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Pseudouridylation of 7SK snRNA promotes 7SK snRNP formation to suppress HIV-1 transcription and escape from latency

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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 12 May 2016

Thank you for the submission of your manuscript to EMBO reports. I have now read and discussed your work with my colleagues here, and I regret to say that we all agree that it is not well suited for us.

We note that your study reports that 7SK snRNA is pseudouridylated at U250. You show that 7SK U250G binds 7SK RNP complex components less, that DKC1 knockdown decreases the pseudouridylation of 7SK RNA and also reduces the interaction of 7SK with 7SK RNP complex components but increases the association of SEC with Tat, that DKC1 depletion increases Tat-dependent HIV promoter activity and SEC and Pol II binding to the HIV promoter, and that DKC1 knockdown activates HIV LTR-driven GFP expression by 85%.

We recognize that your findings suggest that 7SK snRNA pseudouridylation is required for 7SK RNP complex formation, and that reducing 7SK pseudouridylation might activate HIV from latency by freeing P-TEFb from the 7SK RNP complex. However, we also note that endogenous HIV activation by DKC1 knockdown or 7SK U250G is not demonstrated, and the physiological relevance of the regulation of 7SK RNA pseudouridylation therefore remains unclear. It also remains unknown in which context or in response to which stimulus 7SK RNA would be de-pseudouridylated to activate HIV. We think that the manuscript is not sufficiently developed for consideration for publication here, and we have therefore decided not to proceed with in-depth review.

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Please note that we publish only a small percentage of the many manuscripts submitted to us, and therefore only subject to external review those that have a good chance of timely acceptance. I am sorry to disappoint you on this occasion and thank you once more for your interest in our journal.

Appeal 13 May 2016

Thank you for taking the time to carefully consider our manuscript. However, I'm very disappointed by your decision of not sending the manuscript out for external review. I respectfully disagree with your view on the two main points that you mentioned have influenced your decision.

First, we indeed have demonstrated in Fig. 4G and 4F that an ENDOGENOUS latent HIV provirus present in Jurkat-2D10 cells can be activated by DKC1 knockdown (KD). Activation of latency is known to require multiple steps and usually the reversal of the repressive chromatin state is the first step,. Our data in Fig. 4 show that although DKC1 KD alone was not super efficient to activate this ENDOGENOUS latent HIV provirus (likely due to the fact that activation of P-TEFb alone does not do much to change the chromatin state), it significantly facilitated two other chemical activators, JQ1 and prostration, to do so. This result is consistent with our in vivo transcription (Fig. 4A, B & C) and ChIP (Fig. 4D) assays conducted in HeLa cells showing that DKC1 KD activates P-TEFb by releasing it from 7SK snRNP to form the SEC on HIV LTR for activation of viral transcription.

Regarding your request to demonstrate the physiological context in which 7SK snRNA is de-pseudouridylated, as there is currently no evidence demonstrating that this modification can be reversed and that no enzyme has been identified to catalyze the de-pseudouridylation step, the suggested experiment simply cannot be done at this stage.

When this work was presented at a recent international conference, it was very well received, which encourages me to ask your kind reconsideration of your decision. I hope you will be kind enough to give this manuscript a chance to been reviewed by experts in the field. I will accept whatever editorial decision thereafter.

2nd Editorial Decision 02 June 2016

Thank you for the submission of your manuscript to EMBO reports. We have now received the enclosed reports on it.

As you will see, the referees acknowledge that the findings are potentially interesting, however, they also raise concerns and make suggestions for how the study could be strengthened and improved. Referee 2 points out that endogenous proteins should be examined, that the experiments and results need to be described in much more detail, and that quantifications and statistical analyses are missing.

Given the constructive comments, we would 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. Especially all points raised by referee 2 should be addressed. Please also submit a complete point-by-point response to all referee concerns. 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 25,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when

discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

In this manuscript, Zhao et. al provide solid evidence for a major post transcriptional modification of 7SK RNA. The authors show that most of 7SK is pseudouridylated at a specific site in HeLA cells by the DKC1 containing H/ACA snoRNP. Pseudouridylation at U250 is required for the P-TEFb containing 7SK RNP formation. Reducing 7SK RNP through DKC1 knock down leads to increased P-TEFb-SEC levels and enhanced Tat transactivation. Finally, DKC1 knock down potentiates the action of two latency-reversing agents, JQ1 and prostratin in the 2D10 Jurkat post-integrative latency model. The topic is of importance, the experiments are well designed and support the conclusions. However, it is unclear if 7SK pseudouridylation could be modulated through signal transduction and serve to fine tune SEC levels. This can be easily shown by using 7SK RNP disrupting agents such as HMBA or cell stress and analysis of 7SK pseudouridylation levels.

Minor concerns
In figure 4A and D, the levels of Tat-HA should be controlled for with HA immunoblot.
In figure 3F and 3G the authors focus on the AFF1 containing SEC complex but in figure 4D AFF4 occupancy at the HIV-1 promoter is shown by ChIP. Please explain

Referee #2:

The authors have followed up on a previously published finding that 7SK is pseudouridylated at U250. They show that most of the 7SK in cells is modified and that knockdown of DKC1 reduces the modification and reduces the interaction of P-TEFb and HEXIM1 with 7SK and increases the interactions of P-TEFb with Tat, Brd4 and SEC. Most of the results were obtained using transient and stable transfection assays followed by tagged-protein IPs.
Specific comments:
1. Fig. 1F, 1G and 1H are not properly referred to in the text.

2. In Fig. 2 it is not clear what WT means. Is WT actually a mutant except that U250 is not changed? This is necessary and should be described. If so, the RNA should not be called WT as the endogenous 7SK is WT. The reduction in tagged Cdk9 and tagged HEXIM1 is not quantified. What is the evidence that this is significant as indicated in the text? Why are there so many small panels shown? It would be better to show entire gels. How many times were the experiments performed? Were the results quantified? How reproducible were the findings? The experiments shown are not described in the methods. How was NE prepared? It is not clear how cells transfected with a tagged

protein and a different tagged RNA were normalized. As described, the results do not strongly support the conclusion: "Pseudouridylation of 7SK RNA on U250 is required for efficient formation of 7SK RNP." This would require quantification of IP results.

3. In Fig. 3. How was NE prepared? Normalizing 7SK to GAPDH in 3D needs explanation. 7SK is an RNA and GAPDH is a protein. The details of the experiments (methods are missing) are critical to evaluate the results. What is the timing for the knockdown, the loss of modification of U250, and the expression of Tat?

4. Tat, when overexpressed in cells, has been shown to cause release of P-TEFb from the snRNP and the formation of a Tat/P-TEFb complex that can associate with SEC. I don't understand how reduction of U250 modification increases the interaction between Cdk9 and Tat. It should be maximal already.

5. Fig. 4. It would be useful to show + Tat for some of the other promoters to show that the Tat effect is specific for the LTR?

5. Examination of the effect of DKC1 on the endogenous 7SK snRNP using another method besides IP would improve the study.

Overall, this is a potentially interesting study, but the use of such artificial analyses leaves one wondering what would happen in normal cells. Can the interactions shown in 3F be seen in an assay that separates complexes based on size? This would demonstrate the relative amounts of Cdk9 in the various complexes. How are the results with DKC1 KD different from other conditions that cause release of P-TEFb from the 7SK snRNP?

Referee #3:

This work describes the functional study of the pseudouridine modification in 7SK RNA. This abundant non-coding RNA has a well characterized function to modulate the availability of several cellular proteins needed for example for HIV transcription. Pseudouridine is the most abundant modification in cellular RNA. Recent pseudo-seq has hinted at a specific modification site in the 7SK RNA. The authors show that this modification is indeed present in the 7SK RNA from the cell, and it is modified through a H-ACA-snoRNP. They also performed functional studies comparing modified and less modified 7SK RNA and demonstrate that the U230 modification is indeed functionally relevant for its snoRNP and gene expression. Functional studies of specific RNA modifications are still sparse, this well-designed and well-conducted work provides a new insight on our understanding of RNA modifications.

1. My main comment is on the hypothesis that pseudoU230 is mainly there to stabilize 7SK structure. An alternative explanation is that this modification has little effect on 7SK structure, rather a protein directly contacts the modified base. This point should be clarified by comparative structural mapping using in vitro transcript (no modification) and purified, refolded 7SK RNA (with modification) from the cell.

2. The authors could add a figure on the evolutionary conservation of the U230 residue, and any potential phylogenetic support of the secondary structure around this residue.

1st Revision - authors' response 14 July 2016

Thank you very much for organizing the review of our manuscript and giving us a chance to improve our work. Here, I would like to submit a revised version with the altered and new texts all marked in blue and our point-by-point response to the reviewers' comments attached below. In addition to the multiple clarifications, correction of omissions, and quantification of the binding data that we have done in response to the reviewers' criticisms, several new pieces of data in Figures EV1 to EV4 have been added to address Reviewer #2's request for analysis of endogenous proteins (Fig. EV1), use of an alternative method to document the disruption of 7SK snRNP by DKC1 KD (Fig. EV2), demonstration of Tat's specificity toward the HIV-1 LTR (Fig. EV4), as well as

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Reviewer #1's suggestion to examine the modification status of 7SK RNA upon stress-induced disruption of 7SK snRNP (Fig. EV3). These new data have provided additional mechanistic insights into the role and regulation of 7SK RNA pseudouridylation by the DKC1-box H/ACA RNP. Through these revisions, we have addressed all the concerns raised by the reviewers and hope it is improved sufficiently to make it suitable for publication in your esteemed journal.

I am very thankful for your time, effort and careful consideration of our paper. Please let me know if additional information is needed.

Our point-by-point response to the reviewers' comments

Referee #1:

"In this manuscript, …… This can be easily shown by using 7SK RNP disrupting agents such as HMBA or cell stress and analysis of 7SK pseudouridylation levels."

This is an excellent question. To answer it, we treated the HeLa-based F1C2 cells stably expressing CDK9-F with i-CDK9 and DRB, which have been reported previously and confirmed here (Supplemental Fig. EV3A) to disrupt the 7SK snRNP. However, both treatments failed to alter the pseudouridylation state of U250 in 7SK snRNA as detected by 1D-TLC (Fig. EV3B).

Minor concerns
"In figure 4A and D, the levels of Tat-HA should be controlled for with HA immunoblot."

Since the Tat-HA cDNA used in transfection was too low (20 ng per well of the 6-well plate) to allow the protein levels to be determined by Western blotting, we performed qRT-PCR to detect the mRNA levels of Tat-HA and normalized them to those of GAPDH in the same cell. The data have been presented in revised Fig. 4C and 4E.

"In figure 3F and 3G the authors focus on the AFF1 containing SEC complex but in figure 4D AFF4 occupancy at the HIV-1 promoter is shown by ChIP. Please explain."

Since we were not able to obtain a suitable anti-AFF1 antibody for the ChIP assay, only the anti-AFF4 ChIP was performed. To make the analysis to the SEC formation more complete, we have now added the AFF4 panel to both revised Figure 3F and 3G.

Referee #2:

"The authors have followed …… Most of the results were obtained using transient and stable transfection assays followed by tagged-protein IPs."

"Specific comments:
1. Fig. 1F, 1G and 1H are not properly referred to in the text."

We thank the reviewer for pointing out these errors, which have been corrected in the revised manuscript.

"2. In Fig. 2 it is not clear what WT means. …… This would require quantification of IP results."

WT refers to an engineered 7SK RNA species that carries a sequence replacement (nt 216-221) to allows discrimination from endogenous 7SK RNA by primer extension. Except for this change, the rest of the RNA has wild-type sequence including the entire region at and around U250 as shown in Fig. 2A. Taken the reviewer suggestion, we have changed 'WT' in the revised text to 'tagged 7SK containing WT sequence in the U250 region" to be very precise.

Suggested by the reviewer, we have quantified the levels of tagged 7SK RNA in entire Fig. 2 and the numbers, which are indicated in the revised figure, indeed show significant changes as described in the text. Since space is very limited in this figure due to the format requirement of the journal, we have no choice but to show these relatively small size panels. In the new supplemental Fig. EV1 that investigates the binding of endogenous CDK9 to WT and the three 7SK RNA mutants (mut1, 2 &

3), we have a lot more space to show the entire gel of primer extension of the tagged 7SK.

All the experiments were done at least 3 times and the representative results are shown in the figures. More details related to the methods of co-transfection, co-IP and primer extension have been added to the revised text. The preparation of nuclear extracts followed the classic procedure by Dignam et al. (Dignam et al. 1983. *Nucleic Acids Res* **11:** 1475-1489), which is now cited in the revised text. The same amount of cDNA expressing the various tagged proteins was co-transfected into HeLa cells together with an equal amount of the construct expressing either tagged WT or mutant 7SK RNA. In fact, primer extension and Western analyses of the tagged 7SK RNA and protein in NE in each panel already show that their expression levels were generally the same or highly comparable. As mentioned above, the levels of the tagged 7SK RNA in each of the panel have now been quantified, and all three 7SK mutants showed over 50% decrease in binding to CDK9, HEXIM1 and hnRNP R. These results support the conclusion that "Pseudouridylation of 7SK RNA on U250 is required for efficient formation of 7SK RNP."

“3. In Fig. 3. How was NE prepared? Normalizing 7SK to GAPDH in 3D needs explanation. 7SK is an RNA and GAPDH is a protein. The details of the experiments (methods are missing) are critical to evaluate the results. What is the timing for the knockdown, the loss of modification of U250, and the expression of Tat?”

As mentioned above, the preparation of nuclear extracts followed the classic procedure by Dignam et al. In Fig. 3D, the levels of 7SK snRNA were normalized to the mRNA levels of GAPDH in the same cells, and we have relabeled the Y-axis to “7SK snRNA/GAPDH mRNA” to make it super clear. The KD of DKC1 was generally induced by DOX for 5 days and the loss of modification at U250 was detected 5 days post DOX induction and these have been added to the revised text. For the Tat-Flag IP, the expression of DKC1-specific shRNA was first induced by DOX for 3 days and then the cells were transfected with the Tat-Flag cDNA and harvested 2 days post transfection. The time line has been added to the revised legend of Fig. 3.

“4. Tat, when overexpressed in cells, has been shown ..... It should be maximal already.”

Only when overexpressed, which is impossible to achieve under real infection conditions, Tat is shown to cause release of P-TEFb from 7SK snRNP. We previously used ~20 ug of the Tat-expressing plasmid per 150 mm dish to see this effect. Here, under conditions that allow only a moderate level of Tat expression (2 ug/150 mm dish), the amount of Tat was not enough to completely disrupt the snRNP, and the DKC1 KD-induced 7SK snRNP disruption further enhanced the formation of the Tat-SEC complex. The detailed experimental conditions are now described in the revised legend to Fig. 3.

“5. Fig. 4. It would be useful to show + Tat for some of the other promoters to show that the Tat effect is specific for the LTR?”

Tat is a sequence-specific transactivator and requires the TAR element in the HIV-1 LTR for its function. In response to the reviewer’s request, we have conducted the suggested experiment and the result is shown in Fig. EV4, which confirms that the Tat effect is specific for the LTR.

“5. Examination of the effect of DKC1 on the endogenous 7SK snRNP using another method besides IP would improve the study.”

We have conducted a glycerol gradient analysis in Fig. EV2 to show that the DOX-induced DKC1 depletion caused HEXIM1, and to a lesser degree, CDK9 to move out of fractions 9-15 (indicated by a box in Fig. EV2) that correlated with the large size 7SK snRNP to the top of the gradient that contained only smaller size complexes and free proteins.

“Overall, this is a potentially interesting study …… DKC1 KD different from other conditions that cause release of P-TEFb from the 7SK snRNP?“

As mentioned above, a glycerol gradient has been done before and after DKC1 KD and the result is shown in Fig. EV2. Both DKC1 KD and certain stress-inducing agents such as i-CDK9 and DRB can induce the disruption of 7SK snRNP. However, as indicated in Fig. EV3 (in response to the first

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reviewer's question), we have found that unlike DKC1 KD, the drug-induced 7SK snRNP disruption did not alter the pseudouridylation state of U250 in 7SK snRNA.

Referee #3:

"This work describes …… this well-designed and well-conducted work provides a new insight on our understanding of RNA modifications."

We appreciate the reviewer's favorable comments and thank him/her for reviewing this manuscript.

"1. My main comment is on the hypothesis that pseudoU230 ….. purified, refolded 7SK RNA (with modification) from the cell."

We have indeed tried very hard to obtain some structural insights into how the modification may affect the overall structure of 7SK snRNA. However, due to technical difficulties, mostly the extreme difficulty of obtaining sufficient quantity of purified, refolded 7SK RNA with modification from the cell, we had to abandon these efforts. Regarding the possibility that a protein may directly contact the modified U250, since no known subunits (HEXIM1, LARP7, MePCE, CDK9 and CycT1) of the mature 7SK snRNP have been shown to directly bind to the middle region of 7SK containing the modified base, we have raised as a second possibility in Discussion that an unknown protein that interferes with the formation of the complete 7SK snRNP may recognize the "unmodified" U250. Upon modification, this inhibitory factor is gone to allow the formation of the RNP. This is consistent with the reviewer's idea that the modification at U250 may directly influence the binding of a protein factor.

"2. The authors could add a figure on the evolutionary conservation of the U230 …… support of the secondary structure around this residue."

Because a previous study by Gruber et al. (J Mol Evol **66**, 107-115, 2008) has extensively discussed the evolutionary conservation of U250 and its flanking region in vertebrates (see Fig. 5C therein) as well as their influence on forming the so-called stem B around this residue, we decide to cite this paper in revised results and discussion sections to support the argument that both U250 and its flanking sequences are highly conserved in vertebrates and it is possible that Ψ250 also exists in other vertebrate 7SK RNA and that it controls 7SK RNP formation as in humans.

3rd Editorial Decision 22 July 2016

Thank you for the submission of your revised manuscript to our journal. We have now received the comments from the referee that was asked to assess it, and I am hapy to tell you that s/he supports the publication of your revised work.

However, a few changes are still needed. Your manuscript has 4 main figures at the moment and is therefore a scientific report. For reports, the discussion and results sections must be combined. An alternative option would be to move 2 EV figures to the main manuscript file and change the manuscritp into an article. But given the single panel EV figures, I think it makes more sense to combine the results and discussion sections.

Please specify "n" and the error bars for figures 3A and EV4 in the legends.

Figures 1, EV2 and EV3 need to be changed from landscape into portrait format.

I further see a lot of white lines on all gel pictures. I am not sure whether this is a defect of the figure file conversion. Please check your original and uploaded figures. If the original figures do not have these lines, it might be OK.

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. Please let me know if you have any questions.

REFEREE REPORTS

Referee #2:

The authors have addressed my comments and I believe the other reviewers' comments in a satisfactory way.

3rd Revision - authors' response 27 July 2016

I'm very glad to learn that Reviewer #2 supports the publication of our revised manuscript. Here, I would like to submit a new version that addresses the remaining issues raised in your letter.

We have (1) added a two-sentence summary of the main findings and their significance after the abstract page; (2) provided two bullet points highlighting key results; (3) specified "n" and the error bars for figures 3A and EV4 in the legends; (4) converted all figures into the portrait format; (5) included a synopsis image that is 550x200-400 pixels large to accompany the paper; and (6) carefully checked the submitted figures and found no white lines in the gel images. Through these revisions, it is my hope that the manuscript is for publication in your esteemed journal.

I am very thankful for your time, effort and careful consideration of our paper. Please let me know if additional information is needed.

4th Editorial Decision 28 July 2016

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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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Qiang Zhou

Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42682V3

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)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	See legends of figure 3 and 4 (p18-19).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	All experiments are done using immortalized cell lines.
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.	To minimize the effects of subjective bias, some experiments were performed by different lab members.
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?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We did not test for normality.
Is there an estimate of variation within each group of data?	Yes. See legends of Figure 3 and 4 (p18-19).
Is the variance similar between the groups that are being statistically compared?	Yes. See legends of Figure 3 and 4 (p18-19).

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).	See Materials and Methods (p11)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Information of cell lines are provided in Materials and Methods (p13), cells are routinely tested for mycoplasma.

* 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.	NA
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
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
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).	
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.	No