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In vivo structures of an intact type VI secretion system revealed by electron cryotomography

Yi-Wei Chang, Lee A. Rettberg, Davi R. Ortega, and Grant J. Jensen

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Transaction Report:

(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 06 March 2017

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees acknowledge the potential interest of the findings. Referee #1 supports publication of the manuscript in its present form, whereas referees #2 and #3 raise a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn. I think all the points of both referees should be addressed in a revised version. Further, please also add the requested information on processing details and the methods (referee #2) and also strengthen the discussion of the structural observations by better relating the findings of this paper to what is currently known about the structure and function of the T6S apparatus (referee #3).

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be fully addressed in the revised manuscript and in a complete point-by-point response. Acceptance of your 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:

This very clearly written manuscript describes a series of tomographic studies of type VI secretion systems in vivo. Until now information about the sheath components of this system have been limited to visualising the contracted state, as this is the state that is adopted on purification. By studying the system with a cell, the authors are able to visualise the extended conformation of the sheath at moderate resolution allowing first insights into the process of contraction/extension for this important system. As such the manuscript is suitable for publication in EMBO Reports. The authors also identify intriguing antennae associated with the system which seem structural analogous to the fibres found in bacteriophage T4 but cannot identify a protein component of the type VI system likely to adopt this type of structure. This is also an exciting, novel, result. The manuscript is clearly written, the figures are also very clear and prior literature is well acknowledged. The authors are to be congratulated on a beautiful piece of work.

Referee #2:

This is a beautiful electron cryotomography (ECT) study of the type VI secretion system (T6SS) in Myxococcus xanthus. Impressively, the authors presented the most amazing ECT images of the T6SS after screening over 1,000 reconstructions of intact M. xanthus cells. By using sub-tomogram averaging, the authors not only reveal the baseplate structure, but also present two strikingly different conformations of the sheath. The methods and structures presented in the manuscript are clearly important and should be greatly welcomed by the growing and exciting field of T6SS and ECT. However, this manuscript is poorly written and the figures are not well presented, particularly considering the amazing structures shown in Figure 1 and other Figures. Sub-tomogram averaging is the key for this manuscript. However, the details of the processing are lacking. In fact, the methods are poorly described or presented in suboptimal format. The authors spend tremendous efforts in modeling. However the resolution of the averages is not high enough for reliable modeling. More importantly, the strength of the study is not well highlighted or presented.

Specific concerns:

1. The sub-tomogram average of the baseplate from the extended T6SS is shown in Fig. 1c. How about the sub-tomogram average of the baseplate from the contracted T6SS, as similar numbers of tomograms were identified. It should be very informative to compare those two structures.

2. Helical symmetry of the extended sheath appears to be reliable. It will be important to validate the results.

3. In contrast, the helical symmetry of the contracted sheath is not obvious. What's the helical symmetry? Since several contracted sheath structures were determined at high resolution, it will be critical to compare your structure with others.

4. Again, the available structural structures of contracted sheath may be helpful to build your model.

5. The model building of the extended sheath is not convincing and also very confusing.

6. Baseplate structures will be far more interesting than the structures presented in Figure 4 and Figure 5, unless the modeling is reliable.

Referee #3:

In this work, Chang et al. present the in vivo structure of the Myxococcus xanthus type VI secretion system (T6SS) in its extended state and point out the presence of fiber-like structures and an extracellular cap - two previously unobserved features of the T6SS. The T6SS is a protein translocation pathway capable of delivering protein toxins to both eukaryotic and/or prokaryotic cells. Understanding its structure is of significant interest because depending on the bacterium, T6SSs can play a direct role in virulence (via host cell targeting) or in shaping important microbial communities such as the human gut microbiome (via bacterial competition). The tomographic imaging in stunning but I have some concerns regarding the highly speculative conclusions drawn from the analysis of certain parts of the structural data (outlined below). Furthermore, I think the

discussion of some of the structural observations could be strengthened by better relating the findings of this paper to what is currently known about the structure and function of the T6S apparatus.

Major points:

- The images of surface structures that resemble phage tail fibers are intriguing but the lack of identified genes encoding such proteins is problematic. Because of this, I think the language describing this feature needs to be toned down significantly (or perhaps taken out altogether until further substantiated by mutational analyses). If the authors can define the genetic basis for these fiber structures, then I would be a lot more enthusiastic about this work. Additionally, the authors do not adequately relate these fiber-like structures to the current models for the assembly of the T6S apparatus. For example, in bacteriophage, tail fibers are connected directly to the baseplate whereas the structures identified in this work would presumably interact with the membrane complex. How do the authors envisage this would occur? It was recently suggested, based on biochemical data, that the soluble region of the TssM protein cycles between a partially surface exposed state and an entirely periplasmic state as the T6SS extends and contracts (see the extended data in PubMed ID: 26200339). Does this mean the "tail fibers" would transiently dissociate from extracellular TssM during cycles of extension and contraction? On a related note, it should be mentioned that the extracellular density in Fig 1C could correspond to extracellular TssM. Alternatively, if the authors feel their structural data disagree with the extension/retraction model put forth in 26200339 as it pertains to the extracellular localization of TssM, then this should be elaborated upon significantly.

- Are there any PAAR domains encoded in the Myxococcus xanthus genome? None are apparent in Fig. S1, but this should be a simple enough analysis to perform. If none are identified, then this begs the question of what comprises the density that the authors refer to as the PAAR and cargo? In general, the authors do not perform an adequate bioinformatics analysis of T6SS genes in this bacterium. For example, a Blast search of the vgrG gene within the T6 operon reveals a second VgrG encoded by the MXAN_5573 gene, which is not pointed out by the authors. Are there other T6 genes that could explain some of the structural observations such as the presence of cargo at the end of spike complex?

- The authors should use the dimensions of the recent low-res EM structure of the PAAR-containing protein Tse6 which interacts with the tip of VgrG (PMID: 26456113) to substantiate their claim that there is ample room for cargo proteins within the baseplate structure observed in their cryotomographic images.

1st Revision - authors' response 16 March 2017

Please find below our point-by-point responses to the referee’s comments. In our revised manuscript, we have added the details of our sub-tomogram averaging procedure to the Methods section. We have also added a new figure (Fig. EV3) to describe the determination of the helical symmetries of the sheath averages. Finally, we have performed comprehensive bioinformatics analyses to identify possible homologues of the PAAR-repeat protein and bacteriophage T4 tail fiber proteins in the *M. xanthus* genome. Although we could not find homologs of the tail fibers, we were able to find five putative PAAR-repeat protein homologs. These new results are reported in the new Fig. EV1, and we have added a new author, Dr. Davi Ortega, who designed and performed these searches. We have also used the referee's comments to correct and clarify the manuscript where needed. We hope you and the reviewers will now find it suitable for publication.

Referee #1:

This very clearly written manuscript describes a series of tomographic studies of type VI secretion systems in vivo. Until now information about the sheath components of this system have been limited to visualising the contracted state, as this is the state that is adopted on purification. By studying the system with a cell, the authors are able to visualise the extended conformation of the sheath at moderate resolution allowing first insights into the process of contraction/extension for this important system. As such the manuscript is suitable for publication in EMBO Reports. The authors

also identify intriguing antennae associated with the system which seem structural analogous to the fibres found in bacteriophage T4 but cannot identify a protein component of the type VI system likely to adopt this type of structure. This is also an exciting, novel, result. The manuscript is clearly written, the figures are also very clear and prior literature is well acknowledged. The authors are to be congratulated on a beautiful piece of work.

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We have now added the details of the sub-tomogram averaging procedure to the Methods section.

The authors spend tremendous efforts in modeling. However the resolution of the averages is not high enough for reliable modeling.

The resolutions of our extended and contracted sheath sub-tomogram averages are 24Å and 28Å, respectively, but because there are already atomic models available for both the contracted and extended states of a related pyocin structure and a contracted T6SS structure from another organism, and all these share clear sequence homology to the M. xanthus T6SS, building the models and rigid-body-fitting the proteins into the sub-tomogram averages was straightforward. This led to new insights, however, such as the location of the recycling domains of TssB/TssC, which are not present in pyocins and have therefore not been seen before in the extended state. We agree however that our resolution is not high enough to allow us to model and study detailed interactions. Thus even though we saw that in the extended sheath the recycling domains are interacting with the neighboring protofilament, we did not propose specific residues that might be involved in the interaction.

More importantly, the strength of the study is not well highlighted or presented.

Unfortunately we're not sure what the Reviewer considers the main strength of the study.

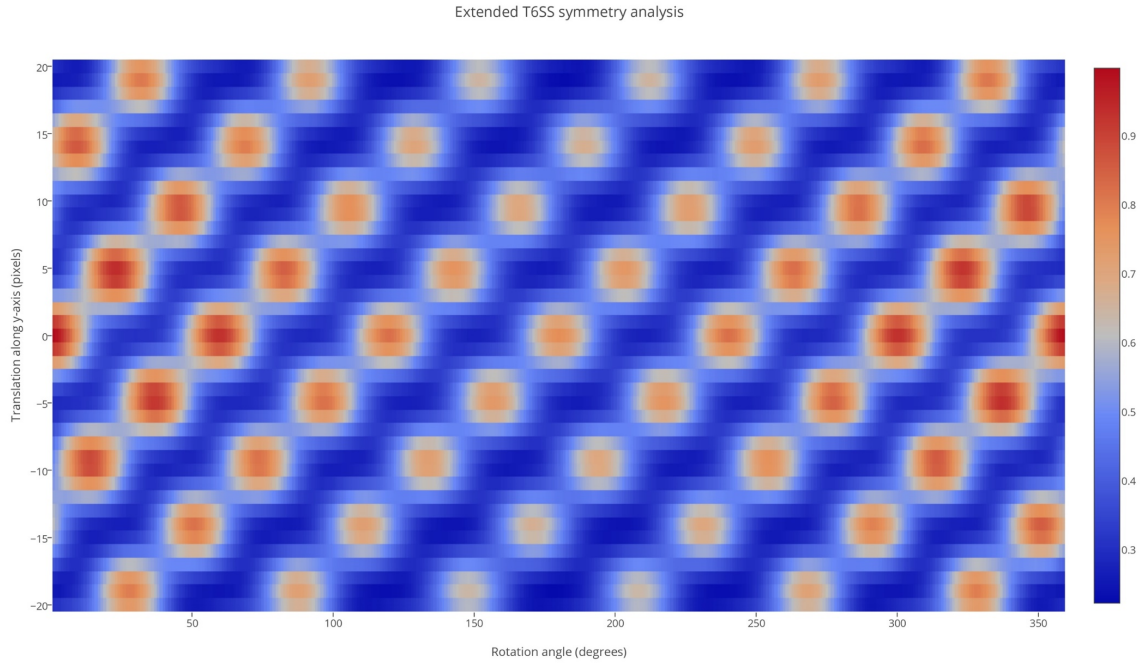
Specific concerns:

1. The sub-tomogram average of the baseplate from the extended T6SS is shown in Fig. 1c. How about the sub-tomogram average of the baseplate from the contracted T6SS, as similar numbers of tomograms were identified. It should be very informative to compare those two structures.

We agree that it would be very informative to generate a sub-tomogram average of the baseplate of the contracted T6SS and compare it with that of extended T6SS. Unfortunately the majority of the contracted sheaths we observed in our tomograms were no longer attached to the cell envelope. We therefore did not have enough images of the baseplate to generate a sub-tomogram average. This observation matches with the previous fluorescence microscopy study (Basler et al, Nature 2012), in which the fluorescently-labeled T6SS sheaths detached from the cell envelope quickly after contraction.

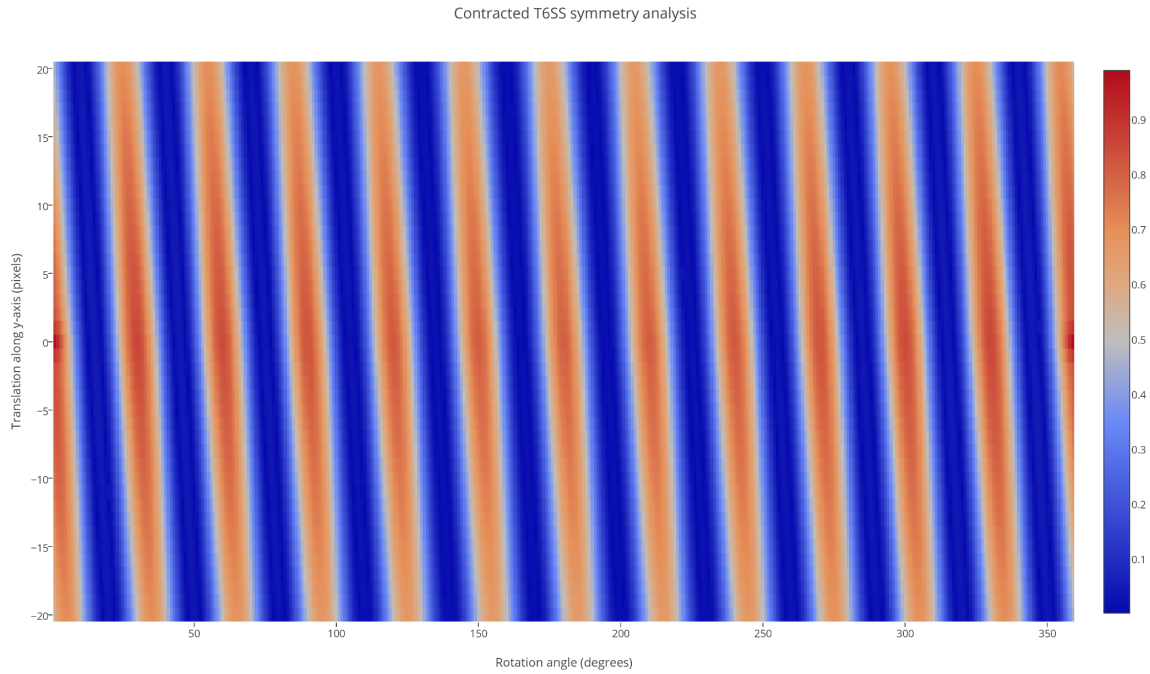
2. Helical symmetry of the extended sheath appears to be reliable. It will be important to validate the results.

The helical symmetry (a rise of 37Å and a right-handed rotation of 22 degrees) of the extended sheath was identified by rotating and translating the sub-tomogram average density to study its autocorrelation. The resulting plot is shown here, and is now also added to the manuscript.



3. In contrast, the helical symmetry of the contracted sheath is not obvious. What's the helical symmetry? Since several contracted sheath structures were determined at high resolution, it will be critical to compare your structure with others.

Due to the fact that there was no clear feature resolved along individual ridges in our contracted sheath average, we were not able to identify the helical symmetry using the same autocorrelation analysis described above. Only the angle of the ridges (corresponding to the angle of the orange pattern shown in the plot below) was apparent, but this was almost identical to the high-resolution structure of the V. cholerae contracted sheath solved previously (Kudryashev et al, Cell 2015). The plot and description are now added to the manuscript.



4. Again, the available structural structures of contracted sheath may be helpful to build your model.

Yes, as stated above, the sheath models built in this study were indeed based on the high-resolution structure of the contracted sheath in V. cholerae (Kudryashev et al, Cell 2015). We have tried to highlight this point in the revised manuscript.

5. The model building of the extended sheath is not convincing and also very confusing.

To study the orientation of the TssB/TssC heterodimer in the extended sheath, we first took the heterodimer structure from the previously solved high-resolution structure of the V. cholerae contracted sheath (Kudryashev et al, Cell 2015) and swapped its sequence to that of M. xanthus sheath proteins. We then concatenated it with the crystal structure of the TssB C-terminal two helices (missing in the V. cholerae structure) and removed all side chains. This model was then fitted as a rigid body to one lobe of the extended sheath density, and subsequently replicated to populate the whole density. During this process, none of the atoms were moved individually. The resulting model agreed very well with the high-resolution structure of the extended pyocin structure in regions where homologous sequences were present, boosting confidence. We have tried to clarify this in the revised manuscript.

6. Baseplate structures will be far more interesting than the structures presented in Figure 4 and Figure 5, unless the modeling is reliable.

We agree that baseplate structures are very interesting and that is why we carefully searched through >1,650 tomograms to identify the 16 suitable baseplate densities that were used to generate the first sub-tomogram average of the extended T6SS's baseplate shown in Figure 5. As mentioned above, we were not able to determine a baseplate structure for the contracted state. As in our response to the earlier point, we believe that the process of generating the models shown in Figure 4 is reliable, since we only performed rigid body fitting of the whole TssB/TssC dimer to a density map of 24 Å resolution, and only discussed subunit orientations (rather than detailed residue-residue interactions). Figure 5 is also interesting, as it shows how the known structures of the membrane complex, spike complex, and sheath/hcp tube can be fit to the overall density of the extended T6SS, giving a first overall view of the intact machine.

Referee #3:

In this work, Chang et al. present the in vivo structure of the Myxococcus xanthus type VI secretion system (T6SS) in its extended state and point out the presence of fiber-like structures and an extracellular cap - two previously unobserved features of the T6SS. The T6SS is a protein translocation pathway capable of delivering protein toxins to both eukaryotic and/or prokaryotic cells. Understanding its structure is of significant interest because depending on the bacterium, T6SSs can play a direct role in virulence (via host cell targeting) or in shaping important microbial communities such as the human gut microbiome (via bacterial competition). The tomographic imaging in stunning but I have some concerns regarding the highly speculative conclusions drawn from the analysis of certain parts of the structural data (outlined below). Furthermore, I think the discussion of some of the structural observations could be strengthened by better relating the findings of this paper to what is currently known about the structure and function of the T6S apparatus.

Major points:

- The images of surface structures that resemble phage tail fibers are intriguing but the lack of identified genes encoding such proteins is problematic. Because of this, I think the language describing this feature needs to be toned down significantly (or perhaps taken out altogether until further substantiated by mutational analyses). If the authors can define the genetic basis for these fiber structures, then I would be a lot more enthusiastic about this work.

That the tail-like fibers exist is proved beyond question by the images. They are clearly part of the T6SS because they were always seen in clusters immediately above the extended sheaths. We agree it would be incredibly useful to identify the genes responsible for these fibers, but unfortunately in our bioinformatics searches for protein homologs of bacteriophage T4 long and short tail fiber components (gp34, gp 35, gp36, gp37 and gp12) in M. xanthus, no significant candidates were identified. We have therefore followed the referee's comment to tone down the language and we report the bioinformatics result in the revised manuscript.

Additionally, the authors do not adequately relate these fiber-like structures to the current models for the assembly of the T6S apparatus. For example, in bacteriophage, tail fibers are connected directly to the baseplate whereas the structures identified in this work would presumably interact with the membrane complex. How do the authors envisage this would occur?

Due to the crowded densities in the periplasm compared with the extracellular space, in the current data we were not able to trace the antennae density across the outer membrane and visualize whether they attach to the membrane complex or the baseplate like the bacteriophage tail-fibers. We don't know how this occurs. We have now added this point to the manuscript.

It was recently suggested, based on biochemical data, that the soluble region of the TssM protein cycles between a partially surface exposed state and an entirely periplasmic state as the T6SS extends and contracts (see the extended data in PubMed ID: 26200339). Does this mean the "tail fibers" would transiently dissociate from extracellular TssM during cycles of extension and contraction?

As shown in Fig. 5d, the six antennae do not intersect with the outer membrane in the same position but are rather spread apart. Therefore, there is likely no direct connection between the antennae and the axial extracellular cap. The antennae probably span the outer membrane and connect to other parts of the membrane complex or baseplate. If this is the case, the antennae would not need to dissociate from the anchored components during cycles of extension and contraction, but of course we don't know this for sure at this point.

On a related note, it should be mentioned that the extracellular density in Fig 1C could correspond to extracellular TssM. Alternatively, if the authors feel their structural data disagree with the extension/retraction model put forth in 26200339 as it pertains to the extracellular localization of TssM, then this should be elaborated upon significantly.

Thank you for this valuable point. We have now added to the manuscript that the extracellular cap density could involve the extracellular part of TssM.

- Are there any PAAR domains encoded in the Myxococcus xanthus genome? None are apparent in Fig. S1, but this should be a simple enough analysis to perform. If none are identified, then this begs the question of what comprises the density that the authors refer to as the PAAR and cargo?

Prompted by this comment and the one below, we performed an extensive bioinformatics analysis on the T6SS-related genes in M. xanthus. Using PSSM models of the PAAR-like superfamily in the Conserved Domain Database (CDD) as templates, five genes outside of the T6SS gene cluster were found to encode proteins homologous to the PAAR-repeat protein. It is therefore possible that one or multiple of these proteins form the spike tip. We have now added an extensive explanation of this result to the manuscript and a new author, Dr. Davi Ortega, who designed and performed the searches.

In general, the authors do not perform an adequate bioinformatics analysis of T6SS genes in this bacterium. For example, a Blast search of the vgrG gene within the T6 operon reveals a second VgrG encoded by the MXAN_5573 gene, which is not pointed out by the authors.

As the reviewer points out, a blast search of the VgrG sequence against M. xanthus proteome does reveal a second similar protein. However, without performing a more extensive analysis we will not be able to conclude how likely it is that the protein is actually involved in T6SS. The same challenge applies to the analysis of all the other T6SS genes. For example, using BLAST, we can find several AAA+ ATPases in the M. xanthus proteome with sequence similar to ClpV, but it is exceedingly unlikely that all are involved in the T6SS. One way to reliably identify potential additional components of the T6SS is to use phylogenetic profiling to find which candidates co-evolve with the T6SS core genes. Building good bioinformatics tools to track such trends is actually an independent project currently in progress in the lab, so we cannot say more at this time, but we have now summarized the known T6SS genes in the new Fig. EV1 with added information about the possible PAAR-repeat proteins found.

Are there other T6 genes that could explain some of the structural observations such as the presence of cargo at the end of spike complex?

As mentioned in the response to the earlier point, through a more elaborated bioinformatics analysis, we found that there are five genes (MXAN_0044, MXAN_1303, MXAN_1813, MXAN_2100 and MXAN_7133) outside of the T6SS gene cluster that encode different sized proteins exhibiting homology to the PAAR-repeat protein (see new Fig. EV1). It is therefore possible that one or more of these proteins form the spike tip with different sizes and/or interact with cargo proteins.

- The authors should use the dimensions of the recent low-res EM structure of the PAAR-containing protein Tse6 which interacts with the tip of VgrG (PMID: 26456113) to substantiate their claim that there is ample room for cargo proteins within the baseplate structure observed in their cryotomographic images.

Thank you very much for the suggestion. We have now added this point to the manuscript.

2nd Editorial Decision 05 April 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.

Referee #2: The manuscript is much improved and suitable for publication.

Referee #3: I would like to thank the authors for taking into account my comments regarding their initial submission. The bioinformatics look much improved; I am happy with the manuscript as is.

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Corresponding Author Name: Grant Jensen
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Manuscript Number: EMBOR-2017-44072V1

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?
  - are there adjustments for multiple comparisons?
  - 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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B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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D- Animal Models

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9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
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14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
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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	PAGE 13
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).	NA
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
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
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.	NA

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