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Remodeling of ER-exit sites initiates a membrane supply pathway for autophagosome biogenesis

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(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.)

Initial point-by-point response submitted by authors 30 May 2017

Thank you for considering a revised version of our manuscript entitled" Remodeling of ER-exit sites initiates a membrane supply pathway for autophagosome biogenesis" to *EMBO Reports*. We also thank the reviewers for providing suggestions to improve the current work. Below please find our point-by-point responses to the reviewers' questions.

Referee #1:

Following up on previous work showing SEC12, an activator of COPII, relocates to ERGIC under starvation conditions, Ge et al. investigate in their manuscript "Remodeling of ER-exit sites initiates a membrane supply pathway for autophagosome biogenesis" how SEC12 relocates from the ERES to the ERGIC. The authors use confocal microscopy to show that SEC12 structures are larger in starvation conditions. The authors further use Structured Illumination Microscopy and STORM to examine the enlarged SEC12 structures, which they conclude represent enlarged ERES and some localization to the ERGIC. The authors find that this relocation of SEC12 requires both CTAGE5 and FIP200. While their confocal images, knockdowns, knockouts, and immunoprecipitation data

appears convincing, their confocal image quantification and super-resolution imaging is unsatisfactory. I suggest the following major revisions:

• *Figure 1 confocal images and similar images in other Figures used to quantify SEC12 compartment size. The images in the Figures appear saturated. Saturated images will result in artificially large measured compartment sizes and an increase in label density can, for example, be wrongly interpreted as an increase in compartment size. This quantification was used in Figures 1, 3, 5, and 7 to provide evidence in a change in compartment size. Please confirm that the quantification was performed on confocal images that were not saturated and clarify this in the methods section, or repeat the experiment under conditions where the images were not saturated.*
Response: We thank the reviewer for pointing this issue. The images we used for quantification were not saturated. We have clarified this in the methods section in the revised version of the manuscript.

• *Size quantification of SEC12 structures in Figure 1B (and similar Figures throughout the manuscript). The smallest structures seem to be smaller than the diffraction-limited resolution, which is not possible using confocal microscopy and therefore represent an image analysis artifact (probably caused by dim structures where only 1-3 sub-diffraction sized pixels were above the detection threshold?). Additionally, the way the plots are represented leads to a "saturation" effect: the number of dots in about the bottom ten lines of dots (smaller than about 0.1 micron squared) look virtually identical since they overlap too much within each line to be separable. The presentation is therefore not appropriate. I am also struggling with the seemingly low difference in the red lines and the huge error bars which makes me wonder how the low p-values were achieved. (This problem is most obvious in Figure EV1.) What the authors probably want to show is, that the fraction of compartments which is reasonably big, i.e. bigger than a certain threshold (say bigger than 0.2 microns squared), is significantly larger in the starvation case. I therefore suggest an alternative representation which eliminates the described problems and also brings the point across more clearly: pick a reasonable size threshold, count how many of the compartments are bigger and use simple bar graphs which show the relative fraction of compartments above the threshold in the starvation and control cases instead of showing all data points. A similar approach is suggested for Fig. 1D, etc.*
Response: We thank the reviewer for the helpful suggestion. We took the reviewer's suggestion and revised the way of quantification. We set a threshold of 0.1 mm^2 in area and 0.04 mm^3 in volume (see more details in the new Method section). We counted the fraction of compartments above the threshold as a measurement of SEC12-ERES remodeling under different conditions. By this approach, we now believe that remodeling of SEC12-ERES is more clearly presented in the revised manuscript.

• *Figure 1E. The main text reports: "Surface modeling of the SEC12-ERES and the ERGIC indicated that individual enlarged SEC12-ERES wrapped around the surface of the tubulovesicular*

ERGIC with a preference for the protrusions (Fig 1E and Fig EV Movie 1)." It is unclear what the authors mean by "protrusions" or "tubulovesicular". Since the z-resolution of the image should be about 2 to 3-fold worse, the tubular appearance of the ERGIC may be an artifact of the imaging approach. There also appears to be a systematic offset between the red and the green color in the z-direction, probably caused by chromatic aberrations, which should be corrected by post-processing if shown. The iso-surface representation used in Figure 1E is generally not a good choice since the brighter channel is hiding the dimmer channel at any particular location and therefore hides any potential co-localization. I suggest to eliminate Figure 1E and instead focus on a better and potentially quantitative analysis of the zoomed in regions in Figure 1C to prove the point of the SEC12 signal wrapping around the ERGIC53 signal.

Response: We thank the reviewer for the suggestion. We have removed this panel and the video from the current manuscript. We also quantified the volume increase of SEC12-ERES based on the reviewer's suggestion above. In addition, we counted the number of SEC12-ERES showing an obvious elongation along the ERGIC. In each cell, we found around ten elongated SEC12-ERES structures after starvation compared with less than two before starvation. The point of SEC12 wrapping around the ERGIC is based on the appearance of some cup-shaped structures we found in the SIM and STORM images shown in the new Fig1. Not all elongated or enlarged SEC12 wraps around the ERGIC. We modified the description in the revised manuscript to tone down the claim that SEC12 wraps around the ERGIC to more accurately reflect our observation.

• *The STORM image in Figure 2B shows fairly convincing colocalization between SEC12 and ERGIC53, but this is not well supported by either Figure 2C or Figure EV3. The signal levels in EV3 in particular are so weak that the white arrows mostly point to single localization events which at a first glance could easily be explained by background or cross-talk. The current colocalization interpretation between these two proteins is therefore tentative at best. Please either include more convincing examples where more molecules colocalize, or provide a more quantitative analysis of the data which proves the statistical significance of the observed few co-localization events.*

Response: We thank the reviewer for the suggestion. We have added more colocalization images to support our conclusion in the revised manuscript (Fig EV4).

• *Figure 7 C and E. Some asterisks appear to be placed incorrectly to mark cells. Please check this and confirm that it did not affect the analysis of LC3 puncta in these images.*

Response: We thank the reviewer for pointing out this. We have corrected the labeling errors.

Referee #2:

This manuscript addresses the role of ERES, ERGIC and sec12 in the formation of autophagosomes building on the previous work of Ge et al. over the past 3 or 4 years. In a further refinement of their original model they here propose a role for the COPII activator sec12-positive sites on ER exit sites (ERES) which contribute to an ERGIC-dependent autophagosome biogenesis pathway. The data

presented supports their proposed model but falls short of being convincing. There are several important experiments which do not have robust effects and should be quantified. The final major point concerning FIP200 and sec12 is merely a descriptive analysis of the interaction and does not provide any molecular insight into their question.

Specific points:

1. Introduction mentions TRAPPIII. In yeast it was my understanding that this complex is specific for autophagy (the other TRAPP complexes involved in tethering). In addition, since the manuscript deals with mammalian cells the role of the mammalian TRAPPIII complex should also be discussed.
Response: We have revised the description of TRAPPIII in the introduction part to cover its role in both yeast and mammalian cells. Corresponding references have been added too.

2. The authors include in their model and experiments the concept that the autophagic PI3K is required for relocation of COPII to ERGIC (p. 2 and 3) and they show inhibition with wortmannin produces an enlarged ERES. They also test Atg14 depletion in Fig 5D which eliminates Sec12 from the ERGIC. To test if the activity of the PI3K complex is required the authors should use one of the newly developed Vps34 inhibitors to support the role of the Vps34 PI3K complex in recruitment of sec12.
Response: We thank the reviewer for the helpful suggestion. We have performed experiments with three newly reported VPS34 specific inhibitors in the revised work (Fig 4E). All inhibitors inhibit relocation of SEC12 and SAR1 to the ERGIC upon starvation. In addition, we also used the three inhibitors in the IF experiment to determine the remodeling of SEC12-ERES (Fig EV2). Similar results were obtained as with 3-MA and wortmannin. We conclude that VP34-PI3K is not required for the starvation-induced remodeling of SEC12-ERES but required for a downstream event facilitating the relocation of SEC12 to the ERGIC.

3. Throughout the manuscript they use flag-tagged Sec12 for co-ips eg cTAGE and FIP200 - data showing interaction using endogenous Sec12 must be provided. This will at least confirm interaction of the endogenous protein (although not direct interaction). If necessary an enriched pool of membranes (ERES, ERGIC or COPII) might be used as starting material to increase chances of detection and provide more detail to the model in Fig. 8.
Response: We thank the reviewer for the suggestion. We have performed coIP experiment to confirm the association between FIP200 and SEC12 at endogenous levels of these proteins in the revised work (Fig 5C, D). This further confirms the association between FIP200 and SEC12. In addition, we observed an enrichment of endogenous FIP200 at the ERES/ERGIC region, which is distinct from the PAS, under starvation conditions (Fig 6). This suggests that FIP200 associates with the ERES/ERGIC-localized SEC12 under starvation conditions. We have also added the new indication in the discussion part regarding the model in Fig 8.

4. The starvation dependent recruitment of sec12 to the ERGIC should be quantified. Fig, 3F is not well reproduced in Fig. 3G or Fig. 5D and the protein control sec22 is overexposed in the ERGIC fractions.

Response: We have added the quantification of the relocation in the revised work (see more details in the Figures and Legends). We have replaced Fig 2G, corresponding to the previous 3G, with a new set of panels to more clearly show the requirement of CTAGE5 in the relocation of SEC12 to the ERGIC. Although the starvation-induced relocation of SEC12 to the ERGIC presented in Fig 4D (the previous 5D) is not as dramatic as Fig 2F (previously 3F) due to different experiments, the effect is still clear and significant (>2 folds of increase after starvation).

5. In Fig. 4A and B the authors still have flux despite the cTAGE because they see an increase of LC3-II with BafA. What is also strange is what happens to all the LC3 in the KO cells? There is no LC3 at all detectable. The other assays are not particularly informative- p62 blot is overexposed, and the actin is over exposed, and for both FIP200 and LC3 puncta there is still a starvation response although dampened. This data does not support a requirement for cTAGE (as stated in section head) but simply shows cTAGE slightly modulates the extent of autophagic response.

Response: We have replaced Fig 3B (previous Fig 4B) with new panels to more clearly show the effect of CTAGE5 on LC3 lipidation and autophagic flux. We also did the quantification of LC3 lipidation to show a ~50% and 70% decrease of the autophagic flux in CTAGE5KD and KO cells under starvation conditions (compare lane4 and 8 in 3A and 3B). Moreover, P62 is not degraded in CTAGE5KO cells (Fig 3B). The data indicate an important role of CTAGE5 in regulating LC3 lipidation and autophagy. For IF image, we have mentioned that the LC3 change is more dramatic than FIP200 and we concluded that CTAGE5 may preferentially affect LC3 lipidation over PAS formation. Even through the data support the importance of CTAGE5 in autophagosome biogenesis, we agree with the reviewer that CTAGE5 may not be absolutely required because we still observe a low level of autophagosome biogenesis in the absence of CTAGE5 (Fig 3). Therefore, we have modified the section head as “CTAGE5 modulates LC3 lipidation and autophagosome biogenesis under starvation”.

6. In Fig. 5 both FIP200 and Atg14 result in decreased amounts of sec22B in the total. This is not reflected in the ERGIC isolation. How can the authors explain this considering in the ERGIC fractions sec22b levels are identical over the different conditions?

Response: In addition to the ERGIC, SEC22B also resides on the ER. It is likely that the slight total decrease of SEC22B that we observed resulted from the decrease of the ER-localized SEC22B. The ERGIC-localized SEC22B is not affected because the level of SEC22B from each treatment is similar in the ERGIC fraction. We also quantified the percentage of SEC12 relocation to the ERGIC to clearly indicate the defect of SEC12 relocation in FIP200 or Atg14 KD cells.

7. Fig. 6A the wortmannin treatment did not inhibit LC3-II lipidation suggesting it did not work and no conclusions can be made about this. The levels of FIP200, ULK1 and ATG13 under the various conditions in the flag-IP should be quantified.

Response: We quantified the amount of LC3 lipidation. Wortmannin treatment reduced ~50% of LC3 lipidation (2.3 versus 1.2). We also quantified the amount of FIP200, ULK1 and ATG13 that associates with SEC12 under the indicated conditions (Fig 5A). The association between FIP200 and SEC12 increased ~ 3 fold, and further increased another ~2 fold in the presence of wortmannin. The amount of ULK1 that associates with SEC12 is in general much lower than that of FIP200 and not increased by starvation. ATG13 showed a reversed trend of association with SEC12 under the treatments.

The siRNA knockdown of ULK1/2 is not complete so no conclusion about the ULK1/2-FIP200-Atg13 complex can be made in Fig. 6B. Finally, the experiment in Fig. 6 does not allow the conclusion to be made that FIP200 acts independently- it just says the detection of ULK1/2 and ATG13 are not enriched in the flag IP making them hard to detect.

Response: Our data support that FIP200 associates with SEC12 independent of ULK and ATG13. 1) The ULK knockdown showed a >75% decrease of ULK protein levels. With this KD efficiency, we have observed a blockage of autophagy (Fig 4A). So we should observe a change of FIP200-SEC12 complex if its formation requires ULK. However, we did not observe a change of FIP200-SEC12 complex when ULK proteins were reduced >75% (Fig 5D). This favors the notion that ULK is not required for FIP200-SEC12 complex formation; 2) Complete depletion of ATG13 by KO did not reduce but increased FIP200 association with SEC12 (Fig 5E), demonstrating that ATG13 is not required for FIP200-SEC12 complex formation; 3) KD of FIP200 but not of ULK or ATG13 abolished starvation-induced remodeling of SEC12-ERES demonstrating that ULK or ATG13 are not required for SEC12 remodeling upon starvation; 4) In the new Fig 6, FIP200 is enriched in both PAS and ERES/ERGIC, while SEC12 is only enriched in the ERES. This indicates that the ERES pool of FIP200 associates with SEC12. However, it has been demonstrated that PAS-localized FIP200 associates with ULK and ATG13. Together, these data support the notion that FIP200 forms a complex with SEC12, which is distinct from the previously reported FIP200/ATG13/ULK complex.

8. Page 6, 4th paragraph the authors make a conclusion that "The above data were consistent....the ULK kinase complex." This has been shown by many researchers and it is not clear what new point the authors are making.

Response: We took the reviewer's suggestion and removed this sentence from the manuscript.

9. Fig. 8 does not give any mechanistic information about the interaction of the C-terminal domain of FIP200 and its interaction with sec12. How does this domain affect ERES expansion in line with their model? FIP200 is also not illustrated in the model shown in Fig. 8.

Response: We have clear evidence indicating the requirement of FIP200 for the remodeling of SEC12-ERES. However, at the current stage, it is still not clear if FIP200 binds directly to SEC12 or how FIP200 facilitates ERES enlargement. Our future direction is to clarify the model by a more detailed mechanistic study on FIP200 and SEC12 association. Even though we have not provided a detailed scenario, we have made a significant advance by identifying the starvation-induced remodeling of the ERES and the consequence of remodeling, and, furthermore, we identified two factors involved. We hope the reviewer appreciates that these advances are significant enough to publish in *EMBO Reports*.

Referee #3:

In this manuscript, the authors reported that starvation-induced remodeling of ERES facilitates the relocation of SEC12 to the ERGIC, they further illustrated the molecular mechanism underlying the SEC12 relocation. The membrane source of autophagosome is one of the fundamental questions in the autophagy field. Previous work from the same group reported starvation induced generation of ERGIC-derived COPII vesicles is the membrane template for LC3 lipidation and relocation of SEC12 from the ERES to the ERGIC triggers assembly of COPII vesicles on the ERGIC. Illustrating the molecular mechanism of SEC12 relocation provides further support to their model. This is a well-done study and for most part the data are compelling.

1) Does another autophagy stimulus (such as rapamycin treatment or glucose starvation) induce remodeling of ERES via relocation of SEC12? Is CTAGE5 required for autophagy induced by such stimulus?
Response: Both rapamycin treatment and glucose starvation increased ERES. We did not put these data into the current manuscript because we think they are beyond the scope of this work. A detailed investigation of multiple stimuli on ERES-remodeling is a future direction of our work.

2) Does Sar1 relocate to the ERGIC after starvation? If so, does the relocation of Sar1 also dependent on FIP300 and CTAGE5?
Response: We also probed SAR1 in the relocation experiments as shown in Fig 2 and 4. SAR1 relocation is also dependent on FIP200 and CTAGE5.

3) Does FIP200 localize on ERES and ERGIC?
Response: We analyzed the localization of FIP200 under starvation conditions. As shown in Fig 6, we found a pool of FIP200 enriched at the ERES/ERGIC region, suggesting that FIP200 may localize on the ERES/ERGIC.

4) Which part of Sec12 binds to FIP200?
Response: We mapped the region of SEC12 that associates with FIP200. As shown in Fig EV7, the C-terminal fragment of the SEC12 cytoplasmic domain associates with FIP200.

5) Although not essential, TEM analysis on APEX tagged Sec12 expressing cells can be very helpful to illustrate the remodeling of ERES.

Response: We thank the reviewer for the suggesting the experiment. We tagged SEC12 with APEX. The tagged SEC12 could not be correctly targeted to the ERES. It will be our future effort to optimize the condition and establish this new TEM assay.

1st Editorial Decision 21 June 2017

Thank you for the submission of your revised manuscript to EMBO reports. We sent your study back to the same referees who evaluated an earlier version of your manuscript for The EMBO Journal and asked them to assess it for potential publication in EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all three referees are very positive about the study and support publication in EMBO reports. Referee #1 requests minor changes to the abstract and text.

From the editorial side, there are also several things that we need before we can proceed with the official acceptance of your study.

- Please reformat the references to match the numbered style of EMBO reports. The respective EndNote file can be downloaded from our Author guidelines if required.

- Please include the figure legends in the main manuscript document file as two separate sections (Figure legends, Expanded View Figure legends).

- Supplementary/additional data: please note that only up to 5 images can be submitted as Expanded View. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

- Please label all figures with their respective number (Figure 1, Figure EV1 etc).

- Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

- I would also suggest making the scale bars a bit thicker to ensure that they are visible in the final print version of the figures.

REFEREE REPORTS

Referee #1:

Ge et al have addressed most of this referee's concerns except for these remaining two points.

1. The abstract should be changed to reflect the point agreed upon in the original list of points, point 5. The authors should replace "cTAGE5, is required" with "cTAGE5, modulates".
2. The data to address point 3, the endogenous IP has been added to Figure 5 but the text has not changed and the new data is not mentioned on page 7.

Referee #2:

Authors have addressed my queries satisfactorily. I believe this manuscript is ready for publication.

Referee #3:

The manuscript is a revised version of an earlier manuscript. The authors addressed the concerns I had with the previous manuscript sufficiently. They substantially improved their image quantification and data representation. I recommend this manuscript for publication.

1st Revision - authors' response 23 June 2017

Thanks very much for the prompt handling of our manuscript and providing further suggestions to revise. Specific points below:

Editor:

- Please reformat the references to match the numbered style of EMBO reports. The respective EndNote file can be downloaded from our Author guidelines if required.

Response: we have downloaded the EndNote file from EMBO Reports and the reference style has been changed.

- Please include the figure legends in the main manuscript document file as two separate sections (Figure legends, Expanded View Figure legends).

Response: we have modified the structure accordingly.

- Supplementary/additional data: please note that only up to 5 images can be submitted as Expanded View. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Response: we changed EV3 and 4 to Appendix Figures S1 and S2 and kept the rest of 5 EV Figures in the main text. The nomenclature has been changed and the Appendix has been generated accordingly. See yellow highlighted parts in the new manuscript.

- Please label all figures with their respective number (Figure 1, Figure EV1 etc).

Response: we have labeled all figures.

- Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

Response: we have added the SD, the n (50-100 cells/experiment and five experiments each) and Two-tailed T test as the approach for statistics in the figure legends.

- I would also suggest making the scale bars a bit thicker to ensure that they are visible in the final print version of the figures.

Response: we thank the editor for the suggestion. We have thickened the scale bars in the Figure 1D and E. The other bars are 2 pt thickness and we think this should be appropriate.

Referee #1:

Ge et al have addressed most of this referee's concerns except for these remaining two points.

1. The abstract should be changed to reflect the point agreed upon in the original list of points, point 5. The authors should replace "cTAGE5, is required" with "cTAGE5, modulates".

Response: we have added : “modulates” before “autophagosome biogenesis” in the abstract. We still kept the “ is required” to indicate the requirement of CTAGE5 in the other functions indicated in this sentence.

2. The data to address point 3, the endogenous IP has been added to Figure 5 but the text has not changed and the new data is not mentioned on page 7.

Response: we thank the reviewer for pointing this out. We have added the description of endogenous IP in the revised manuscript.

Referee #2:

Authors have addressed my queries satisfactorily. I believe this manuscript is ready for publication.

Referee #3:

The manuscript is a revised version of an earlier manuscript. The authors addressed the concerns I had with the previous manuscript sufficiently. They substantially improved their image quantification and data representation. I recommend this manuscript for publication.

Response: we thank all reviewers for the positive response.

2nd Editorial Decision 27 June 2017

Thank you for submitting a revised version of your manuscript to EMBO reports and for incorporating all requested changes. I am now writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few remaining minor issues/corrections have been addressed:

1) Appendix and Figures:

- I noticed in the legend of Appendix Fig. S1 that you mention arrows that indicate ERGIC-localized SEC12, but the corresponding arrows are not part of the figure.

- Fig. 6: the magnified view lacks scale bars.

2) Main text:

- On page 4 you refer to Fig 1C-F, but Fig 1 does not have a panel F.

- Please update the callouts to the Appendix figures to "Appendix Fig S1" or "Appendix Fig S2" instead of Appendix Fig 1.

3) Finally, please provide an ORCID iD for Dr. Schekman.

2nd Revision - authors' response 27 June 2017

The authors made the requested changes and submitted the final version of their manuscript.

3rd Editorial Decision 28 June 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Liang Ge and Randy Schekman
Journal Submitted to:	EMBO Reports
Manuscript Number:	EMBOR-2017-44559V1

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.

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.

USEFUL LINKS FOR COMPLETING THIS FORM

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http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

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?	We quantified 50-100 cells randomly captured and repeated the experiment five times (see all figure legends).
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.	We randomly capture pictures (See page13 paragraph 1).
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

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).	See page 11 paragraph 2
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	See page 11 paragraph 5 and 6

* 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).	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. 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

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.	NA