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Substrate Recruitment of γ-Secretase and Mechanism of Clinical Presenilin Mutations Revealed by Photoaffinity Mapping

Akio Fukumori and Harald Steiner

Corresponding author: Harald Steiner, Ludwig-Maximilians-University Munich & German Center of Neurodegenerative Diseases

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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 17 March 2016

Thank your for submitting your manuscript to The EMBO Journal. Three referees have now seen your study and their comments are provided below.

As you can see, the referees find the work exciting and suitable for publication in the EMBO Journal. They also find the data of very high quality and raise only minor issues. I am therefore very happy to invite a revised version. The raised concerns should be straightforward to address, but let me know if we need to discuss any of them in further details.

REFEREE COMMENTS

Referee #1:

This manuscript uses genetically encoded photocrosslinkers to probe the mechanism of substrate recognition by the gamma-secretase (GS) complex. The mechanism of action and principles of substrate recognition by intramembrane proteases are hotly debated at present, and the subject of this manuscript is thus very relevant and interesting. The approach Fukumori and Steiner have chosen is biochemically very challenging, but it is very suitable for the purpose and the authors have done an excellent job. All the data are of very high quality, presentation is clear, manuscript is concise and logically structured and the data fully support authors' conclusions.

Benzoylphenylalanine is introduced in each position of the GS substrate C100 (derived from APP), and interactions with individual GS subunits are revealed by UV-induced crosslinking and western blotting. Recombinant purified components in detergent micelles were used throughout. Using elegant, logical and well controlled experiments, the authors conclude that GS substrates contact the enzyme at several successive exosites before interacting with the active site and undergoing scission. Furthermore, they analyse the effect of the early onset Alzheimer disease mutations in presenilin on substrate recognition and cleavage specificity, whose causes were hitherto unexplained. They find that in the two Alzheimer's mutants analysed, the binding of C100 to GS is shifted in a way that is consistent with a spectrum of cleavage products biased towards Abeta42 that GS-s with the Alzheimer mutations exhibit. Finally, the authors map the low resolution substrate interaction 'map' onto the recently derived 3D structure of GS (Fig.7). This model is less satisfying as it is consistent with multiple pathways of substrate access from the exosite(s) to the cataytic site. But this is probably the maximal level of detail this biochemical approach can provide, unless the authors would be able to map the cross-linking sites to specific TMDs of GS by mass-spectrometry. As a minor point, the abstract seems a bit clumsy in places. For example, "Despite structural information,..." made me pause. I guess the authors meant more explicitly "Despite the availability of structural information on intramembrane protease apoenzymes, ...". So a reformulation might be considered. Second, "pathomechanism" is an extreme neologism and "pathological mechanism" should be used instead. Third, "mechanistic model of how these subunits interact dynamically to mediate..." There is not much information about the dynamics in the manuscript, the word "dynamics" thus sounds as a biochemical cliche, and I suggest to remove it from the above sentence unless the authors can justify otherwise.

In summary, this is a beautiful and solid piece of work bringing novel and important information.

Referee #2:

This is a tour de force using elegant biochemistry to interrogate the binding of APP C99 gamma-secretase and elucidate how this interaction occurs and how the interaction is altered by inhibitors and FAD-linked Presenilin mutations. The data reveal precise molecular insights into the interaction between gamma-secretase and substrate and provide that reveal a number of surprising features. This study represents a huge amount of work. Many outside the intramembrane protease and Alzheimer's fields will find this of interest simply for its technical "wow" factor. There are few interpretative issues and one experimental issue that could be addressed to strengthen the manuscript.

1. Experimental. Mutations in APP or PS1 can alter gamma-secretase cleavage. IN AD the key issue is the ratio of Ab42 produced. The BpA containing constructs are analyzed for overall cleavage but how the artificial amino acid alters ratios of Abetas produced is not clear. I hate to suggest that the authors measure Abeta42:40 ratios form all constructs but it would seem important to sample this for some. Indeed, it is sort of quantum mechanics like question by trying to measure the output have you altered the system? I think putting some parameters around this is important to establish the potential confound that this introduces. My own guess would be that there will be a relationship between reduced cleavage and ABeta 42 ratios.
2. I think this comment does nto accurately reflect the literature: In the majority of cases, PS FAD mutants display a partial loss of function affecting the efficiency with which !-secretase processes the TMD of C99 32,35,46. This manifests in an increased ratio of A"42 and A"43 species over A"40, the predominant A" species generated by g-secretase 32,34. These mutations are not all loss of function but loss of processivity, so we do now the mechanism just not at the level of molecular interactions.
3. A few sentences such as the following seem to miss something "Unlike expected from previous biochemical data showing that PEN-2 is in close proximity to the PS1 CTF and possibly in vicinity to the catalytic site 40, PEN-2 locates very distant from the active site in the atomic g-secretase structure 11."

Referee #3:

The authors have introduced a photocrosslinkable amino acid at 68 residues in the APP-C99 substrate and tested which ones can be cross linked to γ-secretase. They combine this approach with a series of clever experiments for instance showing that certain cross links can be competed or blocked by active site inhibitors, while other cross links are increased. The first type of binding sites are likely defining interactions between substrate and active site, while the other define potential exosites on Nicastrin, Pen2 and PSEN-NTF. They also perform a very clever substrate-binding chase, further confirming the identity of the exosites. They show that the cross linking interactions are affected by clinical mutations.
This is without doubt an impressive piece of work. I like very much the creative way the authors have worked with the cross-linking technique and have invented novel ways to probe the function of gamma-secretase complex. I agree with the authors that the current structures that were recently published provide little insight in how gamma-secretase works, and the type of study performed here is an important complementary approach to structural studies. Such studies will push the field to generate structures of the complex that provide real insight into function (the currently available structure is in an inactive conformation, although a recent paper in eLife provides further insights).
Overall this paper deserves publication in an excellent journal. I consider the work of the highest quality, the questions addressed highly relevant and the research as highly original. I do not think that for this manuscript additional work is needed, but I have a series of questions and remarks that should be addressed.
-in the abstract the authors say that they identified "all" relevant residues... This is of course an overstatement
-in the introduction not always the correct publications are cited, and the authors switch to reviews when citing work from other groups while citing original papers when citing work from the own group. E.g. the Notch cleavage (Imbibo et al is cited) and the APP-CTF cleavage (Lichtenthaler et al is cited) is both work pioneered by De Strooper et al.; the progressive (stepwise) processing of APP-CTF (Morishima-kawashima is cited) was originally work from Ihara et al, the need of complex formation and additional components to get active gamma-secretase has been work from many groups (Edbauer et al is cited but also the group of Iwatsubo and many others should be given credit). There are probably other citations that need to be verified as well.
- page 4, Chapso solubilized gamma-secretase comes with its problems: the complex is destabilized and the assay is tricky as longer Abeta peptides tend to be generated. Thus a little bit more critical presentation of the assay would be appropriate. Can the authors exclude artificial cross links or lack of cross links because the use of detergent?
- When the first cross linking experiments are described (page 4) the fact that the APH1 bands are non- specific should be discussed more explicitly (it would help the reader)
-page 5 "finally further analysis suggested that the active site of γ-secretase in the PS1 NTF and CTF locates at the epsilon cleavage sites in C99" deserves more explanation. Also the figure sup 3 is unclear and the legend does not provide sufficient explanation to make this piece of data clearly understandable.
Discussion: I am not sure that all interpretation of the data is as straight forward as the authors claim. We know that presenilin is a conformational flexible protein and that GSI can stabilize the structure. It is not clear to me to what extent GSI really compete with binding of substrate or to what extent they alter the conformation of the protease, making certain residues no longer exposed and indirectly affecting the cross linking. Some more discussion and proposal of alternative interpretations might be considered.
Page 10 "Based on increased substrate crosslinking... we speculate..close to active site or come close to it.." I do not see the logic in this sentence/conclusion. Please reevaluate.
Page 11, when discussing the structure, the recent paper in eLife from Scheres should be taken into account.
Page 11-12 when discussing the effect of clinical mutations on the binding of substrate the authors should indicate that their proposal is based on experiments with only two mutations and that the effect on shift in cleavage site is only shown with one of the two mutations. There are alternative mechanisms possible (loss of processivity) and it is also not excluded that combination of effects are playing.
In conclusion, this is a very exciting and interesting study, the story is overall well written, and it will impact the further study of gamma-secretase and likely the broader field of intramembrane proteolysis. A careful editing of the text might make the work ready to go.

1st Revision - authors' response 22 April 2016

Response to Reviewer 1:

The reviewer stated: „*The mechanism of action and principles of substrate recognition by intramembrane proteases are hotly debated at present, and the subject of this manuscript is thus very relevant and interesting. The approach Fukumori and Steiner have chosen is biochemically very challenging, but it is very suitable for the purpose and the authors have done an excellent job. All the data are of very high quality, presentation is clear, manuscript is concise and logically structured and the data fully support authors' conclusions". ... "In summary, this is a beautiful and solid piece of work bringing novel and important information."*

Response: We thank the reviewer for his very positive overall assessment of our work.

As a minor point, the abstract seems a bit clumsy in places. For example, "Despite structural information,..." made me pause. I guess the authors meant more explicitly "Despite the availability of structural information on intramembrane protease apoenzymes, ...". So a reformulation might be considered.

Response: We reworded this sentence (see page 2).

Second, "pathomechanism" is an extreme neologism and "pathological mechanism" should be used instead.

Response: We replaced the term "pathomechanism" as suggested (see page 2).

Third, "mechanistic model of how these subunits interact dynamically to mediate..." There is not much information about the dynamics in the manuscript, the word "dynamics" thus sounds as a biochemical cliche, and I suggest to remove it from the above sentence unless the authors can justify otherwise.

Response: We used this adverb to further emphasize that we found a stepwise transfer of substrate to the active site, which is consistent with a dynamic process. But we agree that it may not be needed here and thus removed "dynamically" to avoid the impression of using a cliché-like word as suggested (see page 2).

Response to Reviewer 2:

The reviewer stated: *"This is a tour de force using elegant biochemistry to interrogate the binding of APP C99 gamma-secretase and elucidate how this interaction occurs and how the interaction is altered by inhibitors and FAD-linked Presenilin mutations. The data reveal precise molecular insights into the interaction between gamma-secretase and substrate and provide that reveal a number of surprising features. This study represents a huge amount of work. Many outside the intramembrane protease and Alzheimer's fields will find this of interest simply for its technical "wow" factor. There are few interpretative issues and one experimental issue that could be addressed to strengthen the manuscript".*

Response: We thank the reviewer for his very positive evaluation of our work and his excitement.

1. Experimental. Mutations in APP or PS1 can alter gamma-secretase cleavage. IN AD the key issue is the ratio of Ab42 produced. The BpA containing constructs are analyzed for overall cleavage but how the artificial amino acid alters ratios of Abetas produced is not clear. I hate to suggest that the authors measure Abeta42:40 ratios form all constructs but it would seem important to sample this for some. Indeed, it is sort of quantum mechanics like question by trying to measure the output have you altered the system? I think putting some parameters around this is important to establish the potential confound that this introduces. My own guess would be that there will be a relationship between reduced cleavage and ABeta 42 ratios.

Response: Since Aβ40 and Aβ42 are downstream in the processing cascade of C99 and products of the longer Aβ43 or Aβ45/46 we did not focus on the generation of these and only evaluated the generation of AICD, which is the direct cleavage product of our C99 substrate variants. However, as suggested by the reviewer, we have now additionally also measured Aβ42/40 ratios for the C99-Bpa substrates. This analysis, presented in the form of Aβ42/(Aβ40+Aβ42) ratios in Appendix Fig S1, showed that the large majority of the mutants do not cause increased Aβ ratios showing that introduction of Bpa *per se* does not cause pathogenic APP processing. Only very few mutants caused ratio increases, mostly at positions known previously to cause relative increases in Aβ42 generation when mutated to the Bpa-related phenylalanine residue (Lichtenthaler et al., PNAS 1999).

In contrast to what the reviewer guessed, we could not see a correlation between reduced overall cleavage activity and increased Aβ42 generation. This is consistent with previous observations from several labs showing that endopeptidase (ε-site cleavage) and trimming activity (ζ- and γ-site cleavages) are not necessarily coupled (e.g. Moehlmann et al., PNAS 2002, Quintero-Monzon et al. Biochemistry 2011, Chavez-Guitierrez et al., EMBO J. 2012, Szaruga et al. J. Exp. Med., 2015).

We also discussed in the new paragraph “Limitations” that the introduction of Bpa, which naturally creates a mutant substrate, represents an unavoidable, intrinsic limitation of this approach (see page 14).

2. I think this comment does nto accurately reflect the literature: In the majority of cases, PS FAD mutants display a partial loss of function affecting the efficiency with which !-secretase processes the TMD of C99 32,35,46. This manifests in an increased ratio of A"42 and A"43 species over A"40, the predominant A" species generated by g-secretase 32,34. These mutations are not all loss of function but loss of processivity, so we do now the mechanism just not at the level of molecular interactions.

Response: As suggested by the reviewer we have reworded this sentence to better highlight the loss of processivity of the PS FAD mutants (see page 13).

3. A few sentences such as the following seem to miss something "Unlike expected from previous biochemical data showing that PEN-2 is in close proximity to the PS1 CTF and possibly in vicinity to the catalytic site 40, PEN-2 locates very distant from the active site in the atomic g-secretase structure 11."

Response: We split this sentence into two hoping that the content is now clearly understandable by this separation.

Response to reviewer 3:

The reviewer stated: *“This is without doubt an impressive piece of work. I like very much the creative way the authors have worked with the cross-linking technique and have invented novel ways to probe the function of gamma-secretase complex” … “Overall this paper deserves publication in an excellent journal. I consider the work of the highest quality, the questions addressed highly relevant and the research as highly original. I do not think that for this manuscript additional work is needed, but I have a series of questions and remarks that should be addressed.” … “In conclusion, this is a very exciting and interesting study, the story is overall well written, and it will impact the further study of gamma-secretase and likely the broader field of intramembrane proteolysis. A careful editing of the text might make the work ready to go.”*

Response: We thank the reviewer for his high regard of our work and his very positive evaluation.

-in the abstract the authors say that they identified "all" relevant residues... This is of course an overstatement

Response: Upon re-evaluation, we agree that this statement should be toned down and have thus removed “all” and also “relevant” (see page 2).

-in the introduction not always the correct publications are cited, and the authors switch to reviews when citing work from other groups while citing original papers when citing work from the own group. E.g. the Notch cleavage (Imbibo et al is cited) and the APP-CTF cleavage (Lichtenthaler et al is cited) is both work pioneered by De Strooper et al.; the progressive (stepwise) processing of APP-CTF (Morishima-kawashima is cited) was originally work from Ihara et al, the need of complex formation and additional components to get active gamma-secretase has been work from many groups (Edbauer et al is cited but also the group of Iwatsubo and many others should be given credit). There are probably other citations that need to be verified as well.

Response: We have carefully checked all citations and included now more original citations for the key work in the field including those mentioned by the reviewer (see pages 3 and 4).

- page 4, Chapso solubilized gamma-secretase comes with its problems: the complex is destabilized and the assay is tricky as longer Abeta peptides tend to be generated. Thus a little bit more critical presentation of the assay would be appropriate. Can the authors exclude artificial cross links or lack of cross links because the use of detergent?

Response: We are not aware of any evidence in the literature for a potential destabilisation of the γ-secretase complex by CHAPSO, whose use as one of the few known detergents preserving the activity of the complex is the gold standard in the field. We know that some laboratories have observed a trend for the generation of longer Aβ peptides (typically Aβ42/43) by CHAPSO-solubilized γ-secretase. However, this is not seen by all laboratories and was also not observed by Li et al., PNAS 2000 who were the first to report this assay. The reasons underlying this phenomenon are currently unclear and might be of technical nature. To reflect these issues, we deleted the term “well-established experimental system“ (see page 5).

With respect to substrate crosslinking using the CHAPSO system, we have shown in Fig 2B that the C99/γ-secretase crosslinks occurring in CHAPSO are not observed when the complex is dissociated with Triton detergent. Thus, artificial crosslinks are excluded. Although major impacts by detergent on the γ-secretase structure have so far not been observed, minor shifts of transmembrane domains were noted in the structure from digitonin compared to that from amphiphol (Sun et al., 205). It can thus not be excluded that the exposure of the crosslink sites identified here using active CHAPSO-solubilized γ-secretase might potentially change with such alternative complex-maintaining detergents or when γ-secretase would be reconstituted in model membranes. To provide a more critical presentation of our assay system used for crosslinking, we have added a few sentences in the Discussion dealing with these issues in the new paragraph “Limitations” (see page 15).

- When the first cross linking experiments are described (page 4) the fact that the APH1 bands are non- specific should be discussed more explicitly (it would help the reader)

Response: We have added a separate sentence in the Results section to more explicitly deal with this issue (see pages 5 and 6). The arguments, why we concluded that this subunit does not play a role in substrate binding are laid out in full in the Discussion section (see page 10).

-page 5 "finally further analysis suggested that the active site of γ-secretase in the PS1 NTF and CTF locates at the epsilon cleavage sites in C99" deserves more explanation. Also the figure sup 3 is unclear and the legend does not provide sufficient explanation to make this piece of data clearly understandable.

Response: We have reworded the sentence in question and modified the supplementary Fig 3 (Fig EV3) and the corresponding legend. We hope that clarity and understandability is now improved (see pages 7 and 30).

Discussion: I am not sure that all interpretation of the data is as straight forward as the authors claim. We know that presenilin is a conformational flexible protein and that GSI can stabilize the structure. It is not clear to me to what extent GSI really compete with binding of substrate or to what extent they alter the conformation of the protease, making certain residues no longer exposed and indirectly affecting the cross linking. Some more discussion and proposal of alternative interpretations might be considered.

Response: We agree with the reviewer that reduced substrate binding in the presence of inhibitor may not necessarily be the consequence of competition with substrate binding. After carefully reading the manuscript again, we realize that we indeed did not explicitly discuss this point. We have thus clearly stated the alternative possibilities of inhibitor-induced conformational changes in the Discussion now (see page 11).

Page 10 "Based on increased substrate crosslinking... we speculate..close to active site or come close to it.." I do not see the logic in this sentence/conclusion. Please reevaluate.

Response: After careful reading, we agree with the reviewer that this sentence may indeed not be fully clear. The reasoning why we speculated that exosite-bound substrates could be close to the active site has now been rewritten and moved further up in the Discussion (see page 10). In this new place, the increased substrate crosslinking at exosites that we observe is also discussed in the context of the recent eLife paper from the Scheres laboratory, which shows a high resolution γ-secretase structure in the presence of DAPT (Bai et al., 2015). We hope that our logic is now better understandable.

Page 11, when discussing the structure, the recent paper in eLife from Scheres should be taken into account.

Response: We have included the recent eLife paper from the Scheres laboratory (Bai et al., 2015) and discussed their findings in the context of our work (see pages 10, 11, 12).

Page 11-12 when discussing the effect of clinical mutations on the binding of substrate the authors should indicate that their proposal is based on experiments with only two mutations and that the effect on shift in cleavage site is only shown with one of the two mutations. There are alternative mechanisms possible (loss of processivity) and it is also not excluded that combination of effects are playing.

Response: We agree with this point of the reviewer and have extended the corresponding paragraph of the Discussion section and state that more PS FAD mutants need to be investigated in order to know whether these mutants display C99 substrate-binding shifts in a common way or whether each mutant will exert individual effects on substrate binding (see page 14). We also stated that besides altered substrate positioning, an altered active site conformation or reduced processivity or a combination of these effects can be mechanistically effective for the generation of longer Aβ species by PS FAD mutants. We do not think, however, that altered substrate binding and reduced processivity are necessarily different mechanisms. In fact, altered substrate binding by PS FAD mutants could also underlie the reduced processivity that is observed for these mutants. FAD mutants may not only alter the interaction with the initial substrate C99 as shown here in our study, but also that of the subsequently generated intermediate Aβ species. This view is supported by previous work from Okochi et al., Cell Reports 2013, who showed that binding of Aβ42 as a substrate for Aβ38 is weakened by PS FAD mutants. We have proposed this link of altered substrate binding with reduced processivity in the Discussion section (see page 14).

Accepted 26 April 2016

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers, and I am happy to inform you that we have now accepted your article for publication in The EMBO Journal.

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: Harald Steiner
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-94151R

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).

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?	As no statistic tests were applied, no power test was performed before. The biochemical experiments showed only very little inter-experimental variations, therefore a common number of 3 experiments was performed (see Figure legends).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	N/A
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	N/A
Is there an estimate of variation within each group of data?	N/A
Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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http://www.selectagents.gov/

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 paragraph Antibodies of the Materials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Sources of the cell lines were described in Materials and Methods section. The cells were not authenticated recently. No mycoplasma contamination was detected by PCR analysis.

* 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.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

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

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	N/A
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).	N/A
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).	N/A
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	N/A
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.	N/A

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.	N/A