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Conformational Change of Syntaxin Linker Region induced by Munc13 Initiates SNARE Complex Formation in Synaptic Exocytosis

Shen Wang, Ucheor Choi, Jihong Gong, Xiaoyu Yang, Yun Li, Austin Wang, Xiaofei Yang, Axel Brunger and Cong Ma

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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 25 October 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see, referee #2 and #3 appreciate your analyses and provide constructive input on how to further strengthen your data. However, it is also clear from the reports (referee #1 and #2) that the broader advance provided by your findings is currently not sufficiently compelling, and this criticism needs to be addressed when revising your work.

REFEREE REPORTS

Referee #1:

In this article, the authors identify two conserved residues in the Syntaxin 1 linker region, based on known crystal structures, which they convincingly show to be important for the action of Munc13. They used a series of well carried out and controlled experiments with recombinant proteins, liposomes, electrophysiological recordings using cultured neurons, and a very nice FRET assay. However, the results shown here, despite being so convincing, are looking at a small detail of a molecular mechanism and do not provide any groundbreaking new concept in the field.

Referee #2:

The authors investigate here the interaction of syntaxin 1 with the exocytosis cofactor Munc18. This is a well-known molecular interaction, which has been discussed in several hundred publications already. Nevertheless, the exact molecular details continue to be of great interest, and publications on the increasingly fine details of the Munc18, Munc13 and syntaxin1 interactions appear at a great pace. They work presented here follows the following basic model: closed syntaxin1 interacts with Munc18, a step that is involved in vesicle priming, and is later opened by interaction with Munc13, a model that the authors have recently verified by studying the Munc13-syntaxin1 interaction (Yang et al., 2015). The authors now switch their attention to the Munc18-syntaxin1 interaction, and suggest that the linker region of the latter, and especially residues R151 and I155, is essential for the function of Munc18, Munc13 and syntaxin1.

The work is convincing, and covers this problem both in vitro, using purified proteins or reconstituted fusion reactions, and in cultured cells, relying on protein expression. The authors should nevertheless check a few minor issues:

- In Figure 2C, the example recording shown for Syx-1A RIAA is not consistent with the quantification shown (the amplitude of the recording shown is far smaller than what would be expected from the quantification).

- The IPSCs shown in Figure 2B appear to have somewhat different kinetics. The Syx-1A I155A (incorrectly labeled as I555A; same error in Figure 2C) recording decays faster than the others, while the "none" recording decays slower. Are these differences consistent? If so, this should be analyzed, and the potential effects on vesicle priming should be discussed.

- Do the two mutations change the rigidity of the linker region? Could the authors discuss this issue?

- The authors should make a stronger effort in presenting the novelty of their work. Otherwise, the result, stated blandly, is only that the linker region of a protein which changes conformation is important in the conformation change. This is expected. It is also not unexpected that a protein that stabilizes one of the conformations (Munc18) interacts with the linker region. The authors should therefore emphasize more strongly how their newly discovered interactions add to the Munc-syntaxin field.

Referee #3:

In this study, Wang et al. have carried out a series of in-vitro experiments in order to clarify the still somewhat enigmatic activation pathway of the neuronal SNARE protein syntaxin-1. Previous work by several laboratories suggested that, starting from an inactive Munc18/syntaxin complex in a closed conformation, the CATCHR protein Munc13 activates the complex (partial opening?) in order to make it accessible to the formation of SNARE complexes. This step can be bypassed in a "classical" mutant in which two residues connecting the linker between the SNARE-domain and the Habc-domain of syntaxin are exchanged (LE mutant), resulting in a preferentially open conformation even though it is still closed when bound to Munc18-1.

Taking these observations as a point of departure, the authors have now generated several new mutants in the linker of syntaxin in order to shed light on the conformational changes during syntaxin activation. One of the new syntaxin mutants phenocopies the LE mutant, whereas a second mutant (referred to as RIAA) does not interact at all with the MUN domain. Similarly, a mutant of the MUN domain (referred to as NF) was used that does not bind to syntaxin.
Without repeating the rather sophisticated experiments in detail, the results show that the MUN domain does not open the closed Munc18/syx complex but rather results in re-arrangements in the linker region that cause higher reactivity towards SNARE assembly. Moreover, the authors show that such intermediate closed but more reactive structure is also present in the LE-mutant even in the absence of the MUN domain, thus providing a molecular explanation for the observation why the LE mutant is able to partially bypass the dependence of exocytosis on (M)unc13.

Generally, this is an interesting study that adds to the understanding of a possible scenario for SNARE activation in the neuronal system. Most of the experiments are of high quality and generally support the conclusions, and they also confirm earlier work not only from the Brunger but also from other labs (not necessarily commonplace in this competitive field). Central to the study are the single molecule FRET experiments that allow for a determination of closed vs open structure of syntaxin. Thus, in my opinion the paper should be acceptable provided the following issues are addressed:

1. The authors used a 15 residue peptide in order to study binding to the MUN fragment in a reduced system. Here, additional controls are needed, such as measuring whether the peptide also binds to Munc18-1 (which it should not), and whether the peptide effectively competes with the binding of full-length syntaxin.

2. In the single molecule experiments shown in Fig. 4, I do not understand why the authors add the syntaxin RIAA mutant to the mix. What was the expectation here? It would be much more interesting to generate CC and MN mutants with this variant also and then look for conformational changes.

Minor points:
The authors state that the MUN domain only binds to assembled Munc18-1/syntaxin complexes but not to free SNAREs. This contradicts earlier work by the Rizo laboratory (Guan et al. (2008) Biochemistry 47, 1474). Please explain.
Fig. 1 A, B, C: The yellow labels are extremely difficult to see on a print and also on the screen. Please replace with darker color to yield more contrast. Similarly, the labeling size in some figures (particularly in Fig. 4) is so small that it can only be read with a magnifying glass on a full-page printout.

1st Revision - authors' response 10 December 2016

We thank the Editor for giving us an opportunity to revise our manuscript. In the revised version, we extensively rewrote and expanded the Introduction and the Discussion with an effort in presenting the novelty of our work more clearly.

Referee #1:

In this article, the authors identify two conserved residues in the Syntaxin 1 linker region, based on known crystal structures, which they convincingly show to be important for the action of Munc13. They used a series of well carried out and controlled experiments with recombinant proteins, liposomes, electrophysiological recordings using cultured neurons, and a very nice FRET assay. However, the results shown here, despite being so convincing, are looking at a small detail of a molecular mechanism and do not provide any groundbreaking new concept in the field.

First, we thank the referee #1 for agreeing that our results are convincing.

Second, we believe that our observation of a local conformational change in the syntaxin-1 linker region induced by Munc13-1 provides an important new insight of the molecular mechanism of Munc13. Our insights add to the understanding of a possible scenario for SNARE activation in the neuronal system. The key points are:

i) a working model of Munc13-1 in syntaxin-1 activation for SNARE complex formation is proposed in this work. Munc13-1 binds the Munc18-1/syntaxin-1 complex without opening syntaxin-1, but instead it induces local re-arrangements in the syntaxin-1 linker region that cause higher reactivity towards neuronal SNARE complex assembly.

ii) a Munc18-1/syntaxin-1/Munc13-1 intermediate identified in this work might be the key for the transition from the closed Munc18-1/syntaxin-1 complex to the ternary SNARE complex. Such an intermediate, globally closed conformation is also present in the more reactive structure of the LE

mutant of syntaxin-1 even in the absence of Munc13-1, thus providing a molecular explanation for the observation why the LE mutant is able to partially bypass the dependence of Munc13 on synaptic exocytosis.

iii) the Munc13-1/syntaxin-1 interaction identified in the present work adds a new regulatory layer to the established Munc18-1/syntaxin-1 interactions, and suggests a Munc18-1/syntaxin-1/Munc13-1 network that allows exquisite regulation of synaptic exocytosis.

iv) our results suggest that the Munc18-1/syntaxin-1/Munc13-1 interactions would preclude formation of the "dead-end" syntaxin-1/SNAP-25 (2:1) complex, promoting the proper 1:1:1 stoichiometry of the ternary SNARE complex.

To emphasize these points, we rewrote the Introduction and the Discussion in the revised manuscript.

Referee #2:

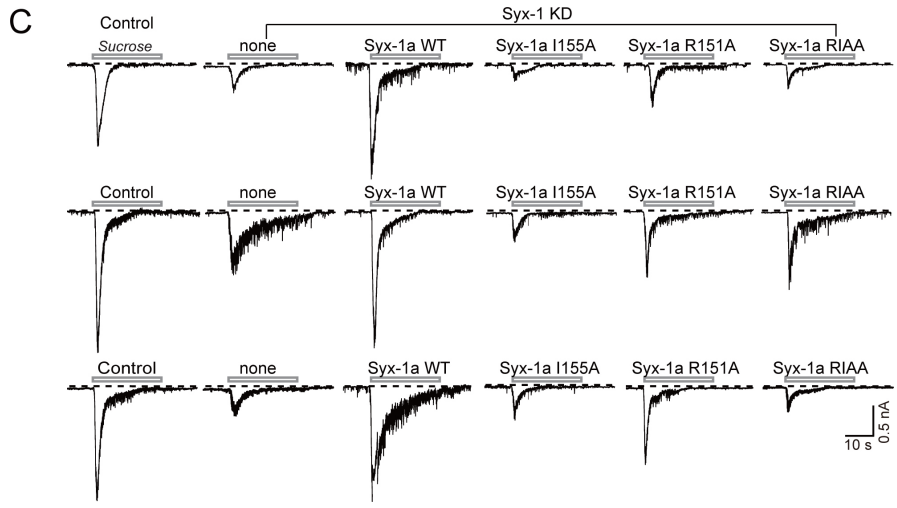
The authors investigate here the interaction of syntaxin 1 with the exocytosis cofactor Munc18. This is a well-known molecular interaction, which has been discussed in several hundred publications already. Nevertheless, the exact molecular details continue to be of great interest, and publications on the increasingly fine details of the Munc18, Munc13 and syntaxin1 interactions appear at a great pace. They work presented here follows the following basic model: closed syntaxin1 interacts with Munc18, a step that is involved in vesicle priming, and is later opened by interaction with Munc13, a model that the authors have recently verified by studying the Munc13-syntaxin1 interaction (Yang et al., 2015). The authors now switch their attention to the Munc18-syntaxin1 interaction, and suggest that the linker region of the latter, and especially residues R151 and I155, is essential for the function of Munc18, Munc13 and syntaxin1.

The work is convincing, and covers this problem both in vitro, using purified proteins or reconstituted fusion reactions, and in cultured cells, relying on protein expression.

We thank the referee #2 for the positive comments.

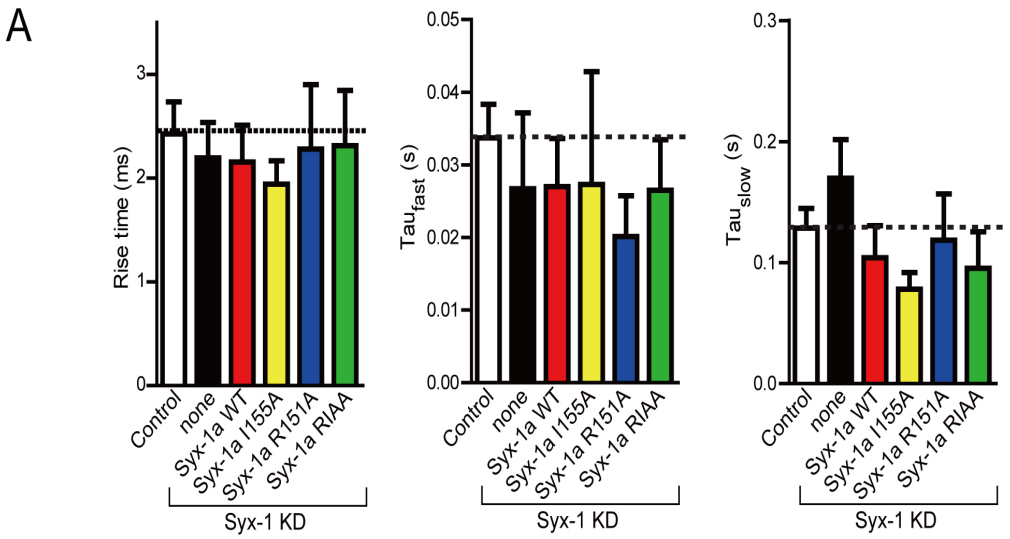
The authors should nevertheless check a few minor issues:
- In Figure 2C, the example recording shown for Syx-1A RIAA is not consistent with the quantification shown (the amplitude of the recording shown is far smaller than what would be expected from the quantification).

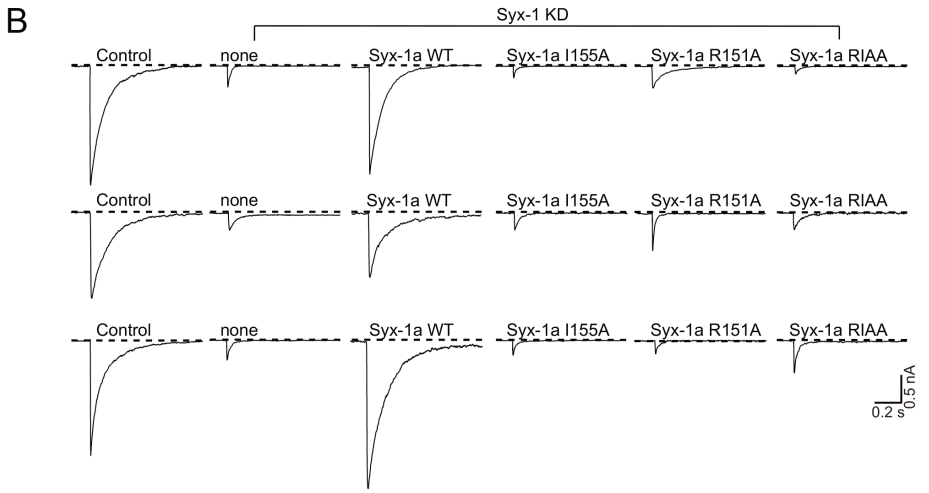
We thank referee #2 for pointing this out. We have added another example recording for Syx-1a RIAA to keep it consistent with the quantification. To illustrate the variability of RRPs, we also included three additional example recordings for each group shown in the revised Fig 2C and the new Appendix Fig S1C, also see below C.



- The IPSCs shown in Figure 2B appear to have somewhat different kinetics. The Syx-1A I155A (incorrectly labeled as I555A; same error in Figure 2C) recording decays faster than the others, while the "none" recording decays slower. Are these differences consistent? If so, this should be analyzed, and the potential effects on vesicle priming should be discussed.

We apologize for the typo. We have corrected it as "Syx-1a I155A" in both Figures 2B and 2C. We also performed more extensive kinetic analyses. We measured the rise time of the IPSCs and observed no difference among all the conditions. We then fitted decay time constants τ_{fast} and τ_{slow} to the data. Again, we did not detect significant differences. We have provided additional example traces for "none" and "Syx-1a RIAA" (new Appendix Fig S1B and also see below B). More variations of IPSCs in τ_{fast} measurement in "none" and "Syx-1a R151A" were observed, indicating a potential synchronous release impairment in these groups (new Appendix Fig S1A and also see below A).





- Do the two mutations change the rigidity of the linker region? Could the authors discuss this issue?

The RI mutant of syntaxin-1 probably does not change the conformation of the linker region because it has a similar elution volume as the wild-type (WT) syntaxin-1 observed by gel-filtration chromatography (Fig 1B and Table EV1). In contrast, the LE mutations (L165A/E166A and L169A/E170A) in the short linker helix resulted in substantially lower elution volumes than WT syntaxin-1 (Fig 1B and Table EV1). This suggests that the two LE mutations, but not the RI mutation, destabilize the linker region, and perhaps lead to the H3 domain self-association. We now discuss these points in the Results.

- The authors should make a stronger effort in presenting the novelty of their work. Otherwise, the result, stated blandly, is only that the linker region of a protein which changes conformation is important in the conformation change. This is expected. It is also not unexpected that a protein that stabilizes one of the conformations (Munc18) interacts with the linker region. The authors should therefore emphasize more strongly how their newly discovered interactions add to the Munc-syntaxin field.

We thank the referee #2 for the excellent suggestions. We rewrote the Introduction and the Discussion as detailed in the response to referee #1 above.

Referee #3:

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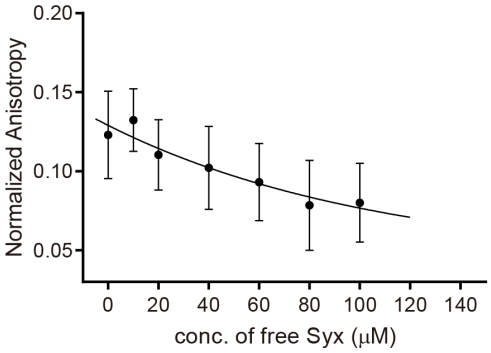
We thank the referee #3 for the positive assessment of our work.

1. The authors used a 15 residue peptide in order to study binding to the MUN fragment in a reduced system. Here, additional controls are needed, such as measuring whether the peptide also binds to Munc18-1 (which it should not), and whether the peptide effectively competes with the binding of full-length syntaxin.

We thank the referee #3 for the excellent suggestions and performed the additional control experiments, which are shown in Fig 3 and Table EV3 in the revised manuscript. Indeed, the peptide does not bind to Munc18-1 (Fig 3C). In addition, the cytoplasmic region of syntaxin-1 (referred to as Syx, residues 2–253) competes with the peptide (Appendix Fig S2 and see below). The concentrations of MUN-BC fragment and rhodamine B-labeled syntaxin-1 peptide were constrained

to 60 μM and 50 nM, respectively. Data plots were presented as means ± SEM (n=10). A non-linear curve fit was performed by using one-phase exponential decay without constraints. Our results show that Syx competes with the peptide for binding to the MUN-BC fragment. In Fig 3B, we used 75 μM Syx (the value of $T_{1/2}$ in the pilot experiment) to illustrate the binding competition between Syx and the peptide.



2. In the single molecule experiments shown in Fig. 4, I do not understand why the authors add the syntaxin RIAA mutant to the mix. What was the expectation here? It would be much more interesting to generate CC and MN mutants with this variant also and then look for conformational changes.

In Figs 1–3, we show that the RI residues in the syntaxin-1 linker region are involved in Munc13-1 MUN domain binding and MUN's catalytic function. Thus, in our smFRET experiments shown in Fig 4, we investigated whether the RIAA mutation accordingly influences the ability of MUN for changing conformations of syntaxin-1.

As described in our smFRET experiments (Fig 4), we designed two FRET labeling pairs on syntaxin-1: E35C and S249C (referred to as syntaxin-1-**CC**, Fig 4A) to monitor relative movements between the H_{abc} and the H3 domain; S95C and S171C (referred to as syntaxin-1-**MN**, Fig 4A) to monitor relative movements between the syntaxin-1 linker region and the H_{abc} domain. Thus, the CC and the MN described in our experiments are not mutations; they represent the engineered labeling sites for detecting conformational changes of syntaxin-1 by smFRET.

Minor points:
The authors state that the MUN domain only binds to assembled Munc18-1/syntaxin complexes but not to free SNAREs. This contradicts earlier work by the Rizo laboratory (Guan et al. (2008) Biochemistry 47, 1474). Please explain.

Our co-flotation experiments suggest that the MUN domain binds preferably to the Munc18-1/syntaxin-1 complex rather than isolated syntaxin-1. This might arise because the binding between the MUN domain and free syntaxin-1 is weak. It is also possible, or even likely, that other Munc18-1/MUN interactions might help to increase the MUN/syntaxin-1 binding affinity and enhance the catalytic activity of the MUN domain. This result does not contradict with the previous finding that the MUN domain binds to membrane-anchored SNARE complexes (Guan et al, 2008, Biochemistry 47, 1474), because after opening syntaxin-1, it is likely that the MUN domain interacts with the assembled SNARE complex (e.g., via binding with the H3 domain and/or the other SNAREs) (Ma et al, 2011, Nat. Struct. Mol. Biol. 18, 542 and unpublished data in Brunger lab), stabilizing the SNARE four-helical bundle, which is important for membrane fusion.

Fig. 1 A, B, C: The yellow labels are extremely difficult to see on a print and also on the screen. Please replace with darker color to yield more contrast. Similarly, the labeling size in some figures (particularly in Fig. 4) is so small that it can only be read with a magnifying glass on a full-page printout.

We apologize and we have improved the quality of Figures 1 and 4 in the new version.

2nd Editorial Decision 22 December 2017

Dear Dr. Ma,

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by two of the original referees again whose comments are enclosed. As you will see, the referees appreciate the introduced changes, and I am thus happy to accept your manuscript in principle for publication in The EMBO Journal.

REFEREE REPORTS

Referee #2:

The authors have addressed all of the points I raised in my original review. I am therefore happy to suggest that the manuscript be published.

Referee #3:

During revision, the authors have carefully addressed all points raised in the review, including additional experiments that further strengthen the interpretation of the authors. For these reasons, I recommend acceptance of the revised manuscript.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Cong Ma
Journal Submitted to: the EMBO Journal
Manuscript Number: EMBOJ-2016-95775

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.

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).

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?	For each in vivo experiment, at least 3 independent cultures, around 15 cells were analyzed (pp.43, lines 926-928; pp.48, lines 1039-1040); For each in vitro experiment, at least 3 independent measurements were shown and analyzed (pp.42, lines 907-908, 911-912; pp.44, lines 940-941, 946-947; pp.47, lines 1002-1004, 1012-1013; For single molecule experiments, two subsets of an equal parition of the data was used to show the mean values ± SD for the observed FRET efficiency values for all molelcules for each different conditions (pp. 45, lines 969-970; pp.49, lines 1050-1051).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	In each experiment, we cultured neurons from at least 3 pups (pp.43, lines 924-925; pp.48, lines 1036-1037).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We didn't exclude any data from the analysis.
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.	All the experiments had been done blindly: all the conditions had been coded when the experimenter collected the data.
For animal studies, include a statement about randomization even if no randomization was used.	All wild type mice were randomly used (pp.25, lines 542-544).
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.	All the experiments had been done blindly: all the conditions had been coded when the experimenter collected the data.
4.b. For animal studies, include a statement about blinding even if no blinding was done	All wild type mice were randomly picked up (pp.25, lines 542-544).
5. For every figure, are statistical tests justified as appropriate?	Statistical analysis were performed with Student's t tests comparing test to control samples (pp.42, lines 905-906; pp.43, lines 926-928; pp. 45, lines 969-970, see also Appendix Table S2-S3; pp.47, lines 1002-1004; pp.48, lines 1039-1040; pp.49, line 1050, see also Appendix Table S2-S3).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The data are normal distribution, thus Student's t tests can be used for comparing test to control samples.
Is there an estimate of variation within each group of data?	In each group of data, SEMs were shown or used for analysis (pp.42, lines 907-908; pp.43, lines 926-928; pp.44, lines 943-944; pp. 45, lines 966-968, see also Appendix Table S2-S3; pp.47, lines 1002-1004; pp.48, lines 1036-1037; pp.49, lines 1047-1049).

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Is the variance similar between the groups that are being statistically compared?	Yes, the variance between groups are similar except we mentioned the difference in the manuscript.

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).	non applicable
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293 cells were purchased from ATCC, no mycoplasma contamination was found. (pp.25, lines 538-539)

* 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.	Wild type mice were fed by mouse facility of South-Central University for Nationalities.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	non applicable
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.	All animal procedures used were approved by South-Central University for Nationalities institutional review boards.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	non applicable
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.	non applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	non applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	non applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	non applicable
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.	non applicable
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.	non applicable

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	non applicable
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).	non applicable
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).	non applicable
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	non applicable
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.	non applicable

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.	non applicable