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Phosphatidylinositol 4,5-bisphosphate controls Rab7 and PLEKMH1 membrane cycling during autophagosome-lysosome fusion.

Takashi Baba, Daniel J. Toth, Nivedita Sengupta, Yeun Ju Kim and Tamas Balla.

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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 31st August 2018

Thank you for submitting your manuscript on a role for PI(4,5)P2 in regulating Rab7 and PLEKHM1 association with late endosomes and autophagosome-lysosome fusion to The EMBO Journal. Your study has been sent to three referees for evaluation, and we have now received reports from them, which are enclosed below for your information.

As you can see, the referees concur with us on the overall interest of your findings. However, they also raise major points that need to be addressed before they can support publication in The EMBO Journal. In particular, referee #1 is concerned that the study lacks mechanistic insight on how PI(4,5)P2 controls Rab7 activation. Furthermore, referees #1 and #2 ask you to further investigate the selective impact of PI(4,5)P2 on PLEKHM1 as well as the existence of different Rab7 pools on endosome that confer specificity towards certain effectors. Referee #2 also points out that the effects of "low" versus "high" constitutively active Rab7 levels on lysosome tubulation require further analysis. Referee #3 points out that a better knockdown of PIP4KIIb is required to assess the role of endosomal PI(4,5)P2 pool and requests you to properly quantify and analyze the colocalization experiments.

Addressing these issues through decisive additional data as suggested by the referees would be essential to warrant publication in The EMBO Journal. Given the overall interest of your study, I would like to invite you to revise the manuscript in response to the referee reports.

REFEREE REPORTS

Referee #1:

In this manuscript Baba and co-workers report that the highest levels of endosomal PI4P are found on Rab7-positive late endosomes. Acute conversion of PI4P to PI(4,5)P2 on endosomes was found

to cause dissociation of Rab7 and its effector PLEKHM1 from endosome membranes whereas two other Rab7 effectors, Vps35 and RILP, were not affected. CRISPR-mediated deletion of the PI 4-kinase PI4K2A led to decreased endosomal levels of PI(4,5)P2 as expected, and was associated with impaired Rab7 inactivation and increased numbers of LC3-positive puncta.

The manuscript is well written and contains fluorescence microscopy and BRET data of good quality, including adequate quantifications. However, although promising data are reported, the manuscript suffers from the lack of mechanistic data that could explain how PI(4,5)P2 controls Rab7 activation and how this selectively impacts on PLEKHM1 and not on other Rab7 effectors.

Major points:

1. The authors speculate that PI(4,5)P2 might control a Rab7 GAP. However, they state that they have tried sRNA-mediated knockdown of 9 candidate GAPs without success. As the authors explain, this might be due to incomplete knock-down or the existence of redundancies among Rab7 GAPs. One could also consider the alternative mechanistic explanation, that a Rab7 GEF might be negatively regulated by PI(4,5)P2. Even though the authors must be commended for their efforts, it is unfortunate that this crucial issue has not been resolved.

2. It is surprising that different Rab7 effectors are differentially controlled by PI(4,5)P2, and the authors need to offer a mechanistic explanation for this. Are there different Rab7 pools on endosomes with differential sensitivity to PI(4,5)P2 regulation?

Referee #2:

The manuscript by Baba et al. describes the inactivation of Rab7 by PI(4,5)P2 in late endosomes or lysosomes. Specifically, they report that PI(4,5)P2 formation from PI4P releases the effector PLEKHM1 from the late endosomes or lysosomes. Because PLEKHM1 functions as a tether between autophagosomes and lysosomes, the authors attribute the abnormal fusion of these compartments reported to result from silencing PI4K2A to the abnormal formation of PI(4,5)P2, a process they propose is mediated by PIP5Kgamma.

The authors designed and implemented a number of ingenious, often complex approaches to visualize the phosphoinositides and their effectors in the Rab7 compartment. Some of these require overexpression of several constructs, which may have altered the normal physiology of the cells.

The experiments and many of the principal findings address an important biological question and provide independent (although somewhat indirect) evidence that PI(4,5)P2 can indeed be generated in late endocytic compartments. There are nevertheless several conceptual and technical aspects that need to be addressed.

1) The main problem I encountered relates to the key observation that autophagosomes fail to fuse normally with lysosomes in PI4K2A-deficient cells, presumably as a result of abnormal PLEKHM1 behavior. If I understood correctly, the lack of PI4P and hence of PI(4,5)P2 should result in higher levels of Rab7 and greater interaction with PLEKHM1. Because the latter is a tether between autophagosomes and lysosomes, I would have expected excess fusion between these compartments, while the opposite was found both by the authors and earlier by Yin's group. Unless I have misunderstood the entire model, these findings are internally inconsistent and rather paradoxical. These observations need to be reconciled; otherwise, the rationale for studying PLEKHM1 is moot.

2) The reason(s) why only PLEKHM1 was affected, while other effectors like RILP and Vps35 were not is unclear. If the total amount of Rab7 associated with the membrane, an indication of its state of activation, is reduced by increasing PI(4,5)P2, as implied by figure 3, why would RILP binding be normal? If the inability to detect changes in RILP were due to the need for overexpression, at least the behavior of endogenous RILP should have been affected. This would have resulted in a change in the subcellular distribution of lysosomes, since RILP links to dynein. Yet, no changes in distribution were found, as stated specifically in the text.

3) The authors found that Rab7 compartments tubulate when constitutively active Rab7 was expressed "at low levels". What happens at "higher levels"? How do "low levels" compare to the levels required to detect enhanced activity in the RILP pull-down assay? Is there precedent for active Rab7-induced tubulation, or are the lysosomes enlarged yet remain spherical?

4) It is puzzling that PI(4,5)P2 formation, as judged using the BRET assay, is only inhibited by ≈30% in the PI4K2A KO cells, which have an ≈80% reduction in PI4P formation. How is this explained, and are the functional phenotypes described in Figure 6 justified by this rather modest change in PI(4,5)P2?

5) Why is the effect of angiotensin transient? Is plasma membrane PI(4,5)P2 restored despite the continued presence of the agonist?

6) The authors ruled out a significant role for PIP5Kbeta based on silencing experiments, but the effectiveness of the silencing process was never validated. If the available antibodies are not useful to detect the endogenous enzyme, the authors should perform qPCR determinations.

7) The extent of the reduction in PI(4,5)P2 formation reported in cells where PIPKgamma was silenced (only about 50%) is not proportional to the decrease in the levels of the enzyme. How is this accounted for?

8) The authors state that silencing PIPK beta and gamma had no discernible effect on plasma membrane PI(4,5)P2. Is all the membrane PI(4,5)P2 then generated by the alpha isoform? Is this consistent with other findings in the literature?

9) The last section of the paper analyzing the effect of Vps34 inhibitors on PI4P formation in late compartments is rather incomplete and only tangentially relevant to the main issue of this paper. I strongly advise the authors to delete or expand this section by defining how exactly PI3P directs PI4K2A to late compartments.

Referee #3:

This manuscript by Baba et al. presents important discovery that reveal a novel role for endosomal PI4P and PI4,5P2 in the regulation of Rab7 activity in endolysosomal membrane trafficking. Through an elegant set of experiments, the authors were able to show that PI4KIIA and PIP5KIγ control a highly dynamic late endosomal phosphoinositide signaling nexus involving PI4P and PI4,5P2. While loss of this signaling nexus (PI4KIIA knockout) caused Rab7 activation, amplification of this pathway (increasing late endosomal PI4,P2) were found to stimulate Rab7 inactivation and its release from the membrane. Interestingly, upon elevation of endosomal PI4,5P2, the authors observed specific endosomal disassociation of the Rab7 effector PLEKHM1, a protein involved in autophagosome-lysosome fusion. These results are consistent with the accumulation of unacidified autophagosomes observed in this study upon knockout of PI4KIIA and in previous reports regarding roles of PI4KIIA and PIPKIgamma. Overall, this is an important study that reveals a novel function of late endosomal PI4,5P2. The experiments were well designed and controls were appropriate. Nevertheless there are few issues to address.

1. Does knockdown of PIPKIγ cause issues of Rab7 inactivation?
2. Page 9, Line 8, the authors stated "a significantly smaller increase". This reviewer can appreciate the difficulty of such experiments given the extremely low levels of intracellular PI4,5P2 and the high background signal, but it is recommended that the term "significantly" be supported by statistical analysis.
3. In Fig. 3E and 3F, had the authors tried to more fully knocking down PI4KIIb to see if that could fully remove endosomal PI4,5P2.
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8. The paper is generally well written but is complex and dense in some places (results section) and some of the abbreviations could be better defined.

1st Revision - authors' response 15th November 2018

We would like to thank the Reviewers for their insightful comments, constructive criticisms and valuable suggestions. Below are our point by point responses to the specific points raised.

Referee #1:

In this manuscript Baba and co-workers report that the highest levels of endosomal PI4P are found on Rab7-positive late endosomes. Acute conversion of PI4P to PI(4,5)P2 on endosomes was found to cause dissociation of Rab7 and its effector PLEKHM1 from endosome membranes whereas two other Rab7 effectors, Vps35 and RILP, were not affected. CRISPR-mediated deletion of the PI 4-kinase PI4K2A led to decreased endosomal levels of PI(4,5)P2 as expected, and was associated with impaired Rab7 inactivation and increased numbers of LC3-positive puncta.

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Major points:

1. The authors speculate that PI(4,5)P2 might control a Rab7 GAP. However, they state that they have tried sRNA-mediated knockdown of 9 candidate GAPs without success. As the authors explain, this might be due to incomplete knock-down or the existence of redundancies among Rab7 GAPs. One could also consider the alternative mechanistic explanation, that a Rab7 GEF might be negatively regulated by PI(4,5)P2. Even though the authors must be commended for their efforts, it is unfortunate that this crucial issue has not been resolved.

We have now performed additional experiments in further attempts to identify Rab7 GAPs that might play a role in the regulation of autophagosome-lysosome fusion controlled by PI(4,5)P2. These experiments included overexpression of selected TBC1D proteins to assess their localization and their localization responses to PI(4,5)P_2 production as well as their effects on Rab7 status and distribution. We also knocked down individual TBC1D proteins and looked at their effect on acidification of LC3 positive structures. These experiments showed that depletion of multiple TBC1D proteins impaired autophagosome-lysosome fusion and their overexpression had differential effects on the position of Rab7 endosomes. These new data are shown in EV Figures 5-7 and discussed on pages 13-14 of the Results section. Unfortunately, none of these efforts were able to pinpoint a single TBC1D protein as the mediator of the PI(4,5)P2 effect on Rab7. However, given that PI(4,5)P_2 has been shown to affect the GAP activity of Arf1 GAPs via allosteric regulation and not by acting as localization signals, and the large number of putative Rab7 GAP proteins, it may require a lot more efforts to identify the protein targets of such regulation. We would like to point out, that many otherwise excellent studies have been unable to identify the targets of endosomal PI(4,5)P2 and our studies are the first to show that Rab7 cycling is affected by this lipid in Rab7 endosomes.

As for the possible involvement of GEFs, we are not aware of any literature showing inositol lipid inhibitory effect on GEFs of any small G-proteins; rather the opposite is reported. In fact, these GEF activities have been shown to respond to PI3P, an interesting link to VPS34. However, our data on Vps34 has been omitted following Reviewers suggestion. Nevertheless, we also cloned the Rab7 GEFs, Mon1 and Ccz1 and investigated their distribution and association with Rab7-NI both in control and PI4K2A K/O cells. We found no indication for enhanced association of these proteins with the Rab7 compartment in the K/O cells. We decided not to include these data in the manuscript to avoid overwhelming the manuscript with negative data.

2. It is surprising that different Rab7 effectors are differentially controlled by PI(4,5)P2, and the authors need to offer a mechanistic explanation for this. Are there different Rab7 pools on endosomes with differential sensitivity to PI(4,5)P2 regulation?

We believe that the differential regulation of the various Rab7 effectors by the various GAPs and perhaps PI(4,5)P2 is based on two principles. First, the localization of the GAPs is likely to be an important factor as the numerous putative Rab7 GAPs most likely affect different subsets of Rab7 pools dedicated to specific effectors. This explains why the Vps35 is seemingly not affected as it shows co-localization only with a minority of the Rab7 positive endosomes which may not be detected by our methods. Equally importantly, however, is the affinity of the Rab7 effector to the activated Rab7. This is exemplified by RILP, which is one of the strongest Rab7 effectors. Unlike PLEKHM1 or Vps35, RILP expression completely changes the localization of the Rab7 endosomes (as already described by many investigators). Our data suggest that when we acutely produce PIP2 on the Rab7 endosomes, RILP shows only a transient decrease (if at all) and, in fact, increases its association with Rab7. We believe that this increase reflects RILP grabbing the Rab7 pool that has been liberated from its other effector by PIP2 and which becomes available upon a new round of GTP binding. We have added a paragraph to the Results (page 12) addressing this question but limited its scope as these are mostly speculations.

Referee #2:

The manuscript by Baba et al. describes the inactivation of Rab7 by PI(4,5)P2 in late endosomes or lysosomes. Specifically, they report that PI(4,5)P2 formation from PI4P releases the effector PLEKHM1 from the late endosomes or lysosomes. Because PLEKHM1 functions as a tether between autophagosomes and lysosomes, the authors attribute the abnormal fusion of these compartments reported to result from silencing PI4K2A to the abnormal formation of PI(4,5)P2, a process they propose is mediated by PIP5Kgamma.

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1) The main problem I encountered relates to the key observation that autophagosomes fail to fuse normally with lysosomes in PI4K2A-deficient cells, presumably as a result of abnormal PLEKHM1 behavior. If I understood correctly, the lack of PI4P and hence of PI(4,5)P2 should result in higher levels of Rab7 and greater interaction with PLEKHM1. Because the latter is a tether between autophagosomes and lysosomes, I would have expected excess fusion between these compartments, while the opposite was found both by the authors and earlier by Yin's group. Unless I have misunderstood the entire model, these findings are internally inconsistent and rather paradoxical. These observations need to be reconciled; otherwise, the rationale for studying PLEKHM1 is moot.

The Reviewer has raised an important point. PLEKHM1 was chosen because of its association with GABARAP, the protein, previously shown to interact with PI4K2A and its central role in autophagy regulation. What our data show is that PLEKHM1 is dissociated from the bulk Rab7 pools by increasing PI(4,5)P_2 and without this effect cells have a difficulty in acidifying their autophagosomes. This suggests that either PLEKHM1 cycling is important for this process, or some additional PI(4,5)P_2-dependent factors are responsible for the acidification defect and increased PLEKHM1 capture by Rab7 endosomes is not the main reason. We have now devoted a paragraph discussing this question (page 16 of Discussion).

It has been shown that PLEKHM not only serves as a tether between the endosomes and autophagosomes but also binds endosomal Rab7 pools unrelated to autophagosomes. Lack of PIP2

could increase the association of PLEKHM1 with a Rab7 compartment that is not linked to autophagosome-lysosome fusion and decrease its availability for that very process. Conversely, PIP2-mediated liberation of PLEKHM1 could increase the availability of PLEKHM1 to autophagosome-lysosome fusion. There are good indications that there are numerous Rab7 pools defined by their localization (including those affecting mitochondrial fission-fusion) and it is not unreasonable to assume that the multitude of Rab7 GAPs are dedicated to specific processes only some of which are under PI(4,5)P2 control. We believe, however, that these arguments are speculative at this point and did not include them in the Discussion. It will take a lot more research to define all of these pools and their role in all the processes regulated by Rab7.

2) The reason(s) why only PLEKHM1 was affected, while other effectors like RILP and Vps35 were not is unclear. If the total amount of Rab7 associated with the membrane, an indication of its state of activation, is reduced by increasing PI(4,5)P2, as implied by figure 3, why would RILP binding be normal? If the inability to detect changes in RILP were due to the need for overexpression, at least the behavior of endogenous RILP should have been affected. This would have resulted in a change in the subcellular distribution of lysosomes, since RILP links to dynein. Yet, no changes in distribution were found, as stated specifically in the text.

Please see our response to Rev. 1 point 2.

3) The authors found that Rab7 compartments tubulate when constitutively active Rab7 was expressed "at low levels". What happens at "higher levels"? How do "low levels" compare to the levels required to detect enhanced activity in the RILP pull-down assay? Is there precedent for active Rab7-induced tubulation, or are the lysosomes enlarged yet remain spherical?

High expression of Rab7 creates a phenotype similar to that caused by expression of Rab7-Q67L. This is characterized by enlarged Rab7 positive vesicles and some tubulation. The pull-down assays are done in a cell population, with cells showing a range expression levels. In the microscope we tried to focus on cells expressing GFP-Rab7 at low levels better mimicking the endogenous situation. We also tried Rab7 antibodies to detect the endogenous proteins, but it is hard to detect tubulation in fixed cells and the signal was not of sufficient quality to make a reliable judgment.

4) It is puzzling that PI(4,5)P2 formation, as judged using the BRET assay, is only inhibited by ≈30% in the PI4K2A KO cells, which have an ≈80% reduction in PI4P formation. How is this explained, and are the functional phenotypes described in Figure 6 justified by this rather modest change in PI(4,5)P2?

We believe that the relationship between PI4P and PIP_2 is not necessarily linear. Also, as shown by recent reports, some PIP_2 is made from PI5P by the type II PIP kinases, although it is not clear whether this happens in Rab7 positive structures. To address this question, we performed additional experiments and knocked down PI4K2B in the PI4K2A K/O cells to eliminate the rest of PI4P on the Rab7 positive endosomes. These data showed a more complete loss of PIP_2 generation in those cells suggesting that the two type II PI4Ks are the major source of PIP_2 in those endosomes. These new data are shown in EV Figure 2E and F.

5) Why is the effect of angiotensin transient? Is plasma membrane PI(4,5)P2 restored despite the continued presence of the agonist?

Angiotensin II receptors show rapid desensitization and internalization. This explains the well-documented partial re-synthesis of PIP_2 even in the continued presence of the ligand.

6) The authors ruled out a significant role for PIP5Kbeta based on silencing experiments, but the effectiveness of the silencing process was never validated. If the available antibodies are not useful to detect the endogenous enzyme, the authors should perform qPCR determinations.

We have added experiments to show the effectiveness of the PIP5K1B silencing. These are shown in EV Figure 3B

7) The extent of the reduction in PI(4,5)P2 formation reported in cells where PIPKgamma was

silenced (only about 50%) is not proportional to the decrease in the levels of the enzyme. How is this accounted for?

Again, we are certain that the amount of enzyme is not necessarily linear with its enzymatic product. For example, we have shown previously that PI4KA silencing (to undetectable levels by WB)) had very minor impact on plasma membrane PI4P levels, even though with a PI4KA inhibitor used at maximally effective concentration, plasma membrane PI4P is eliminated. Similar experiments done by virologists led to the false conclusion that cells do not need PI4KA for proper function, which has since been proven to be wrong.

8) The authors state that silencing PIPK beta and gamma had no discernible effect on plasma membrane PI(4,5)P2. Is all the membrane PI(4,5)P2 then generated by the alpha isoform? Is this consistent with other findings in the literature?

The literature is very confusing regarding the contribution of the various PIP5Ks to plasma membrane PIP2 maintenance. It appears that these enzymes can be redundant in that regard. PIP5Kg K/O mice develops normally but has major problems with CNS function and die perinatally. In one study PIP5Kg was found responsible for the plasma membrane PIP2 pool in HeLa cells, but in other studies PIP2 levels were not changed by PIP5Kg downregulation. This question is now covered in the Results section (page 10).

9) The last section of the paper analyzing the effect of Vps34 inhibitors on PI4P formation in late compartments is rather incomplete and only tangentially relevant to the main issue of this paper. I strongly advise the authors to delete or expand this section by defining how exactly PI3P directs PI4K2A to late compartments.

We agree with the Reviewer and have deleted this section from the manuscript.

Referee #3:

This manuscript by Baba et al. presents important discovery that reveal a novel role for endosomal PI4P and PI4,5P2 in the regulation of Rab7 activity in endolysosomal membrane trafficking. Through an elegant set of experiments, the authors were able to show that PI4KIIA and PIP5KIγ control a highly dynamic late endosomal phosphoinositide signaling nexus involving PI4P and PI4,5P2. While loss of this signaling nexus (PI4KIIA knockout) caused Rab7 activation, amplification of this pathway (increasing late endosomal PI4,P2) were found to stimulate Rab7 inactivation and its release from the membrane. Interestingly, upon elevation of endosomal PI4,5P2, the authors observed specific endosomal disassociation of the Rab7 effector PLEKHM1, a protein involved in autophagosome-lysosome fusion. These results are consistent with the accumulation of unacidified autophagosomes observed in this study upon knockout of PI4KIIA and in previous reports regarding roles of PI4KIIA and PIPKIgamma. Overall, this is an important study that reveals a novel function of late endosomal PI4,5P2. The experiments were well designed and controls were appropriate. Nevertheless there are few issues to address.

1. Does knockdown of PIPKIγ cause issues of Rab7 inactivation?

We have performed additional experiments to address this issue. We found the knock down of PIP5Kg induced Rab7 tubulation and defects in lysosome-autophagosome fusion. These data are now shown in new Figure 6.

2. Page 9, Line 8, the authors stated "a significantly smaller increase". This reviewer can appreciate the difficulty of such experiments given the extremely low levels of intracellular PI4,5P2 and the high background signal, but it is recommended that the term "significantly" be supported by statistical analysis.

We have performed statistical analysis on the data in question. These are now shown in Figure 3F, 5B and EV Figure 2D and F.

3. In Fig. 3E and 3F, had the authors tried to more fully knocking down PI4KIIb to see if that could fully remove endosomal PI4,5P2.

This experiment has been performed and the data are shown in EV Figure 2E and F

4. Fig. 7C shows no clear difference in terms of colocalization of the two channels. Quantification would help here.

These data have been removed as suggested by Reviewer 2.

5. Page 10, 2nd line from the bottom, Figure citation should be "S3A, B" instead of "3A,B".

Thank you for spotting this mistake that has been corrected.

6. Page 13, Line 8, "to strong" should be "too strong".

Again, thank you for spotting this mistake that has been corrected.

7. Since this is a highly dynamic phosphoinositide signaling pathway that has not been previously well appreciated, a working model could be helpful for readers.

We have generated a working model that we added as Appendix Figure S2

8. The paper is generally well written but is complex and dense in some places (results section) and some of the abbreviations could be better defined.

We have made a faithful attempt to improve the text including better definition of the abbreviations.

2nd Editorial Decision 20th December 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below.

As you will see they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address.

REFEREE REPORTS

Referee #2:

The authors have addressed all my initial concerns. In a couple of instances their replies are speculative, but I believe the readers will find them satisfactory, considering the current state of knowledge and available tools.

Referee #3:

The authors have fully addressed all of the comments from this reviewer. The new data with quantitative imaging about PIP5KC knockdown and combined depletion of PI4KIIA/B have substantially strengthen the study by validating the role for endogenous PIP5KC and further clarifying the mechanism of lipid generation. The manuscript now presents a comprehensive analysis of a highly dynamic endosomal PI4P-PI4,5P2 signaling pathway that has not been well-defined in the past. Through a large number of complex but well-controlled experiments, the authors have convincingly shown the presence of endosomal PI4P and PI4,5P2 production and their roles in

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Rab7-mediated membrane trafficking. The creative approaches in visualizing the dynamic pools of endosomal PI4P and PI4,5P2 are highly appreciated and will be instructive to many studies in the field. The manuscript also bridges some gaps in our understanding of endosomal PI4,5P2 signaling and autophagosome-endolysosome fusion. Although direct PI4,5P2 effectors involved in this process are yet to be defined, this study should still be considered as a major contribution to endosomal phosphoinositide signaling.

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Corresponding Author Name: Tamas Balla
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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.
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- 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.
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  - are tests one-sided or two-sided?
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  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The minimum number of experiments that we perform in our quantitative BRET assays is three and all experiments are performed in triplicates. Given the fact that these assays are time-courses with sampling at every 15 or 30 sec, they generate a data-set with hundreds of points for reliable comparisons. We do not have pre-specified effects. We adjuts number of experiments as needed, but also consider whether a change while statistically significant may not mean biologically relevant changes. Therefore, we do not increase experimental numbers to reach significance when the small changes may not be biologically relevant.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We do not exclude experiments or experimental points unless a documented error has been made in the course of the experiment. These are decided before the results are seen.
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.	During BRET analysis, in each repeated experiment the well assignments are different..In microscopy experiment two investigators have chencked the samples and it was not known to the investigator which treatment was applied to the cells.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
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.	I am not sure how this applies to our experiments beyond what is answered under point 3.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	To the best of our knowledge, yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Some staistical test were repeated with non-parametric test when the criteria of normal distribution was questionable.
Is there an estimate of variation within each group of data?	One way ANOVA analyzes the variance within each group using F-test

Is the variance similar between the groups that are being statistically compared?	Yes

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).	Catalogue numbers are provided and in some cases the antibodies are verified experimentally with results shown.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	These are stated in the Method section.

* 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.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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	N/A
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).	N/A
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).	N/A
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.	N/A

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.	N/A