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A pH- and ionic strength-dependent conformational change in the neck region regulates DNGR-1 function in dendritic cells

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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	10 June 2016

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see there is an interest in the analysis. However, both referees also find that the findings need to be significantly extended to consider publication here. Both referees find that you need some more mechanistic insight into how the dimeric state of DNGR-1 affects cross presentation. Referee #2 also suggests an interesting experiment to further support the importance of the neck domain in facilitating cross-presentation. Should you be able to extend the findings and add more mechanistic insight then I am open to consider a revised version. I am aware that this might not be a straightforward task and if you are not able to do so then it is in your best interest to seek publication elsewhere at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://emboj.embopress.org/about#Transparent_Process

Let me know if we need to discuss things further.

REFEREE REPORTS

Referee #1:

In the manuscript, Hanc et al. found that DNGR-1 can dimerize through the neck region of DNGR-1 monomer. Interestingly, they found that the DNGR-1 dimer had two forms. The "type-1 dimer" exists under regular PBS conditions and is reduction-sensitive, while the "type-2 dimer" forms at low pH or ionic strength conditions and becomes reduction-insensitive, and the transition between the two dimeric forms is reversible. They also found that the type-2 dimer had similar activities in F-actin binding and internalization, but could affect the cross-presentation in dendritic cells. Overall the findings are interesting, but the mechanisms for dimerization/oligomerization and cross-presentation are not quite clear.

Major:

1) The type-2 dimer can be formed under low pH and low ionic strength conditions, are the low ionic strength conditions physiologically relevant in the DNGR-1 pathway? Authors suggest that the reduction-insensitive oligomers might be the physiologically relevant forms of DNGR-1, would it be possible to test this in vitro using the full-length proteins? In Fig.6D, Δ6A only shows type-1 interactions (reduction sensitive), does this mean that the transmembrane and cytoplasm domain of DNGR-1 may not be involved in the reduction-insensitive dimerization or oligomerization? If this is the case, then why the ECD of DNGR-1 only shows the reduction-insensitive dimer instead of oligomer in vitro? The neck region mutants, such asΔ4 andΔ6A, do provide some information for DNGR-1 dimerization/oligomerization, but the mechanism of the neck interactions seems still confusing and probably needs to be explored by more experiments.

2) Is the regulation of cross-presentation by DNGR-1 type-2 dimer actin dependent? Have authors tested the role of actin in the regulation? According to the biochemical data in the manuscript,Δ6A only forms type 1 dimer, WT can form both type 1 and type 2 dimer. But in Fig. 8C, KO-Δ6A and KO-WT show similar effects in DC cross-presentation, so how to explain this result? It looks like the oligomeric states of DNGR-1 may affect the cross-presentation, but authors may need to provide more evidence on this, and also investigate the mechanism.

Referee #2:

The ectodomain (ECD + neck) of mouse and human DNGR-1 is made of glycosylated disulphide-bonded dimers. Interestingly, when DNGR-1 ectodomain were subjected to buffers of lower pH and ionic strength, they form reduction resistant dimers when reduction sensitivity was analysed by reducing SDS-PAGE and Western blot. 8 M urea treatment abolished the ability of DNGR-1 to resist reduction. This behaviour was found reversible and the human and mouse DNGR1 necks thought to be responsible for the reduction insensitivity of DNGR-1 dimers. In support of that view, grafting of the DNGR1 neck in place of that found in Dectin-1 induced a reduction-insensitive state in the Dectin-1 ECD. Further analysis of near- and far-UV CD spectra suggested that mutual repositioning of the two neck regions within the dimer was responsible for the observed reduction resistance and for the existence of reduction sensitive ("type-1 dimer") and reduction insensitive ("type-2 dimer") forms. Structure-function analysis of the neck showed that a mutant called N81 - T90 (Δ4) diplays enhanced type-2 dimer formation even under neutral conditions, an observation that also stands when DNGR1 is expressed in its physiological - membrane bound - context. In view of the recent despcription of pH-induced conformational change in the C-type lectin receptor DEC205, the authors showed next that type-2 dimer formation does not affect the ability of DNGR-1 to bind F-actin, signal to NFAT and undergo internalization. Finally, the authors used the DC line MuTu to assess DNGR-1-dependent cross-presentation of dead cell-associated antigens to OT-I. Introduction of the Δ4 mutant into DNGR-1 KO MuTu resulted in a small (less than two-fold) increase in the amounts of IFNγ accumulating in the medium after the incubation with dead cells as compared to MuTu cells expressing WT DNGR-1. Therefore, the switch to type-2 dimer conformation, which is exacerbated in the Δ4 mutant, slightly enhanced the efficiency of the process. As discussed by the authors, conditions present in late endosomes and lysosomes would be predicted to induce a switch to the type-2 dimer conformation accounting the unique routing of DNGR-1 toward cross-presenting compartment. They fairly propose a few lines of experiments

aiming at explaining the mode of action of the DNGR-1 neck and relating it to cross-presentation To solidify their hypothesis the authors may have easily used the "fast track" MuTu expression system to express their Dectin-1 construct with a grafted DNGR1 neck. If their hypothesis is correct (that is the DNGR-1 neck is a evolutionary conserved and autonomous functional module), this will have allow them to readily test in a couple of months whether it confers the chimeric Dectin-1molecules the de novo to capacity to cross-present native OVA targeted to Dectin-1 via an antibody conjugate.

Specific questions

Figure 1. Following reduction the long isoform 'band' appears composed of two bands. This needs to be discussed. Based on Figure 1C, they do not appear to correspond to distinct glycoforms. How are the few multimers assembled (see also Figure 6D)? Is there any additional Cys in the neck ?
Figure 7. Is it necessary to usu pre-activated OVA-specific CD8+ T-cells (OT-I) overnight ?

1st Revision - authors' response   18 August 2016

Reviewer 1

The type-2 dimer can be formed under low pH and low ionic strength conditions, are the low ionic strength conditions physiologically relevant in the DNGR-1 pathway?
We thank the reviewer for pointing out that this has not been clearly stated in the manuscript. The exact ionic strength, especially in the later endocytic compartments is not well described, but for the early endosomes, the combined concentration of salt ions does not appear to exceed 150mM while the pH drops to around 6.2 (Scott et al. Bioessays, 2011). As can be seen in figure 2B, these conditions effectively induce the switch to the reduction-insensitive conformation. We have now added this clarification to the discussion (highlighted in yellow).

Authors suggest that the reduction-insensitive oligomers might be the physiologically relevant forms of DNGR-1, would it be possible to test this in vitro using the full-length proteins?
Unfortunately, full-length transmembrane proteins are not easily amenable to recombinant expression, precluding the experiment that the reviewer is suggesting.

In Fig.6D, Δ6A only shows type-1 interactions (reduction sensitive), does this mean that the transmembrane and cytoplasm domain of DNGR-1 may not be involved in the reduction-insensitive dimerization or oligomerization? If this is the case, then why the ECD of DNGR-1 only shows the reduction-insensitive dimer instead of oligomer in vitro?
We think that the Δ6A mutant is still forming the oligomers, but using our biochemical assay we do not see it because the dimer itself is no longer reduction-resistant, so the whole oligomeric complex falls apart in the presence of reducing agents.

The neck region mutants, such as Δ4 and Δ6A, do provide some information for DNGR-1 dimerization/oligomerization, but the mechanism of the neck interactions seems still confusing and probably needs to be explored by more experiments.
The mechanism of neck interactions is indeed unclear and a detailed description of the forces involved in the conformational change would first require a knowledge of the atomic structure of the neck region. We attempted to crystalize the whole ECD of DNGR-1 but, presumably due to the inherent flexibility of the neck region (see Hanc et al. Immunity, 2015) we never obtained diffracting crystals. There might be other ways to explore the different conformational states through NMR and/or protein structure modelling, but we believe this is beyond the scope of the current study.

Is the regulation of cross-presentation by DNGR-1 type-2 dimer actin dependent? Have authors tested the role of actin in the regulation?
Yes, DNGR-1-mediated cross-presentation, as well as the internalization of the receptor is F-actin dependent. See Hanc et al. Immunity, 2015.

According to the biochemical data in the manuscript, Δ6A only forms type 1 dimer, WT can form both type 1 and type 2 dimer. But in Fig. 8C, KO-Δ6A and KO-WT show similar effects in DC cross-presentation, so how to explain this result?

As we mentioned in the discussion, the biochemical assay that we use to observe the conformational change relies on the fact that part of the neck region protects the disulfide bond from the effects of reducing agents in one of the conformational states. Apparent loss of type 2 dimer formation in the biochemical assay can be caused by two factors – either the ability of the protein to undergo the conformational change has indeed been compromised, or, alternatively, the part responsible for the protection of the disulfide bond has been removed while the ability to undergo the conformational change itself remains unaffected. Based on the fact that the KO-WT and KO-Δ6A cells show similar ability to cross-present, as the reviewer points out, we suggest that the latter is the case for Δ6A. We have now attempted to clarify this further in the discussion (highlighted in yellow).

It looks like the oligomeric states of DNGR-1 may affect the cross-presentation, but authors may need to provide more evidence on this, and also investigate the mechanism.
The mechanism by which dendritic cells and other cell types cross-present exogenous antigens on MHC class I molecules remains poorly understood. Consequently, it is next to impossible to assess how the formation of Type-2 dimers and other DNGR-1 oligomers affects such mechanism. This is a focus of ongoing investigation, which we feel is beyond the scope of this study.

Reviewer 2

To solidify their hypothesis the authors may have easily used the "fast track" MuTu expression system to express their Dectin-1 construct with a grafted DNGR1 neck. If their hypothesis is correct (that is the DNGR-1 neck is a evolutionary conserved and autonomous functional module), this will have allow them to readily test in a couple of months whether it confers the chimeric Dectin-1molecules the de novo to capacity to cross-present native OVA targeted to Dectin-1 via an antibody conjugate.
We thank the reviewer for suggesting this intriguing experiment. Our original conclusion was that the neck region of DNGR-1 was necessary for the proper function of the receptor. However, we did not experimentally address the question of whether it is on its own sufficient to confer the ability to promote cross-presentation to unrelated receptors. The suggested experiment has some caveats that are important to consider. MuTu DC1940 cells already express low levels of Dectin-1 endogenously although this should not be too much of a problem as overexpression of Dectin-1 in RAW cells, which also have low baseline levels of Dectin-1, has previously been shown to enhance Dectin-1 dependent responses (Gantner et al. J. Exp. Med., 2003). More important is the fact that Dectin-1 is expressed as a monomer and its ligand-induced dimerisation has been suggested to be important for receptor function (Rogers et al. Immunity, 2005; Brown et al. Protein Science, 2007). Transplanting the neck region of DNGR-1 makes the receptor constitutively dimeric (see Fig 4B), a feature that could have unpredictable consequences. Nevertheless, we proceeded with the experiment as suggested. We used retroviral transduction of MuTu cells to overexpress WT Dectin-1 or a chimeric protein consisting of the neck region of DNGR-1 and the intracellular, transmembrane and ligand-binding domains of Dectin-1. As a control, we transduced MuTu cells with an empty-vector control virus. We FACS-sorted the transduced cells, so that expression levels of WT Dectin-1 and the chimeric protein were comparable, and we generated a conjugate of anti-Dectin-1 antibody (clone 2A11) covalently bound to an extended SIINFEKL peptide (following the approach detailed in Sancho et al, JCI 2008). Treatment of the control cells with this targeting reagent in a cross-presentation assay resulted in a baseline IFN-γ production by pre-activated OT-I T-cells, presumably due to the low levels of endogenously expressed Dectin-1. When we treated the cells transduced to overexpress WT Dectin-1, we observed approximately 10-fold shift in the dose response compared to the control cells, indicating that overexpression of Dectin-1 has measurable consequences in the assay. However, in the cells transduced with the chimeric protein, we observed no such gain-of-function suggesting that the chimera is inactive.
Antibody-mediated targeting may not allow receptor triggering in the same way as ligand binding. We therefore generated covalent complexes of laminarin, a Dectin-1 ligand, and ovalbumin (as per Xie et al. Biochem. Biophys. Res. Commun. 2010) and used those as an alternative method to targeting ovalbumin to MuTu cells. In line with what we observed using antibody targeting, the ovalbumin present in the laminarin complexes was cross-presented by control cells, as evidenced by production of IFN-γ by pre-activated OT-I T-cells. Overexpression of Dectin-1 caused an improvement in sensitivity as denoted by a shift in the dose-response curve. However, in the cells expressing the chimeric protein, no such improvement was apparent.

Taken together, these data suggest that the neck region of DNGR-1, while necessary for the function of DNGR-1 itself, is of its own not sufficient to confer the ability to promote cross-presentation to unrelated proteins. Importantly, as mentioned above, the data are difficult to fully interpret owing to the constitutively dimeric status of the chimeric protein which might have adverse effects on its ability to function. As such, we include the data here for the reviewer's appraisal, but we mention them in the manuscript only as data not shown (highlighted in yellow).
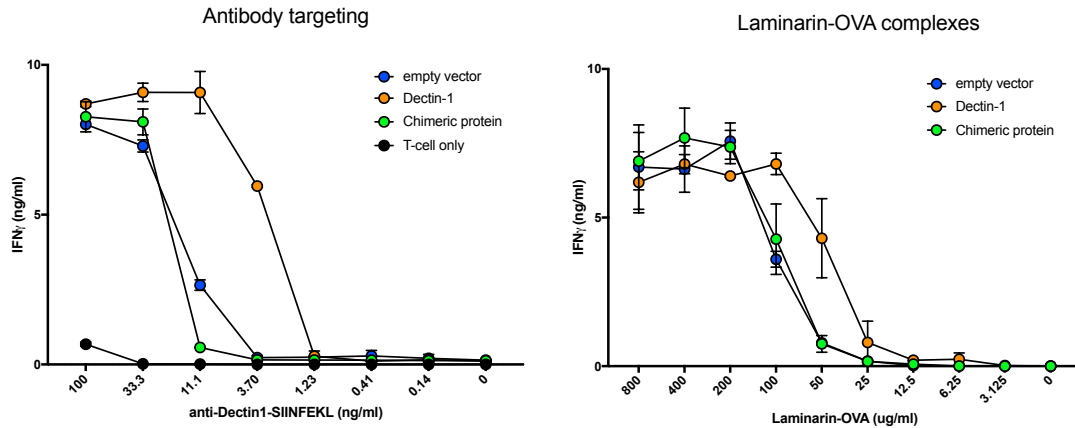

Figure 1. Following reduction the long isoform 'band' appears composed of two bands. This needs to be discussed. Based on Figure 1C, they do not appear to correspond to distinct glycoforms.
We thank the reviewer for pointing out this omission. We think that the two bands do correspond to different glycoforms. The deglycosylation reaction with PNGase F (Fig 1C) that we performed will only remove N-bound glycans that do not contain an α(1-3)-Fucose bound to the core N-acetyl glucosamine. It is thus possible that the two bands correspond to glycoforms that differ in O-glycosylation or α(1-3)-fucosylated N-glycans. We have now clarified this in the text (highlighted in yellow).

How are the few multimers assembled (see also Figure 6D)?
The reduction-sensitive higher oligomers seen in the soluble ECD proteins (Fig 1), probably correspond to a fraction of the protein that failed to fold correctly and aggregated through the cysteines in the CTLD during protein production. Importantly, we could not observe any signs of increased reduction-insensitivity in these oligomers under any conditions, and they are effectively removed by size exclusion chromatography during protein purification. Crucially, they should not be confused with the higher, reduction-resistant oligomers that we observed in the context of the transmembrane protein (Fig 6D). As we speculated in the discussion, the formation of the reduction-resistant oligomers of full-length DNGR-1 could be mediated through the cysteines in the transmembrane or intracellular parts of the protein. We have now included further clarification in the discussion to prevent any confusion (highlighted in yellow).

Is there any additional Cys in the neck?
All murine isoforms contain only one cysteine residue in their neck region. The neck of the human DNGR-1 isoform has an additional cysteine, which is not conserved in other species. Whether this cysteine is also engaged in mediating dimerization of the receptor is currently unknown, but its presence could explain the higher proportion of the misfolded higher oligomers seen in human DNGR-1 ECD compared to the mouse isoforms (Fig 1A).

Figure 7. Is it necessary to use pre-activated OVA-specific CD8+ T-cells (OT-I) overnight?
We and others have invested significant effort into optimizing the in-vitro cross-presentation assay (Iborra et al. JCI, 2012, Hanc et al. Immunity 2015), and find that the sensitivity of pre-activated OT-I T cells increases the robustness of the cross-presentation assay with dead cell-associated antigens compared to using naïve OT-Is. This might be due to the fact that IFNγ production by pre-activated OT-I cells is a direct readout of antigen presentation and not dependent on the activation status of the DCs. An additional advantage, pre-activation also results in T cell expansion, making OT-Is from a single mouse sufficient for one experiment.

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by referee #2 and as you can see below the referee appreciates the introduced changes. I am therefore very pleased to accept the manuscript for publication here.

REFEREE REPORT

Referee #2:

The authors have fairly addressed the points I raised and his rebuttal letter (to be published) is very informative.

EMBO PRESS

Corresponding Author Name: Caetano Reis e Sousa	
Journal Submitted to: EMBO Journal	
Manuscript Number: EMBOJ-2016-94695R	

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 $\chi 2$ 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.

B- Statistics and general methods

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
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	NA
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

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).	anti-FLAG: M2 clone, Sigma Aldrich, catalog number A8592; anti-DNGR-1: clones 7H11, 397 and 1F6 were all described previously in Sancho et al. JCI, 2008.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	MuTu cells were obtained directly from Hans Acha-Orbea who generated the line. 293F cell were obtained from Thermo Fisher (catalog number K900001). B3Z-Syk reporter line was generated previously in the lab (Sancho et al. Nature, 2009). Virus-producing cells (GP-2, FNX, 293FT) are established in the lab. All cell lines were tested and found to be mycoplasma free.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mouse, Methods section under OT-I cells
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Mouse, Methods section under OT-I cells
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.	We comply.

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
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

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	NA
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	We have not used that section.
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.	No.