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RNA activation-independent DNA targeting of the Type III CRISPR-Cas system by a Csm complex

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Transaction Report: This manuscript was transferred from *The EMBO Journal*, where it was originally reviewed. The following report contains those referee comments that were relevant at the time of transfer.

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 28 November 2016

Thank you for the transfer of your reviewed manuscript to EMBO reports. Given that the referees who reviewed your study for The EMBO Journal were not in agreement, I sent it to one more referee, or arbitrator. I also asked referee 1 and 3 for cross-comments on the major points of referee 2. All three reports and the cross-comments are pasted below.

As you will see, both referees 1 and 3 support publication of the study here, and only ask for rather minor revisions. However, they also agree with referee 2 that her/his major concerns should be addressed experimentally. Referee 1 agrees that the first major concern of referee 2 can and should be addressed, and referee 3 agrees that the second major concern should be addressed. I therefore think that both concerns should be addressed experimentally. Please let me know if you disagree, and we can discuss this further. All other suggestions also need to be addressed.

I also noticed that many gels and blots are overexposed, please send us figures with better images, or include original gel/blot images as source data.

I would therefore like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the

completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

The literature on type III CRISPR-Cas complexes (a.k.a. Csm and Cmr complexes) - particularly their targeting activities - has been bewildering in many regards, especially their ability to target RNA, DNA, or both, their requirement for ongoing transcription for DNA targeting, the active site(s) for DNA cleavage, the requirement for RNA target pairing in the allosteric activation of DNA cleavage, the existence (or not) of a protospacer adjacent motif (PAM), the nature of self-nonself discrimination, and other aspects. Consensus has emerged that type III-A/Csm (and III-B/Cmr) systems are indeed dual RNA- and DNA-targeting systems, but even with that general agreement, the other aspects of effector function noted above remain murky, perhaps as a result of genuine functional differences among orthologous systems from different species/strains. In this manuscript, Park et al. analyze the assembly and function of the type III-A/Csm complex from Thermococcus onnurineus, with an emphasis on its DNA targeting activity. Previously, the S. epidermidis Csm complex was shown to have DNA cleavage activity that required ongoing transcription across the targeted region and that depended upon Cas10 Palm domain residues, whereas the S. thermophilus Csm complex was shown to have ssDNA cleavage activity that was cotranscription-independent, required Cas10 HD domain residues, and was suppressed in the absence of RNA target. In the latter case, the RNA-activated DNase activity was non-specific, i.e. the ssDNA was cleaved whether or not it included a crRNA-complementary protospacer.

In T. onnurineus, the authors muddy the waters further by showing that the ToCsm complex exhibits ssDNA cleavage activity that (like in S. thermophilus) is transcription-independent and HD-domain dependent, but this time with no requirement for an RNA target to activate it. Strikingly, however, cleavage only occurs with a DNA that is complementary to the crRNA, and is suppressed when the flanking sequence is made complementary to the crRNA's 5'-tag (previously implicated as a determinant of self-nonself discrimination). The clear implication is that in this instance, the crRNA (within the context of the ToCsm complex) can pair with (and then cleave) the DNA target directly. Along the way they show that ToCsm complex assembly can proceed via discrete subcomplexes that then assemble around the processed crRNA.

Overall, the results are convincing and are likely to have sizable impact on the field. There are a few considerations that could improve the manuscript further.

1. The authors do not address whether interference in cells depends upon ongoing transcription, and whether it requires the HD domain and/or the Palm domain. This is important to consider in relating the current results to the one type III-A system (S. epidermidis) that has been analyzed in depth both genetically and biochemically. It is not clear whether these experiments are feasible in T. onnuirineus itself (probably not), but they are likely doable in E. coli given that the components are well expressed. The experiments about this point are not needed for the current manuscript, but the question of transcription dependence in cells should at least be raised and discussed.

2. There is abundant information available regarding the number of non-template DNA strand nucleotides that are unwound and exposed in prokaryotic transcription bubbles, and that number is considerably smaller than the length of the protospacer. In light of this, the authors should speculate about how this potential difficulty could be circumvented in this model.

3. Figure 3D vs. Figs. 3A-C: the authors clearly show that the HDm and HD/DDm Cas10 mutants are cleavage-defective, but they don't test whether this defect arises due to an inability to bind the target ssDNA (as done for wild-type in Fig. 3A). This should be rectified.

Refere #2:

Papers published recently by three different groups reported that the transcription-dependent DNA silencing by the Type III CRIPSR-Cas system is mediated by the target RNA-activated DNA cleavage activity of Csm/Cmr effector complexes. In the submitted manuscript Woo et al. have confirmed this mechanism by demonstrating the target RNA-activated DNA cleavage activity in vitro for the reconstituted Thermococcus onnurineus Type III-A effector (ToCsm) complex. Consistent with previous reports authors showed that the ssDNA cleavage activity is dependent on the HD-nuclease domain of the ToCsm1 subunit. Authors further claim that they have identified a novel ssDNA cleavage activity independent on the RNA target. Specifically, they show that ToCsm cuts ssDNA containing a target sequence complimentary to crRNA in the absence of the activating target RNA. Authors conclude that this DNA cleavage activity could contribute to the interference against invading nucleic acids by direct ToCsm-mediated cleavage of the DNA target sequence bound to the crRNA. While experiments provided in the manuscript indeed demonstrate ssDNA cleavage independently of target RNA, data does not convincingly show that ToCsm DNA cleavage activity is biologically relevant. The current manuscript version does not significantly advance our understanding of the Type III CRISPR/Cas immunity and should be published in a more specialized journal.

Major problems:

1. Authors show that ToCsm binds target ssDNA target with Kd ~ 1 nM. Although target RNA binding by ToCsm is demonstrated by EMSA, the Kd value for the target RNA binding has to be provided. If ToCsm binds RNA target in pmol range, the gel shift experiments should be performed at low concentrations of radiolabeled RNA. Moreover, binding competition experiments must be performed to compare binding affinities of ssDNA and RNA. During DNA transcription stretches of ssDNA and nascent RNA may become available for ToCsm complex binding through basepairing with crRNA. ToCsm complex partitioning between the two alternative ssDNA and RNA targets will depend on the Kd values for the matching ssDNA and RNA sequences. One cannot exclude that due the differences in RNA and DNA binding affinities ToCsm will predominantly bind to the target RNA and trigger RNA-activated DNA cleavage pathway instead of the direct ssDNA cleavage. This raises the question whether the ssDNA cleavage activity reported in the manuscript ToCsm activity is biologically relevant.

2. In the manuscript authors provide evidence that under the large excess of the To Csm complex in respect to ssDNA, target ssDNA is cleaved by ToCsm in the absence of the activating target RNA, and demonstrate that active site in the HD-domain is responsible for the cleavage. Authors, however, also have to show whether binding of the ssDNA activates the non-target DNA degradation at the HD-domain. To address this question authors have to analyse in trans cleavage of ssDNA lacking the target site by the ToCsm bound to the complimentary ssDNA and compare it to the RNA-activated ssDNA degradation by the ToCsm. Data provided in the manuscript does not exclude that after ToCsm cleavage of ssDNA containing the complementary sequence, ToCsm remains bound to the product and is trapped in the catalytically-dead complex that is unable to turnover ssDNA at the HD domain. In theory, such complex could interfere with non-template ssDNA strand cleavage by the transcript-activated HD-domain (Figure 5).

Minor comments:

1) ToCsm and DNA concentrations must be provided in the Figure legends.
2) Some of the figures are redundant. Consider transfer of Figure 2C; Figure 3 E and F; Figure 4D sections into an extended view section. Data on Csm1 mutations on cleavage activities could also be transferred to an extended view section.
3) Autocorrect function presumably skewed the names of organisms throughout the text: T. thermophilus and S. thermophilus became "T./S.thermophiles" such as in p3/24, p6/24, p8/15), P. furiosus became "P. furious" on p8/16. Please write full names of the organisms when first mentioned in the text (p3/24 and elsewhere).
4) References cited in the text sometimes indicate two first authors, sometimes only one (for instance, p8/13 Jung, An et al, 2015 and p8/24 Jung, et al, 2015), which is confusing. Please unify

according to journal requirements.
5) Please provide values for concentration/time (Figures 2A, 3A-D, 4A, EV5B) either in figures or figure legends. Otherwise, it becomes difficult to compare experiments (e.g., Figure 3A and EV5B). Concentration of target RNA used in the experiment is not specified in Figure 3C.
6) P3/9 Currently, CRISPR-Cas systems are classified into 6 types, not 5 (Shmakov, et al, 2015). A more recent review could be cited (Mohanraju, et al, 2016).
7) P3/13 Mistype: "II/IV" instead of "II/V".
8) P3/20-21 citation seems inconsistent: either cite only the papers that show that both RNA and DNA are targeted by the same Type III system (such as Samai, et al, 2015) or cite RNA targeting papers (Hale, et al, 2009; Tamulaitis, et al, 2014; Staals, et al, 2014) in addition to the DNA targeting ones (Hatoum-Aslan, et al., 2014 and Marraffini & Sontheimer, 2008).
9) P3/25 Please include reference for recently characterized a Type III-A CRISPR-Cas system from S. aureus (Cao, et al, 2016).
10) P4/10 Inaccurate citation: the first paper to demonstrate that RNA is cleaved by the Cmr4 protein in the Cmr complex was Benda, et al, 2014, not Hale, et al, 2014; in case of Csm complex, Tamulaitis, et al, 2014 should be cited alongside Staals, et al, 2014.
11) P4/16 Samai et al paper did not show RNA involvement in the DNA cleavage but reveals the requirement for a coupled transcription, therefore "transcription-coupled" would be a more appropriate phrase than "RNA-coupled".
12) Text does not refer to Figure EV4A anywhere.
13) P12/2-3 The pioneering work on self vs non-self discrimination by Marraffini and Sontheimer discuss the importance of the crRNA 5'-handle non-complementarity only to DNA but not RNA. Therefore either the "RNA-directed" phrase should be omitted from the sentence, or the citation should be expanded appropriately.
14) P13/11 Should be "Csm1" instead of "Cms1".
15) MALS experiment is not described in the "Materials and Methods" section.
16) P24/18 Should be "M13mp18" instead of "M15mp18" instead of.
17) P29 Figure EV6 description: please specify which DNA substrates were used.
18) Please denote the 5' and 3' ends in all the sequences, listed in Table EV1, and consider aligning the text to left, instead of the center.

Referee #3:

Here the authors reconstitute the type IIIA CRISPR effector complex from T. onnurineus and test its biochemical properties in vitro. The complex is one of the smallest type III systems known. RNA is cleaved by backbone cleavage as for all other type III systems studied (Fig 2), and there is also an RNA-activated DNA nuclease activity, non-sequence dependent and mediated by the HD nuclease domain (Fig 2D). crRNA dependent binding of cognate target ssDNA is also demonstrated (Fig3), as is crRNA dependent degradation of ssDNA (Fig3). The nuclease activity is shown to target an area downstream of the sequence match and to be abolished when the 5'-handle is complementary to the target (Fig4).

Overall there is enough of interest here to cater for the generalist audience of EMBO Reports. The literature for Type III systems is already quite complex, and this work suggests that a wider range of activities could exist than seen in other model systems. The quality of the DNA presented and their interpretation stands comparison with several other published studies.

Specific points:

1. Page 6 line 25 "The complex was significantly smaller than that of S. epidermidis or T. thermophiles (Rouillon et al., 2013, Staals et al., 2014) (Figure 1F)"
The complex shown here is from S. solfataricus, not S. epidermidis, though the reference is correct.
2. Quite a number of species names have typos - please check carefully.
3. Figure 2A-C are largely superfluous as the backbone mediated cleavage of type III CRISPR systems is now very well understood. These could be moved to supplemental data to save space.
4. Data in figure 3 should not be described as "RNA-independent ssDNA cleavage", as cognate crRNA is needed. Please revise.
5. Data and model in figure 5 suggest that ToCsm should cut ssDNA whenever it is encountered. This could include DNA replication of target sequences as well as transcription - in fact this could

be more likely. This would rule out a role in the targeting of integrated phage entering a lytic cycle though. Please discuss.

Cross-comments from Referee #1:

I agree with referee #2 that the first major point (the apparent Kd of ToCsm for target RNA, and how it compares to that with DNA) is reasonable, doable, and within the scope of the current study. It is far less clear to me that major point #2 is a substantial concern, and I feel it would be most fair to the authors to leave that suggested experiment to follow-up studies for future manuscripts.

Cross-comments from Referee #3:

1. Authors show that ToCsm binds target ssDNA target with Kd ~ 1 nM. Although target RNA binding by ToCsm is demonstrated by EMSA, the Kd value for the target RNA binding has to be provided. If ToCsm binds RNA target in pmol range, the gel shift experiments should be performed at low concentrations of radiolabeled RNA. Moreover, binding competition experiments must be performed to compare binding affinities of ssDNA and RNA. During DNA transcription stretches of ssDNA and nascent RNA may become available for ToCsm complex binding through basepairing with crRNA. ToCsm complex partitioning between the two alternative ssDNA and RNA targets will depend on the Kd values for the matching ssDNA and RNA sequences. One cannot exclude that due the differences in RNA and DNA binding affinities ToCsm will predominantly bind to the target RNA and trigger RNA-activated DNA cleavage pathway instead of the direct ssDNA cleavage. This raises the question whether the ssDNA cleavage activity reported in the manuscript ToCsm activity is biologically relevant.

I think this point can be addressed in the discussion rather than with new experiments. There are after all situations where there will be target DNA but no target RNA (no transcription). We will not know whether any of the activities reported are biologically relevant, but this is true for almost the whole type III literature so I am content with the data presented and a further discussion to address point 1.

2. In the manuscript authors provide evidence that under the large excess of the To Csm complex in respect to ssDNA, target ssDNA is cleaved by ToCsm in the absence of the activating target RNA, and demonstrate that active site in the HD-domain is responsible for the cleavage. Authors, however, also have to show whether binding of the ssDNA activates the non-target DNA degradation at the HD-domain. To address this question authors have to analyse in trans cleavage of ssDNA lacking the target site by the ToCsm bound to the complimentary ssDNA and compare it to the RNA-activated ssDNA degradation by the ToCsm. Data provided in the manuscript does not exclude that after ToCsm cleavage of ssDNA containing the complementary sequence, ToCsm remains bound to the product and is trapped in the catalytically-dead complex that is unable to turnover ssDNA at the HD domain. In theory, such complex could interfere with non-template ssDNA strand cleavage by the transcript-activated HD-domain (Figure 5).

This is a reasonable point and could be addressed by the authors with a simple experiment.

1st Revision - authors' response 26 January 2017

We are very grateful for the evaluation of our manuscript by the three reviewers and for their constructive comments on our work. We have considered all the points raised by the reviewers and performed additional experiments. In particular, we investigated the binding of the ToCsm complex and revealed a high affinity of the effector complex to the target ssDNA comparable to the target RNA that was confirmed by EMSA competition experiment. We also performed the trans- cleavage assay of the ToCsm complex and demonstrated that the binding of the target ssDNA activates the DNA degradation at the HD-domain of the ToCsm complex, addressing the two major concerns raised by one of the reviewers. Along these comments, we have introduced several changes to the manuscript, which we believe have resulted in a considerable improvement. All these modifications are detailed in our point-by-point responses.

POINT BY POINT RESPONSE

Referee #1

The literature on type III CRISPR-Cas complexes (a.k.a. Csm and Cmr complexes) - particularly their targeting activities - has been bewildering in many regards, especially their ability to target RNA, DNA, or both, their requirement for ongoing transcription for DNA targeting, the active site(s) for DNA cleavage, the requirement for RNA target pairing in the allosteric activation of DNA cleavage, the existence (or not) of a protospacer adjacent motif (PAM), the nature of self-nonself discrimination, and other aspects. Consensus has emerged that type III-A/Csm (and III-B/Cmr) systems are indeed dual RNA- and DNA-targeting systems, but even with that general agreement, the other aspects of effector function noted above remain murky, perhaps as a result of genuine functional differences among orthologous systems from different species/strains. In this manuscript, Park et al. analyze the assembly and function of the type III-A/Csm complex from Thermococcus onnurineus, with an emphasis on its DNA targeting activity. Previously, the S. epidermidis Csm complex was shown to have DNA cleavage activity that required ongoing transcription across the targeted region and that depended upon Cas10 Palm domain residues, whereas the S. thermophilus Csm complex was shown to have ssDNA cleavage activity that was cotranscription-independent, required Cas10 HD domain residues, and was suppressed in the absence of RNA target. In the latter case, the RNA-activated DNase activity was non-specific, i.e. the ssDNA was cleaved whether or not it included a crRNA-complementary protospacer.

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Overall, the results are convincing and are likely to have sizable impact on the field. There are a few considerations that could improve the manuscript further.

1. The authors do not address whether interference in cells depends upon ongoing transcription, and whether it requires the HD domain and/or the Palm domain. This is important to consider in relating the current results to the one type III-A system (S. epidermidis) that has been analyzed in depth both genetically and biochemically. It is not clear whether these experiments are feasible in T. onnuirineus itself (probably not), but they are likely doable in E. coli given that the components are well expressed. The experiments about this point are not needed for the current manuscript, but the question of transcription dependence in cells should at least be raised and discussed.

We appreciate the overall positive and constructive comments from the reviewer. The HD domain of Csm1 is obviously essential for the target RNA independent cleavage activity of ToCsm complex as shown in the manuscript. Considering the ambiguous activities reported by some groups, however, the functional involvement of the Palm domain in targeting remains to be elucidated. We believe that the proposed direct targeting mechanism in T.onnurineus would require transcription process as the transcription dependent DNA cleavage pattern observed in the Type III-A system of S. epidermis (Samai et al, 2015) could be explained well by our results. According to RNA transcript-dependent nuclease mechanism, the non-template strand, in principle, could be cleaved at random positions far apart from the target sequence. Because of the progression of the RNA polymerase, the non-template DNA should be cleaved at a position distant from the target DNA site, unless the effector complex is physically bound to the RNA polymerase and the generated RNA transcript is directly fed into target binding site of the effector complex. However, the Type IIIA complex in S. epidermis was reported to cleave the non-template DNA strand with residual 8 nt and/or 14 nt at the 3' flanking side of the target (Samai et al, 2015). This short residual distance between the position 1 of the protospacer (3' flanking side of the target) and the cleavage site could be interpreted as a result of the direct binding of the target DNA to the effector complex, which correlates with our

observation of the cleavage at the 14 ~ 16 nt away from the position 1 of the protospacer, being consistent to the structural analysis. We briefly described this point in the discussion section of the revised manuscript. It is currently not doable to study the interference function of the ToCsm effector complex inside T.onnurineus, due to the complicated physiological conditions and slow growth of the extremophile. Since the ToCsm complex requires a high temperature for activity (above 50iC), it is also difficult to investigate the cleavage mechanism in E.coli system. We are currently seeking to address the question of transcription dependency of the ToCsm complex in cell.

2. There is abundant information available regarding the number of non-template DNA strand nucleotides that are unwound and exposed in prokaryotic transcription bubbles, and that number is considerably smaller than the length of the protospacer. In light of this, the authors should speculate about how this potential difficulty could be circumvented in this model.

Both the Type I -Cascade and Type II -Cas9 are known to recognize the target DNA on a seed region first, 6~12nt, rather than to bind the whole sequence at a time (Anders et al, 2014; Hayes et al, 2016). Likewise, we speculate that the ToCsm complex could initially recognize a short span of the target ssDNA in the transcription bubble displaced by RNA polymerase as a seed region and sequentially interact to the whole target sequence while the RNA polymerase progress and expose the non-template strand further. We modified the discussion section of the revised manuscript accordingly.

3. Figure 3D vs. Figs. 3A-C: the authors clearly show that the HDm and HD/DDm Cas10 mutants are cleavage-defective, but they don't test whether this defect arises due to an inability to bind the target ssDNA (as done for wild-type in Fig. 3A). This should be rectified.

According to the comment, we performed the EMSA analysis for the effector complexes with mutant forms of Cas10, HDm and HD/DDm, and demonstrated that the ToCsm complexes of these mutants do not have any defect in its ability to bind the target ssDNA (Figure EV4F).

Refere #2:

Papers published recently by three different groups reported that the transcription-dependent DNA silencing by the Type III CRIPSR-Cas system is mediated by the target RNA-activated DNA cleavage activity of Csm/Cmr effector complexes. In the submitted manuscript Woo et al. have confirmed this mechanism by demonstrating the target RNA-activated DNA cleavage activity in vitro for the reconstituted Thermococcus onnurineus Type III-A effector (ToCsm) complex. Consistent with previous reports authors showed that the ssDNA cleavage activity is dependent on the HD-nuclease domain of the ToCsm1 subunit. Authors further claim that they have identified a novel ssDNA cleavage activity independent on the RNA target. Specifically, they show that ToCsm cuts ssDNA containing a target sequence complimentary to crRNA in the absence of the activating target RNA. Authors conclude that this DNA cleavage activity could contribute to the interference against invading nucleic acids by direct ToCsm-mediated cleavage of the DNA target sequence bound to the crRNA. While experiments provided in the manuscript indeed demonstrate ssDNA cleavage independently of target RNA, data does not convincingly show that ToCsm DNA cleavage activity is biologically relevant. The current manuscript version does not significantly advance our understanding of the Type III CRISPR/Cas immunity and should be published in a more specialized journal.

Major problems:

1. Authors show that ToCsm binds target ssDNA target with Kd ~ 1 nM. Although target RNA binding by ToCsm is demonstrated by EMSA, the Kd value for the target RNA binding has to be provided. If ToCsm binds RNA target in pmol range, the gel shift experiments should be performed at low concentrations of radiolabeled RNA. Moreover, binding competition experiments must be performed to compare binding affinities of ssDNA and RNA. During DNA transcription stretches of ssDNA and nascent RNA may become available for ToCsm complex binding through basepairing with crRNA. ToCsm complex partitioning between the two alternative ssDNA and RNA targets will depend on the Kd values for the matching ssDNA and RNA sequences. One cannot exclude that due the differences in RNA and DNA binding affinities ToCsm will predominantly bind to the target RNA and trigger RNA-activated DNA cleavage pathway instead of the direct ssDNA cleavage. This raises the question whether the ssDNA cleavage activity reported in the manuscript ToCsm activity

is biologically relevant.

We appreciate the valuable points raised by the reviewer and the experimental suggestions. As suggested by the reviewer, we performed additional binding experiments of the ToCsm complex toward the target RNA and the target ssDNA, simultaneously. As shown in Figure 3A, EV4D and EV4E, the EMSA analysis revealed that the binding affinity of the ToCsm complex to target ssDNA is similar to the affinity to target RNA, exhibiting approximate Kd value of 1.5 nM (ssDNA) vs 1.3 nM (RNA). We carried out the binding competition experiment with increase of the ToCsm complex concentration, which consistently showed a similar level of interaction to the target ssDNA as to the target RNA (Figure 3D and EV4G,H). We included the experimental results in the revised manuscript accordingly. This high affinity of ToCsm complex toward the target ssDNA is different from the previous report of the SeCsm complex (Tamulaitis et al, 2014) and suggest that the binding activity of ToCsm complex to the target ssDNA is biologically important, which needs further investigation in cells. Alternatively, we cannot exclude the possibility that the ToCsm complex may play a role in the bacterial immunity against ssDNA virus, instead of targeting dsDNA. Recently, some Cas9s were reported to have much higher affinity toward to ssDNA than to dsDNA, revealing the significant divergence of the Crispr system (Ma et al, vol 60, 398-407, Mol. cell, 2015).

2. In the manuscript authors provide evidence that under the large excess of the To Csm complex in respect to ssDNA, target ssDNA is cleaved by ToCsm in the absence of the activating target RNA, and demonstrate that active site in the HD-domain is responsible for the cleavage. Authors, however, also have to show whether binding of the ssDNA activates the non-target DNA degradation at the HD-domain. To address this question authors have to analyse in trans cleavage of ssDNA lacking the target site by the ToCsm bound to the complimentary ssDNA and compare it to the RNA-activated ssDNA degradation by the ToCsm. Data provided in the manuscript does not exclude that after ToCsm cleavage of ssDNA containing the complementary sequence, ToCsm remains bound to the product and is trapped in the catalytically-dead complex that is unable to turnover ssDNA at the HD domain. In theory, such complex could interfere with non-template ssDNA strand cleavage by the transcript-activated HD-domain (Figure 5).

In order to prove that the binding of the target ssDNA activates the non-specific DNA degradation at the HD-domain of the ToCsm complex, we performed the trans-cleavage assay as suggested by the reviewer. First, the ToCsm complex was preloaded with a short 40-nt ssDNA or RNA containing a target sequence that is complementary to the guide sequence of the bound crRNA. These nucleotides shared the same sequence and contained a 5-nt 3' flanking region which is not complementary to the 5' handle of the bound crRNA. The both ToCsm complexes degraded the non-target X174 plasmid DNA (Fig EV5D). Notably, the ToCsm complex containing ToCsm1 (HDm) or ToCsm1 (HD/DDm) could not degrade the non-target DNA, while the effector complex containing ToCsm1 (DDm) degraded this substrate. Next, we performed the same assay with the ToCsm complex loaded with a target ssDNA or RNA lacking the 3' flanking sequence. The both ToCsm complexes could not degrade the plasmid DNA (Fig EV5E). These data clearly demonstrate that binding of the target ssDNA with a 3' flanking sequence that does not base pair with the 5' handle of the bound crRNA activates the nuclease activity of the HD domain. The ratio of protein to substrate used in the cleavage assay was 300:1 (radio-labelled substrate) or 6:1 (unlabeled substrate) in our study, whereas it was 200~250 : 1 (radio-labelled) or 5:1 (unlabeled) in the previously reported biochemical assays (Elmore et al, 2016; Estrella et al, 2016; Kazlauskiene et al, 2016).

Minor comments:

1) ToCsm and DNA concentrations must be provided in the Figure legends.
According to the comment, concentrations of the protein and the substrate used in each experiment were clearly indicated in the material and method section, but not in Figure legends due to the EMBO guidelines; "Experimental details should, where possible, be given in the Materials and Methods section, and not repeated in the figure legends." (page 18, line 13/ 22/ 24) (page 19, line 2/ 5/ 6/ 10/11/13).

2) Some of the figures are redundant. Consider transfer of Figure 2C; Figure 3 E and F; Figure 4D sections into an extended view section. Data on Csm1 mutations on cleavage activities could also be

transferred to an extended view section.
According to the suggestion, we transferred the Figure 2C to the extended view section and rearranged some figures. We kept some of the commented figures in the main section, because it is the first report of Csm complex from T.onnurineus that exhibits the smallest size and subunit ratio. Showing detailed characterization of the nuclease activity of the effector complex may help to identify and understand the target ssDNA cleavage mechanism.

3) Autocorrect function presumably skewed the names of organisms throughout the text: T. thermophilus and S. thermophilus became "T./S.thermophiles" such as in p3/24, p6/24, p8/15), P. furiosus became "P. furious" on p8/16. Please write full names of the organisms when first mentioned in the text (p3/24 and elsewhere).
Names of the organisms were corrected accordingly, and the full names were indicated when mentioned for the first time (page 3, line 23/ 24) (page 6, line 23) (page 8, line 12/ 13).

4) References cited in the text sometimes indicate two first authors, sometimes only one (for instance, p8/13 Jung, An et al, 2015 and p8/24 Jung, et al, 2015), which is confusing. Please unify according to journal requirements.
Citations of the references were corrected accordingly (page 8, line 10/ 20).

5) Please provide values for concentration/time (Figures 2A, 3A-D, 4A, EV5B) either in figures or figure legends. Otherwise, it becomes difficult to compare experiments (e.g., Figure 3A and EV5B). Concentration of target RNA used in the experiment is not specified in Figure 3C.
According to the comment, the reaction conditions and concentrations were indicated as much as possible in figure, figure legend (page 26, line 8) (page 26, line 23) (page 27, line 17) (page 31, line 16~18) and the material and method (page 18, line 2) (page 18, line 13/ 22/ 24) (page 19, line 2/ 5/ 6/ 11/13), respectively. We included additional cartoons for clarity (Figure 3A-D, EV4G and EV4H).

6) P3/9 Currently, CRISPR-Cas systems are classified into 6 types, not 5 (Shmakov, et al, 2015). A more recent review could be cited (Mohanraju, et al, 2016).
According to the comment, we changed the classification to 6 types and the recent review paper was included in the reference (page3 , line9).

7) P3/13 Mistype: "II/IV" instead of "II/V".
It was corrected (page3, line11).

8) P3/20-21 citation seems inconsistent: either cite only the papers that show that both RNA and DNA are targeted by the same Type III system (such as Samai, et al, 2015) or cite RNA targeting papers (Hale, et al, 2009; Tamulaitis, et al, 2014; Staals, et al, 2014) in addition to the DNA targeting ones (Hatoum-Aslan, et al., 2014 and Marraffini & Sontheimer, 2008).
According to the comment, we changed the sentence and cited relevant papers of RNA or/and DNA targeting as references: "the Type III system targets RNA or/and DNA (Hale et al, 2009; Hatoum-Aslan et al, 2014; Marraffini & Sontheimer, 2008; Peng et al, 2015; Samai et al, 2015; Staals et al, 2014; Tamulaitis et al, 2014)" (page3 , line18~20)

9) P3/25 Please include reference for recently characterized a Type III-A CRISPR-Cas system from S. aureus (Cao, et al, 2016).
The recent paper of the Type III-A CRISPR-Cas system from S. aureus was included for reference. (page3, line24)

10) P4/10 Inaccurate citation: the first paper to demonstrate that RNA is cleaved by the Cmr4 protein in the Cmr complex was Benda, et al, 2014, not Hale, et al, 2014; in case of Csm complex, Tamulaitis, et al, 2014 should be cited alongside Staals, et al, 2014.
The citation was corrected accordingly: "The Csm/Cmr complexes possess an RNase activity that cleaves target RNAs at the complementary guide region of crRNA at 6-nt intervals by means of multiple copies of Csm3 in the Csm complex (Staals et al, 2014; Tamulaitis et al, 2014) or Cmr4 in the Cmr complex (Benda et al, 2014)" (page4, line9).

11) P4/16 Samai et al paper did not show RNA involvement in the DNA cleavage but reveals the requirement for a coupled transcription, therefore "transcription-coupled" would be a more

appropriate phrase than "RNA-coupled".
The description was changed accordingly (page4 , line16).

12) Text does not refer to Figure EV4A (revised EV3A) anywhere.
We included an additional description for the figure EV3A accordingly (page7 , line12)

13) P12/2-3 The pioneering work on self vs non-self discrimination by Marraffini and Sontheimer discuss the importance of the crRNA 5'-handle non-complementarity only to DNA but not RNA. Therefore either the "RNA-directed" phrase should be omitted from the sentence, or the citation should be expanded appropriately.
According to the comment, we changed the text about the self vs non-self discrimination and added references: "In previous studies, the complementarity of the repeat-derived 5í-handle of the crRNA was reported to play a key role in self versus non-self discrimination during immunity in Type III system (Marraffini & Sontheimer, 2010) and the base-paring between crRNA 5í-handle and the target RNA 3í-flanking sequence inhibited the nonspecific nuclease activity of the Csm1 in the effector complex (Kazlauskiene et al, 2016)". (page12 , line1~6).

14) P13/11 Should be "Csm1" instead of "Cms1".
It was corrected (page13 , line21).

15) MALS experiment is not described in the "Materials and Methods" section.
According to the comment, we added SEC-MALS section in Materials and Methods (page16 , line8).

16) P24/18 Should be "M13mp18" instead of "M15mp18."
It was corrected (page27 , line7).

17) P29 Figure EV6 (revised EV5C) description: please specify which DNA substrates were used.
According to the comment, we specified the DNA substrate in the figure EV5C and added additional description sentence.

18) Please denote the 5' and 3' ends in all the sequences, listed in Table EV1, and consider aligning the text to left, instead of the center.
It was corrected as suggested (Table EV1)

Referee #3:

Here the authors reconstitute the type IIIA CRISPR effector complex from T. onnurineus and test its biochemical properties in vitro. The complex is one of the smallest type III systems known. RNA is cleaved by backbone cleavage as for all other type III systems studied (Fig 2), and there is also an RNA-activated DNA nuclease activity, non-sequence dependent and mediated by the HD nuclease domain (Fig 2D). crRNA dependent binding of cognate target ssDNA is also demonstrated (Fig3), as is crRNA dependent degradation of ssDNA (Fig3). The nuclease activity is shown to target an area downstream of the sequence match and to be abolished when the 5'-handle is complementary to the target (Fig4). Overall there is enough of interest here to cater for the generalist audience of EMBO Reports. The literature for Type III systems is already quite complex, and this work suggests that a wider range of activities could exist than seen in other model systems. The quality of the DNA presented and their interpretation stands comparison with several other published studies.

We appreciate the overall positive and constructive comments from the reviewer.

Specific points:

1. Page 6 line 25 "The complex was significantly smaller than that of S. epidermidis or T. thermophiles (Rouillon et al., 2013, Staals et al., 2014) (Figure 1F)." The complex shown here is from S. solfataricus, not S. epidermidis, though the reference is correct.
It was corrected as commented (page6 , line23).

2. Quite a number of species names have typos - please check carefully.
We carefully checked typos and corrected them in the revised manuscript (page 3, line 23/ 24) (page 6, line 23) (page 8, line 12/ 13).

3. Figure 2A-C are largely superfluous as the backbone mediated cleavage of type III CRISPR systems is now very well understood. These could be moved to supplemental data to save space.
According to the suggestion, we moved the Figure 2B and 2C to supplemental data and rearranged some figures. We kept the other figures in the main section, because it is the first report of Csm complex from T.onnurineus that exhibits the smallest size and subunit ratio.

4. Data in figure 3 should not be described as "RNA-independent ssDNA cleavage", as cognate crRNA is needed. Please revise.
According to the suggestion, we changed it to either "RNA transcript-independent ssDNA cleavage" or "target RNA -independent ssDNA cleavage" throughout the manuscript.

5. Data and model in figure 5 suggest that ToCsm should cut ssDNA whenever it is encountered. This could include DNA replication of target sequences as well as transcription - in fact this could be more likely. This would rule out a role in the targeting of integrated phage entering a lytic cycle though. Please discuss.
As reviewer pointed out, the ToCsm may theoretically be able to cut any exposed single strand DNA based on the in vitro experiments. However, due to the abundant population of the single strand DNA binding protein (SSB) and its high affinity, the ToCsm complex may be difficult to locate the exposed protospacer region of the single strand in cell during the DNA replication process. Therefore, we speculate that the ToCsm complex may function mainly on transcription but not on DNA replication. This idea was supported by the report that the type III-A of S.epidermis can prevent lytic infection but tolerate lysogenization by temperate phages (Goldberg et al, 2014). We briefly described this point in the discussion section (page15 , line5~9).

Cross-comments from referee 1:

I agree with referee #2 that the first major point (the apparent Kd of ToCsm for target RNA, and how it compares to that with DNA) is reasonable, doable, and within the scope of the current study. It is far less clear to me that major point #2 is a substantial concern, and I feel it would be most fair to the authors to leave that suggested experiment to follow-up studies for future manuscripts.

Please refer to the reply to referee 2

Cross-comments from referee 3:

1. Authors show that ToCsm binds target ssDNA target with Kd ~ 1 nM. Although target RNA binding by ToCsm is demonstrated by EMSA, the Kd value for the target RNA binding has to be provided. If ToCsm binds RNA target in pmol range, the gel shift experiments should be performed at low concentrations of radiolabeled RNA. Moreover, binding competition experiments must be performed to compare binding affinities of ssDNA and RNA. During DNA transcription stretches of ssDNA and nascent RNA may become available for ToCsm complex binding through basepairing with crRNA. ToCsm complex partitioning between the two alternative ssDNA and RNA targets will depend on the Kd values for the matching ssDNA and RNA sequences. One cannot exclude that due the differences in RNA and DNA binding affinities ToCsm will predominantly bind to the target RNA and trigger RNA-activated DNA cleavage pathway instead of the direct ssDNA cleavage. This raises the question whether the ssDNA cleavage activity reported in the manuscript ToCsm activity is biologically relevant.

I think this point can be addressed in the discussion rather than with new experiments. There are after all situations where there will be target DNA but no target RNA (no transcription). We will not know whether any of the activities reported are biologically relevant, but this is true for almost the whole type III literature so I am content with the data presented and a further discussion to address point 1.

Please refer to the reply to referee 2

2. In the manuscript authors provide evidence that under the large excess of the To Csm complex in respect to ssDNA, target ssDNA is cleaved by ToCsm in the absence of the activating target RNA, and demonstrate that active site in the HD-domain is responsible for the cleavage. Authors, however, also have to show whether binding of the ssDNA activates the non-target DNA degradation at the HD-domain. To address this question authors have to analyse in trans cleavage of

ssDNA lacking the target site by the ToCsm bound to the complimentary ssDNA and compare it to the RNA-activated ssDNA degradation by the ToCsm. Data provided in the manuscript does not exclude that after ToCsm cleavage of ssDNA containing the complementary sequence, ToCsm remains bound to the product and is trapped in the catalytically-dead complex that is unable to turnover ssDNA at the HD domain. In theory, such complex could interfere with non-template ssDNA strand cleavage by the transcript-activated HD-domain (Figure 5).

This is a reasonable point and could be addressed by the authors with a simple experiment.

Please refer to the reply to referee 2.

2nd Editorial Decision 15 February 2017

Thank you for the submission of your revised manuscript. We have now received the enclosed report from referee 2 who was asked to assess it. As you will see, s/he still has a few more suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

Please address all suggestions by this referee and interpret and discuss your data more carefully and in a more balanced manner.

1) Alternative interpretations of the cleavage pattern and in cis cleavage activity data should be provided in the Discussion.
2) Tone-down speculations in the Discussion section that ssDNA in the transcription bubble is a target for ToCsm. There is no data to indicate that targeting of the specific DNA sequence is transcription-dependent.
3) Move data from figure EV4 to the main figure 3 as indicated by the referee.

Please note that the source data (currently called raw data) file you sent needs to be split into and uploaded as single files, one per figure or per figure panel. Please also hand-label the size markers on the gels.

Also the EV figures need to be split into separate files and uploaded individually. Each file must have a resolution of at least 300 dpi.
Please also upload the EV table as a separate file.

The reference style needs to be changed to the numbered EMBO reports style, which can be found in Endnote.

Please generate a profile page in our online manuscript submission system (ejp) for all authors on the manuscript.

Please provide an ORCID number for the co-corresponding author.

Please provide a running title and up to 5 keywords for your manuscript.

I also would like to suggest a few minor changes to the title and abstract:
RNA activation-independent DNA targeting of the Type III CRISPR-Cas system by a Csm complex

The CRISPR-Cas system is an adaptive and heritable immune response that destroys invading foreign nucleic acids. The effector complex of the Type III CRISPR-Cas system targets RNA and DNA in a transcription-coupled manner, but the exact mechanism of DNA targeting by this complex remains elusive. In this study, an effector Csm holocomplex derived from Thermococcus onnurineus is reconstituted with a minimalistic combination of Csm1121334151, and shows RNA targeting and RNA-activated single-stranded DNA (ssDNA) targeting activities. Unexpectedly, in the absence of an RNA transcript, it cleaves ssDNA containing a sequence complementary to the bound crRNA guide region in a manner dependent on the HD domain of the Csm1 subunit. This nuclease activity is blocked by a repeat tag found in the host CRISPR loci. The specific cleavage of ssDNA without a target RNA suggests a novel ssDNA targeting mechanism of the Type III system, which could

facilitate the efficient and complete degradation of foreign nucleic acids.

Please let me know whether you agree with these changes.

We are also missing a completed author checklist from you, which can be found at http://embor.embopress.org/authorguide#revision. Please send us the completed list together with your revised manuscript.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible. Do not hesitate to contact me if you have any questions.

REFEREE REPORT

Referee #2:

Two additional experiments (1&2 below) were requested to support authors' conclusions.

1. Although target RNA binding by ToCsm is demonstrated by EMSA, the Kd value for the target RNA binding has to be provided.

In the revised manuscript authors addressed this concern and show that ToCsm binds target RNA with the same affinity as the target DNA. In agreement to this finding, binding competition experiments provided in the revised version demonstrate that target DNA competitor decreases the yield of ToCsm-target RNA complex. Binding data, however is difficult to analyse because they are scattered throughout Figures of the main text and Supplemental materials. Authors have to move sections D, E, G and H of the supplemental Figure EV4 into the Figure 3 of the main text to enable quick comparison of binding data.

2. Authors, however, also have to show whether binding of the ssDNA activates the non-target DNA degradation at the HD-domain. To address this question authors have to analyse in trans cleavage of ssDNA lacking the target site by the ToCsm bound to the complimentary ssDNA and compare it to the RNA-activated ssDNA degradation by the ToCsm.

In the revised manuscript authors provided the requested data. They show that ToCsm bound to the target DNA or RNA is able to degrade circular ssDNA that lacks the target site. These data demonstrate that ToCsm HD-domain exhibits non-specific DNA cleavage activity similar to other Type III-A systems. This raises a question whether the in cis cleavage activity proposed for the ToCsm-target DNA complex (Fig. 5 C-E) is indeed in cis activity or it results from the in trans cleavage. Indeed, data provided in the Fig. 5D show that 3'-labelled DNA fragment is degraded to small labelled fragments. Such degradation pattern implies in trans cleavage activity similar to that reported for the circular ssDNA degradation. In this case the major product observed for the 5'-labelled DNA cleavage (Fig. 5C) could result due to the protection of the 5'-fragment in the ToCsm-DNA complex against in trans degradation rather than in cis cleavage. Authors should discuss this alternative explanation in the revised manuscript.

General comment: In vitro experiments provided in the manuscript demonstrate that the target DNA binding similar to RNA binding activates DNA degradation. However, the interpretation of the cleavage patterns and in cis cleavage activity still raises questions. Therefore, alternative interpretations should be provided in the Discussion.

Authors should turn-down speculations in the Discussion section that ssDNA in the transcription bubble is a target for ToCsm. First, there is no data to indicate that targeting of the specific DNA sequence is transcription dependent. Next, the assumption that ToCsm targets a specific sequence (seed sequence) at the transcription bubble has no experimental or theoretical support. What is the average lifetime of the transcription bubble at the specific sequence? Is it long enough to enable target location through the 3D-diffusion of ToCsm? Therefore, the paragraph on p. 15 (lines (5-14) in the Discussion section should be removed.

2nd Revision - authors' response 19 February 2017

We are glad to note that reviewer #2 is overall satisfied with the revision and suggested some modifications in the discussion. We also appreciate your kind consideration and the suggestion of changes in the title and abstract. We have now addressed these suggestions.

Point-by-point response to the editor/reviewersí comments:

1) Alternative interpretations of the cleavage pattern and in cis cleavage activity data should be provided in the Discussion.

Both the cis and the trans cleavage were stated, and possible physiological relevance of the cis cleavage was discussed. (Page 13: 9-12, Page 14:17-19).

2) Tone-down speculations in the Discussion section that ssDNA in the transcription bubble is a target for ToCsm. There is no data to indicate that targeting of the specific DNA sequence is transcription-dependent.

We rearranged the Discussion section and significantly toned down by stating that "Csm complexes seem to function during transcription. Further investigation is required to know whether the ToCsm complex may target ssDNA in the transcription bubble and whether the cis-acting activity that we observed in in vitro may be functionally relevant in cells ". We removed the description about the transcription bubble and DNA replication as requested. (Page 13:12-16)

3) Move data from figure EV4 to the main figure 3 as indicated by the referee.

We have moved the figure to the main section accordingly. We hope that these changes transformed the manuscript in an acceptable format now, and look forward to hearing your final decision on the acceptance of our manuscript for publication in EMBO Reports.

3rd Editorial Decision 23 February 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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CRISPR-Cas system
Journal Submitted to: EMBO Reports
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- If n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	EM 3D map of ToCsm have been deposited in the PDBe ; EMD-3454 (page 17)
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We provided all raw data and relevant data.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	The crystal structure of cmr complex (PDB ID; 3X1L) and ToCsm1 (PDB; 4UW2) was used for 3D map of ToCsm (EMD-3454) by the 'Fit in map' function of Chimaera software (Figure 1E, Figure EV2D and Figure 7B) (page 17)

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA