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Manuscript EMBO-2016-95534

AKT-Phosphorylated FOXO1 Suppresses ERK Activation and Chemoresistance by Disrupting IQGAP1-MAPK Interaction

Chun-Wu Pan, Xin Jin, Yu Zhao, Yunqian Pan, Jing Yang, R. Jeffrey Karnes, Jun Zhang, Liguo Wang and Haojie Huang

Corresponding author: Haojie Huang, Mayo Clinic College of Medicine

Review timeline:

Submission date:	18 August 2016
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Additional Correspondence:	12 October 2016
Additional Correspondence:	18 October 2016
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Editor: Andrea Leibfried

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 for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below. Another referee has promised a report, and I will forward you this extra report should it be available in the next week.

As you will see, the referees appreciate your findings. A few points need to be strengthened though:
- please add quantitative data and statistical analyses (point 1 of referee 1).
- please add further insight into the mechanism of interaction (referee 3, points 1-3)
- please add further insight into how paclitaxel induces nuclear localization of FOX1 (referee 3, point 4)

REFEREE REPORTS

Referee #1:

This is an excellent, interesting and data-rich manuscript by Chun-Wu Pan et al reporting the role of FOXO proteins tumor suppressors in regulation of IQGAP1 control of Erk signaling. The authors show that AKT-mediated phosphorylation of serine 319 on FOXO1 enhances its binding to

IQGAP1. Further, as IQGAP1 is a hub for activation of the Erk pathway, binding of phospho-FOXO1 blocks IQGAP1-controlled phosphorylation of ERK1/2 (pERK1/2) and interactions with the MAP kinase pathway members. Consistently FOXO1 expression increases pERK1/2 in cells and correlated with pERK1/2 levels in cancer specimens and disease progression. The authors suggest that this set of interactions could be harnessed to overcome chemoresistance in cancer. This is an outstanding and very interesting paper. There are very minor concerns that should be addressed to strengthen the manuscript for publication in EMBO J.

1. The number of replications for each data set should be clearly stated. Most of the Western blotting data is clear but at least three replications should be performed. In addition, key data including select Western blots should be quantified and statistical analysis performed and detailed. This is particularly important for the data showing that binding of phospho-serine319-FOXO1 to IQGAP1 fully blocked Erk signaling. Another examples are Fig. 6D.

2. Some of the writing is a bit awkward. For example the authors could use less first person descriptions. Example pg 9, we sought, we surveyed, we found, we examined. The figure legends need work and the methods need to be clarified through out.

Referee #2:

This is a relevant and technically excellent paper describing a previously unknown cytoplasmic function of the Foxo transcription factor. The starting point for the project is the observation that Foxo degradation is important for tumor formation in some models, even when Foxo is phosphorylated by Akt and cannot get into the nucleus. This finding suggested that Foxo may have a cancer-related cytoplasmic function that would be distinct from its role as a DNA binding transcription factor.

Extensive and high-quality biochemical evidence documents that a specifically phosphorylated form of Foxo can bind to the scaffold protein IQGAP1 and in that way prevent the activation of ERK, the effector kinase downstream of the Ras / Raf / Mek pathway. Molecular mapping experimentation identified the domains on Foxo and IQGAP1 that mediate this interaction. Based on this information a small cytoplasmic Foxo derived phospho-peptide is shown to be sufficient to interact with IQGAP1 and suppress ERK activation. These data are an interesting and unexpected extension of the known molecular function of Foxo.

The biological relevance of this information derives from the fact that tumors in which Akt activation contributes to malignancy, for example in PTEN loss-of-function conditions, and that are than treated with AKT inhibitors frequently acquire resistance, causing a relapse. This resistance can be caused by activation of ERK. The molecular model presented by Pan et al. offers an explanation for this effect: Decreased Akt activity would relieve the interaction of Foxo with IQGAP1 and de-repress ERK activity. In support of this model, tissue micro array analyses show an inverse correlation between ERK and AKT activity in different tumor samples. Consistently ,cell culture and in vivo experiments show that an IQGAP1-inhibitory Foxo phospho peptide can cooperate with an AKT inhibitor to suppress tumor growth.

In conclusion, this paper presents novel and important information on the molecular functions of Foxo transcription factors and also suggests an interesting new mechanism of cancer progression as well as approaches to therapy. The experimental evidence is extensive and convincing.

I recommend acceptance of the manuscript for publication in the EMBO Journal. One suggestion would be to discuss how potential therapeutically approaches based on AKT inhibitors and an IQGAP1 interacting peptide might compare to a simple combination therapy with AKT and MEK inhibitors. This might be relevant especially in light of the fact that IQGAP1 is a very pleiotropically acting protein with effects on multiple signaling pathways. Therefore interfering with its function might have adverse side effect. An expanded discussion covering these points might be interesting to some readers, but is not essential for publication.

Referee #3:

The finding that the ERK pathway scaffold function of IQGAP1 is negatively regulated in a competitive manner by the binding of the AKT-phosphorylated pS319 cytoplasmic population of the FOXO1 transcription factor (and possibly other FOXO family members) is new, as far as I am aware, and the concept that there is crosstalk between the PI3K/AKT and ERK pathways at this level is interesting. I do have a few issues:

1. Most transcription factors are scarce proteins raising the question whether there is enough cytoplasmic FOXO1 to sequester the majority of IQGAP1 away from ERK pathway proteins i.e. what are the relative levels of FOXO1 and IQGAP1 in the cell?

2. It is unclear how pS319 binds to the coiled-coil region of IQGAP1 - coiled-coil regions of proteins are not generally known to serve as phosphobinding sites (I can't think of an example) - or whether the phosphate is directly recognized for binding. In this regard, Glu residues are generally poor phosphomimics for phosphates that are involved in protein-protein interactions, because they are less charged and bulky than phosphate.

3. The other major unanswered mechanistic question is how binding of the short S319E peptide to the coiled-coil region would block the binding of RAF, MEK and ERK to IQGAP1, since these kinases all bind to separate sites downstream of the coiled-coil region.

4. The ability of the S319E FOXO1 peptide to suppress paclitaxel-induced ERK activation and to synergize with docetaxel in a tumor xenograft model is of relevance to cancer therapy and paclitaxel resistance mechanisms, but it is unclear how paclitaxel induces nuclear localization of FOXO1, i.e. does it cause dephosphorylation of the 14-3-3 binding sites responsible for cytoplasmic retention of FOXO1, and more importantly does it lead to dephosphorylation of pS319?

5. It is not obvious to me that S319 is conserved in FOXO3 and FOXO4, which also bind IQGAP1 in a phosphodependent manner apparently.

Additional Correspondence - Author 12 October 2016

I would like to thank you for your time and evaluation of our work and kind invitation for revision. We also sincerely appreciate the thorough evaluation and constructive suggestions provided by each of the reviewers.

We agree fully with the first reviewer that the number of replications for each data set should be clearly stated. However, the reviewer further requested that most of the western blotting data is clear, but at least 3 replications should be performed. Indeed, the current version of our manuscript was the product we had revised for Nature Communications. One of the NC reviewer asked us to repeat all the western blot data in 2 replicates and provided the quantitative data with statistics. It took us 7.5 months to finish those replicate experiments. Therefore, based upon our experience in the past, it would be almost impossible for us to repeat most of the western blotting data in 3 replicates within 90 days. We are happy to follow reviewer's suggestions to repeat most experiments in 3 replicate, but because of time limitation, would it be possible/reasonable for us to have your inputs/permission to repeat some key experiments in 3 replicates and do the statistics? If so, I would greatly appreciate if you could kindly point to us which key experiments should be repeated in 3 replicates, and for the rest of the study 2 replicates should be sufficient, as those shown in the attached figures, which are the quantitative data of 2 replicates for all western blots in the manuscript that were submitted along with our revised manuscript to NC before.

I am sorry for any inconvenience this may cause to you, but your kind reply and constructive suggestions would be greatly appreciated.

Additional Correspondence - Editor 18 October 2016

I would suggest to have three replicates for the blots showing that pS319-Foxo1 binds IQGAP1 and the ones showing that this inhibits IQGAP1-RAF, MEK, ERK interaction. These seem to be the most important, also in light of referee #3's comments.

I have informed referee #1 that not all data will be presented in triplicates. But I would like to suggest to include as mentioned before the quantifications of the duplicates in the supplementary/expanded view section (displayed side-by-side, not averaged).

Please also note that the figure legends should not only include the information on n=2/3 etc, but be much more explicit in describing the actual experiments, constructs used etc (this is also mentioned by one of the referees). Thank you for paying attention to this while revising your manuscript.

1st Revision - authors' response 04 January 2017

Authors' Response to the Comments on the Manuscript EMBOJ-2016-95534

We thank the Editor and all three Referees for their time to evaluate our work and insightful comments, which we have considered thoroughly in generating the revised manuscript.

Dr. Andrea Leibfried, Editor:

As you will see, the referees appreciate your findings. A few points need to be strengthened though:
- please add quantitative data and statistical analyses (point 1 of referee 1).
- please add further insight into the mechanism of interaction (referee 3, points 1-3)
- please add further insight into how paclitaxel induces nuclear localization of FOX1 (referee 3, point 4)

Reply: All these points have been addressed experimentally. Please see below our point-by-point response to the points raised by each Referee.

I would suggest to have three replicates for the blots showing that pS319-Foxo1 binds IQGAP1 and the ones showing that this inhibits IQGAP1-RAF, MEK, ERK interaction. These seem to be the most important, also in light of referee #3's comments. I have informed referee #1 that not all data will be presented in triplicates. But I would like to suggest to include as mentioned before the quantifications of the duplicates in the supplementary/expanded view section (displayed side-by-side, not averaged).

Reply: As kindly instructed by the Editor, we have performed three replicates for the blots showing that pS319-FOXO1 binds IQGAP1 (Fig 1F and Appendix Fig S1C and D) and the ones showing that this peptide inhibits IQGAP1-RAF, MEK, ERK interaction (Fig 3F and Appendix Fig S3D and E).

Also, as instructed, we have performed the quantifications of the duplicates and displayed the quantitative results side-by-side, not averaged in Appendix Fig S1-S6.

Please also note that the figure legends should not only include the information on n=2/3 etc, but be much more explicit in describing the actual experiments, constructs used etc (this is also mentioned by one of the referees). Thank you for paying attention to this while revising your manuscript.

Reply: As instructed, in addition to the information on n-2 or 3, we have provided detailed description of the experiments including constructs used, the concentration of drugs/inhibitors, etc in all the quantitative data (Appendix Fig S1-6).

Referee #1:

This is an excellent, interesting and data-rich manuscript by Chun-Wu Pan et al reporting the role of FOXO proteins tumor suppressors in regulation of IQGAP1 control of Erk signaling. The authors show that AKT-mediated phosphorylation of serine 319 on FOXO1 enhances its binding to IQGAP1. Further, as IQGAP1 is a hub for activation of the Erk pathway, binding of phospho-FOXO1 blocks IQGAP1-controlled phosphorylation of ERK1/2 (pERK1/2) and interactions with the MAP kinase pathway members. Consistently FOXO1 expression increases pERK1/2 in cells and correlated with pERK1/2 levels in cancer specimens and disease progression. The authors suggest that this set of interactions could be harnessed to overcome chemoresistance in cancer. This is an outstanding and very interesting paper. There are very minor concerns that should be addressed to strengthen the manuscript for publication in EMBO J.

Reply: We very much thank the Referee for thorough evaluation of our manuscript and recognizing this is an excellent, interesting and data-rich manuscript and an outstanding and very interesting paper.

1. The number of replications for each data set should be clearly stated. Most of the Western blotting data is clear but at least three replications should be performed. In addition, key data including select Western blots should be quantified and statistical analysis performed and detailed. This is particularly important for the data showing that binding of phospho-serine319-FOXO1 to IQGAP1 fully blocked Erk signaling. Another examples are Fig. 6D.

Reply: As requested, the number of replications for each data set has been clearly stated in Appendix Fig S1-S6.

Also, as suggested by the Editor (see above), we have performed three replicates for the blots showing that pS319-FOXO1 binds IQGAP1 (Fig 1F and Appendix Fig S1C and D) and the ones showing that this peptide inhibits IQGAP1-RAF, MEK, ERK interaction (Fig 3F and Appendix Fig S3D and E).

All the Western blots in the study have been quantified and the data are shown in Appendix Fig S1-S6. Also, as instructed by the Editor (see above), the quantified results are displayed side-by-side, not averaged, and therefore we cannot perform statistical analysis of the quantified results.

2. Some of the writing is a bit awkward. For example the authors could use less first person descriptions. Example pg 9, we sought, we surveyed, we found, we examined. The figure legends need work and the methods need to be clarified through out.

Reply: We thank the Referee for raising these excellent points. The first person descriptions have been reduced substantially. Also, the figure legends and the methods have been clarified throughout the revised manuscript, and majority of methods have been moved to the main text.

Referee #2:

This is a relevant and technically excellent paper describing a previously unknown cytoplasmic function of the Foxo transcription factor. The starting point for the project is the observation that Foxo degradation is important for tumor formation in some models, even when Foxo is phosphorylated by Akt and cannot get into the nucleus. This finding suggested that Foxo may have a cancer-related cytoplasmic function that would be distinct from its role as a DNA binding transcription factor.

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The biological relevance of this information derives from the fact that tumors in which Akt activation contributes to malignancy, for example in PTEN loss-of-function conditions, and that are than treated with AKT inhibitors frequently acquire resistance, causing a relapse. This resistance can be caused by activation of ERK. The molecular model presented by Pan et al. offers an explanation for this effect: Decreased Akt activity would relieve the interaction of Foxo with IQGAP1 and de-repress ERK activity. In support of this model, tissue micro array analyses show an inverse correlation between ERK and AKT activity in different tumor samples. Consistently, cell culture and in vivo experiments show that an IQGAP1-inhibitory Foxo phospho peptide can cooperate with an AKT inhibitor to suppress tumor growth.

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> **Reply**: We thank the Referee for recommending acceptance of the manuscript for publication in the EMBO Journal. We also agree with the Referee that it is important to discuss the advantage of utilizing the FOXO1-derived peptide versus targeting IQGAP1. As suggested, we have emphasized in our discussion (page 19) that IQGAP1 is a very pleiotropically acting protein with effects on multiple signaling pathways. Targeting ERK activation by interfering with the function of IQGAP1 might have adverse side effect. On the contrary, utilization of a small, FOXO1-derived peptide inhibitor of IQGAP1 could specifically inhibit AKT inhibition-induced activation of ERK and drug resistance.

Referee #3:

The finding that the ERK pathway scaffold function of IQGAP1 is negatively regulated in a competitive manner by the binding of the AKT-phosphorylated pS319 cytoplasmic population of the FOXO1 transcription factor (and possibly other FOXO family members) is new, as far as I am aware, and the concept that there is crosstalk between the PI3K/AKT and ERK pathways at this level is interesting. I do have a few issues:

Reply: We very much thank the Referee for thorough evaluation of our manuscript and recognizing the novelty of our findings and the interesting concept of the crosstalk between the PI3K/AKT and ERK via FOXO1-IQGAP1 interaction.

1. Most transcription factors are scarce proteins raising the question whether there is enough cytoplasmic FOXO1 to sequester the majority of IQGAP1 away from ERK pathway proteins i.e. what are the relative levels of FOXO1 and IQGAP1 in the cell?

Reply: We thank the Referee for raising this excellent point. To address this concern, we performed co-IP experimental to examine the binding between FOXO1 and IQGAP1 proteins.

It has been shown previously that in PTEN-null LNCaP cells, FOXO1 is highly phosphorylated by AKT and mainly located in the cytoplasm (Nakamura et al., MCB 20: 8969-82, 2000). IQGAP1 is a protein present in the cytoplasm (Bielak-Zmijewska et al., Dev Biol 322: 21-32, 2008). In agreement with these findings, we found that while cytoplasmic FOXO1 proteins in LNCaP cell lysate were depleted by anti-FOXO1 antibody, approximately 70% of IQGAP1 was depleted by the same antibody (Fig EV2J). This finding is not surprising since IQGAP1 is a pleiotropically acting protein with effects on multiple signaling pathways and different portions of the proteins need to bind to dozens of different protein complexes. Thus, our data suggest that cytoplasmic FOXO1 is enough to bind to the majority of IQGAP1 at least in LNCaP cell lysate examined. This finding is consistent with our data that other FOXO proteins such as FOXO3 also enable to bind to IQGAP1 even in the presence of FOXO1, an indication of a collaborative rather than redundant role of different FOXO factors in sequestering IQGAP1 and regulating pERK1/2 (Fig EV2I).

2. It is unclear how pS319 binds to the coiled-coil region of IQGAP1 - coiled-coil regions of proteins are not generally known to serve as phosphobinding sites (I can't think of an example) - or whether the phosphate is directly recognized for binding. In this regard, Glu residues are generally poor phosphomimics for phosphates that are involved in protein-protein interactions, because they are less charged and bulky than phosphate.

Reply: We agree with the Referee that Glu residues are less charged and bulky than phosphate and therefore are generally poor phosphomimics for phosphates. Like Glu (E), Asp (D) is another acidic amino acid. It has been shown previously that mutation to Glu or Asp functions as a phospho-mimicking residue in different proteins (Bochkareva et al. PNAS 102: 15412-17, 2005; Kaneko et al., Genes Dev 24: 2615-20, 2010; Liu et al., Mol Cell 57: 648-61, 2015). Since the molecule mass of Asp is not as bulky as Glu and better mimics a phosphoserine, we generated S319D mutant of FOXO1 and examined its binding capacity with IQGAP1. We demonstrated that similar to the S319E mutant, S319D also had higher affinity of binding to IQGAP1 than the non-phosphorylable mutant S319A (Fig EV1D and E). Thus, the new data further suggest that S319 phosphorylation is important for FOXO1 binding to IQGAP1.

3. The other major unanswered mechanistic question is how binding of the short S319E peptide to the coiled-coil region would block the binding of RAF, MEK and ERK to IQGAP1, since these kinases all bind to separate sites downstream of the coiled-coil region.

Reply: This is an excellent question. As exemplified in a particular case, it has been shown recently that a single phosphorylation induces protein conformational change and subsequent protein folding (Bah et al., Nature 519: 106-9, 2015). Given that phosphorylated FOXO1 binds to the coiled-coil region whereas RAF, MEK and ERK bind to different domains of IQGAP1 downstream of the coil-coil region, we hypothesized that binding of IQGAP1 by phosphorylated FOXO1 causes conformation changes, which in turn impair IQGAP1 binding with RAF, MEK and ERK proteins. Limited proteolysis assay is often used to determine the changes in protein conformation (Varne et al., FEBS Lett 516: 129-32, 2002). We employed this approach to determine whether binding of a FOXO1 phospho-mimicking peptide induces conformation changes in IQGAP1 protein. We incubated recombinant IQGAP1 proteins with GST-FOXO1-IQBP S319E or GST alone and performed partial digestion of proteins using

trypsin. As shown in Fig EV1G, there were two major proteolytic bands migrated slightly faster than the uncleaved IQGAP1 in the control (GST alone) group, whereas there was only one major band migrated slightly faster than the uncleaved IQGAP1 in the GST-FOXO1-IQBP S319E group. These data suggest that binding of the short S319E peptide causes conformation changes in IQGAP1. Thus, our new data provide experiment supports to our hypothesis that binding of IQGAP1 by phosphorylated FOXO1 causes conformation changes, which in turn impair IQGAP1 binding with RAF, MEK and ERK proteins.

4. The ability of the S319E FOXO1 peptide to suppress paclitaxel-induced ERK activation and to synergize with docetaxel in a tumor xenograft model is of relevance to cancer therapy and paclitaxel resistance mechanisms, but it is unclear how paclitaxel induces nuclear localization of FOXO1, i.e. does it cause dephosphorylation of the 14-3-3 binding sites responsible for cytoplasmic retention of FOXO1, and more importantly does it lead to dephosphorylation of pS319?

Reply: This is an excellent point. It has been shown in breast cancer cells that paclitaxel treatment decreases AKT phosphorylation (S473-p) and activity and FOXO3 phosphorylation at T32 (a 14-3-3 binding site) (Sunters et al. Cancer Res 66: 212-220, 2006). In agreement with this report, we demonstrated that paclitaxel treatment of LNCaP prostate cancer cells also decreased AKT phosphorylation (S473-p) and reduced phosphorylation of T24 and S256 in FOXO1, two 14-3-3 binding sites responsible for cytoplasmic retention of FOXO1 (Fig EV5B). Thus, the result that paclitaxel-induced decrease in phosphorylation of the 14-3-3 binding sites in FOXO1 is consistent with the observation that paclitaxel induces nuclear localization of FOXO1 (Fig EV5A). Moreover, in concordance with decreased AKT phosphorylation, paclitaxel treatment also decreased FOXO1 phosphorylation at S319 (Fig EV5B).

5. It is not obvious to me that S319 is conserved in FOXO3 and FOXO4, which also bind IQGAP1 in a phosphodependent manner apparently.

Reply: This is an excellent point. We performed protein sequencing alignment analysis. As shown in Fig EV2D, S319 phosphorylation site in FOXO1 is homologous to S315 in FOXO3 and S262 in FOXO4. Importantly, this observation is consistent with our finding that FOXO3 and FOXO4 also bind to IQGAP1 and the binding was largely diminished by mutating S315 and S262 to alanine (Fig EV2E and F). These data suggest that binding of FOXO3 and FOXO4 to IQGAP1 is also phospho-dependent.

2nd Editorial Decision 25 January 2017

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by the two original referees again. As you will see below, both referees appreciate the revision and support publication in The EMBO Journal. I am thus happy to accept your manuscript in principle for publication here.

REFEREE REPORTS

Referee #1:

The authors have addressed all of my comments and this outstanding paper should be published at this time.

Referee #2:

The authors have dealt satisfactorily with the minor concerns that I expressed in my previous review. This is an interesting and innovative paper that fits well into 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: Haojie Huang
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2016-95534

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?	All experiments showed in the manuscript have been repeated for at least two times. And the two-sided student T-test was used to determine if the differences were statistically significant, see Materials and Methods for specifics on each type of experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Minimum of 7 age- and sex- matched animals per group were used.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Animals were excluded from analysis only if they became ill or their weight dropped below 90% of their original weight at the start of the experiment. However, no animals were excluded from the current study.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Age and sex matched mice were randomly selected.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes, tumor growth was monitored blindly by living imaging.
5. For every figure, are statistical tests justified as appropriate?	Yes, see Figure Legends and Materials and Methods
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Two-sided student T-test
Is there an estimate of variation within each group of data?	We show standard deviation or standard error of the mean as described in the figure legend.
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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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).	Antibodies used are described in Material and 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.	We described the source of cell lines. All cell lines used in this study were tested for mycoplasma contamination.

* 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.	6-week-old NOD-SCID IL-2-receptor gamma null (NSG) mice were generated in house and used for animal experiments. All mice were housed in standard conditions with a 12 h light/dark cycle and access to food and water ad libitum
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The animal study was approved by the IACUC at Mayo Clinic.
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.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA