THE EMBO JOURNAL

Manuscript EMBO-2016-95669

# USP49 negatively regulates tumorigenesis and chemoresistance through FKBP51- AKT signaling

Kuntian Luo, Yunhui Li, Yujiao Yin, Lei Li, Chenming Wu, Yuping Chen, Somaira Nowsheen, Qi Hu, Lizhi Zhang, Zhenkun Lou and Jian Yuan

*Corresponding author: Jian Yuan, Mayo Clinic*

**Review timeline:**

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| Submission date: | 06 September 2016 |
| Editorial Decision: | 12 October 2016 |
| Revision received: | 30 January 2017 |
| Editorial Decision: | 22 February 2017 |
| Revision received: | 24 February 2017 |
| Accepted: | 08 March 2017 |

Editor: Hartmut Vodermaier

## 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 October 2016

Thank you for submitting your manuscript on USP49 as a new AKT pathway regulator for our consideration. We have now heard back from three expert referees, whose comments are copied below for your information. While the referees are somewhat divided in their overall recommendation, they all acknowledge the potential interest of your new findings, and offer a number of constructive and well-taken suggestions for consolidating and improving the work. Should you be able to satisfactorily address these various points, we would therefore be happy to consider a revised version of the manuscript further for publication in The EMBO Journal.

Please bear in mind that it is our policy to allow only a single round of major revision, making it important to carefully respond to all points raised at this stage. Additional data would not be required for the last point of referee 1; nor for point 6 of referee 2, which in my view would be well-addressed by following up on referee 3's point 3. In any case, should you have any additional questions/comments regarding the referee reports or your revision work, please do not hesitate to get in touch with me ahead of resubmission. If needed, we might also arrange for an extended revision period, during which time the publication of any competing work elsewhere would have no negative impact on our final assessment of your own study.

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REFEREE REPORTS

Referee #1:

 

The manuscript by Luo et al describes the regulation of Akt by FKBP51 and USP49. This group previously demonstrated that FKBP51 can recruit PHLPP to Akt thereby promoting dephosphorylation at Ser473. In this study, they identify the de-ubiquitinase USP49 as a FKBP51-interacting protein, and validate association of endogenous proteins in the SU86 pancreatic cancer cell line. A set of experiments in SU86 cells convincingly showed that USP49 de-ubiquitinates FKBP51 causing its protection from proteasome-mediated degradation. Experiments linking USP49-mediated FKBP51 de-ubiquitination on Akt Ser-473 phosphorylation and activation, as well as tumor promoting consequences, were also compelling. They go on to show that in a cohort of 254 pancreatic cancer specimens, 74% show decreased USP49 protein expression by IHC, and most of these showed low FKBP51 and high Akt Ser473 phosphorylation. Decreased USP49 levels was also associated with poor prognosis. Finally they show that manipulation of USP49 levels influences response to chemotherapies

The manuscript is clearly written and the data is well presented and convincing. Moreover, the concepts should be interesting to the readers of EMBO J. However, before publication the authors need to address the following points:

1. What fraction of FKBP51 and USP49 are co-associated?
2. Is the overexpressed FKBP51 also regulated by USP49? Need to show FKBP51 expression by itself in Fig. 3B.
3. Provide a full table showing USP49, FKBP51 and P-Akt staining in the pancreatic and normal tissue samples.
4. GDC-0941 is not an Akt inhibitor, as suggested in the discussion (it is a PI3K inhibitor).

I also have a couple of suggestions, one which would strengthen the paper, and one that could be speculated upon, with evidence if they have any.

1.Would it be possible to analyze FBBP51, USP49 and P-Akt in the SU86 tumor xenografts? This would strengthen the connection between these three proteins during tumorigenesis.
2. What underlies the decreased expression of USP49 in tumors?

Referee #2:

The manuscript by Luo et al. described a study on identifying USP49 as a novel tumor suppressor by stabilizing FKBP51 and inactivating Akt in pancreatic cancer cells. Biochemical studies have provided strong evidence that USP49 functions as positive regulator of FKBP51 by serving as a deubiquitinase both in vitro and in cells. Functionally, the tumor suppressor role of USP49 in pancreatic cancer has been supported by in vivo tumorigenesis experiments as well as expression analysis in patient samples. In general, the data are of high quality. It is interesting that loss of USP49 expression contributes to chemoresistance in pancreatic cancer cells. However, this study is better fitted for cancer research related journals since the mechanism of action for USP49 is somewhat predictable based on what is known about FKBP51 and Akt in cancer.

Specific comments:
1) In Fig. 3B, it is necessary to probe the expression of FKBP51 using the FKBP51 antibody in addition to the Flag antibody. Since knockdown of USP49 decreases the expression of Flag-FKPB51 as well, it is important to know how much overexpression of FKBP51 is needed to rescue the effect of USP49 depletion.

2) The expression of FKBP51 and phosph-Akt levels need to be analyzed in tumors obtained from in vivo tumorigenesis experiments (as those shown in Fig. 3H).

3) It looks like that the expression of USP49 and FKBP51 in normal pancreas tissue (Fig. 4A and 4E) is high in acinar cells rather than ductal cells. Additional evidence is needed to shown USP49 and FKBP51 are expressed in normal ductal cells.

4) What is the difference between the USP49 high group and the USP49 high censored group in Fig.

 

4G (same for Fig. 4H and 4I)?

5) It has been indicated throughout the entire manuscript that USP49 regulates Akt by regulating FKBP51-PHLPP-Akt axis. However, whether PHLPP is involved in USP49-mediated effects has not been examined directly at all. Additional experiments are needed to show the functional contribution of PHLPP.

6) What are other known substrates of USP49? How those substrates may affect the tumor suppressor function of USP49?

Referee #3:

In this manuscript the authors convincingly showed that USP49 affects the ubiquitylation of FKBP51. However, there are several concerns regarding the effect on Akt:

1. There are two Akt hydrophobic motif phosphatases, PHLPP1 and PHLPP2. PHLPP1 preferentially dephosphorylates Akt2 and Akt3 but not Akt1, whereas PHLPP2 preferentially dephosphorylates Akt1 and Akt3. Since the authors found that only Akt1 is co-purified with FKBP51, it is likely that the phosphatase is PHLPP1. The authors should clarify this issue and determine which PHLPP was identified in the mass spec data. Furthermore, it seems that PHLPP binds very weakly to FKBP51. Since Akt2 is the major isoform in pancreatic cancer cells, it is important that the authors should address these issues.
2. The authors should determine if the phosphorylation of Akt1, Akt2 or both are affected.
3. Since both FKBP51 and USP49 have multiple targets, the authors should determine if the effect on proliferation and tumorigenesis is due to Akt, by expressing phospho-mimetic Akt to show that tumorigenesis is not affected by the knockdown of USP49 and FKBP51.

1st Revision - authors' response 30 January 2017

**Referee#1**:

**The manuscript by Luo et al describes the regulation of Akt by FKBP51 and USP49. This group previously demonstrated that FKBP51 can recruit PHLPP to Akt thereby promoting dephosphorylation at Ser473. In this study, they identify the de-ubiquitinase USP49 as a FKBP51-interacting protein, and validate association of endogenous proteins in the SU86 pancreatic cancer cell line. A set of experiments in SU86 cells convincingly showed that USP49 de-ubiquitinates FKBP51 causing its protection from proteasome-mediated degradation. Experiments linking USP49-mediated FKBP51 de-ubiquitination on Akt Ser-473 phosphorylation and activation, as well as tumor promoting consequences, were also compelling. They go on to show that in a cohort of 254 pancreatic cancer specimens, 74% show decreased USP49 protein expression by IHC, and most of these showed low FKBP51 and high Akt Ser473 phosphorylation. Decreased USP49 levels was also associated with poor prognosis. Finally they show that manipulation of USP49 levels influences response to chemotherapies**

**The manuscript is clearly written and the data is well presented and convincing. Moreover,**

 

**the concepts should be interesting to the readers of EMBO J. However, before publication the authors need to address the following points:**

*We thank the reviewer for the positive and constructive comments.*

**1. What fraction of FKBP51 and USP49 are co-associated?**

*As shown in Fig 1B-C, 15% of the whole cell lysate subjected to Co-IP was loaded as input. We quantified the ratio between input and IP samples and found that about 8% of FKBP51 and USP49 are co-associated.*

**2. Is the overexpressed FKBP51 also regulated by USP49? Need to show FKBP51 expression by itself in Fig. 3B.**

*We appreciate the reviewer's comment. We stably expressed FLAG-FKBP51 in SU86 cells and then infected the cells with lentivirus encoding control or USP49 shRNA. As shown in Fig EV1B, depletion of USP49 also decreased the overexpressed FLAG-FKBP51 level. We also repeated the experiment in Fig 3B and blotted for FKBP51 according to the reviewer's comment.*

**3. Provide a full table showing USP49, FKBP51 and P-Akt staining in the pancreatic and normal tissue samples.**

*We tabulated USP49, FKBP51 and P-Akt staining in the pancreatic and normal tissue samples according to the reviewer's suggestion (Fig 4B).*

**4. GDC-0941 is not an Akt inhibitor, as suggested in the discussion (it is a PI3K inhibitor).**

*Thank you for pointing out our error. The sentence has been edited in the discussion section to reflect the correction.*

**I also have a couple of suggestions, one which would strengthen the paper, and one that could be speculated upon, with evidence if they have any.**

**1. Would it be possible to analyze FBBP51, USP49 and P-Akt in the SU86 tumor xenografts? This would strengthen the connection between these three proteins during tumorigenesis.**

*We appreciate the reviewer's suggestion. We examined the expression of USP49, FKBP51 and phospho-Akt levels in tumors obtained from in vivo tumorigenesis experiments (shown in Fig. 3H). As shown in Fig EV2I, FKBP51 level was dramatically decreased in USP49 depleted samples;*

 

*however, p-AKT was increased in USP49 depleted samples. In addition, overexpression of FLAG-FKBP51 rescued the effect of USP49 depletion on AKT phosphorylation.*

**2. What underlies the decreased expression of USP49 in tumors?**

*So far, we do not have any conclusive data as to the mechanism underlying the decreased expression of USP49 in tumors. In the future, we will examine whether the transcription levels or post translation modifications of USP49 are changed in tumors. However, it is outside the scope of our current work.*

**Referee #2:**

**The manuscript by Luo et al. described a study on identifying USP49 as a novel tumor suppressor by stabilizing FKBP51 and inactivating Akt in pancreatic cancer cells. Biochemical studies have provided strong evidence that USP49 functions as positive regulator of FKBP51 by serving as a deubiquitinase both in vitro and in cells. Functionally, the tumor suppressor role of USP49 in pancreatic cancer has been supported by in vivo tumorigenesis experiments as well as expression analysis in patient samples. In general, the data are of high quality. It is interesting that loss of USP49 expression contributes to chemoresistance in pancreatic cancer cells. However, this study is better fitted for cancer research related journals since the mechanism of action for USP49 is somewhat predictable based on what is known about FKBP51 and Akt in cancer.**

*We thank the reviewer for the positive and constructive comments.*

**Specific comments:**

**1) In Fig. 3B, it is necessary to probe the expression of FKBP51 using the FKBP51 antibody in addition to the Flag antibody. Since knockdown of USP49 decreases the expression of Flag-FKPB51 as well, it is important to know how much overexpression of FKBP51 is needed to rescue the effect of USP49 depletion.**

*According to the reviewer's suggestion, we repeated the experiments in Fig 3B and blotted for FKBP51. We found that the expression level of overexpressed FLAG-FKBP51 in USP49 depleted cells was almost equal to endogenous FKBP51 in control cells, and was able to rescue the effect of USP49 depletion.*

 

**2) The expression of FKBP51 and phosph-Akt levels need to be analyzed in tumors obtained from in vivo tumorigenesis experiments (as those shown in Fig. 3H).**

*We appreciate the reviewer's suggestion. We examined the expression of USP49, FKBP51 and phospho-Akt levels in tumors obtained from in vivo tumorigenesis experiments (shown in Fig. 3H). As shown in Fig EV2I, FKBP51 level was dramatically decreased in USP49 depleted samples; however, p-AKT was increased in USP49 depleted samples. In addition, overexpression of FLAG-FKBP51 rescued the effect of USP49 depletion on AKT phosphorylation.*

**3) It looks like that the expression of USP49 and FKBP51 in normal pancreas tissue (Fig. 4A and 4E) is high in acinar cells rather than ductal cells. Additional evidence is needed to shown USP49 and FKBP51 are expressed in normal ductal cells.**

*We provided additional evidence to show that USP49 and FKBP51 are expressed in both acinar cells and normal ductal cells (Fig 4A, 4D and EV4A-B).*

**4) What is the difference between the USP49 high group and the USP49 high censored group in Fig. 4G (same for Fig. 4H and 4I)?**

*In Fig 4G, some patients with high USP49 expression (or high FKBP51 or p-AKT in Fig 4H-I) were still alive at the last recorded follow up date or lost to follow-up. Since we cannot accurately calculate the survival time for these patients, they were subjected to the censored group. We have added further details in the figure legend to clarify what the censored group entails.*

**5) It has been indicated throughout the entire manuscript that USP49 regulates Akt by regulating FKBP51-PHLPP-Akt axis. However, whether PHLPP is involved in USP49-mediated effects has not been examined directly at all. Additional experiments are needed to show the functional contribution of PHLPP.**

*According to the reviewer's comment, we depleted USP49 or PHLPP1/2 both individually and in combination in SU86 cells. As shown in Fig EV2G, knockdown of USP49 dramatically increased the phosphorylation of AKT on Serine 473 in control cells. However, in PHLPP1/2 knockdown cells, further depletion of USP49 did not affect the phosphorylation of AKT. Furthermore, depletion of USP49 markedly increased pancreatic cancer cell proliferation, while USP49 knockdown had no further effect on proliferation in cells depleted of PHLPP1/2 (Fig EV2H). Taken together, these results suggest that USP49 regulates AKT by regulating FKBP51-PHLPP-Akt axis.*

 

**6) What are other known substrates of USP49? How those substrates may affect the tumor suppressor function of USP49?**

*A previous study suggested that USP49 deubiquitinates H2B and regulates cotranscriptional pre-mRNA splicing. However, the role of USP49 in cancer was unclear. According to the editor's suggestion, to confirm that USP49 regulates tumorigenesis in an AKT dependent manner, we stably overexpressed the phospho-mimetic AKT in SU86 cells and found that knockdown of either USP49 or FKBP51 dramatically increased cell proliferation and tumorigenesis in control cells but not in cells stably overexpressing phospho-mimetic AKT (Fig EV3A-C). These results confirmed that USP49 regulates tumorigenesis in an AKT dependent manner.*

**Referee #3:**

**In this manuscript the authors convincingly showed that USP49 affects the ubiquitylation of FKBP51. However, there are several concerns regarding the effect on Akt:**

*We thank the reviewer for the positive and constructive comments.*

**1. There are two Akt hydrophobic motif phosphatases, PHLPP1 and PHLPP2. PHLPP1 preferentially dephosphorylates Akt2 and Akt3 but not Akt1, whereas PHLPP2 preferentially dephosphorylates Akt1 and Akt3. Since the authors found that only Akt1 is co-purified with FKBP51, it is likely that the phosphatase is PHLPP1. The authors should clarify this issue and determine which PHLPP was identified in the mass spec data. Furthermore, it seems that PHLPP binds very weakly to FKBP51. Since Akt2 is the major isoform in pancreatic cancer cells, it is important that the authors should address these issues.**

*Previous studies reported amplification and/or overexpression of AKT2 in 10–20% of primary pancreatic carcinomas and pancreatic cancer cell lines (1-4). The pancreatic cancer cell lines PANC1 and ASPC1 exhibited 30- and 50-fold amplification of AKT2, respectively, and high levels of AKT2 RNA and protein (1). However, we utilized the pancreatic cancer cell line SU86 in our purification experiments, which has not been shown to have amplification of AKT2. To address the reviewer's concern, we examined the protein levels of PHLPP1/2 and AKT1/2 in SU86 cells and found that all these proteins are expressed in SU86 cells (Fig EV1A). Furthermore, we confirmed that FKBP51 interacts with PHLPP1/2, AKT1/2 and USP49 in SU86 cells by co-IP assay (Fig EV1A). When we performed the purification experiment, cells stably expressing FLAG-tagged FKBP51 were lysed with high salt NETN buffer (300 mM NaCl). Cell lysates were diluted 1:1 with NET buffer (NETN buffer without NaCl) and incubated with anti-FLAG beads overnight at 4°C.*

 

*After washing with NETN buffer five times, the bound proteins were eluted with FLAG peptide.The high salt NETN buffer lysed the cells more completely. For example, some chromatin or membrane proteins need to be extracted by high salt buffer. However, the high salt buffer may also disrupt the interaction, even if we dilute the salt concentration after the lysis step. It is possible the purification method lead to a loss of some binding partners. We used mass spectrometry a screening method to identify novel FKBP51 binding proteins to initiate the project. Our mass spectrometry data may not have covered all FKBP51 binding proteins. However, we did validate all identified partners using co-IP experiments as shown in Fig EV1A [normal salt NETN buffer (150mM NaCl) was used as the lysis buffer in Co-IP experiment]. PHLPP1 was identified in the mass spectrometry experiment. The table in Figure 1 has been edited to clarify this point.*

**2. The authors should determine if the phosphorylation of Akt1, Akt2 or both are affected.**

*To determine whether the phosphorylation of Akt1 or Akt2 was regulated by USP49, we immunoprecipitated AKT1 or AKT2 in control and UPS49 knockdown cells and examined the p-AKT(S473) level in the immunoprecipitation samples. As shown in Fig EV2A-B, the Ser 473 phosphorylation of both AKT1 and AKT2 was increased in USP49 depleted cells, which suggests that USP49 regulates both AKT1/2 activation.*

**3. Since both FKBP51 and USP49 have multiple targets, the authors should determine if the effect on proliferation and tumorigenesis is due to Akt, by expressing phospho-mimetic Akt to show that tumorigenesis is not affected by the knockdown of USP49 and FKBP51.**

*We stably overexpressed the phospho-mimetic AKT in SU86 cells and found that knockdown of either USP49 or FKBP51 dramatically increases cell proliferation and tumorigenesis in control cells but not in cells stably overexpressing the phospho-mimetic AKT (Fig EV3A-C). These results confirm that USP49 regulates cell proliferation and tumorigenesis in an AKT dependent manner.*

**References:**

1. J. Q. Cheng *et al.*, AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc Natl Acad Sci U S A* **89**, 9267-9271 (1992).
2. W. Miwa *et al.*, Isolation of DNA sequences amplified at chromosome 19q13.1-q13.2 including the AKT2 locus in human pancreatic cancer. *Biochem Biophys Res Commun* **225**, 968-974 (1996).
3. B. A. Ruggeri, L. Huang, M. Wood, J. Q. Cheng, J. R. Testa, Amplification and overexpression of the AKT2 oncogene in a subset of human pancreatic ductal adenocarcinomas. *Mol Carcinog* **21**, 81-86 (1998).
4. D. A. Altomare, J. R. Testa, Perturbations of the AKT signaling pathway in human cancer. *Oncogene* **24**, 7455-7464 (2005).

 

2nd Editorial Decision 22 February 2017

Thank you for submitting your revised manuscript for our editorial consideration. Two of the original referees have now once more assessed the study, and I am pleased to inform you that they both consider the key concerns satisfactorily addressed and have no further reservations regarding publication. We shall therefore be happy to accept the manuscript for The EMBO Journal, pending correction of a number of outstanding editorial issues:

- We still need you to complete our author checklist (see link below), and upload it with the final manuscript.

- Please introduce scale bars in the micrographs shown in Figures 3d, 4a,c, EV4.

- Please slightly re-write the first parts of the Material & Methods section (in particular the descriptions for co-IP, protein stability, DUB, cell survival, cell proliferation and colony formation assays), since they currently appear to be near-verbatim copies of respective passages from earlier publications by your group and others. This is important to avoid potential post-publication complications such as possible (self-)plagiarism allegations.

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REFEREE REPORTS

Referee #1:

The authors have adequately addressed my comments.

Referee #3:

In the revised manuscript the authors have adequately addressed all my concerns.

2nd Revision - authors' response 24 February 2017

Thank you for accepting our manuscript. We have made some changes according to your suggestion. We also made a schematic image and completed the checklist and uploaded them in the system.

3rd Editorial Decision 08 March 2017

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it 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

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|---|
| Corresponding Author Name: Jian Yuan |
| Journal Submitted to: EMBO Journal |
| Manuscript Number: EMBOJ-2016-95669 |

**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? | As described in Figure legend (Figure 2,3,4, 5, EV2,EV3,EV5 ) |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | No statistical method was used to predetermine sample size |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | Yes In Method section page 17. |
| 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. | Yes In Method section page 17. |
| For animal studies, include a statement about randomization even if no randomization was used. | Randomization does not apply to this study |
| 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. | Yes In Method section page 17. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | Yes In Method section page 17. |
| 5. For every figure, are statistical tests justified as appropriate? | Yes (in figure legends) |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | Yes |
| Is there an estimate of variation within each group of data? | In Methods section: Data are expressed as mean ± standard errors of the mean (SEM). Statistical analyses were performed with the Student's t-test or ANOVA. Statistical significance is represented in figures by: *, p<0.05; **, p<0.01. |
| Is the variance similar between the groups that are being statistically compared? | In Methods section: Data are expressed as mean ± standard errors of the mean (SEM). Statistical analyses were performed with the Student's t-test or ANOVA. Statistical significance is represented in figures by: *, p<0.05; **, p<0.01. |

## C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
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). | In Supplementary methods |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | In Methods section page 14 |

* 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. | 5-week-old male athymic nude NCr nu/nu (NCI/NIH) mice. In page 16 |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | All the animals were purchased from the National Cancer Institute and maintained and treated under specific pathogen–free conditions. Experiments were performed under the approval of the Institutional Animal Care and Use Committee at Mayo Clinic. The statement is included in Method section |
| 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. | confirmed |

## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | Not 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. | Not applicable |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | Not applicable |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | Not applicable |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | Not 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. | Not 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. | Not applicable |

## F- Data Accessibility

| | |
|---|---|
| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | Not 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). | Not 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). | Not 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.<br><br>Examples:<br>**Primary Data**<br>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<br>**Referenced Data**<br>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<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208 | Not 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. | Not applicable |

## G- Dual use research of concern

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| 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. | Not applicable |