48 \pm 0.05$ min$^{-1}$ and $0.42 \pm 0.08$ min$^{-1}$, respectively. To gain further insight into the role of SiRe_0244 in cOA degradation, we compared the cOA degradation ability of SiRe_0244 with the main ring nuclease in *S. islandicus* Rey15A, SiRe_0455 (Figures 6D and S1D). The results show that with 1 mM cOA as substrate, the degradation rate of 20 nM SiRe_0244 was even higher than that of 5 mM SiRe_0455. The data indicate that SiRe_0244 is hyperactive for cOA degradation and could rapidly deactivate Csx1.

**DISCUSSION**

Type III CRISPR-Cas systems produce second messenger to activate cellular RNA degradation activity to confer anti-viral immunity (Kazlauskiene et al., 2017; Niewoehner et al., 2017). The second messenger has to be degraded to switch off the immune response (Athukoralage et al., 2018). On the other hand, virus may degrade the second messenger to subvert type III CRISPR immunity for the benefit of the virus (Athukoralage et al., 2020b). The cOA degrading enzymes belong to the ring nuclease family, which employs the CARF domain to specifically degrade cA4 using a metal-independent mechanism. In addition, some Csm6 and Csx1 proteins can employ their HEPN domains to degrade cOA (Foster et al., 2020; Garcia-Doval et al., 2020; Jia et al., 2019; Smalakyte et al., 2020), and the activity is much higher than that of the CARF domain of cellular ring nucleases (Jia et al., 2019; Smalakyte et al., 2020), indicative of a role in controlling type III immunity as well. On the other hand, compared to the low cOA degradation efficiency of cellular ring nucleases (Athukoralage et al., 2020a), cOA synthesis is very efficient. We have shown that the type III-B system could efficiently synthesize cOA using a cooperative substrate-binding mechanism (Han et al., 2018). Consistently, a recent study shows that type III CRISPR systems could amplify the signal from the viral transcript to the cOA level (Athukoralage et al., 2020a). Further, several situations may lead to a high level of viral transcript and excess cOA, including late expression of the target gene (Jiang et al., 2016), high dosage of viral infection, and high expression of the target gene. Thus, the ring nucleases may not be enough to switch off type III immunity on time. Other cOA-degrading enzymes should be required, especially for the organisms where Csx1/Csm6 do not degrade cOA, such as *S. solfataricus* (Athukoralage et al., 2018) and *S. islandicus* (Molina et al., 2019).

In this study, we identified metal-dependent and membrane-associated cOA degradation activity from the CE of *S. islandicus*. Comparison of the metal-dependent activity and the metal-independent activity, the latter of which is likely to be performed by ring nucleases (Athukoralage et al., 2018), suggests that the two activities cooperate to remove cOA. Specifically, the metal-independent activity efficiently degrades low-level cOA, whereas the metal-dependent activity accelerates cOA clearance at a high cOA level (Figure 2). Thus, the metal-dependent activity may play an important role in controlling type III immunity when high-level cOA is synthesized.
Consistently, we identify that SiRe_0244, a membrane-associated DHH-DHHA1 family nuclease (MAD), uses a metal-dependent mechanism to degrade cOA and deactivate type III CRISPR RNase effector. Compared to the ring nuclease SiRe_0455, MAD shows three distinct features: (1) it is hyperactive for cOA degradation (Figure 4D); (2) it is associated with the cell membrane (Figure 5G). The three features provide important insight into how MAD may contribute to cOA degradation in vivo. First, the hyperactivity endows MAD with the ability to degrade high-level cOA, in agreement with the finding that the metal-dependent activity of the CE efficiently clears high-level cOA. Second, the spatial organization may restrict the access of MAD to nascent cOA and make it degrade only the cOA that diffuses to the cell membrane. Nevertheless, when a virus forms foci near the periphery of cells (e.g., SIRV2) (Martínez-Alvarez et al., 2017), MAD may be close to the center of cOA synthesis. Together, these data suggest that MAD may act as the second cOA-degrading enzyme after the ring nuclease and degrade excess cOA that exceeds the degradation capacity of ring nuclease (Figure 7).

Third, due to the non-specificity of MAD, the cOA degradation can be compromised by RNA as substrate competitors. Indeed, mRNA might be delivered to the periphery of cells for degradation in Sulfolobus and some bacteria, since their RNA degradation is also associated with the cell membrane (Evgenieva-Hackenberg et al., 2011). However, RNA degradation of type III CRISPR signaling pathway might facilitate the cOA degradation by MAD by removing RNA competitors, implying that MAD could more efficiently degrade cOA at the late stage of the type III immune response.

Noticeably, S. solfataricus, from which the first ring nuclease has been identified, encodes a MAD homolog (Sso2457, 94% identity to SiRe_0244). Moreover, biochemical studies have shown that the ring nucleases are not sufficient to degrade high-level cOA in S. solfataricus (Athukoralage et al., 2020a). These findings indicate that S. solfataricus, and probably other related organisms, may also employ the metal-dependent activities to control type III immunity when high-level cOA is synthesized. The reason why the metal-dependent activity was not identified in S. solfataricus is possibly that a metal-free condition and 30% saturation ammonium sulfate treatment have been applied in the experiments (Athukoralage et al., 2018). Currently, we cannot conclude that MAD is the sole metal-dependent membrane-associated cOA-degrading enzyme. Other membrane-associated cOA-degrading enzymes, if any, should act like MAD (i.e., degrading high-level cOA after the ring nuclease to switch off type III immunity). Nevertheless, the specific contributions of the ring nuclease and membrane-associated nuclease to controlling type III immunity in different situations should be clarified by further studies.

We further show that MAD is a general nuclease, with both RNA and DNA as its substrates. This feature resembles the bacterial strand-alone DHH-DHHA1 family proteins, which degrade nanoRNA, pAp, single-strand DNA, and cyclic-di-NMP and play multiple functions (He et al., 2016; Huynh and Woodward, 2016). Therefore, MAD is likely to play additional functions. On the other hand, the subcellular organization of MAD resembles that of GdpP, a membrane-associated protein that specifically degrades cyclic-di-AMP (Rao et al., 2010). In GdpP, the DHH and DHHA domains are fused to a
transmembrane domain. However, as a stand-alone protein, how MAD is associated with the membrane is unknown. Undoubtedly, it will be important to investigate the additional functions of MAD and the mechanism and functional implication of its subcellular location.

DHH family proteins are not usually associated with CRISPR-Cas systems. A few exceptions are those associated or even fused with the divalent cation channel CorA, a transmembrane protein (Shmakov et al., 2018). Nevertheless, a representative DHH protein that is associated with CorA does not cleave cOA (Figure S7), indicating that it plays a different role than Sulfolobus MAD. More interestingly, type III systems tend to be linked with membrane-associated proteins (Shah et al., 2019; Shmakov et al., 2018). It is expected that more functional connections between the type III CRISPR-Cas system and membrane-associated proteins and/or DHH family proteins would be discovered.

In addition to type III CRISPR-Cas systems, many bacteria and some archaea encode numerous and diverse nucleotide-centric second messenger systems (Burroughs et al., 2015; Lowey et al., 2020; Whiteley et al., 2019), many of which are involved in defense against viral infection (Cohen et al., 2019). Different signaling systems, if encoded by the same organism, may cross-talk at the second messenger level. A good example is cA3, which is synthesized by many type III systems (Grüsschw et al., 2019; Kazlauskien et al., 2017) but not sensed by CRISPR accessory CARF domain proteins. Nevertheless, cA3 acts as the signaling molecule to activate a DNase implicated in aborting infection (Lau et al., 2020). For many new second messengers, their effectors and degraders remain to be identified. Given that DHH-DHHA1 nucleases have low substrate specificity, they might function as a common degrader for different second messengers. As numerous new nucleotide-centric second messengers are discovered, identification and characterization of enzymes degrading them would be important for full understanding of bacterial and archaeal defense mechanisms.
REFERENCES


### STAR METHODS

#### KEY RESOURCES TABLE

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RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Wenyuan Han (hanwenyuan@mail.hzau.edu.cn).

Materials Availability
Plasmids and other unique reagents generated in this study are available upon request.

Data and Code Availability
This study did not generate any unique datasets or code.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

The archaeal host S. islandicus E233S1 were grown at 78°C in SCV medium (basic salts medium supplemented with 0.2% sucrose, 0.2% casamino acids, 0.2% uracil and a vitamin mixture (Deng et al., 2009). Uracil was removed when the cells carried a pSeSD-derived expression plasmid. Escherichia coli DH5α and Rosetta (DE3) were used for plasmid cloning and protein expression, respectively. E. coli cells were grown in LB medium, and antibiotics were supplemented when desired.

Expression of SiRe_0244 and its variants, SiRe_1902 and SiRe_0198 was performed in S. islandicus E233S1 and induced by arabinoose. Expression of SiRe_0053, SiRe_0455 and HsaDHH was carried out in Rosetta (DE3) and induced by IPTG.

METHOD DETAILS

Isolation of cOA-degrading fraction from S. islandicus cell extract
Sulfolobus islandicus Rey15A E233S1 (DpyrEF,ΔlacS) (Deng et al., 2009) was grown in SCVU medium (0.2% sucrose, 0.2% Casamino acids, 5 ml/ml of vitamin mixture solution and 20 mg/ml uracil) as describe previously (Han et al., 2017c) and cell mass were collected from 8 l late exponential phase culture by centrifuge at 7000 rpm for 10 min. The cells were resuspended by 50 mL bufferA (20 mM Tris, pH 8.0, 500 mM NaCl) and lysed by French press. Cell debris was removed by centrifuge at 12000 rpm for 20 min at 4°C. The cell extract was supplemented with ammonium sulfate up to 30% saturation and precipitated proteins were separated by centrifuge at 12000 rpm for 20 min at 4°C. Then, the supernatant was stepwise supplemented with more ammonium sulfate up to 40%, 50%, 60%, 75% and 90% saturation and precipitated proteins were pelleted with centrifuge as described above. The precipitated proteins were dissolved by bufferB (20 mM Tris, pH 9.0), dialyzed to bufferB and analyzed by cOA degradation assay.

Then, the 30% saturation fraction was loaded onto a Q FF column (GE Healthcare, Waukesha, WI, USA) and the proteins bound to Q FF was eluted by bufferC (20 mM Tris, pH 9.0, 1 M NaCl). To remove all proteins that are affinitive to Q FF, flow through (FT) fraction was collected and further loaded onto Q FF column for 10 times with bound proteins eluted by bufferC at each time as well. The FT fractions and eluted fractions (high salt elution, HE) were analyzed by cOA degradation assay. The FT fraction from DEAE column was loaded onto a Superdex 200 increase prepacked column (GE Healthcare, Waukesha, WI, USA) pre-balanced with bufferB.

Then, the active fractions (8-, 9-ml) from size exclusion chromatography (SEC) were mixed with 0.5% Triton X-100 and the mixture was incubated for 1 h at 4°C. Then, the mixture was diluted by bufferB for 5 times and loaded onto Q FF column pre-equilibrated with bufferB containing 0.1% Triton X-100. Protein was eluted with a linear gradient of bufferC containing 0.1% Triton X-100 across 5 column volumes (CV). Then, the active fractions were dialyzed to bufferD (20 mM Mes 6.0, 50 mM NaCl) and loaded on to S FF column (GE Healthcare, Waukesha, WI, USA) pre-balanced with bufferD. Bound proteins were eluted by a linear gradient of bufferE (20 mM Mes 6.0, 1 M NaCl, 0.1% Triton X-100) across 5 CV. The active fraction (7#) was sent to Novogene (Beijing, China) for mass spectrometry analysis and used for further activity analysis.

cOA degradation assay
Radioactive cOA was synthesized in a 10 μL mixture containing 20 mM Mes (pH 6.0), 5 mM MnCl₂, 5 mM DTT, ca. 1 nM α32P-ATP (PerkinElmer, Waltham, MA, USA), 100 μM ATP, about 20 nM Cmr-α, and 200 nM target RNA as described previously (Han et al., 2018). The mixture was diluted for 50 times, heated at 95°C for 5 min and directly used as substrate in cOA degradation assay.
The cOA degradation activity of *Sulfolobus*-derived samples, including cell extract, Csx1, Sire_0455, cOA-degrading enzyme candidates, Sire_0244 and its variants, the fractions generated from the isolation process and the fractions isolated by ultra-centrifugation, was analyzed by similar experimental conditions. One μL of radioactive cOA was incubated with these samples in the presence of 20 mM Mes (pH 6.0), 5 mM MnCl₂ and 5 mM DTT at 70°C and the reactions were stopped by addition of 2X RNA loading dye, followed by heated at 95°C for 5 min and cooled on ice. Then, the reaction mixtures were loaded onto a 18% denaturing polyacrylamide gel, and the radioactivity was detected by exposing the gel to a phosphor screen and scanning the screen with a Fujifilm FLA-5100 (FUJIFILM Life Science, Tokyo, Japan). Specifically, 200 nM Csx1 was incubated with cOA for 10, 20 and 40 min (Figure S1A); 2 μL of the fractions generated from the isolation process was incubated with cOA for 20 min (Figure 1); about 0.15 mg/ml cell extract was incubated with 80 nM cOA (Figure 2) and about 0.45 mg/ml cell extract was used for 1 μM and 5 μM cOA (Figure 2); about 3×10⁶ S. islandicus E233S1 cells (intact cells) or the cell extract from equivalent cell mass was incubated with cOA for 10 min (Figure S1C); 1 μL of the gel filtration fractions of cOA degrading enzyme candidates was incubated with cOA for 20 min (Figure S3); 1 μL of the gel filtration fractions of Sire_0244 and its variants was incubated with cOA for 4 min (Figures 5C and S5); about 20 nM of Sire_0244 and its variants were incubated with cOA for 5 min (Figure 5F). To analyze the effects of ssRNA on the cOA degradation by Sire_0244 (Figure S6E), about 80 nM cOA was incubated with 20 nM Sire_0244 in the presence of 1.4 ng/μl of SS1-42 (Table S3). When required, unlabelled cOA was supplemented in the reaction mixtures to a final concentration of 1 μM or 5 μM as indicated. Moreover, EDTA and other metal ions were used to replace MnCl₂ to analyze the metal-dependency of the reaction if applicable.

To analyze the cOA degradation of the *Halonatronum saccharophilum* DHH family protein, 1 μL of radioactive cOA was incubated with 1 μM enzyme in the presence of 50 mM Tris-HCl at pH 7.5, 5 mM MnCl₂, 100 mM NaCl and 5 mM DTT at 37°C for indicated time. Then, the reactions were stopped by addition of 2X RNA loading dye and analyzed by denaturing polyacrylamide gel electrophoresis as described above.

**Nuclease assay of Sire_0244**

To investigate whether Sire_0244 degrades ApA > p, the cleavage product of cA₄ by ring nuclease, we incubated 80 nM cOA with 5 μM Sire_0455 for 20 min in the presence of 20 mM Mes (pH 6.0), 5 mM MnCl₂ and 5 mM DTT at 70°C. Then, an aliquot was heated at 95°C for 5 min, cooled at room temperature, supplemented with 20 nM Sire_0244 and incubated again at 70°C for 5 min. Incubation of cOA with Sire_0244 alone was carried out as control. At last, the reactions were stopped by addition of 2X RNA loading dye and analyzed by denaturing polyacrylamide gel electrophoresis as described above.

To analyze the cleavage of ssRNA, ssDNA, dsRNA and dsDNA by Sire_0244, FAM-labeled ssRNA and ssDNA oligos were purchased from GenScript (Piscataway, NJ, USA) (Table S3). Double-stranded RNA and DNA were generated by annealing of FAM-labeled strand and the complementary strand (Table S3). The RNA complementary strand was generated by *in vitro* transcription using a dsDNA template (Table S3). The reaction mixture contained 20 mM Mes (pH 6.0), 5 mM MnCl₂, 5 mM DTT, 100 nM each substrate and 20 nM Sire_0244. The reaction was stopped at indicated time points by addition of 2X RNA loading dye and heat treatment. Then, the reaction was analyzed by denaturing gel electrophoresis and the gel was scanned by Fujifilm FLA-5100. Remaining substrate was quantified by ImageJ.

**FastAP treatment of cOA cleavage products**

The cOA degradation reaction, containing 1 μL of radioactive cOA, 20 mM Mes (pH 6.0); 5 mM MnCl₂; 5 mM DTT and 2 μL of 7#, was stopped at desired time points by cooling on ice. Then, aliquots of the reaction mixtures were added into 10 μL FastAP reaction mixtures, containing 1 μL of 10X Buffer and 1 μL FastAP (Thermo Fisher Scientific, Waltham, MA, USA), which were then incubated at 37°C for 30 min and analyzed by denaturing gel electrophoresis. Mock treatment using water instead of FastAP was performed as controls. In addition, the cOA cleavage products by Sire_0244 were also treated with FastAP by a similar procedure.

**Isolation and characterization of membrane-enriched fraction**

Cells from 300 mL early exponential phase culture were collected and resuspended by 10 mL of bufferF (20 mM HEPES, pH 8.0, 500 mM NaCl, 20 mM imidazole), followed by sonication to lyse cells. Then, the cell lysate was subjected to centrifuge at 7000 g for 10 min to remove intact cells and cell debris. Membrane-enriched fraction was isolated using a modified published method (Lower et al., 2000; Roppelt et al., 2010). Specifically, the cell extract (CE) was ultracentrifuged at 150,000 g for 75 min at 4°C to generate the cytoplasm-enriched fraction (supernatant, Sup) and membrane-enriched fraction (pellet, P). The pellet was dissolved in 1 mL bufferF containing 0.5% Triton X-100 (Sangon Biotech, Shanghai, China) (P₆M) or 0.1% n-Dodecyl-β-D-Maltopyranoside (DDM, RHAWN, Shanghai, China) (P₃DM) and diluted for 5 times by bufferF. Then, the fractions, including CE, Sup and P, were analyzed by SDS-PAGE, cOA degradase assay and western blot as indicated.

In the cOA degradation assay, the fractions were incubated with 1 μL of radioactive COA and 5 μM unlabelled COA in the presence of 5 mM MnCl₂ or 1 mM EDTA at 70°C for 20 min. Then, the reactions were analyzed as described above. The experiments were repeated at least three times.

**Western blot**

CE, Sup and P (P₆M, P₃DM) fractions of cells expressing His-tagged Sire_0244 were analyzed by SDS-PAGE and proteins separated on the gel were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) using Trans-Blot SD. Semi-Dry Transfer Cell
(Bio-Rad, Hercules, CA, USA). The membrane was blocked by 6% milk, followed by incubation with anti-His antibody (GenScript, Piscataway, NJ, USA) and secondary antibody (Goat Anti-Rabbit IgG, GenScript), successively. After removing unspecific binding, the second antiserum was detected using the Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA). Hybridization signals were recorded by exposure of the membrane to MFChemibis 3.2 imaging device (DNR, Jerusalem, Israel). CE of S. islandicus E233S1 containing pSeSD plasmid (Peng et al., 2017) and semi-purified His-tagged SiRe_0244 after nickel affinity chromatography were set as negative (NC) and positive control (PC), respectively.

**Immunofluorescence microscopy**

To visualize SiRe_0244 in cells, the antiserum against SiRe_0244 was raised in rabbit by DIAAN (Wuhan, China). S. islandicus cells from 3 mL early exponential phase culture were collected by centrifugation at 3000 g for 10 min. The cells were washed with phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4), resuspended in 300 μL PBS buffer. Cold ethanol was added into the cell solution up to a final concentration of 70% (v/v) to fix the cells. Then, the cells were successively incubated in 1 mL PBSM buffer (PBS containing 3% milk) at room temperature for 2 h, 1 mL PBSM buffer containing 1 μl anti-SiRe_0244 antiserum at 4°C for overnight and 1 mL PBSM buffer containing 1 μL commercial secondary antibody Dylight 488 Affinipure Goat anti-Rabbit IgG (H+L) (EarlOx Life Sciences, Millbrae, CA) at room temperature for 2 h. Before each incubation, the PBSM buffer was washed with PBS buffer for three times. After incubation with the secondary antibody, the cells were washed with PBS buffer for three times as well, resuspended in 30 μL PBS containing 0.4 μg/ml DAPI and incubated for 10 min on ice. At last, 3 μL of the cell solution was dropped onto a slide and photos were captured by Leica TCS SP8 STED microscope system.

**Expression and purification of proteins used in the study**

To express COA-degrading enzyme candidates and SiRe_0455, the genes encoding them were amplified using the primers listed in Table S1 from the genomic DNA of S. islandicus E233S1. The primers were synthesized by Tsingke (Beijing, China). The gene fragments were inserted into pET30a and a shuttle vector pSeSD (Peng et al., 2012) (Table S2) to express them in E. coli BL21 Rosetta (DE3) strain and S. islandicus E233S1, respectively. Expression in E. coli BL21 Rosetta (DE3) was induced by 500 mM IPTG at 16 °C for 24 h, while expression in S. islandicus E233S1 was induced by 0.2% arabinose as described previously (Peng et al., 2012). The gene encoding *H. saccharophilum* DHF family protein was synthesized by Tsingke (Beijing, China) and inserted in PET28a (Table S2).

The purification procedure for the protein was similar. Specifically, cell mass of 1 L *E. coli* or 3 L *islandicus* culture was collected by centrifuge. Cells were resuspended in 50 mL bufferF (20 mM HEPES pH 7.5, 20 mM Imidazole, 500 mM NaCl) and lysed by French press. Cell extract was loaded onto a 1 mL HisTrap HP column (GE Healthcare, Waukesha, WI, USA) and His-tagged proteins were eluted with bufferF containing 300 mM imidazole after washing the column with bufferF. Then, the elution fractions were concentrated and further purified by size exclusion chromatography (SEC) in bufferG (20 mM Tris-HCl pH 7.5, 250 mM NaCl), using a Superdex 200 column (GE Healthcare, Waukesha, WI, USA). The fractions collected during SEC were analyzed by SDS-PAGE and COA degradation assay.

SiRe_0244 and its variants were purified with DDM as detergent. Cell extract was prepared from 8 L cells expressing His-tagged SiRe_0244 as described above. Then, the cell extract was mixed with 0.1% DDM at 4 °C for 12 h, followed by loaded onto a 1 mL HisTrap HP column. The column was washed with 50 mL bufferF containing 60 mM imidazole and 0.02% DDM. Then, His-tagged SiRe_0244 was eluted by bufferF containing 300 mM imidazole and 0.02% DDM. Fractions containing His-tagged SiRe_0244 were concentrated and further purified with SEC in bufferG containing 0.02% DDM. The fractions from SEC were analyzed by western blot, SDS-PAGE and COA degradation assay. Plasmids expressing mutated SiRe_0244 were constructed by SOE-PCR using the primers listed in Table S1 and mutated proteins were purified with Ni affinity chromatography and SEC as well. Protein concentration was measured by BCA kit (Thermo Scientific, Waltham, MA, USA) and the concentration of SiRe_0244 was estimated by quantification of the protein band in SDS-PAGE gel.

Csx1 (Han et al., 2017b) and Cmr-α complex (Han et al., 2017a) were expressed and purified as described previously.

**Construction of structural model of SiRe_0244**

The structural model of SiRe_0244 was constructed with Swiss-model (Waterhouse et al., 2018) (https://swissmodel.expasy.org/) using a well-characterized *Mycobacterium tuberculosis* Rv2837c (PDB: 5iju) as the template. The model was constructed as a monomer structure to simplify discussion.

**Csx1 deactivation assay**

Labeled and unlabeled COA was synthesized in the same reaction mixture as described above except that ca. 1 mM 32P-ATP was used for labeled COA synthesis. Then, the reaction mixtures were diluted for 5 times and used as substrate for the COA degradation assay by SiRe_0244, for which the 10-μl reaction mixture contained 20 mM Mes (pH 6.0), 1 mM MnCl2, 5 mM DTT, 2 μl labeled/unlabeled COA and 20 nM SiRe_0244. The reaction was stopped at desired time points by supplementing 10 μl of 2X RNA loading dye (labeled COA) or 5 μl of 10 mM EDTA (unlabeled COA). Then, reactions with labeled COA were analyzed by denaturing gel electrophoresis to monitor COA degradation, while reaction mixtures containing unlabeled COA were tested for their capability to activate Csx1. Specifically, 1.5 μl of the reaction mixtures was added into a 10-μl reaction mixture containing 20 mM Mes pH 6.0, 1 mM EDTA, 5 mM DTT, 100 nM FAM-labeled ssRNA substrate (synthesized by Tsingke, Table S3) and 100 nM Csx1. Reaction mixtures were incubated for 24 h, while expression in *E. coli* strain and *S. islandicus* (DE3) were induced by 0.2% arabinose as described previously (Peng et al., 2012). The gene encoding *H. saccharophilum* DHF family protein was synthesized by Tsingke (Beijing, China) and inserted in PET28a (Table S2).
at 70°C for 20 min and analyzed by denaturing gel electrophoresis. The gel was scanned by Fujifilm FLA-5100. Remaining ssRNA substrate in the presence of each treated cOA ([S]_treated cOA) was quantified to calculate Csx1 activity. Moreover, remaining substrate in the presence of mock-treated cOA by bufferG containing 0.02% DDM instead of SiRe_0244 and in the absence of cOA was also quantified as positive control ([S]_PC) and negative control ([S]_NC), respectively. The Csx1 activation by each treated cOA relative to mock-treated cOA was calculated by the following equation:

\[ \text{Relative activation} = \frac{[S]_{NC} - [S]_{treated cOA}}{[S]_{NC} - [S]_{PC}} \]

The resulting data were plotted against cOA treatment time points and the derived cure was fitted into an exponential curve by Origin-Pro 2017 software (OriginLab Co., Northampton, MA, USA) to calculate the rate constant of cOA degradation and Csx1 deactivation. The experiments were performed in three independent repeats.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Quantification of remaining substrate in the cOA degradation assay was performed with ImageJ. Fitting was carried out with Origin-Pro 2017 software. Error bars show standard deviations of three independent experiments.