THE
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JOURNAL

Anoctamin 8 Tethers Endoplasmic Reticulum and Plasma Membrane for Assembly of Ca2+ Signaling Complexes at the ER/PM compartment

Archana Jha, Woo Young Chung, Laura Vachel, Jozsef Maleth, Sarah Lake, Guofeng Zhang, Malini Ahuja and Shmuel Muallem

Review timeline:

Submission date:	26th Dec 2018
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Editor: Daniel Klimmeck

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 5th Feb 2019

Thank you for the submission of your manuscript (EMBOJ-2018-101452) to The EMBO Journal. Your manuscript has been sent to three referees, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your work, although they also express a number of issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail, referee #2 states that the claims on direct ANO8 interactions with Ca2+ signaling components are not sufficiently supported by the data (ref#2, pt.1). In addition, referee #3 points out that the molecular control and chronology of ANO8 mediated tethering and its association with STIM1 remain unclear at this stage and need to be characterised in more detail. Further, the referees list a number of issues related to missing controls, comprehensive data access as well as data illustration that would need to be conclusively addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments.

REFEREE REPORTS:

Referee #1:

The manuscript by Jha et al. entitled "Anoctamin8 Tethers the Endoplasmic Reticulum and Plasma Membranes to Assemble Ca2+ Signaling Complexes at ER/PM Junctions" aims at elucidating the role of anoctamin8 (ANO8) in the regulation of CRAC channel activity. Specifically, focus is

initially laid on STIM1-STIM1 interaction as well as clustering and STIM1-Orai1 interaction and channel activation at PIP2-rich domains. Moreover, ANO8 facilitates SCDI by markedly enhancing SERCA2-mediated Ca2+ influx into the ER. ANO8 is actually involved in the assemble of all core Ca2+ signaling proteins into complexes at ER/PM junctions for the control of fundamental properties of Ca2+ signaling.
The manuscript is very interesting and timey. It unravels a novel role of ANO8 in Ca2+ signaling based on STIM1/Orai1 utilizing an impressive number of complimentary techniques and approaches. Experiments appear carefully conducted and the conclusions drawn are justified. I have only some minor points to be addressed by the authors to strengthen their manuscript.
1) The translocation of significant portion of ANO8 to the PM is shown in Fig. EV4b. Is ANO8 really translocated or does it stay anchored at the ER? Does this require the presence of both STIM1 and Orai1 or does it work with either of them?
2) The observation of ANO8-mediated recruitment of SERCA2 pumps to ER/PM junction to initiate Ca2+ uptake into the junctional ER is linked to an increase in slow Ca2+-dependent inactivation (p11, l1 from the bottom). Is it correct to assume that Orai1 channel inactivation actually occurs as the increase in the ER Ca2+ concentration de-activates STIM1?
3) A schematic model would help the reader to keep the overview about the many proteins affected by ANO8.

Referee #2:

The manuscript entitled, "Anoctamin8 Tethers the Endoplasmic Reticulum and Plasma Membranes to Assemble Ca2+ Signaling Complexes at ER/PM Junctions" reveals Ano8 as a previously unknown regulator of ER-PM junctions. As apparently universal features of all mammalian cells, new insights into the mechanisms that control ER-PM junction formation is inherently significant. The Ano family was initially screened based on homology to the yeast gene Lst2. It is remarkable that out of this family, only Ano8 regulated SOCE, however, as discussed below, it was disappointing that the results of the screen were not presented. It is further shown that Ano8 associates with multiple Ca2+ signaling components including STIM1, Orai1, SERCA2, PMCA and IP3R. By coordinating these many Ca2+ signaling components, Ano8 increases the efficiency of IP3-induced ER Ca2+ depletion and ER Ca2+ refilling. While these findings are generally exciting, I have several comments below which, if addressed, could increase the impact of this study.

Specific comments:

1. Can the authors comment on whether or not Ano8 directly interacts with all of these Ca2+ signaling components vs. promoting the formation of ER-PM junctions where these proteins interact? As written, it is not entirely clear which is driving this process.

2. I found the presentation of the data to be unusually vague, with some data only partially analyzed and/or described. The results section needs to be written much more carefully, with details fully explained.

3. The initial screen of the Ano family is not shown properly. In figure EV1, the effect of siRNA of each member of the Ano family on current is shown except for siAno8. In Figure 1, the effect of Ano8 o/e on current is shown. While I appreciate the significance of the finding, the complete dataset should be shown. In addition, it would be helpful if the Ano family members in figure EV1 are presented in an organized fashion, instead of the random presentation currently presented.

4. In discussing channel inactivation in figure 1, there is no analysis or detailed description. I recognize that this point is revisited later in the paper, but, it is difficult to accept as presented.

5. STIM1 is a dimer at rest. As such, I am mildly surprised that basal FRET between S1-CFP and S1-YFP is as low as it is. I have no problem with Ano8 modifying ER depletion-induced STIM-STIM interaction, but, perhaps the authors can comment on the low basal FRET.

6. There is a statement that "Ano8 is predicted to have 10 TMD, is located in the ER, and has a long

cytoplasmic C terminus rich in basic residues that has putative PI(4,5)P2 interacting motifs (Figs. 4, EV4 and EV8)." There is data in these figures that Ano8 associates with PIP2, but no sequence or structural information. Please clarify.

Referee #3:

Summary
Calcium signaling within cells requires a precise spatial coordination of a variety of molecular players localized in the ER, the plasma membrane and the cytosol. ER-plasma membrane contacts, in particular, have emerged as key nodes in this regulation, via the partnership of the ER sensor STIM1 with the plasma membrane ion channel Orai1. Recently, several other components of ER-plasma membrane contacts have been identified, with two of them, VAP (two isoforms) and E-Syt (three isoforms), being conserved from yeast to mammals (neither STIM1 nor Orai1 are present in yeast). Whether another component of yeast ER-plasma membrane contacts in yeast, Ist1, has a functional equivalent in mammals remained unclear. Mammalian genomes express multiple homologues of Ist1, the so-called anoctamines, whose functions include Cl- channel activity and lipid scramblase activity. At least some of these proteins, however, function at the plasma membrane, and thus are unlikely to be functional equivalent of Ist1. This study identifies anoctamine 8 (ANO8) as an ER protein that participates in ER-plasma membrane tethering and that is required for the organization of the machinery required to respond to Ca2+ store depletion (both STIM1-ORAi-dpendent Ca2+ influx and SERCA-dependent ER Ca2+ loading). The study is of significant interest but several issues require further attention before publication.

All FRET images indicate that the major increase in FRET occurs in the central region (Golgi complex area) of the cell. Based on the authors' interpretation of the significance of this FRET change, the increase should occur at the cell periphery (ER-plasma membrane junctions)

Fig. EV4 is critical to the message of the paper. It should be a main figure and, if possible, a more compelling field B should be shown.

I am puzzled by the increase in both Orai1 and STIM1 in the plasma membrane (surface accessible). STIM1 functions from inside the cells and should not be accessible to surface biotinylation.

The study does not clarify whether ANO8 tethering activity is regulated, and if so in which way. Do the authors think that ANO8 preassembles with STIM-STIM in the ER before making contact with the PM to increase the likelihood of establishing a contact site? Or is ANO8's tethering activity independently regulated? Is ANO8 recruited to ER-plasma membrane contacts under conditions in which STIM1-Orai1 contacts do not form?

For the discussion:

The manuscript does not address a potential channel function of scramblase function of ANO8. This issue should at least be discussed.

Is ANO8 ubiquitously expressed?

Minor:

in the text 877RREAFKR803 : correct 803 to 883.

1st Revision - authors' response 16th Mar 2019

Response to reviewers' comments

We are grateful to all reviewers for the positive evaluation of the manuscript and the constructive comments. Below we provide a detail response to all comments and the changes made to address them. In response to several of the comments we rearranged some of the Figures.

Referee #1:

The manuscript by Jha et al. entitled "Anoctamin8 Tethers the Endoplasmic Reticulum and Plasma Membranes to Assemble Ca2+ Signaling Complexes at ER/PM Junctions" aims at elucidating the role of anoctamin8 (ANO8) in the regulation of CRAC channel activity. Specifically, focus is initially laid on STIM1-STIM1 interaction as well as clustering and STIM1-Orai1 interaction and channel activation at PIP2-rich domains. Moreover, ANO8 facilitates SCDI by markedly enhancing SERCA2-mediated Ca2+ influx into the ER. ANO8 is actually involved in the assemble of all core Ca2+ signaling proteins into complexes at ER/PM junctions for the control of fundamental properties of Ca2+ signaling.

The manuscript is very interesting and timey. It unravels a novel role of ANO8 in Ca2+ signaling based on STIM1/Orai1 utilizing an impressive number of complimentary techniques and approaches. Experiments appear carefully conducted and the conclusions drawn are justified. I have only some minor points to be addressed by the authors to strengthen their manuscript.

Response: Thank you for finding "The manuscript is very interesting and timey…. Experiments appear carefully conducted and the conclusions drawn are justified."

1) The translocation of significant portion of ANO8 to the PM is shown in Fig. EV4b. Is ANO8 really translocated or does it stay anchored at the ER? Does this require the presence of both STIM1 and Orai1 or does it work with either of them?

Response: We think that part of ANO8 translocates to the junctions rather than moving from the ER to the plasma membrane. To better illustrate this and in response to the other reviewers' comments, we replaced the images of stimulated cells (now are shown in Figure 1g, j) to show that some ANO8 is retain in the ER. We also include Z scans of TIRF images of ANO8 when expressed alone (Figs. 1g-h) and together with STIM1 (Fig. 1k) to show that a) presence of ANO8 puncta at the junctions in unstimulated cells in the presence and absence of STIM1, b) clustering of ANO8 at the TIRF plan in response to store depletion in the absence of STIM1, c) co-expression with STIM1 increases the clustering of ANO8 at the ER/PM junctions, and d) the relationship between ANO8 and STIM1 puncta. The new findings and the clustering of ANO8 at the junctions are now described more clearly in the text (page 5, second paragraph).

2) The observation of ANO8-mediated recruitment of SERCA2 pumps to ER/PM junction to initiate Ca2+ uptake into the junctional ER is linked to an increase in slow Ca2+-dependent inactivation (p11, l1 from the bottom). Is it correct to assume that Orai1 channel inactivation actually occurs as the increase in the ER Ca2+ concentration de-activates STIM1?

Response: This is indeed our explanation for the effect of SERCA on the SCDI that is SARAF-independent and inhibited by CPA. This interpretation is supported by the finding that preventing ER Ca^{2+} accumulation by activation of the IP_3 receptors and by TPEN inhibits the SERCA-dependent SCDI. We attempted to clear this in the text, Fig. 9 beginning of second paragraph and page 11 end of first paragraph.

3) A schematic model would help the reader to keep the overview about the many proteins affected by ANO8.

Response: Thank you. We include such a model in the new Figure 8 and discuss it in page 13 last paragraph.

Referee #2:

The manuscript entitled, "Anoctamin8 Tethers the Endoplasmic Reticulum and Plasma Membranes to Assemble Ca2+ Signaling Complexes at ER/PM Junctions" reveals Ano8 as a previously unknown regulator of ER-PM junctions. As apparently universal features of all mammalian cells, new insights into the mechanisms that control ER-PM junction formation is inherently significant. The Ano family was initially screened based on homology to the yeast gene Lst2. It is remarkable

that out of this family, only Ano8 regulated SOCE, however, as discussed below, it was disappointing that the results of the screen were not presented. It is further shown that Ano8 associates with multiple Ca2+ signaling components including STIM1, Orai1, SERCA2, PMCA and IP3R. By coordinating these many Ca2+ signaling components, Ano8 increases the efficiency of IP3-induced ER Ca2+ depletion and ER Ca2+ refilling. While these findings are generally exciting, I have several comments below, which, if addressed, could increase the impact of this study.

Response: Thank you for finding the work "generally exciting"

Specific comments:

1. Can the authors comment on whether or not Ano8 directly interacts with all of these Ca2+ signaling components vs. promoting the formation of ER-PM junctions where these proteins interact? As written, it is not entirely clear which is driving this process.

Response: Thank for pointing this out. We revised the text to indicate that the most likely scenario is that ANO8 does not interact directly with all the proteins but rather assembles them into complexes at the ER/PM junctions. The end of page 13, first paragraph now reads "It is not clear at present whether ANO8 directly interacts with any of the core Ca2+ signaling proteins in the complexes, perhaps except for STIM1, since it affects STIM1-STIM1 clustering in the absence of Orai1. However, it is more likely that the ANO8-mediated formation and stabilization of ER/PM junctions (Fig. 2) is sufficient for assembly of the core Ca2+ signaling proteins into complexes in response to cell stimulation." This is also evident from the model in Figure 8 added in response to reviewer 1 comments.

2. I found the presentation of the data to be unusually vague, with some data only partially analyzed and/or described. The results section needs to be written much more carefully, with details fully explained.

Response: We substantially revised the results section in an attempt to clarify any uncertainty. We hope that the revised results section in now clear.

3. The initial screen of the Ano family is not shown properly. In figure EV1, the effect of siRNA of each member of the Ano family on current is shown except for siAno8. In Figure 1, the effect of Ano8 o/e on current is shown. While I appreciate the significance of the finding, the complete dataset should be shown. In addition, it would be helpful if the Ano family members in figure EV1 are presented in an organized fashion, instead of the random presentation currently presented.

Response: This Figure was moved to the supplement. We now include the results with siANO8 both in supplementary Figure 1 and as a panel in Figure 3. However, please note that the ANOs are not presented randomly. Rather, we grouped and present separately all the experiments done with siRNA and the experiments done with ANOs overexpression. The experiments are shown in order within each group.

4. In discussing channel inactivation in figure 1, there is no analysis or detailed description. I recognize that this point is revisited later in the paper, but it is difficult to accept as presented.

Response: Thank you. We now show analysis of the increase in channel density and the change in the rate of SCDI. We also moved description of the results with siSARAF (now Figure EV1) and it is discussed together with Figure 1a. The text now states the % increase in Ca^{2+} influx in relation to Figure 1b and the legend indicates that the confocal and TIRF experiments are of at least 4 experiments with similar results. All FRET data are shown as mean ± SEM. We revised the text to indicate these changes and describe in more detail channel inactivation when first mentioned.

5. STIM1 is a dimer at rest. As such, I am mildly surprised that basal FRET between S1-CFP and S1-YFP is as low as it is. I have no problem with Ano8 modifying ER depletion-induced STIM-STIM interaction, but perhaps the authors can comment on the low basal FRET.

Response: In these experiments the resting FRET efficiency between STIM1-CFP and STIM1-YFP was 0.05. With STIM1 and Orai1 the resting FRET efficiency was 0.02. Considering that in resting

conditions, the interaction between STIM1 and Orai1 is minimal, there is indeed some STIM1-STIM1 interaction at rest. However, importantly, FRET was analyzed at the cell periphery, where the level and interaction of STIM1-STIM1 is minimal at rest and markedly increases upon store depletion. The methods now specify that FRET was analyzed at the cell periphery (Page 15, FRET measurements, end of first paragraph).

6. There is a statement that "Ano8 is predicted to have 10 TMD, is located in the ER, and has a long cytoplasmic C terminus rich in basic residues that has putative PI(4,5)P2 interacting motifs (Figs. 4, EV4 and EV8)." There is data in these figures that Ano8 associates with PIP2, but no sequence or structural information. Please clarify.

Response: The sequence is given in supplementary Figure 4 (original EV9). We now indicate in page 11, end of first paragraph that the structure was predicted by ROBETTA using the structure of ANO1 as a template.

Referee #3:

Summary
Calcium signaling within cells requires a precise spatial coordination of a variety of molecular players localized in the ER, the plasma membrane and the cytosol. ER-plasma membrane contacts, in particular, have emerged as key nodes in this regulation, via the partnership of the ER sensor STIM1 with the plasma membrane ion channel Orai1. Recently, several other components of ER-plasma membrane contacts have been identified, with two of them, VAP (two isoforms) and E-Syt (three isoforms), being conserved from yeast to mammals (neither STIM1 nor Orai1 are present in yeast). Whether another component of yeast ER-plasma membrane contacts in yeast, Ist1, has a functional equivalent in mammals remained unclear. Mammalian genomes express multiple homologues of Ist1, the so-called anoctamines, whose functions include Cl- channel activity and lipid scramblase activity. At least some of these proteins, however, function at the plasma membrane, and thus are unlikely to be functional equivalent of Ist1. This study identifies anoctamine 8 (ANO8) as an ER protein that participates in ER-plasma membrane tethering and that is required for the organization of the machinery required to respond to Ca2+ store depletion (both STIM1-ORAi-dpendent Ca2+ influx and SERCA-dependent ER Ca2+ loading). The study is of significant interest but several issues require further attention before publication.

Response: Thank you for finding “The study is of significant interest”

All FRET images indicate that the major increase in FRET occurs in the central region (Golgi complex area) of the cell. Based on the authors' interpretation of the significance of this FRET change, the increase should occur at the cell periphery (ER-plasma membrane junctions)

Response: Please note that all FRET experiments were carried out on a confocal system with maximally opened pinhole to maximize photons capture, while recording close to the cell surface rather than the middle of the cell. Thus, the microscope was used in a “widefield mode”, which makes does not exclude some out of focus light. Thus, the images shown represent a maximal projection of the captured signal, rather than precise localization. This setup makes it impossible to discriminate among cell organelles and the plasma membrane region. To overcome this limitation, we only analyzed the FRET signal at the cell periphery, where the FRET signal is driven by proteins at the ER-PM junctions. This is now indicated in the methods section, page 15, FRET measurements, end of first paragraph.

The study is of significant interest
Fig. EV4 is critical to the message of the paper. It should be a main figure and, if possible, a more compelling field B should be shown.

Response: Thank you. The images are now part of Figure 1i and 1j, new images of stimulated cells are included together with confocal and TIRF-Z scan images of puncta at the ER/PM junctions of resting (new Figs. 1g, 1h, 1k).

I am puzzled by the increase in both Orai1 and STIM1 in the plasma membrane (surface accessible). STIM1 functions from inside the cells and should not be accessible to surface biotinylation.

Response: STIM1 is not in the plasma membrane, but rather is associated with the Orai1 that is present in the plasma membrane. In the biotinylation assay the lysines of all plasma membrane proteins are biotinylated. Avidin pules down all biotinylated proteins and any protein attach to them that remained so after cell solubilization. In this case Orai1 is biotinylated and is pulled down by avidin. STIM1 and all other proteins that are in a stable complex with Orai1 are also pulled down. We attempted to clear this point in the text, and page 6, first paragraph now reads "The results in Figs. 2i-l indicate that ANO8 increased the PM level of Orai1 (Fig 2j) and increased the level of STIM1 in the junctions where it interacts with the PM-localized Orai1 and was thus pulled-down together with Orai1 (Fig 2k).".

The study does not clarify whether ANO8 tethering activity is regulated, and if so in which way. Do the authors think that ANO8 preassembles with STIM-STIM in the ER before making contact with the PM to increase the likelyhood of establishing a contact site? Or is ANO8's tethering activity independently regulated? Is ANO8 recruited to ER-plasma membrane contacts under conditions in which STIM1-Orai1 contacts do not form?

Response: Thank you for pointing this. To address these points, we revised Figure 1 to include images and TIRF-Z scan images with cells expressing ANO8 alone and ANO8 with STIM1. The findings show ANO8 puncta at the TIRF field in resting cells that are increased in response to store depletion. Moreover, STIM1 increases the puncta at the plasma membrane both in resting and store-depleted cells. Thus, the tether function of ANO8 does respond to store depletion and it appears that when interacting with STIM1, the STIM1-ANO8 complex translocates to the ER/PM junctions better than ANO8 alone. This is now discussed in the manuscript, page 5 end of second paragraph.

For the discussion:

The manuscript does not address a potential channel function of scramblase function of ANO8. This issue should at least be discussed.

Response: We now indicate that we could not detect a cation or anion current by ANO8. This is not surprising as most ANO8 is localized in the ER even when in the junctions. The discussion, end of the first paragraph now reads '' However, unlike other ANOs that are primarily expressed in the plasma membrane and function as Cl- channels, we could not detect change in anion or cation currents by expressing ANO8 in HEK cells. However, this does not exclude the possibility that ANO8 functions as a Cl- channel and lipid flipase in the ER, the primary localization of ANO8.".

Is ANO8 ubiquitously expressed?

Response: Gene expression appears ubiquitous with protein expression seems to be highest in the lung, brain, adrenal, breast, and pancreas (https://www.genecards.org/cgi-bin/carddisp.pl?gene=ANO8). This is indicated in the manuscript, discussion, first paragraph.

Minor:

in the text 877RREAFKR803 : correct 803 to 883.

Response: Thank you. This was corrected.

2nd Editorial Decision 2nd Apr 19

Thank you for submitting the revised version of your manuscript. Your revised study has now been re-evaluated by two of the original referees, please find their comments enclosed below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly favour of publication. Please note also that while referee #1 was not able at this time to re-assess the work, we have carefully analysed your point-by-point response and adjustments of the manuscript

towards this referee at editorial level and concluded that these concerns are also addressed satisfactorily.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues regarding formatting and data representation, as outlined below, which need to be adjusted at re-submission.

Please also consider the remaining minor comments of referee #3 as to data discussion and a more intuitive layout for the model in figure 8.

REFEREE REPORTS:

Referee #2:

The writing of this manuscript is much improved, however, some issues remain that should be addressed.

1. The idea of including a diagram is a good one, however, I found figure 8 to be very confusing. There are many, many players here and what is really being proposed is not clear as presented. In addition, some of the shapes are difficult to accept. The transition between resting and activated STIM1 is particularly challenging. Considerably more thought is needed into organizing this diagram in a way that informs the reader.

2. Although it doesn't change the result, it seems extremely odd to me that 8 members of the anoctamin family were screened by siRNA and 4 were screened by overexpression. Can you provide any explanation for this experimental strategy?

3. In response to my query about the topology of Ano8, the authors wrote that the sequence is given in supplemental figure 4. That is not an accurate statement.

Referee #3:

I am happy with the revision and the manuscript can be accepted

2nd Revision - authors' response 3rd Apr 2019

Referee #2:

1. The idea of including a diagram is a good one, however, I found figure 8 to be very confusing. There are many, many players here and what is really being proposed is not clear as presented. In addition, some of the shapes are difficult to accept. The transition between resting and activated STIM1 is particularly challenging. Considerably more thought is needed into organizing this diagram in a way that informs the reader.
The Figure was considerably simplified by removing many of the proteins that were shown more than once. In addition, the STIM1 complexes were trimmed to show only one extended dimer. Finally, we now clearly divide the assembly of the complexes to two steps and mark them as Stip 1: Store depletion and Step 2: Translocation.

2. Although it doesn't change the result, it seems extremely odd to me that 8 members of the anoctamin family were screened by siRNA and 4 were screened by overexpression. Can you provide any explanation for this experimental strategy?
There was no mystery to this. For the ANOs siRNA that did not work well in down regulating expression we used the proteins as an alternative.

3. In response to my query about the topology of Ano8, the authors wrote that the sequence is given in supplemental figure 4. That is not an accurate statement.
Thank you. This was corrected.

The authors performed all requested editorial changes.

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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: Shmuel Muallem
Journal Submitted to: EMBO
Manuscript Number: EMBOJ-2018-101452R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

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?	Experiments were performed at least 4 times or until statistical significance was obtained
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA, no animals were used.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No expperiments were excluded when no experimental error was noted.
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. No animals were used and when transfected cells were used, the cells were indentified by a flourescent tag so that experimentalist knew cell inentity. There is no other way to perform these experiments.
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.	Yes. Experimental conditions were always compared to appropriate controls and in all experiments only two groups were compared, control and experimental.
Is there an estimate of variation within each group of data?	Yes and data is shown as m+/-sme.
Is the variance similar between the groups that are being statistically compared?	yes. Experimental conditions were always compared to appropriate controls and in all experiments only two groups were compared, control and experimental.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	These are all provided in the appendix
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The text indicates that HEK293 cells were obtained from ATCC and are routinely verified to be clear of mycoplasma

* 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. No data was generated that need to be deposited in a public depository site.
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.	No