EMBO reports

Modulation of phagosome phosphoinositide dynamics by a *Legionella* phosphoinositide 3-kinase

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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.)

1st Editorial Decision 29th Jul 2020

Dear Prof. Qiu,

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all points need to be addressed, I will not detail them here.

Nevertheless, please carefully address major point 1 of referee #3, regarding improper referencing of previous research. During cross-commenting all three referees agreed that the identification of MavQ as a lipid kinase was first reported by Urbanus et al. (Urbanus et al., 2016, Mol Syst Biol 12:893 - reference 37 of your manuscript), and that these findings need to be summarized in the introduction section, and that the respective part in the results of the present manuscript describing similar data needs to be re-written. Given your expertise and proficiency, and given the fact that you cite the paper in question in another context, two referees suspected during cross-commenting that ignoring these previous findings was not an oversight, but rather happened deliberately. We therefore encourage you to commit yourself to taking greater care in this regard during revision, and in future manuscripts.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted

to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines: http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

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

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

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please also note our new reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Please add up to 5 key words to the title page.
I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling

Editor
EMBO Reports

Referee #1:

In this study, Li et al. identify and characterize a novel effector protein, MavQ, of the facultative intracellular pathogen Legionella pneumophila. Based on an HHpred analysis revealing a C-terminal conserved kinase motif, MavQ was identified as a putative (lipid) kinase. Upon ectopic production, the effector protein is toxic for yeast, and mutations in amino acid residues predicted to be catalytically important abolish toxicity. Moreover, purified MavQ is active as a wortmannin-insensitive phosphoinositide (PI) 3-kinase, which preferentially uses phosphatidylinositol (PtdIns) as a substrate. The effector protein associates with the Legionella-containing vacuole (LCV), but is not essential for intracellular replication of L. pneumophila. Intriguingly, the PtdIns 3-kinase MavQ seems to function together with the bacterial PtdIns3P 4-kinase LepB and the PtdIns(3,4)P2 3-phosphatase SidF to produce PtdIns4P on the LCV membrane. In contrast, the previously identified L. pneumophila PtdIns 3-kinase LegA5 does not seem to affect the production of PtdIns4P on LCVs along with LepB and SidF. Overall, the study documents the characterization of a novel L. pneumophila PtdIns 3-kinase implicated in PI conversion during LCV maturation.

This is an interesting study of high relevance to a broad readership. The manuscript is well-written and concise, and the story unravels in a straightforward manner. A few rather minor points should be addressed to further strengthen the work.

Specific points:

1) Fig. 3c: A positive control is missing for the inhibition of PI 3-kinase activity in vitro by wortmannin.

2) Fig. 4c: The L. pneumophila mavQ mutant strain does not show a (pronounced) intracellular growth phenotype. It would be informative to test intracellular replication of the mavQ-lepB double mutant and - if available - a mavQ-lepB-sidF triple mutant strain.

3) Fig. 4a, 5b and 6d: Consider zooming-in to increase the validity and resolution of the images.

4) The paragraphs, wherein Fig. 5a and Fig. 4c-d are called-out are placed oddly in the manuscript. The flow of the text would be improved, if the figures were called-out in the sequence they are already arranged. Accordingly, consider ordering the paragraphs in the sequence Fig. 4a-b, Fig. 4c-d, Fig. 5a and Fig. 5b-d.

5) Some statements should be referenced/phrased more precisely, and some additional references should be discussed:
- l. 152 and l. 241: Ref. #43 should be cited.
- l. 238: Technically, it has not been shown that the interception of the LCV with ER-derived vesicles results in pathogen vacuole expansion. The statement should be phrased more cautiously.
- l. 244/245: Rab10 has been previously implicated in intracellular growth of L. pneumophila by RNA interference (Hoffmann et al. (2014) Cell Microbiol 16:1034-52). This reference should also be cited.
- l. 267-270: The accumulation of PtdIns4P on LCVs (PI conversion from PtdIns3P to PtdIns4P) also involves capturing Golgi-derived, PtdIns4P-positive vesicles (Weber et al. (2018) mBio 9:e02420-18). This reference should be discussed and cited.
- l. 302: PtdIns3P-binding effector proteins have been previously identified : Ref. #44; Jank et al.

(2012) Cell Microbiol 14:852-68; Finsel et al. (2013) Cell Host Microbe 14:38-50. These references should also be cited here.

6) Typos/wording:
- l. 39: ... to a lesser extent ...
- l. 43: ... small GTPases of the Arf ...
- l. 135: In response to wortmannin, a specific inhibitor of eukaryotic PI3Ks, fluorescence signals were ...
- l. 166: ... whether MavQ participates in ...
- l. 209: ... MavQ and SidP interact in ...
- l. 214: ... ectopically produced MavQ ...
- l. 266: Thus, we propose a model ...
- l. 276: ... that this effector protein ...

Referee #2:

The MS by Li et al. reported the characterization of Legionella effector MavQ functions as a PI-3-knase specifically catalyzes the production of PtdIns3P from PtdIns. The authors performed elegant biochemical assays convincingly demonstrates the specific activity of MavQ. The authors further provided evidence to show that MavQ works together with other two previous identified PtdIns metabolizing enzymes (LepB and SidF) to facilitate the establishment of mature LCV, which is enriched with PtdIns4P. Overall, this is a nice piece of work and is an important addition towards our understanding of the control of phosphoinositides on the surface of LCV by legionella effectors.

Major points:

1, This paper presented data to show that the activity of MavQ is not affected by another effector SidP, which is contradictory to the previously published data (Urbanus et al. 2016). Does SidP suppress the toxicity of MavQ in yeast? It is necessary to provide these data particularly there are discrepancies between this and previous published manuscript.
2, Although the MavQ-LepB-SidF axis is possible mechanism for the accumulation of PtdIns4P on LCV, it is also possible that MavQ generates PtdIns3P on the LCV and provides an anchor for some other effectors such as SetA that associate with LCV through its specific binding with PtdIns3P. It would be idea to test this possibility by analyzing the localization of SetA or other PtdIns3P specific effectors on the LCV. Or at least discuss this possibility.
3, This paper presented evidence to show that unlike MavQ, Leg5A, a Legionella PI-3 kinase reported previously, does not affect the accumulation of PtdIns4P on the LCV suggested by the LCV-localization of SidC. This observation is certainly against our intuition. There is no difference of PtdIns3P produced by MavQ or Leg5A. Is there any difference in term of the activity/localization, etc. of these two enzymes?

Minors:
1. In figure 3b and 6c, the Y-axis is not clearly defined either in the methods section or in the legends. Is it the ratio of fluorescent intensity on the puncta vs. total intensity within the whole cell? Need to be clarified. Related to this,
Line 215, "Approximately 70% of GFP-2xFYVE uctate distribution was observed in MavQ ....." It is not clear what does this 70% mean here.

2. Line 104-106, "Because the PI kinase activity associated with LepB_NTD is also....PI metabolism." The logic of this sentence is not correct. The kinase activity of Lep_NTD is toxic does not suggest MavQ is also a kinase. Needs to be revised.

3. Line 137-139, "......pattern of GFP-2FYVEhrs was restored in cells treated with wortmannin." The GFP-2FYVEhrs pattern is never changed in this experiment. Need to change "restored" with a different word.

Referee #3:

The manuscript by Li et al describes the finding that MavQ is a PI3P kinase. Deletion of MavQ phenocopies the deletion of LepB, a PI4P kinase that adds a D4-phosphate to PI3P to create PI3,4P and SidF, a phosphatase that removes the D3-phospate from PI3,4P and PI3,4,5P, with regards to SidC localization on the LCV (indicative of PI4P pools at the LCV), suggesting they function in the same pathway. The sequential action of MavQ, LepB and SidF on PtdIns indeed creates PI4P lipids. Surprisingly, SidP a PI3P phosphatase which was previously shown to rescue the yeast growth defect by MavQ and shown to physically interact with MavQ, does not inhibit the activity of MavQ in in vitro reactions and the SidP deletion strain does not phenocopy the SidF deletion strain.

Major concerns:

1) Insufficient citation of prior work that informs the current study: The presentation of data in Figure 1 and 2, along with the framing of the introduction, give the false impression that this part of the manuscript is original work based on original insights. Specifically, in 2016 Urbanus et al (PMID: 27986836, ref. 37 of the current manuscript) showed via HHPred analysis that MavQ has homology to kinases including PI kinases (shown in figure 3C of that paper), that MavQ is toxic to yeast (shown in Figure 3B) and that when the predicted active site is mutated (specifically residues D147 and D160) this toxicity is alleviated (shown in Figure 3D). Urbanus et al hypothesized, based on the homology to the kinases, the fact that MavQ binds specific PIPs (Figure S3D) and the functional link with SidP (a PI3P phosphatase) that MavQ is a PI kinase. Those authors then go on test this hypothesis using an ADP-Glo assay (Figure 3E) which showed that MavQ can hydrolyze ATP, moreover the activity was stimulated by the addition of phosphoinositide (PtdIns) and is absent in the D147/D160 mutant.

To be clear, simply modifying this manuscript to reference past work (Ref 37) is insufficient. The text describing the prediction of MavQ function must be changed, the figures repeating past experiments must be retitled to describe where they are merely confirming past work versus adding to it. The present work is an important mechanistic extension of ref 37., thus please frame it as such. The introduction must mention that MavQ has been previously proposed to be a PI kinase based on all the same reasons that the authors use to start their current paper.

2) Figure 4a: how many hpi are these images taken? Does the first panel show superinfection or replication of the bacterium? If replication, how do the authors explain replication only 2hpi (Fig 4b)? If superinfection, how representative is this image for an infection (which MOI was used here? 2?).

3) Figure 5b ("Representative images of SidC anchoring on LCVs") is not altogether representative. As presented, the images for the delta-mavQ strain show no SidC positive vacuoles, yet the

quantification done in 5c clearly shows that even in this genetic background >50% of the LCVs are SidC+. Presentation of the data this way oversells the phenotype and gives a misleading impression of its severity. Show both classes of LCVs (SidC+ and SidC-) for each strain and put percentages in the frame.

4) Figure 5c: is the difference between delta-mavQ and delta-mavQ + pMavQ statistically significant? If not, how can one claim rescue?

More broadly, given the subtleties of these phenotypes, how were the samples blinded from the observer prior to quantification?

5) Figure 6b: Is the ADP-Glo assay subject to saturation? According to the manufacturer's website, the one example PI kinase saturates 3.6 x10^5 (https://www.promega.ca/products/cell-signaling/kinase-assays-and-kinase-biology/adp-glo-kinase-assay/?catNum=V6930). Without such a curve, is unclear that the authors are measuring MavQ activity within a linear range. As such, it is impossible to interpret the reduction in RLUs observed with SidP or whether increasing SidP by 10x would have an effect. Similarly, the PTase activity of SidP has the potential to complicates this assay, providing more substrate for MavQ.

The TLC assays performed in Fig2 might be a better approach.

6) Figure 6d: Why would anyone have predicted MavQ to inhibit SidP activity given that the interaction between the two proteins occurs within a non-catalytic C-terminal domain of SidP (ref 37)?

7) Figure 6d,e: It is unclear why the deletion of SidP is anticipated to give any phenotype in these assays. Wouldn't a more appropriate assay be the overexpression of SidP or the double mutant?

The authors themselves recognize the likely nuances of the biology underlying the interaction between MavQ and SidP (discussion, lines 292-304) but none of this nuance is present in the results section describing figure 6. Instead, the results section claims "... further suggesting that SidP plays no role in the activity of MavQ during L. pneumophila infection." That statement is simply not supported by the data contained within this manuscript. The incongruence between the certitude of the results and the more measured (probably correct) interpretation of the data in the discussion is hard to understand.

Minor/Specific comments:
Line 88: There are now more than 41 species described in the Legionellae genus. Extend to the 58 species described in Gomez-Valero et al, 2019.

Line 92 : "amino portion" amino-terminal portion?

Line 100: Ref 36 does not belong in this sentence (though this sentence will likely not exist in a reframed version of the manuscript that adequately addresses the contributions of past work to the present study).

Line 136: "In response to wortmannin, the inhibitor specifically against eukaryotic PI3Ks"
This misses a reference, in addition, wortmannin is not specific to PI3P kinases, it also inhibits other kinases like PI4KA and B (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3143478/#R8 and references therein), though with a higher IC50.

Line 150: "It is established that LCVs are enriched with PtdIns4P and many Dot/Icm substrates are associated with the bacterial phagosome via specifically binding to this PI." - This statement lacks references.

Line 172: " The association of the LCV will enable" - The association of MavQ with the LCV?

Lines 242-243: Is DrrA localization dependent on MavQ in the same way that SidC is?

Figure 3A: the merge column doesn't include the mCherry signal - no red for mCherry or yellow for co-localization.

Figure 3B: the description in the figure legend and axis is unclear: does this show the percent of cells that have vesicle-like localization or is this the ratio of vesicle-localized vs cytoplasmic GFP-2XFYVE within a cell?

Figure 4 and 5, why is the story line jumping from Figure 3 to Figure 5A to Figure 4, back to Figure 5?

Figure 4D shows a western blot of MavQ levels in strains used for infection, but the MavQ mutant (last lane of 4D) isn't used in the experiments shown in Figure 4.

Dear editor,

Thanks for handling our manuscript EMBOR-2020-51163V1. We appreciated the constructive suggestions made by the reviewers, which have been greatly helpful in improving our manuscript. We have modified the manuscript by adding new experimental results and careful revision in accordance with their comments. Our response to each of their comments is as follows. Please note that all changes in the manuscript are highlighted in green are also included in the “response to review” document.

Point by point responses

Response to Referee #1:

Specific points:

1) Fig. 3c: A positive control is missing for the inhibition of PI 3-kinase activity in vitro by wortmannin.

Response: Thanks for the advice. We have used the p110α as the positive control for the inhibition of PI 3-kinase activity by wortamannin in the revised Figure 3C and 3D.
Lines 150-152 of the revised manuscript: “In contrast, the activity of p110α, the mammalian PI3K , which is known to be sensitive to wortmannin (Kumar & Doss, 2016), is inhibited **(Fig 3C and D)**”

2) Fig. 4c: The L. pneumophila mavQ mutant strain does not show a (pronounced) intracellular growth phenotype. It would be informative to test intracellular replication of the mavQ-lepB double mutant and - if available - a mavQ-lepB-sidF triple mutant strain.

Response: Thanks! We tested the intracellular growth phenotype of a mutant lacking both *mavQ* and *lepB*. As shown in Figure EV1, the deletion of *mavQ* and *lepB* did not cause significant growth defect in BMDMs.

Lines 213-215 of the revised manuscript: "Consistent with this phenotype, a mutant lack of both *mavQ* and *lepB* did not significantly affect the intracellular replication of *L. pneumophila* (**Fig EV1**)."

3) Fig. 4a, 5b and 6d: Consider zooming-in to increase the validity and resolution of the images.

Response: Thanks! We have added zoomed-in images of the LCVs in each of these figures to more clearly show the association of the relevant proteins with the bacterial phagosomes.

4) The paragraphs, wherein Fig. 5a and Fig. 4c-d are called-out are placed oddly in the manuscript. The flow of the text would be improved, if the figures were called-out in the sequence they are already arranged. Accordingly, consider ordering the paragraphs in the sequence Fig. 4a-b, Fig. 4c-d, Fig. 5a and Fig. 5b-d.

Response: Thanks! We have reorganized these paragraphs with the addition of a new subtitle in the Results section. Please find the revision of this part in the new subtitle "**MavQ is associated with the LCV and is dispensable for the intracellular growth of *L. pneumophila***"

5) Some statements should be referenced/phrased more precisely, and some additional references should be discussed:

- l. 152 and l. 241: Ref. #43 should be cited.

Response: Thanks! The reference has been added in the revised manuscript.

- l. 238: Technically, it has not been shown that the interception of the LCV with ER-derived vesicles results in pathogen vacuole expansion. The statement should be phrased more cautiously.

Response: Thanks! We have rephrased the statement.
Line 255 of the revised manuscript: "Vesicles originating from the ER have been suggested to be one major source of the membrane materials needed to compensate the expansion of LCVs containing multiplying bacteria (Kagan & Roy, 2002)."

- l. 244/245: Rab10 has been previously implicated in intracellular growth of L. pneumophila by RNA interference (Hoffmann et al. (2014) Cell Microbiol 16:1034-52). This reference should also be cited.

Response: Thanks! We have added the reference.

- l. 267-270: The accumulation of PtdIns4P on LCVs (PI conversion from PtdIns3P to PtdIns4P) also involves capturing Golgi-derived, PtdIns4P-positive vesicles (Weber et al. (2018) mBio 9:e02420-18). This reference should be discussed and cited.

Response: Thanks! We have revised the manuscript according to your suggestion.
Lines 291-292 of the revised manuscript: "Indeed, the accumulation of PtdIns4P on the LCVs also involves capturing Golgi-derived PtdIns4P-positive vesicles (Weber *et al*, 2018)"

- l. 302: PtdIns3P-binding effector proteins have been previously identified : Ref. #44; Jank et al. (2012) Cell Microbiol 14:852-68; Finsel et al. (2013) Cell Host Microbe 14:38-50. These references should also be cited here.

Response: Thanks! We have added these references.

6) Typos/wording:
- l. 39: ... to a lesser extent ...
- l. 43: ... small GTPases of the Arf ...
- l. 135: In response to wortmannin, a specific inhibitor of eukaryotic PI3Ks, fluorescence signals were ...
- l. 166: ... whether MavQ participates in ...
- l. 209: ... MavQ and SidP interact in ...
- l. 214: ... ectopically produced MavQ ...
- l. 266: Thus, we propose a model ...
- l. 276: ... that this effector protein ...

Response: Thanks! We have corrected the Typos/wording according to the suggestions. Please find relative changes in the lines 44, 50, 143, 224, 233, 287, 299 of the revised manuscript.

Response to Referee #2:

Major points:

1, This paper presented data to show that the activity of MavQ is not affected by another effector SidP, which is contradictory to the previously published data (Urbanus et al. 2016). Does SidP suppress the toxicity of MavQ in yeast? It is necessary to provide these data particularly there are discrepancies between this and previous published manuscript.

Response: Thanks! We have added the yeast suppression data in the revised manuscript **(Fig EV2)**.
Lines 226-229 of the revised manuscript: "In our yeast suppression assay, wild-type SidP, the catalytically inactive mutant $SidP_{R560K}$ as well as the carboxyl fragment of SidP ($SidP_{664-822}$) each indeed suppresses the toxicity of MavQ **(Fig EV2).**"

2, Although the MavQ-LepB-SidF axis is possible mechanism for the accumulation of PtdIns4P on LCV, it is also possible that MavQ generates PtdIns3P on the LCV and provides an anchor for some other effectors such as SetA that associate with LCV through its specific binding with PtdIns3P. It would be idea to test this possibility by analyzing the localization of SetA or other PtdIns3P specific effectors on the LCV. Or at least discuss this possibility.

Response: Thanks for the suggestion. We obtained the SetA antibody from Dr. Xiaoyun Liu from Peking university (Wang et al. Cell Discov. 2018 Oct 9;4:53. doi: 10.1038/s41421-018-0055-9. eCollection 2018.). However, due to its poor reactivity, immunostaining of the LCV with SetA antibody failed to obtain any discernible fluorescent signal. Instead, we have discussed the possibility in the revised manuscript.
Line 301-305 of the revised manuscript: "PtdIns3P is also an important anchor for some effector proteins that harbor PtdIns3P-binding domains, allowing their association with the LCV (Finsel *et al*, 2013; Jank *et al*, 2012; Nachmias *et al*, 2019). Therefore, MavQ may play a role in the localization of PtdIns3P-binding effector proteins on the LCV through its compartmentalized generation of PtdIns3P."

3, This paper presented evidence to show that unlike MavQ, Leg5A, a Legionella PI-3 kinase reported previously, does not affect the accumulation of PtdIns4P on the LCV suggested by the LCV-localization of SidC. This observation is certainly against our intuition. There is no difference of PtdIns3P produced by MavQ or Leg5A. Is there any difference in term of the activity/localization, etc. of these two enzymes?

Response: Thanks! We did observe the association of LegA5 on the LCV, but at a percentage considerably lower than that of MavQ (panel A). Results reported in the previous study (Ledvina *et al.* Cell Host Microbe 24, 285, 2018) did not conclusively demonstrate that LegA5 functions as a PI3K. LegA5 catalyzes the formation of PIP, but whether the product is PI3P is not clear. We have used the TLC assay to characterize the product of LegA5. Consistent with the previous study, LegA5 indeed catalyzes the production of PIP from PtdIns. However, only a small fraction of the PIP produced by this protein can be hydrolyzed into PtdIns by the 3' phosphatase MTM or be used by LepB-N to generate PtdIns(3,4)P2 (panel B and C). In addition, a portion of PIP generated by LegA5 can be hydrolyzed into PtdIns by Sac1 (the phosphatase dephosphorylates PtdIns4P and to a lesser extent PtdIns3P) (panel B and C). Taken together, our data indicate that the product of LegA5 likely is a mixture of PtdIns3P and PtdIns5P. Therefore, its contribution on the lipid composition of LCV requires further investigation. The exact biochemical activity of LegA5 and its role in *L. pneumophila* pathogenesis is currently being investigated in our laboratory. [Figures for referees not shown.]

Minors:

Response:

1. In figure 3b and 6c, the Y-axis is not clearly defined either in the methods section or in the legends. Is it the ratio of fluorescent intensity on the puncta vs. total intensity within the whole cell? Need to be clarified. Related to this, Line 215, "Approximately 70% of GFP-2xFYVE puctate distribution was observed in MavQ ....." It is not clear what does this 70% mean here.

Response: Thanks! The ratio is calculated by the cells with the puncta distribution (vesicle-like structure) of GFP-2xFYVE in the total cells counted. We have changed the Y-axis title as "2xFYVE localized to vesicle-like structures (% cells)". In addition, we have revised the sentence (lines 233-236 of the revised manuscript) into "In MavQ and SidP co-transfected cells treated with wortmannin, approximately 70% of the cells showed vesicular localization of GFP-2xFYVE$_{Hrs}$, which was similar to samples transfected to express MavQ alone **(Fig 6D)**"

2. Line 104-106, "Because the PI kinase activity associated with LepB_NTD is also....PI metabolism." The logic of this sentence is not correct. The kinase activity of Lep_NTD is toxic does not suggest MavQ is also a kinase. Needs to be revised.

Response: Thanks! This sentence has been deleted in the revised manuscript.

3. Line 137-139, "......pattern of GFP-2FYVEhrs was restored in cells treated with wortmannin." The GFP-2FYVEhrs pattern is never changed in this experiment. Need to change "restored" with a different word.

Response: Thanks! We have changed the word “restored” into “not affected”.

Response to Referee #3:

Major concerns:

1) Insufficient citation of prior work that informs the current study: The presentation of data in Figure 1 and 2, along with the framing of the introduction, give the false impression that this part of the manuscript is original work based on original insights. Specifically, in 2016 Urbanus et al (PMID: 27986836, ref. 37 of the current manuscript) showed via HHPred analysis that MavQ has homology to kinases including PI kinases (shown in figure 3C of that paper), that MavQ is toxic to yeast (shown in Figure 3B) and that when the predicted active site is mutated (specifically residues D147 and D160) this toxicity is alleviated (shown in Figure 3D). Urbanus et al hypothesized, based on the homology to the kinases, the fact that MavQ binds specific PIPs (Figure S3D) and the functional link with SidP (a PI3P phosphatase) that MavQ is a PI kinase. Those authors then go on test this hypothesis using an ADP-Glo assay (Figure 3E) which showed that MavQ can hydrolyze ATP, moreover the activity was stimulated by the addition of phosphoinositide (PtdIns) and is absent in the D147/D160 mutant.

To be clear, simply modifying this manuscript to reference past work (Ref 37) is insufficient. The text describing the prediction of MavQ function must be changed, the figures repeating past experiments must be retitled to describe

where they are merely confirming past work versus adding to it. The present work is an important mechanistic extension of ref 37., thus please frame it as such. The introduction must mention that MavQ has been previously proposed to be a PI kinase based on all the same reasons that the authors use to start their current paper.

Response: Thank you very much! We sincerely accept the criticisms on the presentation of this part of our manuscript. In the revision we have extensively revised the text according to the comments. The major changes include:

(i). In the introduction part: "An earlier study by Urbanus *et al* (Urbanus *et al*, 2016) reported that the *L. pneumophila* Dot/Icm effector MavQ (Lpg2975) (Huang *et al*, 2011) is a PI kinase, but the exact PI product synthesized by MavQ and the biological roles of MavQ during *L. pneumophila* infection has not yet been defined. Here, we demonstrate that MavQ catalyzes the formation of PtdIns3P using PtdIns as substrate. We also found that MavQ works together with LepB and SidF to sequentially synthesize PtdIns4P on the LCV surface."

(ii). We have deleted the subtitle "MavQ habors a functional kinase motif at its N-terminal region" in the results section. Some of the contents have been integrated into the section under the subtitle "**MavQ is a phosphatidylinositol specific phosphoinositide 3-kinase (PI3K)**". The description on the HHpred analysis has been completely deleted in the revised manuscript.

(iii). In the results section: Lines 105-108, "It has been demonstrated that MavQ is a kinase potentially participated in the modulation of host PI metabolism (Urbanus *et al.*, 2016). However, neither the PI products generated by MavQ nor the biological roles of MavQ during *L. pneumophila* infection has been elucidated. " Lines 110-112, "It has been shown that MavQ is lethal to yeast (Burstein *et al*, 2015; Urbanus *et al*., 2016). Consistent with previous study (Urbanus *et al.*, 2016), mutations in residues predicted to be important for catalysis or ATP binding abolished its yeast toxicity **(Fig 1A)**."

2) Figure 4a: how many hpi are these images taken? Does the first panel show superinfection or replication of the bacterium? If replication, how do the authors explain replication only 2hpi (Fig 4b)? If superinfection, how representative is this image for an infection (which MOI was used here? 2?).

Response: Thanks! The images in Figure 4a were taken from samples infected with Legionella for 2 hour at an MOI of 2. Apparently, the bacteria have not grown at 2 hour post infection. The image for the WT sample represents two vacuoles.

3) Figure 5b ("Representative images of SidC anchoring on LCVs") is not altogether representative. As presented, the images for the delta-mavQ strain show no SidC positive vacuoles, yet the quantification done in 5c clearly shows that even in this genetic background >50% of the LCVs are SidC+. Presentation of the data this way oversells the phenotype and gives a misleading impression of its severity. Show both classes of LCVs (SidC+ and SidC-) for each strain and put percentages in the frame.

Response: Thanks! We have revised Figure 5b according to your suggestion. We provided representative images of SidC+ and SidC- for the delta-mavQ sample.

4) Figure 5c: is the difference between delta-mavQ and delta-mavQ + pMavQ statistically significant? If not, how can one claim rescue?

Response: Thanks! We have calculated the p value (p=0.042) between delta-mavQ and delta-mavQ + pMavQ. p<0.05 indicates significant difference between the two samples.

More broadly, given the subtleties of these phenotypes, how were the samples blinded from the observer prior to quantification?

Response: Thanks! Authors were blinded for quantitative analysis. One lab members coded the treatment or bacterial strains used for the experiments and the other performed the experiments, including infection, processing and quantitation.

5) Figure 6b: Is the ADP-Glo assay subject to saturation? According to the manufacturer's website, the one example PI kinase saturates 3.6 x10^5 (https://www.promega.ca/products/cell-signaling/kinase-assays-and-kinase-biology/adp-glo-kinase-assay/?catNum=V6930). Without such a curve, is unclear that the authors are measuring MavQ activity within a linear range. As such, it is impossible to interpret the reduction in RLUs observed with SidP or

whether increasing SidP by 10x would have an effect. Similarly, the PTase activity of SidP has the potential to complicates this assay, providing more substrate for MavQ.

The TLC assays performed in Fig2 might be a better approach.

Response: Thank you very much! We have performed the TLC assays as you suggested. Please find the data in revised Fig. 6C.
Lines 230-233 of the revised manuscript: "However, adding recombinant SidP into reactions containing PtdIns and MavQ did not significantly affect its kinase activity, even in reactions in which the molar ratio between SidP and MavQ was 10: 1 **(Fig 6B and C)**".

6) Figure 6d: Why would anyone have predicted MavQ to inhibit SidP activity given that the interaction between the two proteins occurs within a non-catalytic C-terminal domain of SidP (ref 37)?

Response: Thanks for pointing out this problem. We have deleted the Fig. 6d of the original manuscript.

7) Figure 6d,e: It is unclear why the deletion of SidP is anticipated to give any phenotype in these assays. Wouldn't a more appropriate assay be the overexpression of SidP or the double mutant?

Response: Thanks! We have performed the experiment with the overexpression strain. Please find the data in the Figure EV3.
Lines 237-239 of the revised manuscript "deletion or overexpression of *sidP* does not affect the association of SidC with the LCV **(Figs 6E and EV3)**".

The authors themselves recognize the likely nuances of the biology underlying the interaction between MavQ and SidP (discussion, lines 292-304) but none of this nuance is present in the results section describing figure 6. Instead, the results section claims "... further suggesting that SidP plays no role in the activity of MavQ during L. pneumophila infection." That statement is simply not supported by the data contained within this manuscript. The incongruence between the certitude of the results and the more measured (probably correct) interpretation of the data in the discussion is hard to understand.

Response: Thanks! We have revised the part as following:

Lines 236-239 of the revised manuscript: Thus, SidP does not interfere with the activity of MavQ, at least with regard to its PI3K enzymatic activity. Consistent with these observations, deletion or overexpression of *sidP* does not affect the association of SidC with the LCV **(Figs 6E and EV3)**. Thus, it appears that SidP does not affect the PI3K activity of MavQ in biochemical assays. The biological relevance of its ability to suppress MavQ toxicity awaits further investigation.

Minor/Specific comments:

Line 88: There are now more than 41 species described in the Legionellae genus. Extend to the 58 species described in Gomez-Valero et al, 2019.

Response: Thanks! We have revised this part according to your suggestion. Lines 103-105 of the revised manuscript: "MavQ is a Dot/Icm substrate that is widely distributed in *Legionella* spp., and genes coding for this protein are present in 71 out of the 80 sequenced genomes covering 58 *Legionella* species and subspecies (Gomez-Valero *et al*, 2019)."

Line 92 : "amino portion" amino-terminal portion?

Response: Thanks! The sentence has been deleted in the revised manuscript.

Line 100: Ref 36 does not belong in this sentence (though this sentence will likely not exist in a reframed version of the manuscript that adequately addresses the contributions of past work to the present study).

Response: Thanks! Actually, the yeast toxicity of MavQ (lpg2975) was firstly described in Ref #36 of the original manuscript. Please the result image from the ref. 36 below. [Figure for referees not shown.]

Line 136: "In response to wortmannin, the inhibitor specifically against eukaryotic PI3Ks"
This misses a reference, in addition, wortmannin is not specific to PI3P kinases, it also inhibits other kinases like PI4KA and B (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3143478/#R8 and references therein), though with a higher IC50.

Response: Thanks! We have deleted the word "specific". We also added a reference.

Line 150: "It is established that LCVs are enriched with PtdIns4P and many Dot/Icm substrates are associated with the bacterial phagosome via specifically binding to this PI. " - This statement lacks references.

Response: Thanks! We have added the references.

Line 172: " The association of the LCV will enable" - The association of MavQ with the LCV?

Response: Thanks! This sentence is no longer existed in the revised manuscript.

Lines 242-243: Is DrrA localization dependent on MavQ in the same way that SidC is?

Response: Thanks! Similar to SidC, the association of DrrA/SidM on the LCV is strictly dependent on its PI4P binding domain. Therefore, we believed that MavQ will also affect the localization of DrrA/SidM as well as other effector proteins which harbor PI4P binding domain.

Figure 3A: the merge column doesn't include the mCherry signal - no red for mCherry or yellow for co-localization.

Response: Thanks! We have added mCherry signals in the “merge” column of Figure 3A.

Figure 3B: the description in the figure legend and axis is unclear: does this show the percent of cells that have vesicle-like localization or is this the ratio of vesicle-localized vs cytoplasmic GFP-2XFYVE within a cell?

Response: Thanks! The ratio is calculated by the cells with the puncta distribution (vesicle-like structure) of GFP-2xFYVE in the total cells counted. We have changed the Y-axis title as “2xFYVE localized to vesicle-like structures (% cells)”.

Figure 4 and 5, why is the story line jumping from Figure 3 to Figure 5A to Figure 4, back to Figure 5?

Response: Thanks! We have reorganized the paragraphs with the addition of a new subtitle in the Results section.

Figure 4D shows a western blot of MavQ levels in strains used for infection, but the MavQ mutant (last lane of 4D) isn't used in the experiments shown in Figure 4.

Response: Thanks! The last lane of Figure 4D has been cropped.

1st Revision - Editorial Decision **18th Nov 2020**

Dear Prof. Qiu

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. Nevertheless, referees #1 and #3 have remaining concerns and suggestions to improve the manuscript I ask you to address in a final revised version. Please also provide a point-by-point response that addresses the remaining points of the referees.

Further, I have these editorial requests I ask you to address:

- Please provide a more active title with not more than 100 characters (including spaces).

- Please have your final manuscript (including the methods section and the figure legends) carefully proofread by a native English speaker (see also the referee reports). There are still typos and grammatical errors present.

- Please add a formal "Data Availability section" to the manuscript after the methods section. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated or deposited').

- Please provide all the microscopic images with scale bars of similar style and thickness. Please define their size only in the respective figure legend. Presently, some scale bars are too small (see e.g. Fig. 6E).

- Please sequentially call out each of the single panels of Fig EV4 in the manuscript text.

- Please make sure that regarding data quantification and statistics, the number "n" for how many independent experiments (biological vs technical replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is specified in the respective figure legends (ALSO in the Appendix). Please provide statistical testing where applicable. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

- Could statistical testing be performed for the data in Figs. 2A/B, 4B (presently partial), 4D and EV1?

- In the figure legends you state several times that the data shown are from 'independent experiments'. Please indicate in each case if these were biological or technical replicates, and how many.

- Please name the 'Methods' section 'Materials and Methods'.

- Please add a TOC (table of contents) to the Appendix file with page numbers. Best split this table in three (Bacterial strains S1, plasmids S2, primers S3), and change the callouts in the manuscript text. Please name the tables Appendix Table Sx and use this as callout in the manuscript text.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with

changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The study provides novel and intriguing mechanistic insights into how L. pneumophila steers the production of PtdIns4P on the Legionella-containing vacuole. The authors did a thorough and convincing job experimentally revising the manuscript and addressing the reviewers' points. In particular, the authors also addressed the concerns regarding the acknowledgment of previous work and the overall novelty of the current study.
A few typos should still be corrected:
- l. 106: ... MavQ is a kinase potentially participating in ...
- l. 111: Consistent with a previous study ...

Referee #2:

the revision is satisfactory

Referee #3:

I appreciate the efforts of the authors to address my initial review. I have a few outstanding concerns and several suggestions as outlined below.

Major comments
1) I remain somewhat unconvinced with the in vitro data presented in Fig 6B,C. In my original comment, I suggested that the ADP-Glo assay may be subject to saturation, limiting the ability of this assay to detect a reduction in kinase activity. Similarly, by using catalytically active SidP in these assays, the pool of PtdIns is likely being increased, providing more substrate for the kinase. The new results, in Fig 6C are a significant improvement, but they are complicated by the inclusion

of a catalytically active SidP. Presumably the increase in PtdIns is due to this activity, but it a clearer result would have followed from using the SidP catalytically dead mutant (or the C-terminus) both of which were shown previously to inhibit MavQ acitivity in vivo.

2) Referencing could be improved. Consider referencing primary literature when possible.

3) The new Fig 5b is improved. Consider including % of each class in the figure itself to better illustrate how representative each class is.

Specific comments:

Line 58: "By controlling the activation cycle of Rab1, these two effectors facilitate the fusion of ER-derived vesicles to the LCV (Arasaki et al, 2012)" - Arasaki et al 2012 describes DrrA/SidM function, not LepB.

Line 71: "These lipids are minor phospholipid (less than 10%) of the organellar membranes localizing on their cytoplasmic surfaces." - consider "minor phospholipid components"

Line 105: "It has been demonstrated that MavQ is a kinase potentially participated in the modulation of host PI metabolism (Urbanus et al., 2016)." - consider "participating" instead of "participated"

Line 110: "It has been shown that MavQ is lethal to yeast (Burstein et al, 2015; Urbanus et al., 2016)." - Missing reference Nevo et al 2013 (PMID 24272784), which is the original paper where MavQ toxicity in yeast was shown and is referred to in Burstein et al. for the yeast toxicity of MavQ/Lpg2975.

Lines 114-120: This section is an important extension but inadequately frames prior work. Urbanus et al showed, using the same ADP-GLO assay, that there was a basal level of kinase activity and that was increased in the presence of PtdIns. They also showed that this was absent in the kinase motif mutant D160A. The inclusion of other PIs in the current work is indeed an important extension but should mention that the data is in agreement with previous data.

Line 142: "In mammalian cells, GFP-2xFYVEHrs localizes to the early endosomal membrane through interacting with PtdIns3P (Gaullier et al., 1998)" - "by interacting" or "through interaction"

Line 213: "Consistent with this phenotype, a mutant lack of both mavQ and lepB did not significantly affect the intracellular replication of L. pneumophila (Fig EV1)." - "the mutant lacking both mavQ and lepB"

Line 254: "Vesicles originating from the ER have been suggested to be one major source of the membrane materials needed to compensate the expansion of LCVs containing multiplying bacteria (Kagan & Roy, 2002). " to support?

Line 348: "The integrative yeast plasmid pSB157 (Tan & Luo, 2011) was used to express MavQ in S. cerevisiae strain W303a (Tan et al., 2011)"

These are not appropriate references for the plasmid or the strain. W303 was created by Rodney Rothstein who refers to Thomas and Rothstein 1989 (https://doi.org/10.1016/0092-8674(89)90584-9) in his own papers. In the in previous papers from Dr. Luo (Shen et al., 2009) the plasmid was

referred to with " was cloned into pSB157 (Fazzio and Tsukiyama, 2003) (courtesy of Sue Biggins, Fred Hutchinson Cancer Research Center, Seattle, WA)".

Line 397: MavQ or its point mutants were inserted into pSB157 (Tan & Luo, 2011) which contains a galactose-inducible promoter and transformed into the S. cerevisiae strain W303a (Tan et al., 2011). See comment above.

In addition the supplementary table with bacterial strains and plasmids mentions pSB157m and a different reference, to Qiu et al 2016. No modification of the plasmid was described in that paper (or the pSB157m name mentioned).

Line 403: "To determine the influence of SidP on the yeast toxicity of MavQ, wild-type SidP, SidPR560K and SidP664-822 were cloned into p425GPD and transformed into W303 (pSB157::MavQ). The spotting of the yeast cells on selection medium containing glucose or galactose were performed similarly as described above."- Source or reference for p423GPD not available in the supplementary plasmid table. Reference for the active site mutant is Toulabi et al, reference for the C-terminal fragment that will rescue MavQ is Urbanus et al.

Line 453: "E7, 1: 10,000); anti-ICDH (1: 20,000) (Xu et al., 2010), anti-MavQ (1: 1000), anti-SidC "
Xu et al doesn't describe anti-ICDH but refers to Liu et al. This paper only mentions use, but not source or reference.

Figures:
Figure 3B and figure legend:
Figure 3B shows GFP-FYVE localized to vesicle-like structures in % cells, the figure legend describes ratios of GFP-2XFYVE localized to vesicle like structures. Please clarify how the ratio of GFP-2XFVYE is represented by % cells. Please also define the +wort conditions.

Figure 4:
Legend 4A: define scale bar.

Figure 6:
Legend 6D: define + wort

Supplementary table S1:
The references of Berger and Isberg and Dumenil and Isberg are not in the main references list or properly described in the supplementary table. Please add a reference list to the table.

p425GPD entry is missing
GFP-2xFYVEHrs - Dong et al does not describe the construction of this plasmid. Please provide an appropriate description and reference (Cao et al 2008 PMID 18524850 described the construction of pEGFP-2xFYVEHrs using the pEGFP-C2 vector from clonetech). In addition, in the Methods the plasmid is described as peGFP-2xFYVEHrs and in the table as GFP::2FYVEHrs
pRS47S reference should be Merriam et al 1997 (PMID 9169800)
pSB157 - Sue Biggings Fred Hutchinson Cancer Research Center, Seattle, WA
W303 - R. Rothstein and appropriate reference
pCMV-4XFLAG - the CMV plasmid mentioned in Xu et al 2010 was pFLAG-CMV from Sigma, no modifications of this backbone were described to warrant a reference to this paper.
pcDNA3.1mCherry - Addgene.

Addgene on citation:
"These plasmids were created by your colleagues. Please acknowledge the Principal Investigator, cite the article in which the plasmids were described, and include Addgene in the Materials and Methods of your future publications.
• For your Materials & Methods section: pcDNA3.1-mCherry was a gift from David Bartel (Addgene plasmid # 128744 ; http://n2t.net/addgene:128744 ; RRID:Addgene_128744)
• For your References section: A Network of Noncoding Regulatory RNAs Acts in the Mammalian Brain. Kleaveland B, Shi CY, Stefano J, Bartel DP. Cell. 2018 Jul 12;174(2):350-362.e17. doi: 10.1016/j.cell.2018.05.022. Epub 2018 "Jun 7. 10.1016/j.cell.2

Dear Dr. Breiling,

Thanks for handling our manuscript EMBOR-2020-51163V2. We appreciated the positive comments by the reviewers. We have revised the manuscript according to their suggestions. Our responses to each of their comments is as follows. Please note that all changes in the manuscript are highlighted in yellow are also included in the “response to review” document.

Response to the editor:

- Please provide a more active title with not more than 100 characters (including spaces).

Response: Thanks. We have revised the title into “**Modulation of phagosome phosphoinositide dynamics by a *Legionella* phosphoinositide 3-kinase**”.

- Please have your final manuscript (including the methods section and the figure legends) carefully proofread by a native English speaker (see also the referee reports). There are still typos and grammatical errors present.

Response: Thanks. We have asked a colleague who is a native English speaker to proofread our manuscript.

- Please add a formal "Data Availability section" to the manuscript after the methods section. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated or deposited').

Response: Thanks. We have added the “Data availability” section after the Methods section.

- Please provide all the microscopic images with scale bars of similar style and thickness. Please define their size only in the respective figure legend. Presently, some scale bars are too small (see e.g. Fig. 6E).

Response: Thanks. We have revised some of the figures by adjusting the scale bars.

- Please sequentially call out each of the single panels of Fig EV4 in the manuscript text.

Response: Thanks. We have revised the manuscript text as your suggestion.

- Please make sure that regarding data quantification and statistics, the number "n" for how many independent experiments (biological vs technical replicates) were performed, the bars and error bars (e.g. SEM, SD) and the

test used to calculate p-values is specified in the respective figure legends (ALSO in the Appendix). Please provide statistical testing where applicable. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis
- In the figure legends you state several times that the data shown are from 'independent experiments'. Please indicate in each case if these were biological or technical replicates, and how many.

Response: Thanks. We have revised our manuscript according to your suggestions. Please find the relative changes in the figure legends.

- Could statistical testing be performed for the data in Figs. 2A/B, 4B (presently partial), 4D and EV1?
Response: Thanks. We have added the *p* values of Figs. 2A/B and 4D. We have followed the methods and data presentation used by numerous papers in the *Legionella* field to present data on the intracellular growth of the bacteria. Our data presentation of Figs. 4D and EV1 are in line with those commonly used in the field.

- Please name the 'Methods' section 'Materials and Methods'.
Response: We have changed "Methods" into "Materials and Methods".

- Please add a TOC (table of contents) to the Appendix file with page numbers. Best split this table in three (Bacterial strains S1, plasmids S2, primers S3), and change the callouts in the manuscript text. Please name the tables Appendix Table Sx and use this as callout in the manuscript text.
Response: Thanks. We have revised the Appendix file according to your suggestions.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.
Response: Thanks. We have revised our manuscript according to comments. Please find those modifications in our final manuscript using track changes.

Response to Referee #1:

A few typos should still be corrected:
- l. 106: ... MavQ is a kinase potentially participating in ...

- l. 111: Consistent with a previous study ...
Response: We have revised the typos according to the suggestions.

Response to Referee #3:

Major comments

1) I remain somewhat unconvinced with the in vitro data presented in Fig 6B,C. In my original comment, I suggested that the ADP-Glo assay may be subject to saturation, limiting the ability of this assay to detect a reduction in kinase activity. Similarly, by using catalytically active SidP in these assays, the pool of PtdIns is likely being increased, providing more substrate for the kinase. The new results, in Fig 6C are a significant improvement, but they are complicated by the inclusion of a catalytically active SidP. Presumably the increase in PtdIns is due to this activity, but it a clearer result would have followed from using the SidP catalytically dead mutant (or the C-terminus) both of which were shown previously to inhibit MavQ acitivity in vivo.

Response: Thanks for the comment. Actually, when we set up the samples for the kinase assay, we were worried that the phosphatase activity of SidP may potentially influence our interpretation of the results. In lanes 5 and 6 of the Fig. 6C, the amount of PtdIns increased in the presence of 5xSidP or 10xSidP. We believed that the increased PtdIns was due to the catalytic activity of SidP. Indeed, 10xSidP itself in the kinase buffer can hydrolyze significant amount of PtdIns3P (lane 7). In the source file of Fig. 6C, we have two other samples which measure the ability of 5x$SidP_{R560K}$ and 10x$SidP_{R560K}$ to hydrolyze PtdIns3P in the kinase buffer. We did not show these two lanes in the Figure used in the main text of the R1 version of the manuscript. We have now included these two lanes in the revised figure (lanes 8 and 9) (please see below).[Figure for referees not shown.]

2) Referencing could be improved. Consider referencing primary literature when possible.
Response: Thanks. We have changed the references by using primary literature when appropriate according to your suggestions.

3) The new Fig 5b is improved. Consider including % of each class in the figure itself to better illustrate how representative each class is.
Response: Thanks. We have revised Fig 5b according to your suggestion.

Specific comments:

Line 58: "By controlling the activation cycle of Rab1, these two effectors facilitate the fusion of ER-derived vesicles to the LCV (Arasaki et al, 2012)" - Arasaki et al 2012 describes DrrA/SidM function, not LepB.
Response: Thanks. We have changed the sentence into “By controlling the activation cycle of Rab1, SidM/DrrA facilitates the fusion of ER-derived vesicles to the LCV”.

Line 71: "These lipids are minor phospholipid (less than 10%) of the organellar membranes localizing on their cytoplasmic surfaces." - consider "minor phospholipid components"
Response: Thanks. We have changed into “minor phospholipid components” in the revised text.

Line 105: "It has been demonstrated that MavQ is a kinase potentially participated in the modulation of host PI metabolism (Urbanus et al., 2016)." - consider "participating" instead of "participated"
Response: Thanks. We have revised the word as your suggestion.

Line 110: "It has been shown that MavQ is lethal to yeast (Burstein et al, 2015; Urbanus et al., 2016)." - Missing reference Nevo et al 2013 (PMID 24272784), which is the original paper where MavQ toxicity in yeast was shown and is referred to in Burstein et al. for the yeast toxicity of MavQ/Lpg2975.
Response: Thanks. We have added the reference (PMID: 24272784).

Lines 114-120: This section is an important extension but inadequately frames prior work. Urbanus et al showed, using the same ADP-GLO assay, that there was a basal level of kinase activity and that was increased in the presence of PtdIns. They also showed that this was absent in the kinase motif mutant D160A. The inclusion of other PIs in the current work is indeed an important extension but should mention that the data is in agreement with previous data.
Response: Thanks. We have revised this part according to your suggestion.

Line 120. “These observations are in good agreement with previous data (Urbanus et al., 2016).”

Line 142: "In mammalian cells, GFP-2xFYVEHrs localizes to the early endosomal membrane through interacting with PtdIns3P (Gaullier et al., 1998)" - "by interacting" or "through interaction"
Response: Thanks. We have revised the word as your suggestion.

Line 213: "Consistent with this phenotype, a mutant lack of both mavQ and lepB did not significantly affect the intracellular replication of L. pneumophila (Fig EV1)." - "the mutant lacking both mavQ and lepB"
Response: Thanks. We have changed “a” into “the”.

Line 254: "Vesicles originating from the ER have been suggested to be one major source of the membrane materials needed to compensate the expansion of LCVs containing multiplying bacteria (Kagan & Roy, 2002). " to support?
Response: Thanks. We have revised the word as your suggestion.

Line 348: "The integrative yeast plasmid pSB157 (Tan & Luo, 2011) was used to express MavQ in S. cerevisiae strain W303a (Tan et al., 2011)"
These are not appropriate references for the plasmid or the strain. W303 was created by Rodney Rothstein who refers to Thomas and Rothstein 1989 (https://doi.org/10.1016/0092-8674(89)90584-9) in his own papers. In the in previous papers from Dr. Luo (Shen et al., 2009) the plasmid was referred to with " was cloned into pSB157 (Fazzio and Tsukiyama, 2003) (courtesy of Sue Biggins, Fred Hutchinson Cancer Research Center, Seattle, WA)".
Line 397: MavQ or its point mutants were inserted into pSB157 (Tan & Luo, 2011) which contains a galactose-inducible promoter and transformed into the S. cerevisiae strain W303a (Tan et al., 2011).
See comment above.
In addition the supplementary table with bacterial strains and plasmids mentions pSB157m and a different reference, to Qiu et al 2016. No modification of the plasmid was described in that paper (or the pSB157m name mentioned).
Response: Thank you very much. We have made the following changes.
1. pSB157m was modified from the integrative yeast plasmid pSB157 (Fazzio & Tsukiyama, 2003) (courtesy of Sue Biggins, Fred Hutchinson Cancer Research Center, Seattle, WA) with the addition of a Flag tag and several restriction sites for inserting genes of interest.
2. We have also made changes in the table S2.

Line 403: "To determine the influence of SidP on the yeast toxicity of MavQ, wild-type SidP, SidPR560K and SidP664-822 were cloned into p425GPD and

transformed into W303 (pSB157::MavQ). The spotting of the yeast cells on selection medium containing glucose or galactose were performed similarly as described above."- Source or reference for p423GPD not available in the supplementary plasmid table. Reference for the active site mutant is Toulabi et al, reference for the C-terminal fragment that will rescue MavQ is Urbanus et al.
Response: Thanks. We have added the references.

Line 453: "E7, 1: 10,000); anti-ICDH (1: 20,000) (Xu et al., 2010), anti-MavQ (1: 1000), anti-SidC "
Xu et al doesn't describe anti-ICDH but refers to Liu et al. This paper only mentions use, but not source or reference.
Response: Thanks. We have changed the reference into Liu & Luo, 2007.

Figures:
Figure 3B and figure legend:
Figure 3B shows GFP-FYVE localized to vesicle-like structures in % cells, the figure legend describes ratios of GFP-2XFYVE localized to vesicle like structures. Please clarify how the ratio of GFP-2XFVYE is represented by % cells. Please also define the +wort conditions.
Response: Thanks. We are sorry that the description may cause potential misunderstandings. And we have revised the sentence into "Percentage of HeLa cells transfected with the indicated plasmids in which GFP-2xFYVE localized to vesicle-like structures." We have also defined the "+Wort" in the legend.

Figure 4:
Legend 4A: define scale bar.
Response: Thanks. We have defined the scale bar in the legend of Fig. 4A.

Figure 6:
Legend 6D: define + wort
Response: Thanks. We have defined "+Wort" in the legend.

Supplementary table S1:
The references of Berger and Isberg and Dumenil and Isberg are not in the main references list or properly described in the supplementary table. Please add a reference list to the table.
Response: Thanks. We have added a reference list in the Appendix.

p425GPD entry is missing
Response: Thanks. We have added the information of p425GPD in the table S2.

GFP-2xFYVEHrs - Dong et al does not describe the construction of this plasmid. Please provide an appropriate description and reference (Cao et al 2008 PMID 18524850 described the construction of pEGFP-2xFYVEHrs using the pEGFP-C2 vector from clonetech). In addition, in the Methods the plasmid is described as peGFP-2xFYVEHrs and in the table as GFP::2FYVEHrs

Response: We have made the following changes.

1. The reference (Cao et al 2008 PMID 18524850) was added.
2. We have changed the description of peGFP-2xFYVE$_{Hrs}$ in the Methods and the table.
3. The citation of the plasmid in the table S2 was changed to Cao et al 2008.

pRS47S reference should be Merriam et al 1997 (PMID 9169800)

Response: Thanks. We have changed the reference.

pSB157 - Sue Biggings Fred Hutchinson Cancer Research Center, Seattle, WA

W303 - R. Rothstein and appropriate reference

Response: Thanks. We have changed the references.

pCMV-4XFLAG - the CMV plasmid mentioned in Xu et al 2010 was pFLAG-CMV from Sigma, no modifications of this backbone were described to warrant a reference to this paper.

Response: Thank you very much. We have made the following changes.

1. pCMV-4xFlag was constructed based on the backbone of pCMV-Flag (Sigma-Aldrich) with the fusion of additional 3xFlag tag before the multiple cloning site. In order to express proteins in mammalian cells, genes were ligated to pCMV-4xFlag or pcDNA3.1-mCherry (Kleaveland *et al*, 2018) (a gift from David Barte, Addgene plasmid # 128744; http://n2t.net/addgene:128744; RRID:Addgene_128744).
2. We have also made relative changes in the table S2.

pcDNA3.1mCherry - Addgene.

Addgene on citation:

"These plasmids were created by your colleagues. Please acknowledge the Principal Investigator, cite the article in which the plasmids were described, and include Addgene in the Materials and Methods of your future publications.

• For your Materials & Methods section: pcDNA3.1-mCherry was a gift from David Bartel (Addgene plasmid # 128744 ; http://n2t.net/addgene:128744; RRID:Addgene_128744)

• For your References section: A Network of Noncoding Regulatory RNAs Acts in the Mammalian Brain. Kleaveland B, Shi CY, Stefano J, Bartel DP. Cell. 2018 Jul 12;174(2):350-362.e17. doi: 10.1016/j.cell.2018.05.022. Epub 2018

"Jun 7. 10.1016/j.cell.2

Response: Thanks. We have revised the citation of pcDNA3.1mCherry according to your suggestions.

2nd Revision - Editorial Decision 21st Dec 2020

Prof. Jiazhang Qiu
Jilin University
Xi'an Road 5333#
Changchun, Jilin 130062
China

Dear Prof. Qiu,

I have now received the report from the referee that was asked to evaluate your final revised manuscript, which can be found below. As you can see, the referee now fully supports publication of your study. I am thus very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #3:

I am satisfied with the modifications to the manuscript and the response to reviewers. There remain several typos/grammar mistakes in the newly added (green) text that I recommend are corrected prior to publication. Rather than list them here, I recommend editorial review of the new content.

****

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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.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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

B- Statistics and general methods

	Please fill out these boxes ➔ (Do not worry if you cannot see all your text once you press return)
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For the quantification of GFP-FYVE localized to vesicle-like structure or "protein of interest"-positive Legionella-containing vacuoles (LCVs)", at least 100 cells or LCVs were counted. The sample size was chosen based on previous literatures in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	In this study, we only used the bone marrow-derived macrophages (BMDM) prepared from mice.
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.	L. pneumophila strains were grown to the post-exponential phase (OD600nm=3.3-3.8) and were checked for motility before bacterial infection experiments.
For animal studies, include a statement about randomization even if no randomization was used.	In this study, we only used the bone marrow-derived macrophages (BMDM) prepared from mice.
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.	Authors were blinded for quantitative analysis. One lab member coded the treatment or bacterial strains used for the experiments and the other performed the experiments, including infection, processing and quantitation.
4.b. For animal studies, include a statement about blinding even if no blinding was done	In this study, we only used the bone marrow-derived macrophages (BMDM) prepared from mice.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	Yes

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).	The source, cat# as well as citation of the antibodies used in the study were described in the Materials and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HeLa and U937 cells were purchased from ATCC. Both cell lines have been tested for mycoplasma contamination prior to use via PCR.

* 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.	6- to 8-week-old female A/J mice was provided by the Model Animal Research Center of Nanjing University and used to prepare bone marrow-derived macrophages (BMDM).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University (number of permit: SY201902008).
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.	All animal experiments were performed in accordance to local animal handling laws.

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA