EMBO reports

Bur1 functions with TORC1 for vacuole-mediated cell cycle progression

Yui Jin, Natsuko Jin, Yu Oikawa, Ron Benyair, Michiko Koizumi, Thomas Wilson, Yoshinori Ohsumi, and Lois Weisman
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Corresponding author(s): Lois Weisman (lweisman@umich.edu) , Yoshinori Ohsumi (yohsumi@iri.titech.ac.jp), Lois Weisman (lweisman@umich.edu), Yui Jin (jin.y.ah@m.titech.ac.jp)

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Dear Dr. Weisman,

Thank you for the submission of your research manuscript to our journal, which was now seen by two referees, whose reports are copied below.

Referees express interest in the proposed role of kinase Bur1 in yeast cell cycle progression and vacuole TORC1 signaling. However, they also raise important concerns that need to be addressed to consider publication here.

I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript. As the reports are below, and I think all points need to be addressed, I will not detail them here.

Given these positive recommendations, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As

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4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

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Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <http://embor.embopress.org/authorguide#sourcedata>.

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Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

Specific molecular pathways in eukaryotic cells control and optimize the inheritance of membrane-bounded organelles at the time of cell division. The yeast Saccharomyces cerevisiae is an often used model to study organelle inheritance because of its asymmetric cell division. The vacuole, the yeast counterpart of the mammalian lysosome, is actively inherited at the time of cell division and is also necessary for the progression of cell division via signaling through the TORC1 pathway. In this manuscript, Jin and colleagues used a synthetic growth defect screen with a yeast strain defective in vacuole inheritance (vac17Δ) to identify other proteins and pathways associated with vacuole inheritance and cell cycle progression. Using this approach they identified the essential cyclin-dependent kinase Bur1 as critical for cell cycle progression from G1 phase in concert with TORC1. The authors further showed that Bur1 functions together with TORC1, because mutation of bur1 shows high sensitivity to rapamycin, an inhibitor of TORC1, and because both bur1 and TORC1 work through the phosphorylation of Sch9, an AGC family protein kinase necessary for the functionality of TORC1. The authors conclude that multiple pathways converge on Sch9 to control and advance the cell cycle in yeast.

There following issues should be addressed by the authors:

1) The quality of the microscopic images must be greatly improved. It was very difficult to see fluorescence patterns even on a high resolution screen and at enlarged size.

2) The microscopic images used in Fig. 3A were used again in Fig. S5A.

3) Figs. 2A and 2C. Show the position of 1N and 2N DNA.

4) Fig. 4. Transpose panels A and B.

5) p. 9. 2nd paragraph, 2nd line. Give references that report that the double mutants show similar phenotypes.

6) Fig. S5B is not mentioned in the Results or elsewhere. Discuss.

7) p. 15, last paragraph, 3rd line. (Fig. 5C) NOT (Fig. 3C).

8) Throughout the manuscript. 'medium' is singular. 'media' is plural.

Referee #2:

This study investigates the role of kinase Bur1 in yeast cell cycle progression and vacuole TORC1 signaling. Using a mutagenesis screen, they identify a BUR1 mutant that has a synthetic genetic defect with a vacuole inheritance VAC17 mutant. They find that Bur1 functions in Sch9-mediated signaling in parallel with the TORC1 machinery. The C-terminal region of Bur1 contributes to this regulation. They propose that Bur1 has both nuclear and cytoplasmic roles in cell cycle regulation.

This is an interesting and generally well conducted study. Understanding how vacuole regulated cell growth signaling intefaces with the cell cycle and its nuclear signaling is important to understanding cell homeostasis and growth, and this work nicely demonstrates a potential new role for kinase Bur1 in regulating cell cycle progression. However, the study requires more quantification for certain assays. Additionally, it is currently unclear exactly how the Bur1 C-terminal region regulates Bur1 activity. Since this region is missing in the originally uncovered mutant in the screen, it would be mechanistically helpful to understand more about how the C-terminal region of Bur1 works. Finally, the last section of the study that investigates the cytoplasmic and nuclear Bur1 pools appears preliminary and should be examined more.

Major comments:

1) As presented it is unclear what the role of the Bur1 C-terminal region is. Loss of it impacts kinase activity of Sch9, but this could be for several reasons. Does the C-terminal region interact directly with Sch9 biochemically? Alternatively, does expression of only the C-terminal region exhibit a dominant negative effect? Determining whether this is an intra or inter molecular regulation would be helpful.

2) Sub-cellular activity of Bur1: the model is predicated on the idea that Bur1 functions distinctly in the nucleus and cytoplasm. The C-terminal mutant shows slightly less cytoplasmic localization. However, the cytoplasmic Bur1 pool is very small, at least in the images presented in Figure 5A. Does this pool change during the cell cycle? And can this sub-cellular concept be tested further? For example, does introduction of a NES to Bur1-mNG impact function? Alternatively, does introducing nuclear import signals to Bur1 to better hold it inside the nucleus cause defects in Sch9 phosphorylation, etc? If the model is true, anchoring Bur1 to the vacuole via a lipid binding domain for instance may maintain Sch9 regulation.

Minor comments:

1) Figure 2 A,C: Although the quantifications in panels in B and D are the key point, you should mark the y-axis in panels A and C. Are these all the name scale? It is ambiguous as marked now.

2) Figure 2G: here it is observed that the bur1-C vac17 double mutant does not inherit vacuoles similar to vac17-null alone. Can this be quantified? Are the two mutant strains identical in vacuole inheritance behavior? This appears necessary because the vacuole is morphologically fragmented in the double mutant.

3) Figure 3B: It is reported that the bur1 vac17 double mutant has less phosphorylated Sch9-HA compared to WT, but that is not obvious from this blot. Can this be quantified?

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There following issues should be addressed by the authors:

1) The quality of the microscopic images must be greatly improved. It was very difficult to see fluorescence patterns even on a high resolution screen and at enlarged size.

We now repeated many of these experiments and took images with a SpinSR10, Olympus confocal microscope. See figures Fig. 2E, Fig. 3A (old Fig. 2G), and Fig. S6A- (old Fig. 5A). We also split Figure 2 into Figures 2 and 3 to allow us to present larger images.

2) The microscopic images used in Fig. 3A were used again in Fig. S5A.

Thank you for pointing out this error. We now ensured that all images shown are unique to one figure.

3) Figs. 2A and 2C. Show the position of 1N and 2N DNA.

Thank you for this suggestion. We now indicate the 1N and 2N DNA positions. We also added the cell numbers on the Y-axis.

4) Fig. 4. Transpose panels A and B.

We now transposed panels A and B (new Fig. 5B and 5A).

5) p. 9. 2nd paragraph, 2nd line. Give references that report that the double mutants show similar phenotypes.

We add referenced the appropriate reference, PMID: 26322385.

6) Fig. S5B is not mentioned in the Results or elsewhere. Discuss.

Thank you pointing out this error. We now indicate this figure, new-Fig. S6E, in the result section.

7) p. 15, last paragraph, 3rd line. (Fig. 5C) NOT (Fig. 3C).

Sorry for the confusion. Fig. 3C was indicating the figure in our previous publication, Jin and Weisman 2015. We now removed referring to Fig. 3C, and instead indicate the figure in the current manuscript that we are discussing at that point. We rewrote this sentence as follows:

“It is not clear why the Bur1 C-terminal tail is only essential in vacuole inheritance mutants, vac17Δ, vac8Δ, or myo2-N1304D (Fig. 1H, and Fig. S1). This may be due to defects in these mutants in the ability of TORC1 to signal from an immature vacuole (Jin and Weisman, 2015).”

8) Throughout the manuscript. 'medium' is singular. 'media' is plural.

We now use medium where appropriate.

Referee #2:

This study investigates the role of kinase Bur1 in yeast cell cycle progression and vacuole TORC1 signaling. Using a mutagenesis screen, they identify a BUR1 mutant that has a synthetic genetic defect with a vacuole inheritance VAC17 mutant. They find that Bur1 functions in Sch9-mediated signaling in parallel with the TORC1 machinery. The C-terminal region of Bur1 contributes to this regulation. They propose that Bur1 has both nuclear and cytoplasmic roles in cell cycle regulation.

This is an interesting and generally well conducted study. Understanding how vacuole regulated cell growth signaling interfaces with the cell cycle and its nuclear signaling is important to understanding cell homeostasis and growth, and this work nicely demonstrates a potential new role for kinase Bur1 in regulating cell cycle progression. However, the study requires more quantification for certain assays. Additionally, it is currently unclear exactly how the Bur1 C-terminal region regulates Bur1 activity. Since this region is missing in the originally uncovered mutant in the screen, it would be mechanistically helpful to understand more about how the C-terminal region of Bur1 works. Finally, the last section of the study that investigates the cytoplasmic and nuclear Bur1 pools appears preliminary and should be examined more.

Major comments:

1) As presented it is unclear what the role of the Bur1 C-terminal region is. Loss of it impacts kinase activity of Sch9, but this could be for several reasons. Does the C-terminal region interact directly with Sch9 biochemically? Alternatively, does expression of only the C-terminal region exhibit a dominant negative effect? Determining whether this is an intra or inter molecular regulation would be helpful.

These are important questions. We showed in the first submission that the C-terminus of Bur1 is important for its kinase activity in vitro, as well as for growth of mutants with a defect in vacuole inheritance, and for a tor1-delta mutant. During the revision, we found that Bur1 interacts with Sch9, and then discovered that the C-terminus of Bur1 is not required for this interaction. We found that recombinant peptides from either the N- and C terminal regions of Sch9, GST-Sch9 (1-390) and GST-Sch9 (391-824), but not GST alone, pull down Bur1 from yeast lysates (Fig. S5A). Importantly, these GST-Sch9 peptides also pulled down Bur1-ΔC from yeast lysates. These results strongly suggest that the C-terminal region is not critical for Bur1 association with Sch9.

In addition, we tested whether over-expression of the Bur1 C-terminal region exhibited a measurable phenotype. However, over-expression of this region did not affect yeast growth or sensitivity to rapamycin (Fig. S5). This raises the possibility that the Bur1 C-terminus does not contact other proteins, and instead may participate in an intramolecular interaction within Bur1, perhaps to promote kinase activity, and/or Bur1 interaction with other proteins.

2) Sub-cellular activity of Bur1: the model is predicated on the idea that Bur1 functions distinctly in the nucleus and cytoplasm. The C-terminal mutant shows slightly less cytoplasmic localization. However, the cytoplasmic Bur1 pool is very small, at least in the images presented in Figure 5A. Does this pool change during the cell cycle? And can this sub-cellular concept be tested further? For example, does introduction of a NES to Bur1-mNG impact function? Alternatively, does introducing nuclear import signals to Bur1 to better hold it inside the nucleus cause defects in Sch9 phosphorylation, etc? If the model is true, anchoring Bur1 to the vacuole via a lipid binding domain for instance may maintain Sch9 regulation.

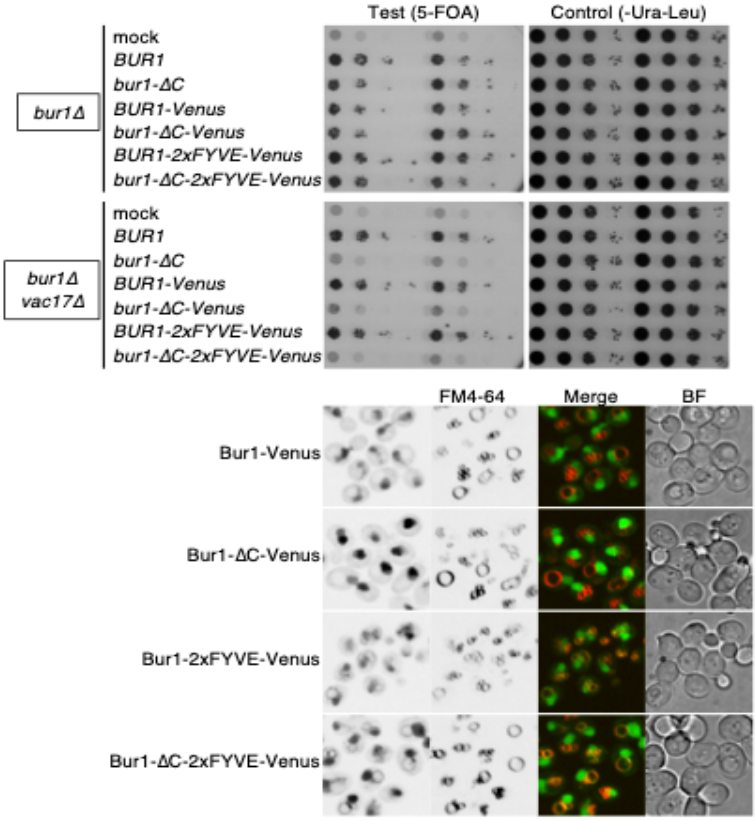
Thank you for these suggestions. We have now performed these new recommended experiments, which reveal that Bur1 must be localized in the nucleus at some step in the activation of the TORC1 pathway.

We tested and found that there was not a dramatic change in the cytoplasmic pool of Bur1 during the cell cycle. However, we observe an increase in nuclear Bur1 at 120 minutes after alpha-factor arrest and release, which corresponds to the second initiation of the cell-cycle (new-Fig. S6D). This is consistent with the possibility that Bur1 has a nuclear role early in the cell-cycle. Note that the initial release of cells from alpha-factor is not identical to G1 phase in asynchronous cells, while initiation of the second cell-cycle is closer to G1 phase in asynchronous cells.

To learn more about the functional significance of the cellular localization of Bur1, we generated mutants to alter Bur1 localization to the nucleus. There is a predicted importin α-dependent nuclear-localization signal (NLS) (14-49 aa) in Bur1 (PMID: 19520826). Deletion of an N-terminal region (2-49 aa) of Bur1 (Bur1-ΔN) results in much less Bur1 in the nucleus, yet this mutant supports yeast growth under normal conditions and in a vacuole inheritance mutant (new-Fig. S6, E and F). This suggests that some functions of Bur1 may occur in the cytoplasm. Interestingly, the Bur1-ΔN mutant did not fully support rapamycin sensitivity of bur1-ΔC (new-Fig. 6, A and B), suggesting that Bur1 functions at nucleus during the TORC1 activation. In support of this, we found that Bur1 fused to an SV40-NLS (PMID 6096007) does not change its rapamycin sensitivity (new-Fig. 6A). Moreover, adding nuclear-export signal (NES) of PKI (PMID 7634336) to Bur1 showed

higher rapamycin sensitivity compared to without the NES (new-Fig. 6B). These results suggest that at least some of Bur1 functions must occur in the nucleus for the activation of TORC1 pathway.

In addition to the above experiments, we also tested whether adding a FYVE domain to Bur1 would increase its association with the vacuole and provide better growth. Indeed, we found that this strain grew at a little better than wild-type. However, expression of Bur1-ΔC-FYVE-Venus did not rescue a growth defect of bur1-ΔC vac17Δ. This suggests that the C-tail has functions beyond anchoring Bur1 to the vacuole membrane or for the cytoplasmic localization of Bur1. Note that the fusion protein was localized both in the nucleus and in the cytoplasm, so we decided to omit this data from the paper.



Minor comments:

1) Figure 2 A,C: Although the quantifications in panels in B and D are the key point, you should mark the y-axis in panels A and C. Are these all the name scale? It is ambiguous as marked now.

As suggested, we remade Fig. 2A and 2C with an indication of 1N and 2N DNA positions (as suggested by referee #1). We also added actual cell numbers to the Y-axis.

2) Figure 2G: here it is observed that the bur1-C vac17 double mutant does not inherit vacuoles similar to vac17-null alone. Can this be quantified? Are the two mutant strains identical in vacuole inheritance behavior? This appears necessary because the vacuole is morphologically fragmented in the double mutant.

Thank you for the suggestion. We re-took images and found that vacuole morphology is similar in all the strains new-Fig. 3. In addition, we assessed vacuole inheritance using pulse-chase FM4-64 staining (new-Fig. S3).

3) Figure 3B: It is reported that the bur1 vac17 double mutant has less phosphorylated Sch9-HA compared to WT, but that is not obvious from this blot. Can this be quantified?

To facilitate observation of Sch9-HA phosphorylation we performed western-blot analysis on samples run with phos-tag SDS-PAGE, and quantitated the results (Fig. S4A). We found that bur1-ΔC showed significantly less phosphorylation, and that the phosphorylation status of Sch9-HA in the bur1-267 mutant was even lower.

Dear Prof. Weisman,

Thank you for submitting your revised manuscript. It has now been seen by both of the original referees.

My apologies for the delay in getting back to you, it took longer than anticipated to receive the referee reports given this busy time of the year.

As you can see, the referees find that the study is significantly improved during revision and recommends publication. However, I need you to address the editorial points below before I can accept the manuscript.

• Please rename the 'Data and materials availability' as 'Data Availability'. Moreover, we note that the text in this section is not accurate. As per our guidelines, the Data Availability section is reserved for the new primary dataset that is generated in this study and deposited in a public data repository. If this is not applicable, please make a statement that no data were deposited in a public database, and the remove the current text.
• Please rename the 'Declared interests' as 'Conflict of Interests'.
• In the Author Contributions section, please abbreviate Y.Oikawa and Y.Ohsumi as Y.Oi. and Y.Oh.
• Please rename 'Supplemental Figure Legends' as 'Expanded View Figure Legends'.
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• For technical reasons, we can only accommodate 5 Expanded View figures. There are currently 6. Please reduce the EV Figure count to 5 (e.g. by combining figures). Alternatively, you can convert EV Figures (some or all) into an Appendix file, which does not have such a limitation (please see https://www.embopress.org/page/journal/14693178/authorguide#expandedview).
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• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors have responded appropriately and adequately to my queries and issues. In particular and most importantly, they have submitted much improved microscopic images as requested in Point 1 of my initial review.

Referee #2:

The revised study has addressed the majority of criticisms. New higher quality images have been added, and there is new data on how the C-term of Bur1 may influence TORC1 signaling. There is also significant new data on the cytoplasmic versus nuclear roles for Bur1, which add to the overall findings of the study. It is now clear that some aspect of Bur1 function must occur in the nucleus.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision **21st Jan 2022**

Dear Dr. Weisman,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz Senyilmaz Tiebe
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

--
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A- Figures

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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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For most of experiments, we did at least three indipendent experiments. For tetrads analysis, we tested al least 25 cells in each category.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	No
5. For every figure, are statistical tests justified as appropriate?	Yes, to our knoledge. For all experiment except Fig EV6B, we used a one-way ANOVA and Tukey post hoc test. For Fig EV6B, we used a paired student t-test.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For Fig 1E, Fig 1G, and Fig EV6B, graphs of frequency had normal distribution.
Is there an estimate of variation within each group of data?	No

USEFUL LINKS FOR COMPLETING THIS FORM

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

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-tumo

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://jjj.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	No

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We indicate antibodies information at 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.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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).	Data are shown in figures and EV figures
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. 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.	We used standardized formats

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No