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Reverse Fountain Flow of Phosphatidylinositol-3,4-Bisphosphate Polarizes Migrating Cells

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received a full set of reviewer reports on your manuscript, which are included below for your information.

As you will see from the comments, all appreciate the novelty of the proposed mechanism of rearward PI(3,4)P2 flow for establishment of PI(3,4)P2 polarity in migrating cells. However, they also point out a number of concerns that would have to get clarified and addressed before they can support publication of the manuscript, especially regarding more rigorous demonstration of vesicle delivery and fusion at the trailing edge. Furthermore, reviewers #1 and #3 indicate that some additional mechanistic insight into the enzymes participating in PI(3,4)P2 production or motor proteins involved would be important to support the proposed concept. I agree with the reviewers, and I would be happy to discuss further whether you would be able to extend the study along these lines.

Based on the overall interest expressed in the reports, I would like to invite you to submit a revised version of your manuscript in which you address the comments of all three referees. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

We have also extended our 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.

Referee #1:

This study by Li et al from the Devreotes lab describes a new mechanism by which migrating cells maintain polarity in their inositol lipid, PI(3,4)P2 distribution, which is high at the trailing end of migrating cells. They use genetically encoded biosensors, one based on a Dictyostelium protein CynA developed in the Devreotes lab and one based on the TAPP1 protein, developed in the Hamond laboratory. They mostly show data from Dictyostelium cells and some from HL60 cells to demonstrate that macropinosomes formed at the leading edge (or rather at most highly active actin areas) moving along microtubules break up into smaller vesicles, which then deliver PI(3,4)P2 lipid to the trailing membrane. This vesicular flow contributes to the polarized distribution of PI(3,4)P2 and appears to be present even when PI(3,4,5)P3 is not produced. The authors also present a mathematical model that is compatible with the experimental observations.

These are interesting new data introducing a concept of inositol lipid distribution by vesicular trafficking flow with directionality relative to the direction of migration. There are a few questions that would be nice to clarify to support this concept. One is the source of PI(3,4)P2 since it is convincingly shown that it is present even when PI(3,4,5)P3 is not produced. Is it made form PI4P by Class II PI3Ks? Are there such enzymes in Dictyostelium? The second is the directionality of the vesicular flow. If these vesicles move along the microtubules, they have to move toward the centrosome first then away from it to reach the trailing edge of the cells. What would be the motor protein that would switch the directionality of the movement? Addressing these questions may go beyond the scope of this initial work, but certainly would be important to prove this concept.

One weakness of the study is that the data on HL60 cells are really not as developed and convincing as those done in the Dictyostelium cells.

Even with these deficiencies, this paper is interesting enough to attract attention from a wide range of readers of The EMBO Journal.

The main problem, however, is the way the findings are presented for a general readership. The manuscript reads as if it was written for the laboratory members, who understand some of these finding without proper explanation. The paper has to go through a significant rewriting to make it more accessible for general readers. Below is the list that I found most striking:

1. The Authors should describe in the Results section that different biosensors of PI(34)P2 and different cells were used for example in in Fig. 1. The rational of using different cells and reporters should be explained. Also, for the general reader, it should be explained that AX3 cells are Dictyostelium cells.

2. The specific mentioning of the construct used in the various experiments would be extremely important as well as the rational of their choice. The diffusion and association-dissociation rate of each one of them might be different. Their affinity to bind PI(3,4)P2 would affect the degradation rate of the lipid. These considerations should be spelled out in each of the experiment.

3. It would be useful to label in each Figure panel the probe used instead of (or in addition to) the label, PI(3,4)P2. Also, when labeled, the labels are inconsistent. For example, in Fig.2A CynA is the label, whereas in the result, it is described as the tandem PH domain of CynA (the same presumably used in Fig. 1A and B). Some (but not all) of this information is available in the Figure Legend, but it would make it easier for the reader to find it in the text and in the Figure itself.

4. There is no description in the text or Figure legend which way cells are migrating. Do the yellow arrows (in some panels) show the point of chemotactic delivery or the direction of the migration? In Figure 1 panel C the reciprocal distribution of F-actin and PI(3,4)P2 reporter does not show the clear polarization seen in the Dictyostelium cells (panel A).

5. Similarly, which way do the cells migrate in Fig. 1F?

6. Line 107: What diffusion was measured? Lateral diffusion in the membrane? Please, clarify. Was the diffusion of the green signal into the photoconverted area also tested? If yes, did it indicate association of the probe from the cytosol or only lateral diffusion?

7. Line 119: Again, what cells were used here? I assume Dictyostelium cells based on the CAR1 receptor on their surface. Which way do they migrate?

8. Line 123: Based on FRAP experiments the authors conclude that there is a cytosolic source of PI(3,4)P2. One would argue that there is a cytosolic source of the PI(3,4)P2 reporter not the lipid itself. This conclusion is in conflict with the photoconversion experiment shown in Fig. 1F, where the conclusion was that there is "little exchange of membrane and cytosolic PI(3,4)P2 biosensor".

9. Line 127: It is not clear what the two different sensors (one is a tandem, the other is a single PH domain od CynA) are supposed to demonstrate. The tandem obviously has the higher affinity and is used in almost all other Dictyostelium experiments. I do not see what additional information we get from Fig. 2D.

10. Line 134. There seem to be no polarized distribution of the TAPP1-based reporter shown in Fig. 2F. In fact, there is almost no membrane localization of the probe. Does this cell migrate? Is there a directionality here?

11. Line 170: Describe here what probe was used for PI(3,4)P2 (not only in the Figure legend). The probe used should be indicated on the Figure as well [not just PI(3,4)P2].

12. Line 179: What concentration of wortmannin was used here? How do Dictyostelium PI3Ks wortmannin sensitivity compares to the mammalian ones?

13. Line 188: What was the concentration of LY294002? Also, in Fig.4 panels F and G, were the cells treated with Latrunculin A? What sensor was used here? There is hardly a membrane localization in panel G. Also, if the membrane/cytosol intensity ratios are about 0.5 (according to the Y axis), does it mean that there was more intensity in the cytosol than in the membrane?

14. Line 194. Again, describe what cells were used here. The tubulin staining is way overexposed. I think some still images from the movies would be useful here. The pictures shown in this Figure really do not demonstrate convincingly the turning of macropinosomes to small satellite vesicles.

15. Also, there is no reference to Fig. 5C in the text. What was this panel supposed to demonstrate? What is LimE on this panel?

16. Line 216: Explain again in the text that AX2 cells are a Dictyostelium line (is this different from AX3?). Was a similar defect in PI(3,4)P2 polarization observed after nocodazole treatment?

17. Fig. 7A: There are a number of small red vesicles in the red channel even before photoconversion and even more after the conversion of the small area. Many of these must be autofluorescent structures, and obviously not originated from the photoconverted signal (some being in another, neighboring cell). This makes it difficult to clearly interpret these results.

18. Fig. 7C. Indicate on the panels that this is a time series and add time stamps.

19. Line 251 and Fig. 7D-F. The Authors should do a better job explaining what panel D shows and what they mean about developed and vegetative cells, a distinction that has never surfaced throughout the manuscript or any of the Figures. Also, there are elements in panel G that appear without any reference during the paper (such as caffeine treatment).

Minor:

-There is reference to HEK293 cells in the Methods, but I did not see any experiment with them.

-Some Figure panels do not have scale bars.

Referee #2:

Li et al., proposed a polarized trafficking model for establishing polarity of PI(3,4)P2 at the cell back in migrating cells. It demonstrates PI(3,4)P2 localizes to macropinosomes along the leading edge protrusions that will eventually be removed and docked to the rear end of these cells resulting in the enrichment of PI(3,4)P2 at the back. Whether formation of the gradient (or polarized patch of some cortical factor) requires localized trafficking is a fundamental question that remains open. The null hypothesis is that localized production (within plasma membrane) coupled with slower degradation would be sufficient to achieve a gradient or travelling wave, without requiring localized delivery through fusion events. Hence, it remains to be determined: 1) whether localized fusion happens; 2) if it happens, what is the contribution of this mechanism compared to the in-plane mechanisms, or why is it necessary. The paper here addressed to some extent question 1 (I have some reservations, see below) and provided genetic evidence for question 2.
Overall, I think the idea is interesting and I am in support of getting the proposal out without it being fully proven if the overstated conclusions could be toned down.
Specific Questions
1. The pattern of PI(3,4)P2 in neutrophil is clearly different from that of the Dictyostelium. I am not convinced that they are polarized at the back or form any gradient similar to Dictyostelium cells.
2. There were little exchange of membrane and cytosolic PI(3,4)P2 biosensor. Is it because of the tandem PH domains that increased binding affinity?
3. Fig 1J. If the authors wish to demonstrate differential rate of degradation at different position of the cell, wouldn't it be better to photoactivate two separate spots of identical size at separate location and observe their rate of decay simultaneously?
4. Figure 2E: There was no visible intracellular vesicle structures (in the GFP channel) before or after photoconversion that colocalize with the structure in the RFP channel. Why is that?
5. The way fusion was defined in Fig 2E/F, Fig 7C was not very rigorous. There are many quantitative assays one would use to demonstrate fusion (content mixing coupled membrane fluorescence dequenching or loss of dyes loaded in the vesicle, phluorin assay, membrane

capacitance, etc.). I am not saying these because I think these experiments need to be included in the current paper. I understand some of these techniques could be specialized or require efforts beyond the scope of one study. However, in the absence of these additional evidence, conclusion on membrane fusion cannot be reached.
6. Figure 3A: Does PI(3,4)P2 dissociate from the vesicle after it is internalized (frame 32s) or does the breaks up into satellite vesicles? Can a membrane marker used in this experiment to trace the fate of the vesicle independent from lipid metabolism like the FM dye in Fig 3F? In Fig 3F, PI(3,4)P2 remains until the end so it does not address my question. This is important as one need to know whether there are multiple waves of synthesis/degradation of PI(3,4)P2 coupled with a single round of micropinocytosis/vesicle pinching into smaller satellite PI(3,4)P2 tagged vesicles/vesicle docking and fusion.
7. Fig 4A is an important experiment that is worth elaborating on. Can the quantification of the time course be shown?
8. Line 277, which data support that the gradient is sharpened with vesicle arrival?

Referee #3:

This interesting manuscript by Li et al describes a very novel route of lipid trafficking in migrating cells that involves micropinocytosis of PIP2 toward the leading edge for delivery at the cell rear to facilitate the generation of a PIP2 gradient. This is intriguing, because older models of cell migration suggested that vesicles might move from the cell rear to the leading edge. The authors further characterize their observation by showing that microtubules are required for rearward accumulation of PIP2 and generate a mathematical model that predicts that insertion of vesicles at the rear is required to generate a stable back zone in chemotaxis. The results and proposed model are interesting and new, but at present the manuscript is lacking in mechanistic explanation of the observations, and it is not clear that rearward flow of PIP2 vesicles is important in determining the chemotactic response.
Major points:
The manuscript lacks definitive proof that the reverse fountain flow is important in the response of cells to chemotactic gradients. At present it is hard to envisage how this could be shown, because the manuscript lacks mechanistic insight into the regulation of PIP2 generation at the leading edge, or how macropinosomes are split into satellite vesicles and targeted to the rear. An understanding of these aspects would greatly enhance the manuscript and provide potential tools to investigate the impact of reverse fountain flow on chemotaxis.
Figure 1: It is not clear if the quantification shown in G/H/I/K/L is from a single cell or several. It is important to know how reproducible this phenomenon is in multiple cells across independent experiments.
Figure 2: Several images are poor quality, e.g. 2A appears very noisy (particularly the CynA panels). In 2C/D the resolution makes it hard to distinguish vesicles from the membrane signal- super resolution or CLEM would allow this distinction to be made.
In Figure 2E, the brightness of the red panels doesn't match that in the merged panels.
The images in 2F are not of high enough quality to support the idea there might be vesicles at the cell rear.
Figure 4: Image quality is poor in A and B, making it difficult to assess the accuracy of their claims. In figure 4D/F, it is not clear why experiments are performed on non-polarized cells? Also, the data suggest that PI3K1/2 knockout does decrease PIP2 at the membrane (2E). This makes the claim that PIP3 is not the source of PIP2 unconvincing.

Figure 5: In panels A/B, it appears that nocodazole treatment inhibits macropinsome formation? Better resolution images are needed to support the claim that macropinosomes break into smaller satellite vesicles that move to the rear.
Figure 7: How is the experiment in 7A different to that in 2J? In 7B, the quantification is confusing. Around 5% of green signal is converted to red, and around 50% of this goes to the membrane- around 2.5% in total. It would be better to reflect this 5%-2.5% rather than mix scales as has been done here.
Minor points:
The authors are very general at times, referring to 'migrating cells' when they have tested their findings in Dictyostelium and neutrophils, but not other migratory cells (e.g. line 94 in the results). They should alter these statements to make clear that at the moment the findings are relevant to Dictyostelium and neutrophils.
N numbers and scale bars are missing from many figures/figure legends.
ROIs to show regions bleached/photoconverted would be useful (e.g. Figure 1F/J, Figure 7A).

To the Editor and Reviewers:

We would like to thank all three reviewers for their helpful and insightful comments. We have rewritten the manuscript extensively, performed additional experiments, reorganized the sequence of the data, added a cartoon diagram and further quantifications, and revised the requested details to manuscript and figures accordingly. Please find below a point-by-point response to the reviewers' comments.

Referee #1:

This study by Li et al from the Devreotes lab describes a new mechanism by which migrating cells maintain polarity in their inositol lipid, PI(3,4)P2 distribution, which is high at the trailing end of migrating cells. They use genetically encoded biosensors, one based on a Dictyostelium protein CynA developed in the Devreotes lab and one based on the TAPP1 protein, developed in the Hamond laboratory. They mostly show data from Dictyostelium cells and some from HL60 cells to demonstrate that macropinosomes formed at the leading edge (or rather at most highly active actin areas) moving along microtubules break up into smaller vesicles, which then deliver PI(3,4)P2 lipid to the trailing membrane. This vesicular flow contributes to the polarized distribution of PI(3,4)P2 and appears to be present even when PI(3,4,5)P3 is not produced. The authors also present a mathematical model that is compatible with the experimental observations.

We would like to thank the reviewer for reading our paper thoroughly and the nice summary of the work.

These are interesting new data introducing a concept of inositol lipid distribution by vesicular trafficking flow with directionality relative to the direction of migration. There are a few questions that would be nice to clarify to support this concept. One is the source of PI(3,4)P2 since it is convincingly shown that it is present even when PI(3,4,5)P3 is not produced. Is it made form PI4P by Class II PI3Ks? Are there such enzymes in Dictyostelium? The second is the directionality of the vesicular flow. If these vesicles move along the microtubules, they have to move toward the centrosome first then away from it to reach the trailing edge of the cells. What would be the motor protein that would switch the directionality of the movement? Addressing these questions may go beyond the scope of this initial work, but certainly would be important to prove this concept.

One weakness of the study is that the data on HL60 cells are really not as developed and convincing as those done in the Dictyostelium cells.

Even with these deficiencies, this paper is interesting enough to attract attention from a wide range of readers of The EMBO Journal.

The main problem, however, is the way the findings are presented for a general readership. The manuscript reads as if it was written for the laboratory members, who understand some of these

finding without proper explanation. The paper has to go through a significant rewriting to make it more accessible for general readers. Below is the list that I found most striking:

We really appreciate this reviewer feels that our work is "interesting enough to attract attention from a wide range of readers" as well as his/her thoughtful comments. According to the reviewer's suggestion, we have rewritten the manuscript extensively, performed additional experiments, reorganized the sequence of the data, added a cartoon diagram and further quantification, and revised the requested details to manuscript and figures.

Origin of PI(3,4)P2: Our data showed that a fraction of PI(3,4)P2 must come from a source other than PI(3,4,5)P3. As the reviewer suggests it could from PI4P by Class II PI3Ks. There are multiple phosphoinositide kinases in *Dictyostelium* that have not been characterized. It is possible that one of these corresponds to a novel PI3K, which is one of the directions that we are pursuing. We have added this in the discussion section.

Microtubule transport: The reviewer raises a very good point on directionality of the vesicular flow. We showed only that macropinosomes were brought inside the cell along microtubules, not that any vesicles traverse MTOC and travel all the way to the back along microtubules. Although we do not know the mechanism, our data clearly showed that macropinosomes break up into satellite vesicles which deliver PI(3,4)P2 to the back based on the following evidence. In the revised Fig. 5B-D, we quantified the relative percentage of green and red signal upon photoconversion. On average, 4.97% of green fluorescence was converted to red while 51.43% of the generated red fluorescence ended up at the back. This suggests that vesicles labelled with PI(3,4)P2 biosensor travel all the way to the back of membrane.

HL60 cells: Please see below how we intend to address the comments raised by the reviewer.

A point-by-point response to the reviewer's further comments follows:

1. The Authors should describe in the Results section that different biosensors of PI(34)P2 and different cells were used for example in in Fig. 1. The rational of using different cells and reporters should be explained. Also, for the general reader, it should be explained that AX3 cells are Dictyostelium cells.

We agree with the reviewer on these comments and apologize for the confusion. First, there are two biosensors for PI(3,4)P2, tPH-CynA and PH-TAPP1, with slightly different patterns in the cells, despite the fact that they are identical on lipid strips and have preference to PI(3,4)P2. Li and Edwards *et al.* previously established in *Dictyostelium* that both tPH-CynA and PH-TAPP1 label the base of macropinocytotic cups and back of the cell membrane (PMID: 30194235, 30591513). However, tPH-CynA labels both and PH-TAPP1 labels the back weakly. We have been able to express tPH-CynA in human HEK293T cells and breast epithelial MCF-10A cells but not in HL-60 cells. Second, we thought it important and useful to show whether this is conserved in other human migrating cells. For this reason, we used cPHX3$_{TAPP1}$-GFP, which was recently developed in the Hammond lab, as the biosensor for PI(3,4)P2 in HL-60 cells. In fact, the distribution of PH-TAPP1 in HL-60 resembles that of PH-TAPP1 in *Dictyostelium.*

Nevertheless, to eliminate the confusion, we have reorganized the figures and moved results of HL-60 cells to extended view figures in the end and provided explanations on the different cell lines and PI(3,4)P2 biosensors used in our revised manuscript. We also added examples of comparing tPH-CynA and PH-TAPP1 in *Dictyostelium* in Appendix Fig S8. Additionally, we provided more examples of cPHX3$_{TAPP1}$-GFP localization in HL-60 in Fig EV1.

AX3 cells are *Dictyostelium* cells has also been explained in revised manuscript and figure legends.

2. The specific mentioning of the construct used in the various experiments would be extremely important as well as the rational of their choice. The diffusion and association-dissociation rate of each one of them might be different. Their affinity to bind PI(3,4)P2 would affect the degradation rate of the lipid. These considerations should be spelled out in each of the experiment.

Having reorganized the paper, we now provide this rationale near the end of the results section where we introduce the second biosensor. Up to that point, most of the experiments used only one biosensor tPH-CynA in *Dictyostelium* cells.

3. It would be useful to label in each Figure panel the probe used instead of (or in addition to) the label, PI(3,4)P2. Also, when labeled, the labels are inconsistent. For example, in Fig.2A CynA is the label, whereas in the result, it is described as the tandem PH domain of CynA (the same presumably used in Fig. 1A and B). Some (but not all) of this information is available in the Figure Legend, but it would make it easier for the reader to find it in the text and in the Figure itself.

Following the reviewer's suggestion, we labeled the probe in the figure panels. All the labels have been made consistent throughout the revised figures and manuscript.

4. There is no description in the text or Figure legend which way cells are migrating. Do the yellow arrows (in some panels) show the point of chemotactic delivery or the direction of the migration? In Figure 1 panel C the reciprocal distribution of F-actin and PI(3,4)P2 reporter does not show the clear polarization seen in the Dictyostelium cells (panel A).

We appreciate the reviewer's constructive comments. We have labeled Front and Back of the cells in each figure panel to denote the direction of their migration. Yellow arrows point to PI(3,4)P2 localization at the back of the cell membrane. We have made these changes in the figures and figure legends of the revised manuscript. We have also moved panel C to Fig EV1 and provided the explanation at that point of the result. As explained above, the issue is due to the biosensors, not the cells.

5. Similarly, which way do the cells migrate in Fig. 1F?

We have labeled Front and Back to the cells in the revised Fig. 1 to denote the direction of their migration.

6. Line 107: What diffusion was measured? Lateral diffusion in the membrane? Please, clarify. Was the diffusion of the green signal into the photoconverted area also tested? If yes, did it indicate association of the probe from the cytosol or only lateral diffusion?

The reviewer has made a very valid point here. We measured the lateral diffusion of the red photoconverted molecules of PI(3,4)P2 on the membrane by comparing the intensity time profiles of the photoconverted molecules and the profiles obtained from the simulation of stochastic diffusion process. The details are provided on the method section. Over time, the red signal spread but did not decrease significantly (less than 10% change over 60s), indicating lateral diffusion. Consistently, the green signal also filled in the bleached area, but we did not

use that data in this calculation. In a separate experiment, we did use the data from green channel; see answer to question 8. To clarify the concepts, we have revised the text.

7. Line 119: Again, what cells were used here? I assume Dictyostelium cells based on the CAR1 receptor on their surface. Which way do they migrate?

Please see explanation above.

8. Line 123: Based on FRAP experiments the authors conclude that there is a cytosolic source of PI(3,4)P2. One would argue that there is a cytosolic source of the PI(3,4)P2 reporter not the lipid itself. This conclusion is in conflict with the photoconversion experiment shown in Fig. 1F, where the conclusion was that there is "little exchange of membrane and cytosolic PI(3,4)P2 biosensor".

We are not arguing that there is a cytosolic source of the reporter. As noted above, following photoconversion there is only 10% loss of the reporter in 60 sec (and less than 20% in the first 5 minutes) (Fig. 1E), indicating that the binding is very stable over 12 seconds. This suggests that the localization of the reporter is equivalent to the localization of the lipid, at least over short time. Therefore, in this experiment the movement of the reporter indicates that the lipid is coming from the cytosol to the membrane.

9. Line 127: It is not clear what the two different sensors (one is a tandem, the other is a single PH domain of CynA) are supposed to demonstrate. The tandem obviously has the higher affinity and is used in almost all other Dictyostelium experiments. I do not see what additional information we get from Fig. 2D.

The purpose of using HALO was because it is brighter and we added this data since it is a clear example that PI(3,4)P2- containing vesicles dock in the back region of *Dictyostelium* cells. Additionally, the use of another tag and monomeric PH-CynA shows that our results do not depend on these features.

10. Line 134. There seem to be no polarized distribution of the TAPP1-based reporter shown in Fig. 2F. In fact, there is almost no membrane localization of the probe. Does this cell migrate? Is there a directionality here?

Please see the explanation above where we discuss how we reorganized the data. We have also added more examples comparing the two biosensors tPH-CynA and cPH-TAPP1 in *Dictyostelium* in Appendix Fig S8. Additionally, we provided more examples of PH-TAPP1 localization in HL-60 in Fig EV1.

11. Line 170: Describe here what probe was used for PI(3,4)P2 (not only in the Figure legend). The probe used should be indicated on the Figure as well [not just PI(3,4)P2]..

We used tPH-CynA as a probe for PI(3,4)P2. We have added descriptions and labels in the results section and figure legends.

12. Line 179: What concentration of wortmannin was used here? How do Dictyostelium PI3Ks wortmannin sensitivity compares to the mammalian ones?

The concentration of wortmannin used here is 5 µM and this information has been added to the revised manuscript. Although both *Dictyostelium* and mammalian PI3Ks show sensitivity to

wortmannin, the mammalian PI3Ks are inhibited by the drug at nanomolar concentrations whereas *Dictyostelium* PI3Ks are inhibited at low micromolar concentrations of wortmannin. (PMID: 8257416, 15809030, 8947549).

13. Line 188: What was the concentration of LY294002? Also, in Fig.4 panels F and G, were the cells treated with Latrunculin A? What sensor was used here? There is hardly a membrane localization in panel G. Also, if the membrane/cytosol intensity ratios are about 0.5 (according to the Y axis), does it mean that there was more intensity in the cytosol than in the membrane?

The concentration of LY294002 used was 5 µM. From looking at the images of undifferentiated HL-60 cells under Latrunculin A treatment from multiple experiments, we think it is clear that there is PI(3,4)P2 signal after PI3K inhibition. This indicates that there is another source of PI(3,4)P2 which is not from PIP3, as in *Dictyostelium.* However, the original signal of PI(3,4)P2 on the membrane were weak, which made the quantification difficult. For this reason, we have removed the claim in the revised manuscript.

14. Line 194. Again, describe what cells were used here. The tubulin staining is way overexposed. I think some still images from the movies would be useful here. The pictures shown in this Figure really do not demonstrate convincingly the turning of macropinosomes to small satellite vesicles.

The cells used in all the revised main figures are *Dictyostelium* cells. We intentionally overexposed the MTOC in order to see the ends of the microtubules and the contact site of microtubules and PI(3,4)P2 vesicles.

The breaking-apart phenomenon is not in this figure. We demonstrated turning of macropinosomes to small satellite vesicles from the movie EV7 and have added still images of breaking apart phenomenon in revised Fig 5A.

15. Also, there is no reference to Fig. 5C in the text. What was this panel supposed to demonstrate? What is LimE on this panel?

We have described this in the revised manuscript. LimE is labelling F-actin and we have added this in the revised manuscript and figure legends.

16. Line 216: Explain again in the text that AX2 cells are a Dictyostelium line (is this different from AX3?). Was a similar defect in PI(3,4)P2 polarization observed after nocodazole treatment?

Ax2 and Ax3 are two different wild-type axenic *Dictyostelium* strains. The reason we used Ax2 cells is because it is the parental strain of RacH mutant. There are considerable similarities between Ax2 and Ax3 strains: both have comparable rates of macropinocytosis, similar microtubular arrangement and sensitivity to nocodazole (PMID: 29440238, 1295696, 6619183). Based on these findings, we would expect a similar defect in PI(3,4)P2 vesicular transportation in Ax2 after nocodazole treatment.

To further verify the similarity of the two strains, we repeated the nocodazole experiment using Ax2 cells and found a similar defect in PI(3,4)P2 vesicular transportation after nocodazole treatment. We have added the data in Appendix Fig S4 and the revised manuscript.

17. Fig. 7A: There are a number of small red vesicles in the red channel even before photoconversion and even more after the conversion of the small area. Many of these must be autofluorescent structures, and obviously not originated from the photoconverted signal (some being in another, neighboring cell). This makes it difficult to clearly interpret these results.

We agree that the small red vesicles seen in the red channel even before photoconversion are background. As you can see, the background signal does not arrange itself in a pattern at the back of the cell membrane. Additionally, the total intensity of red signal increased about 10-fold after photoconversion. Therefore, the pattern must come from photoconversion and not the background.

18. Fig. 7C. Indicate on the panels that this is a time series and add time stamps.

We have indicated on the panels that this is a time series and added time stamps in revised Fig. 5.

19. Line 251 and Fig. 7D-F. The Authors should do a better job explaining what panel D shows and what they mean about developed and vegetative cells, a distinction that has never surfaced throughout the manuscript or any of the Figures. Also, there are elements in panel G that appear without any reference during the paper (such as caffeine treatment).

We have addressed it mostly by revising the cartoons to illustrate our point in revised Fig. 6. We substituted the terms “less polarized” and “more polarized” to refer to vegetative cells and developed cells, respectively.

We have explained the caffeine treatment in the figure legends and added relevant three references (Arai, Shibata et al., 2010, Li et al., 2018, Wang, Senoo et al., 2013) in the revised manuscript. Schematic representation of our previous study demonstrating the mutual inhibition between Ras activity and PI(3,4)P2 which establishes polarity even in immobilized latrunculin A treated cells under different conditions, such as a chemotactic gradient (with a stationary back) or in caffeine treatment (with a rotating back).

Minor:

-There is reference to HEK293 cells in the Methods, but I did not see any experiment with them.

HEK293T cells were used to perform lentivirus transduction of biosensors such as PH-TAPP1 or RFP-LifeAct in HL-60 cells. We have added this in the Methods and Protocols section of the revised manuscript.

-Some Figure panels do not have scale bars.

We have added scale bars throughout the revised figures.

Referee #2:

Li et al., proposed a polarized trafficking model for establishing polarity of PI(3,4)P2 at the cell back in migrating cells. It demonstrates PI(3,4)P2 localizes to macropinosomes along the leading edge protrusions that will eventually be removed and docked to the rear end of these cells resulting in the enrichment of PI(3,4)P2 at the back. Whether formation of the gradient (or polarized patch of some cortical factor) requires localized trafficking is a fundamental question

that remains open. The null hypothesis is that localized production (within plasma membrane) coupled with slower degradation would be sufficient to achieve a gradient or travelling wave, without requiring localized delivery through fusion events. Hence, it remains to be determined: 1) whether localized fusion happens; 2) if it happens, what is the contribution of this mechanism compared to the in-plane mechanisms, or why is it necessary. The paper here addressed to some extent question 1 (I have some reservations, see below) and provided genetic evidence for question 2.
Overall, I think the idea is interesting and I am in support of getting the proposal out without it being fully proven if the overstated conclusions could be toned down.

We thank the reviewer for recognizing the idea and significance of our paper. We have toned down the conclusions in the revised manuscript, performed additional experiments, reorganized the sequence of the data, added cartoon diagram and quantification, and revised the requested details to manuscript and figures accordingly.

The reviewer asked the relative amount of PI(3,4)P2 product at the back of the cell and the delivery. We carried out additional experiments and added following text and Appendix Fig S7 in the revised manuscript.

> "To access how much the vesicular trafficking contributes to the overall production of PI(3,4)P2, we measured turnover before and after Latrunculin A treatment. Latrunculin A acutely blocks macropinosomes formation. After Latrunculin A treatment, there was a 30% decrease of tPH-CynA on the membrane and a photoconversion experiment showed that the degradation rate of PI(3,4)P2 decreased 3-fold (Appendix Fig S7). Together these data suggest that the vesicular trafficking is responsible for more than 50% of the levels of PI(3,4)P2, which is consistent with the estimate made above when vesicular trafficking is blocked by *RacH*- mutant cells (Fig 4)".

A point-by-point response to the reviewer's further comments, including a discussion of new experiments, follows:

Specific Questions
1. The pattern of PI(3,4)P2 in neutrophil is clearly different from that of the Dictyostelium. I am not convinced that they are polarized at the back or form any gradient similar to Dictyostelium cells.

We agree that, using this biosensor PH-TAPP1, the signal at the back of neutrophil is not bright enough. There are two biosensors for PI(3,4)P2, tPH-CynA and PH-TAPP1, with slightly different patterns in the cells, despite the fact that they are identical on lipid strips and have preference to PI(3,4)P2. Li and Edwards *et al.* previously established in *Dictyostelium* that both tPH-CynA and PH-TAPP1 label the base of macropinocytotic cups and the back of the cell membrane (PMID: 30194235, 30591513). However, tPH-CynA labels both and PH-TAPP1 labels the back weakly. We have been able to express tPH-CynA in human HEK293T cells and breast epithelial MCF-10A cells but not in HL-60 cells.

We thought it important and useful to show whether this is conserved in other migrating cells. For this reason, we used cPHX3$_{TAPP1}$-GFP, recently developed in the Hammond lab, as the biosensor for PI(3,4)P2 in HL-60 cells. In fact, the distribution of PH-TAPP1 in HL-60 resembles that of PH-TAPP1 in *Dictyostelium.*

Nevertheless, to eliminate the confusion, we have now reorganized the figures and moved results of HL-60 cells to supplemental figures in the end and provided explanations on the different cell lines and PI(3,4)P2 biosensors used in our revised manuscript. We also added examples of comparing CynA and TAPP1 in *Dictyostelium* in Appendix Fig S8. Additionally, we provided more examples of cPH-TAPP1 localization in HL-60 in Fig EV1. These results are now described in the revised manuscript.

2. There were little exchange of membrane and cytosolic PI(3,4)P2 biosensor. Is it because of the tandem PH domains that increased binding affinity?

We agree with the reviewer that the tandem PH domains likely increased binding affinity. We have previously demonstrated the distribution of monomer, tandem and tetramer PH domain of CynA (PI(3,4)P2 biosensor). That data showed that these biosensors displayed increasingly wider distributions, strongly suggesting that the affinity increased (PMID: 30194235). Our photoactivation data, shown in Fig 1E, suggested that the tandem version dissociates slowly.

3. Fig 1J. If the authors wish to demonstrate differential rate of degradation at different position of the cell, wouldn't it be better to photoactivate two separate spots of identical size at separate location and observe their rate of decay simultaneously?

The reviewer proposed a great experiment which we were happy to try. Unfortunately, some of the signal at the back region diffused very fast towards the front and merged with the signal at the side in 20 seconds. Therefore, we had to combine multiple experiments from different cells where only one location was activated.

4. Figure 2E: There was no visible intracellular vesicle structures (in the GFP channel) before or after photoconversion that colocalize with the structure in the RFP channel. Why is that?

We appreciate this comment. To address this, we have measured the intensity of the two regions before and after photoconversion. We found that 20% of the green signal was converted to red. This may represent some small intercellular vesicles. However, by 95s it become clear that some red vesicles co-localized with green vesicles and were approaching to the membrane and appear to be fusing. These images were captured using a confocal microscope and showed only one plane of the cell. Some out-of-plane vesicle structures could have been photoactivated at time 0 sec and moved into the focal plane by 5 seconds.

5. The way fusion was defined in Fig 2E/F, Fig 7C was not very rigorous. There are many quantitative assays one would use to demonstrate fusion (content mixing coupled membrane fluorescence dequenching or loss of dyes loaded in the vesicle, phluorin assay, membrane capacitance, etc.). I am not saying these because I think these experiments need to be included in the current paper. I understand some of these techniques could be specialized or require efforts beyond the scope of one study. However, in the absence of these additional evidence, conclusion on membrane fusion cannot be reached.

The reviewer is highlighting an issue which is indeed challenging. We agree with the reviewer that we didn't formally prove fusion. Therefore, we have modified our description in the models to allow the possibility that vesicles dock at the back, do not actually fuse, and then slide along the membrane to the front. We think the fusion is more likely, but docking cannot be ruled out.

In addition, we have added more time points of vesicles arriving at the back in Fig 1L, and added a movie to show the dynamics of these events at the back of the cell membrane.

6. Figure 3A: Does PI(3,4)P2 dissociate from the vesicle after it is internalized (frame 32s) or does the breaks up into satellite vesicles? Can a membrane marker used in this experiment to trace the fate of the vesicle independent from lipid metabolism like the FM dye in Fig 3F? In Fig 3F, PI(3,4)P2 remains until the end so it does not address my question. This is important as one need to know whether there are multiple waves of synthesis/degradation of PI(3,4)P2 coupled with a single round of micropinocytosis/vesicle pinching into smaller satellite PI(3,4)P2 tagged vesicles/vesicle docking and fusion.

We proposed that PI(3,4)P2 enriched vesicles break up into smaller satellite vesicles which deliver PI(3,4)P2 to the back based on the following evidence. In the revised Fig 5, we quantified the relative percentage of green and red signal upon photoconversion. On average, 4.97% of the green fluorescence was converted to red while 51.43% of the generated red fluorescence ended up at the back. We have now included our quantification in the revised Fig 5 to clarify our points. This suggests that vesicles labelled with PI(3,4)P2 biosensor travel all the way to the back of membrane before the biosensor dissociates. Thus, our data argues against the alternative possibility that the biosensor dissociates from the macropinosomes and rebinds to smaller vesicles which go to the back. We have added a cartoon showing the two potential alternatives and giving our rationale (Fig 5D).

Last, we did use FM-64 dye to trace the early stage of the macropinosomes, but after washing out FM-64, there were still many vesicles labelled which were difficult to follow.

7. Fig 4A is an important experiment that is worth elaborating on. Can the quantification of the time course be shown?

We have followed the reviewer's suggestion and have presented time-lapse images in revised Fig 3A. We also added the kymograph and line scan quantification in revised Figs 3B and C.

8. Line 277, which data support that the gradient is sharpened with vesicle arrival?

We thank the reviewer for pointing out this imprecise language. Owing to a slower arrival time of PI(3,4)P2 vesicles and a slower decay rate of PI(3,4)P2 on the membrane, the PI(3,4)P2 signal at the back of more polarized cells broadens and extends further to the front. We have revised the text.

Referee #3:

This interesting manuscript by Li et al describes a very novel route of lipid trafficking in migrating cells that involves micropinocytosis of PIP2 toward the leading edge for delivery at the cell rear to facilitate the generation of a PIP2 gradient. This is intriguing, because older models of cell migration suggested that vesicles might move from the cell rear to the leading edge. The authors further characterize their observation by showing that microtubules are required for rearward accumulation of PIP2 and generate a mathematical model that predicts that insertion of vesicles at the rear is required to generate a stable back zone in chemotaxis. The results and proposed model are interesting and new, but at present the manuscript is lacking in mechanistic explanation of the observations, and it is not clear that rearward flow of PIP2 vesicles is important in determining the chemotactic response.

Major points:

The manuscript lacks definitive proof that the reverse fountain flow is important in the response of cells to chemotactic gradients. At present it is hard to envisage how this could be shown, because the manuscript lacks mechanistic insight into the regulation of PIP2 generation at the leading edge, or how macropinosomes are split into satellite vesicles and targeted to the rear. An understanding of these aspects would greatly enhance the manuscript and provide potential tools to investigate the impact of reverse fountain flow on chemotaxis.

We thank the reviewer for the thoughtful suggestions and comments and for recognizing the novelty of the work. We have performed additional experiments, reorganized the sequence of the data, added cartoon diagram and quantification, and revised the requested details to manuscript and figures accordingly.

The reviewer is correct in noting that we have not pinpointed the enzymes that make PI(3,4)P2. However, our data show that a large fraction of PI(3,4)P2 must come from a source other than PI(3,4,5)P3, which is valuable information. There are multiple phosphoinositide kinases in *Dictyostelium* that have not been characterized. It is possible that one of these corresponds to a novel PI3K.

It is also true that we did not show exactly how PI(3,4)P2 breaks up into satellite vesicles, but our data strongly suggests that the PI(3,4)P2 appearing on macropinosomes is delivered to the back. In the revised Fig 5, we quantified the relative percentage of green and red signal upon photoconversion. On average, 4.97% of green fluorescence was converted to red while 51.43% of the generated red fluorescence ended up at the back. We have now represented our quantification in the revised Fig 5C to clarify our points.

This suggests that vesicles labelled with PI(3,4)P2 biosensor travel all the way to the back of membrane before the biosensor dissociates. Our data argue against the alternative possibility that the biosensor dissociates from the macropinosomes and rebinds to smaller vesicles which go to the back. We have added a cartoon showing the two potential alternatives and giving our rationale (Fig 5D).

While the focus of our study was on polarity, this is clearly important for chemotaxis and thus believe that this study will be informative for future studies on directed cell migration.

A point-by-point response to the reviewer's further comments, including a discussion of new experiments, follows:

Figure 1: It is not clear if the quantification shown in G/H/I/K/L is from a single cell or several. It is important to know how reproducible this phenomenon is in multiple cells across independent experiments.

We provided profile from one cell in the revised Fig 1D. We have also measured the decay rate from 10 cells and added the decay rate curve in the revised Fig 1E.

Figure 2: Several images are poor quality, e.g. 2A appears very noisy (particularly the CynA panels).

We have improved the image quality in the revised Fig 1J. We can clearly see from the image at 12.5 sec that CynA signal recovered evenly at the back of the membrane, but CAR1 recovered from the edges. Quantification of several cells provides consistent conclusion from this panel (Fig 1I).

In 2C/D the resolution makes it hard to distinguish vesicles from the membrane signal- super resolution or CLEM would allow this distinction to be made.

We have added more images in the revised Figs 1J and K, and provided the movie EV2 to show membrane docking and fusing. We are working on our protocols to do a super resolution or CLEM in *Dictyostelium* cells.

In Figure 2E, the brightness of the red panels doesn't match that in the merged panels.

We thank the reviewer for pointing this out. We have replaced the images in the revised figure.

The images in 2F are not of high enough quality to support the idea there might be vesicles at the cell rear.

We agree that using biosensor PH-TAPP1, the signal at the back of neutrophil is not bright enough. There are two biosensors for PI(3,4)P2, tPH-CynA and PH-TAPP1, with slightly different patterns in the cells, despite the fact that they are identical on lipid strips and have preference to PI(3,4)P2 (PMID: 30194235, 30591513). Li and Edwards *et al.* have previously established in *Dictyostelium* that both tPH-CynA and PH-TAPP1 labels the base of macropinocytotic cups and back of the cell membrane. However, tPH-CynA labels both and PH-TAPP1 labels the back weakly. We have been able to express tPH-CynA in human HEK293T cells and breast epithelial MCF-10A cells but not in HL-60 cells.

We thought it important and useful to show whether this is conserved in other migrating cells. Hence, we used cPHX3$_{TAPP1}$-GFP, recently developed in the Hammond lab, as the biosensor for PI(3,4)P2 in HL-60 cells. In fact, the distribution of PH-TAPP1 in HL-60 resembles that of PH-TAPP1 in *Dictyostelium.*

Nevertheless, to eliminate the confusion, we have now reorganized the figures and moved results of HL-60 cells to supplemental figures in the end and provided explanations on the different cell lines and PI(3,4)P2 biosensors used in our revised manuscript. We also added examples of comparing CynA and TAPP1 in *Dictyostelium* in Appendix Fig S8. Additionally, we provided more examples of TAPP1 vesicles localization at the back of HL-60 in Fig EV1. These results are now described in the revised version.

Figure 4: Image quality is poor in A and B, making it difficult to assess the accuracy of their claims.
We have followed the reviewer's suggestion and have presented time-lapse images in revised Fig. 3A. We also added the kymograph and line scan quantification in revised Fig. 3B and C.

Expression of both biosensors in this mutant was difficult, making imaging challenging. Nevertheless, the images in Fig. 4B clearly show that the dynamic localization of PI(3,4)P2 remained even in the absence of PIP3. To clarify this, we have labeled Front and Back of the cell in the revised panel.

In figure 4D/F, it is not clear why experiments are performed on non-polarized cells? Also, the data suggest that PI3K1/2 knockout does decrease PIP2 at the membrane (2E). This makes the claim that PIP3 is not the source of PIP2 unconvincing.

It is easier to quantify levels of the biosensor on the membrane using non-polarized cells. We have added this in the manuscript. We agree that the data shows that some of PIP2 is generated from PIP3. However, our point is that PIP3 is not the major source of PI(3,4)P2.

Figure 5: In panels A/B, it appears that nocodazole treatment inhibits macropinsome formation? Better resolution images are needed to support the claim that macropinosomes break into smaller satellite vesicles that move to the rear.

In panels A/B, we are showing the structure of microtubule and the relative localization of macropinosomes and microtubule before and after nocodazole treatment. We are not showing that nocodazole treatment inhibits macropinosomes formation, but our data in panel E suggest that nocodazole inhibits the initial transportation of macropinosomes away from the edge of the cell.

We demonstrated turning of macropinosomes to small satellite vesicles from the movie S7. We have added still images from the movie S7 in revised Fig 5A.

Quantitative information in a new diagram demonstrate that an average 4.97% of green fluorescence was converted to red while 51.43% of the generated red fluorescence ended up at the back, indicating that vesicles labelled with PI(3,4)P2 biosensor travel all the way to the back of membrane before the biosensor dissociates (Fig 5C). Our data argues against the alternative possibility that the biosensor dissociates from the macropinosomes and rebinds to smaller vesicles which go to the back. We have added a cartoon in Fig 5D to propose two potential alternatives and show our rationale.

Figure 7: How is the experiment in 7A different to that in 2J? In 7B, the quantification is confusing. Around 5% of green signal is converted to red, and around 50% of this goes to the membrane- around 2.5% in total. It would be better to reflect this 5%-2.5% rather than mix scales as has been done here.

We think the reviewer is asking about panel 1J, which shows the flashing on the side of the membrane. In contrast, Fig 7A is flashing the macropinosomes at the front. We have now represented the quantification in the revised Fig 5C to clarify our points. Please see explanation above where we clarified the quantification in revised Figs 5C and D.

Minor points:
The authors are very general at times, referring to 'migrating cells' when they have tested their findings in Dictyostelium and neutrophils, but not other migratory cells (e.g. line 94 in the results). They should alter these statements to make clear that at the moment the findings are relevant to Dictyostelium and neutrophils.

We have changed “migrating cells” to “migrating Dictyostelium cells” in the results section and revised our discussion: “Membrane folding, fountain flow model or reverse fountain flow models may operate in specific cells during different migratory modes”.

N numbers and scale bars are missing from many figures/figure legends.

We have added N numbers and scale bars throughout the figures and figure legends.

ROIs to show regions bleached/photoconverted would be useful (e.g. Figure 1F/J, Figure 7A).

We have added ROIs in the yellow box in the revised Fig 1 and Fig 5, and described these in the figure legends.

Thank you for submitting a revised version of your manuscript. Your study has now been seen by all original referees, who find that their main concerns have been addressed and are now in favour of publication of the manuscript. There now remain only a few mainly editorial issues that have to be addressed before I can extend formal acceptance of the manuscript.

Referee #1:

This is a revised version of the manuscript that describes a novel mechanism by which polarized cells maintain a phosphoinositide 3,4-bisphosphate gradient using a vesicular membrane flow from the leading edge toward the trailing edge.

The Authors have addressed my main concerns and reorganized the Figures and improved the flow of the writing. These changes have significantly improved the accessibility of the manuscript. I have only a few remaining comments that only affect the labeling of some panels in Figure EV1.

It is confusing that there were no Figure numbers on the Figure panels. Fig. EV1 was not distinguishable from the main Figures. There are a couple of small labeling issues in Figure EV1:
The labeling of the left top in panel B needs correction.
Also, In panel E, the red labels of the coordinate and the coordinate axes are really not necessary and only distract.

Referee #2:

i find the revision to be greatly improved in its clarity. I do not have further questions and support its publication.

Referee #3:

The authors have done a good job providing more analyses and reorganising the manuscript to answer most of my comments. This will be a valuable addition to the literature.

The authors performed the requested changes.

Referee #1:

This is a revised version of the manuscript that describes a novel mechanism by which polarized cells maintain a phosphoinositide 3,4-bisphosphate gradient using a vesicular membrane flow from the leading edge toward the trailing edge.

The Authors have addressed my main concerns and reorganized the Figures and improved the flow of the writing. These changes have significantly improved the accessibility of the manuscript. I have only a few remaining comments that only affect the labeling of some panels in Figure EV1.

It is confusing that there were no Figure numbers on the Figure panels. Fig. EV1 was not distinguishable from the main Figures. There are a couple of small labeling issues in Figure EV1:

The labeling of the left top in panel B needs correction.

Also, In panel E, the red labels of the coordinate and the coordinate axes are really not necessary and only distract.

We thank the review for point this out. We have corrected the labeling of the left top in panel B.

The red labels in panel E are showing the 3D information. We have cropped the labels out as suggested.

Referee #2:

i find the revision to be greatly improved in its clarity. I do not have further questions and support its publication.

Referee #3:

The authors have done a good job providing more analyses and reorganising the manuscript to answer most of my comments. This will be a valuable addition to the literature.

2nd Revision - Editorial Decision 18th Nov 2020

Editor accepted the manuscript.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Peter Devreotes
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-105094

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 $\chi 2$ 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?	The sample size for this study was chosen based on the existing literature in the field, and from previous experience; we believe it is within the range that are generally accepted in the field.
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.	NA
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
Is there an estimate of variation within each group of data?	Yes

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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).	We used Mouse anti α-Tubulin monoclonal antibody fromSigma Aldrich, the Catlog number is T9026. This information is shown in manuscript
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Dictyostelium cells do not acquire mycoplasma contamination. All Dictyostelium cell lines were taken from authenticated frozen stocks at Devreotes lab. HL-60 cell line is from Dr. Orion Weiner lab at UCSF. We have not tested for mycoplasma contaminiation in HL-60 cell line.

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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.	On acceptance, an archived version of the simulation code supporting this article will be made available through the Johns Hopkins University Data Archive with the following doi:xxx (https://archive.data.jhu.edu).

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