EMBO reports

Aster-C Coordinates with COP I Vesicles to Regulate Lysosomal Trafficking and Activation of mTORC1

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1st Editorial Decision 27th Jan 2020

Dear Dr. Shi,

Thank you for submitting your manuscript to our journal. We have now received three referee reports, which are copied below.

Please accept my apologies for this unusual delay in getting back to you. It took longer than usual to receive the full set of referee reports due to the recent holiday season.

As you can see, the referees express interest in the analysis. However, they also raise a number of concerns that need to be addressed to consider publication here. I find the reports constructive and well informed, and addressing these concerns will strengthen the manuscript. In general, referees find that more support into the proposed mechanism by which Aster-C regulates mTORC1 signaling is required. I noted that referee #3 suggests removing COPI vesicle part of the manuscript. However, I recommend trying to strengthen also this part as per referee recommendations during revision, and discussing later whether it is still necessary to omit.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the 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 or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1. A data availability section providing access to data deposited in public databases is missing (where applicable).
2. Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter plots in these cases.

Supplementary/additional data: 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 labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

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. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<http://embor.embopress.org/authorguide>).

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <http://embor.embopress.org/authorguide#expandedview>.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <http://embor.embopress.org/authorguide#sourcedata>.

8) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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.

Kind regards,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

Summary:
In this manuscript, Zheng et al. investigate the mechanism by which amino acids induce the translocation of mTORC1 to lysosomal membranes. The work centers on the interesting finding that knockout (KO) of Aster-C, an ER resident protein with known roles in cholesterol trafficking, increases mTORC1 signaling in cells cultured in complete media as well as those starved or stimulated with amino acids. Consistent with elevated mTORC1 signaling, Aster-C KO increases mTOR localization to lysosomes during AA starvation. Thus, Aster-C functions to restrict mTORC1 signaling. Through co-IP experiments, the paper finds that Aster-C interacts with mTOR and the GATOR2 complex (but not the GATOR1 complex) more strongly in AA starved cells than in AA stimulated cells. Further experiments lead the authors to propose a model in which Aster-C sequesters mTOR and GATOR2 on ER membranes in the absence of AAs. AA stimulation then causes release of mTOR and GATOR2 from Aster-C concurrent with the translocation of mTOR to lysosomal membranes in a manner dependent on COP I vesicle function, non-muscle myosin function, and actin polymerization.

General comments:
This manuscript benefits from many interesting observations that have potential to significantly improve our understanding of how mTOR traffics to lysosomal membranes during AA stimulation. Moreover, it seeks to define where mTOR localizes within the cell in the absence of sufficient amino

acids and how mTOR is delivered to lysosomal membranes upon AA stimulation. These questions are important but remain poorly understood. Currently, the field explains that mTOR exhibits a diffuse cytosolic location in the absence of AAs and translocates to lysosomal membranes in response to AA stimulation.....somehow. While the findings have potential to represent a highly significant advance, several major concerns limit enthusiasm for the manuscript in its current form. For example, insufficient data support the key conclusion that Aster-C sequesters mTOR and GATOR2 on ER membranes in the absence of AAs, other possible mechanisms for how Aster-C KO increases mTORC1 signaling require further consideration, and key experimental results should be shown in the same cell type and corroborated in another cell type.

Specific Critiques:
1) Little supporting evidence from localization/imaging studies substantiate the claim that Aster-C sequesters mTOR and GATOR2 on ER membranes in the absence of AAs. In addition to imaging studies, the authors could employ biochemical fractionation to determine whether mTOR and GATOR2 associate with ER membranes in an AA-sensitive manner. Such an approach could also be used to determine whether Aster-C co-IPs with mTOR and GATOR2 in the ER membrane fraction in an AA-sensitive manner.

2) In Figure 1, the paper investigates whether the ability of Aster-C to suppress mTORC1 signaling is related to the known role of Aster-C in cholesterol trafficking. This line of query is important, as the Zoncu lab has published two papers demonstrating that lysosomal cholesterol activates mTORC1 and that ER-lysosome contacts enable cholesterol sensing by mTORC1 (Lim et al 2019 Nature Cell Bio 21: 1206; and Castellano et al 2017 Science 355: 1306). Curiously, these papers are not cited. Unfortunately, how cholesterol depletion and add-back affects mTORC1 signaling, as monitored by phosphorylation of S6K1 and 4EBP1, in wild type vs. Aster-C KO cells cannot be evaluated because the cholesterol depleting drug MCD curiously ablated the expression of total S6K1 and 4EBP1 proteins; thus of course there are no phospho signals in these lanes.

3) As shown in Figure EV4 and explained in the Abstract, Aster-C KO induced dissociation of Tsc2 off lysosomal membranes in the absence of amino acids. While convincing and very interesting, this result makes me question several key conclusions of the manuscript pertaining to the mechanisms by which Aster-C KO increases mTORC1 signaling. As Demetriades et al (2014) published that amino acid withdrawal recruits Tsc2 to lysosomes (which inhibits Rheb and thus mTORC1) through Tsc2 binding to GDP-loaded RagA/B proteins, the result in EV4 makes one wonder whether an important mechanism by which Aster-C KO increases mTORC1 signaling is by modulating guanine nucleotide loading on Rag GTPases, specifically either increasing GTP-loading on RagA/B or decreasing GDP-loading on RagC/D. The authors explain that the results in EV4 provide further evidence that "mTORC1 was hyper-activated by Aster-C deficiency". Yes, I agree, but the results also suggest that Aster-C KO may increase RagA/B-GTP loading, which of course would recruit mTOR to the lysosomal surface, a well-established mechanism. Thus, the more novel question would be how Aster-C KO affects the guanine nucleotide loading state of Rag GTPases.

4) Figure EV1 shows that Aster-C KO increases Akt phosphorylation on T308, the activation loop site whose phosphorylation activates Akt. This result puts into question the mechanism proposed by that authors that Aster-C KO increases mTORC1 signaling. Active Akt phosphorylates Tsc2 to induce Tsc2 dissociation off lysosomal membranes (Menon et al 2014). Thus, Aster-C KO could increase mTORC1 signaling by this known mechanism and not by loss of mTOR sequestration on a non-lysosomal subcellular site.

5) The immunofluorescence (IF) microscopy experiments examining the subcellular localization of

mTOR, Aster-C, and CopA and the biochemistry experiments examining how Aster-C KO affects mTORC1 signaling were only performed in one cell type, C12C12 myoblasts, which limits potential impact of this work. Moreover, all the interesting, AA sensitive interactions between Aster-C, mTOR, and GATOR2 were examined in a different cell type, HEK293T cells. Impact would be strengthened if the key experiments regarding mTOR subcellular localization, activity, and interactions were assessed in the same cell type and then extended to at least one other cell type. For example, Does Aster-C KO in HEK293T cells (or other cell types) increase mTORC1 signaling in a way consistent with how Aster-C interacts with mTOR and GATOR2 depending an AA levels? Does Aster-C interact with mTOR and GATOR2 in an amino acid repressible manner in C2C12 myoblasts?

6) To extend the point above, Figure 6 makes the interesting observation that Aster-C interacts with non-muscle myosin (MYH10) in HEK293T cells, KO of MYH10 increases mTORC1 signaling in MEFs (similar to KO of Aster-C in C2C12 cells), and inhibition of MYH10 function with BBS (blebbistatin) induces co-localization of mTOR with COPA in C2C12 cells. These experimental results need to be corroborated in the same cell type, and preferably confirmed in a different cell type. For example, it would be simple to determine whether KO of MYH10 in MEFs also induces co-localization of mTOR with COPA to phenocopy the effect of BBS in C2C12 cells. Moreover, Figure 7 makes the interesting observation that actin polymerization is required for mTOR to form puncta on actin cables during AA stimulation. For reasons that remain unclear, this experiment was done in Cos-7 cells. Why not examine C2C12 cells? Also, does actin de-polymerization reduce mTOR puncta formation on lysosomes (co-stain with Lamp1/2)?

7) Insufficient data support the conclusion that Aster-C restricts mTORC1 activation by sequestering mTORC1 on ER membranes in the absence of AA. While mTOR co-IPed with Aster-C, raptor did not. Either Aster-C interacts with unassembled mTOR, Aster-C interacts with an unknown mTOR complex, or the raptor interaction was below the level of detection for some technical reason.

8) The data shown in Figure EV1 are quite surprising. EV1 shows that amino acid starvation potently reduced Akt phosphorylation on T308 and S473 while AA stimulation robustly increased these phosphorylation events. Note that Pdk1 phosphorylates Akt T308 while mTORC2 phosphorylates S473; in addition, Akt S473 phosphorylation can boost Pdk1-mediated Akt T308 phosphorylation in many cellular contexts. Abundant evidence in the literature (as well as data from this reviewer's lab) indicates that AAs do not modulate Akt phosphorylation on T308 and S473. Interestingly, published work indicates that acidic pH of cell culture media inhibits mTORC1 and mTORC2. In addition, work form this reviewer's lab indicates that feeding cells with media at basic pH (pH 9-10) is sufficient to robustly increase Akt T308 and S473 phosphorylation. Of concern, addition of MEM amino acids to buffers such as KRPH or D-PBS increases the pH to 9-10. Thus, KRPH + MEM amino acid solution must be pHed back down to 7.4 and if it is not, then KRPH + MEM AAs will increase Akt phosphorylation. While the Materials and Method sections states that the KRPH buffer -/+ amino acid solutions were indeed pHed back to 7.4, this reviewer wonders whether this step was missed.

Referee #2:

Zhang et al. report a novel function fort the lipid binding protein Aster-C in regulating mTORC1

activation. Aster-C interacts with mTORC1 and its deletion increases mTORC1 signaling while preventing mTORC1 inactivation under starvation. The authors identify COPA and COPG as Aster-C binding proteins and present evidence that COP proteins mediate lysosomal translocation of mTORC1 upon amino acid stimulation in an actin-dependent process.

It has been unclear where mTORC1 localizes upon release from lysosomal membranes during amino acid starvation. Zhang et al. propose that mTORC1 in amino acid-starved cells is sequestered at lysosomal membranes in a process that depends on the ER-resident protein Aster-C. This is an interesting study that sheds light on one of the central questions regarding mTORC1 signaling. The data are of overall high quality and phenotypes are often confirmed by biochemical and cell biological approaches and with both pharmacological and genetic manipulations. The results are clearly presented in a well-organized manuscript. A few comments that would strengthen the author's conclusions are detailed below.

1. To conclusively show that mTORC1 localizes to the ER during amino acid starvation, the authors should demonstrate co-localization or co-fractionation of mTORC1 with ER markers specifically under this condition.

2. As the authors point out, COP I vesicles are small and not normally visible with conventional microscopy. By contrast, they identify COP I containing structures of a size similar to that of lysosomes. The authors should discuss the possible nature of these structures (e.g. COP I vesicles vs. COP I containing organelles or membrane domains).

3. It is surprising that Aster-C co-IPs with mTOR but not Raptor or Rictor (Figure EV2C). How do the authors explain a pool of mTOR that is neither part of mTORC1 nor mTORC2?

4. The autophagy phenotypes are not very convincing (Figure EV2A,B). Lysosomal lipid droplets and LC3-II are increased in Aster-C KO cells. This suggests increased induction of autophagy or decreased degradation of autophagic cargo. However, Aster-C KO cells have increased mTORC1 activity, which blocks autophagy induction. How do the authors explain this?

5. Related to the above point, Ulk1 phosphorylation is completely lost in Aster-C KO (Figure EV2B). The authors do not specify the phospho-site. Is this the the mTORC1 phospho-site on Ulk1? If yes, why is phosphorylation not increased in the KO? To functionally probe for ULk1 activity in the KO, an LC3-based flux assay would be informative.

6. Figure EV7: It would be nice to show redistribution of COPA from ER to lysosomes in the same cell type and experiment.

7. Methods and figure legends lack some experimental details (specific antibody phosphosites, concentrations for several inhibitors, qPCR primer sequences).

8. In the discussion of ARF1 and mTORC1 signaling, the authors should comment on Jewell et al., 2015.

9. On p. 14, the authors misleadingly relate their findings on the role of actin in amino acid-mediated mTORC1 activation to Jacinto et al., who study functions of mTORC2 in regulating actin dynamics.

Referee #3:

The manuscript by the Shi group reports several upstream regulators of mTORC1 localization upon amino acid starvation and stimulation. They propose that Aster C sequesters mTORC1 in the ER during starvation conditions while COP I vesicles and actin dynamics participates in delivering mTORC1 to the lysosomal membranes upon stimulation. The manuscript contains several interesting observations suitable for publication in Embo reports. However, the attempt in identifying the whole pathway from Aster C to COP I and actomyosin contractility is ambitious and not fully supported by the experimental data. One suggestion may be to strengthen the Aster C-ER-TSC2-mTORC1 part and leave out the COP I vesicle-actomyosin part for future in depth analysis.

1) The claim that mTOR interacts with Aster C in the ER is based on co-IP experiments using overexpressed Aster C and mTOR (Fig. 2). This should be confirmed using endogenous proteins and immunofluorescence studies. In addition, mTOR is also present in mTORC2 complexes that have been reported in different subcellular compartments, including ER. The authors should perform immunofluorescence detection of mTORC1-specific localization in the ER, together with Aster C. Tag-versions of the proteins should be expressed at levels comparable to endogenous by retroviral transduction.

2) Again, the co-localization of COPA and mTOR is based on massive overexpression of ectopic proteins by transient transfection of C2C12 cells (Fig. 3D). The authors should switch to retroviral vectors in order to achieve stable expression similar to endogenous.

3) The immunofluorescence localization of mTORC1 should be analyzed in ARF1 knockout cells, and not only after the pharmacological inhibition that may be unspecific (Fig. 5A). The effect on mTORC1 activation is minor in ARF1 knockout cells as compared to pharmacological agents (compare Fig. 5D with 5B and C).

4) MYH10 knock-out appears to induce the up-regulation of mTORC1 independent on nutrient availability (Fig. 6B). No resistance to starvation is detected. This is different to the Aster C knockout studies shown in Fig. 1A, for instance. My interpretation would be that MYH10 has a general effect on mTORC1 activity, independent to nutrient sensing and Aster C function.

Response to Reviewers' comments:

We would like to thank all the reviewers for their comments and suggestions which have significantly improved the quality of our manuscript. In response to the comments and suggestions, we have provided a substantial amount of new data to address all the major concerns raised by the reviewers, as detailed in this rebuttal.

Referee #1:

Summary:
In this manuscript, Zheng et al. investigate the mechanism by which amino acids induce the translocation of mTORC1 to lysosomal membranes. The work centers on the interesting finding that knockout (KO) of Aster-C, an ER resident protein with known roles in cholesterol trafficking, increases mTORC1 signaling in cells cultured in complete media as well as those starved or stimulated with amino acids. Consistent with elevated mTORC1 signaling, Aster-C KO increases mTOR localization to lysosomes during AA starvation. Thus, Aster-C functions to restrict mTORC1 signaling. Through co-IP experiments, the paper finds that Aster-C interacts with mTOR and the GATOR2 complex (but not the GATOR1 complex) more strongly in AA starved cells than in AA stimulated cells. Further experiments lead the authors to propose a model in which Aster-C sequesters mTOR and GATOR2 on ER membranes in the absence of AAs. AA stimulation then causes release of mTOR and GATOR2 from Aster-C concurrent with the translocation of mTOR to lysosomal membranes in a manner dependent on COP I vesicle function, non-muscle myosin function, and actin polymerization.

General comments:

This manuscript benefits from many interesting observations that have potential to significantly improve our understanding of how mTOR traffics to lysosomal membranes during AA stimulation. Moreover, it seeks to define where mTOR localizes within the cell in the absence of sufficient amino acids and how mTOR is delivered to lysosomal membranes upon AA stimulation. These questions are important but remain poorly understood. Currently, the field explains that mTOR exhibits a diffuse cytosolic location in the absence of AAs and translocates to lysosomal membranes in response to AA stimulation.....somehow. While the findings have potential to represent a highly significant advance, several major concerns limit enthusiasm for the manuscript in its current form. For example, insufficient data support the key conclusion that Aster-C sequesters mTOR and GATOR2 on ER membranes in the absence of AAs, other possible mechanisms for how Aster-C KO increases mTORC1 signaling require further consideration, and key experimental results should be shown in the same cell type and corroborated in another cell type.

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to imaging studies, the authors could employ biochemical fractionation to determine whether mTOR and GATOR2 associate with ER membranes in an AA-sensitive manner. Such an approach could also be used to determine whether Aster-C co-IPs with mTOR and GATOR2 in the ER membrane fraction in an AA-sensitive manner.

Re: First of all, we would like to thank the reviewer for the insightful suggestion and comments. As suggested, we carried out subcellular fractionation analysis to determine the subcellular localization of mTOR and GATOR2 under starvation and in response to amino acids stimulation. Please see the detailed method of subcellular fractionation in the Methods part of the revised manuscript. HEK293T cells transiently transfected with FLAG-tagged Aster-C were fractionated into the cytosol (Cyto), rough ER (RER), lysosomal (Lyso), and microsomal (Micro) fractions by differential centrifugation, followed by western blot analysis of Aster-C, mTOR, WDR24 and Mios (for GATOR2 complex). The blot was also probed with anti-Sec63, LAMP1, and GAPDH antibodies as biomarkers for RER, lysosomal, and cytosol proteins, respectively. As shown in Fig. R1A, Aster-B colocalized well with mTOR and the GATOR2 complex (as shown by the blots of WDR24 and Mios) at RER during amino acids starvation (-AA). In response to AA stimulation, both mTOR and GATOR2 complex, but not Aster-C, translocated from the RER to lysosomes (Fig.R1A, also see Figure 2B in our revised manuscript). In contrast, Aster-C is exclusively localized at the RER (Fig.R1A), which is consistent with our findings from confocal imaging analysis (Figure 2A in the revised manuscript).

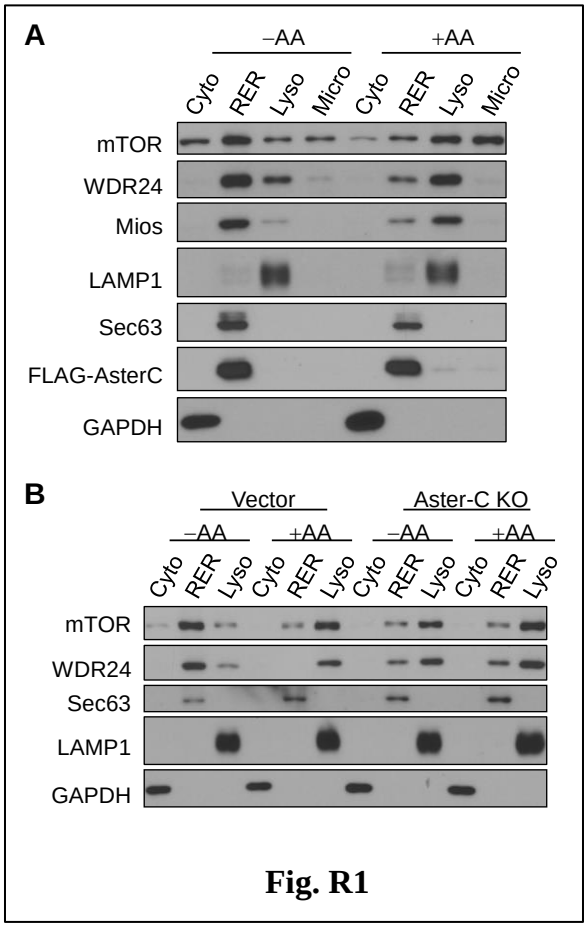

Fig. R1

Using the subcellular fractionation analysis, we next addressed the critical issue whether Aster-C regulates mTORC1 trafficking and activation in C2C12-Vector and Aster-C knockout cells (KO). In support of the findings from the 293T cells, most of the mTOR and WDR24 proteins were localized in the RER during AA starvation, and translocated to lysosomes in response to AA stimulation (Fig. R1B, also see Figure 2C in our revised manuscript). In contrast, most of the mTOR and WDR24 protein in Aster-C KO cells were localized at lysosomes during AA starvation, and were not responsive to AA stimulation (Fig. R1B). Together, these findings lend further support to our hypothesis and conclusion that mTOR translocates from the RER to lysosomes in response to AA stimulation, and Aster-C is

required for the retention of mTORC1 and GATOR2 at the RER. We did not carry out the suggested co-IP analysis, because we believe the fractionation process, which took several hours to complete, would have disrupted any sensitive protein-protein interactions. Additionally, the buffer conditions used for subcellular fractionation are quite different from those for co-IP analysis, which would also render any results difficult to interpret.

2) In Figure 1, the paper investigates whether the ability of Aster-C to suppress mTORC1 signaling is related to the known role of Aster-C in cholesterol trafficking. This line of query is important, as the Zoncu lab has published two papers demonstrating that lysosomal cholesterol activates mTORC1 and that ER-lysosome contacts enable cholesterol sensing by mTORC1 (Lim et al 2019 Nature Cell Bio 21: 1206; and Castellano et al 2017 Science 355: 1306). Curiously, these papers are not cited. Unfortunately, how cholesterol depletion and add-back affects mTORC1 signaling, as monitored by phosphorylation of S6K1 and 4EBP1, in wild type vs. Aster-C KO cells cannot be evaluated because the cholesterol depleting drug MCD curiously ablated the expression of total S6K1 and 4EBP1 proteins; thus of course there are no phospho signals in these lanes.

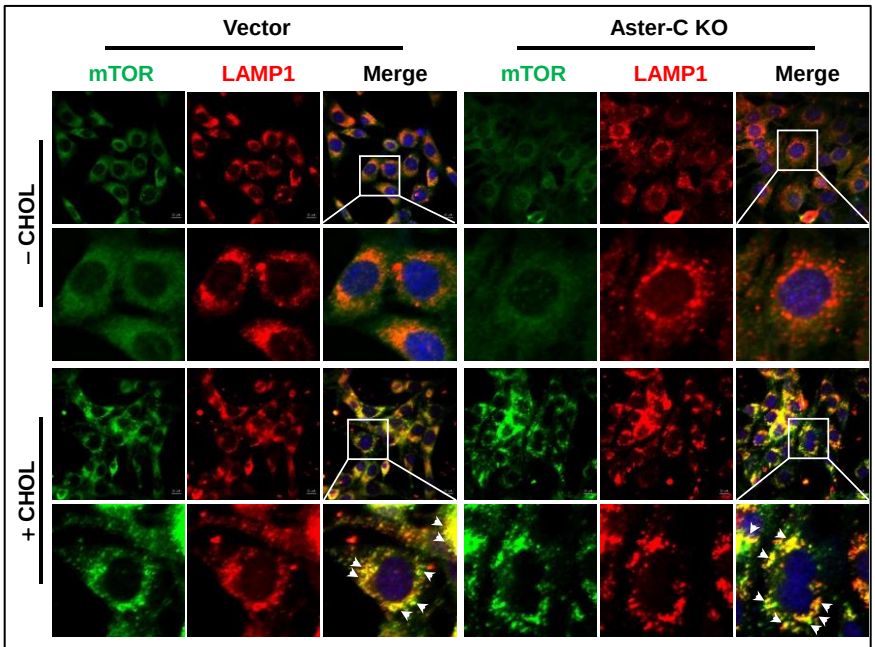

Fig. R2

Re: We agree with the review that the findings of these two papers are very important contributions to our understanding on how cholesterol regulates mTORC1 activity. We have cited the two papers in our revised manuscript (Please refer to page 6 of our revised manuscript, references 11 and 12). In response to this comment, we determined whether Aster-C deficiency abolish cholesterol-stimulated mTORC1 activation by immunofluorescent analysis of the endogenous mTOR in C2C12 cells using the identical experimental conditions as we used for western blot analysis in Figure 1H of the revised manuscript. In support of the powerful effect of cholesterol on mTORC1 activation, cholesterol depletion by MCD (-CHOL) not only caused mTOR disassociation from lysosomes in vector control cells, but also in Aster-C KO cells (Fig. R2, also see Figure 1G in our revised manuscript). Remarkably, cholesterol replenishment stimulated lysosomal association of mTOR both in vector control as well as in Aster-C KO cells (Fig.R2, arrows

indicated the co-localization of mTOR and LAMP1). These results lend further support to our interpretation of the western blot results (Figure 1H in our revised manuscript) that Aster-C regulates mTORC1 trafficking and activation that is independent from its projected role in cholesterol transport.

3) As shown in Figure EV4 and explained in the Abstract, Aster-C KO induced dissociation of Tsc2 off lysosomal membranes in the absence of amino acids. While convincing and very interesting, this result makes me question several key conclusions of the manuscript pertaining to the mechanisms by which Aster-C KO increases mTORC1 signaling. As Demetriades et al (2014) published that amino acid withdrawal recruits Tsc2 to lysosomes (which inhibits Rheb and thus mTORC1) through Tsc2 binding to GDP-loaded RagA/B proteins, the result in EV4 makes one wonder whether an important mechanism by which Aster-C KO increases mTORC1 signaling is by modulating guanine nucleotide loading on Rag GTPases, specifically either increasing GTP-loading on RagA/B or decreasing GDP-loading on RagC/D. The authors explain that the results in EV4 provide further evidence that "mTORC1 was hyper-activated by Aster-C deficiency". Yes, I agree, but the results also suggest that Aster-C KO may increase RagA/B-GTP loading, which of course would recruit mTOR to the lysosomal surface, a well-established mechanism. Thus, the more novel question would be how Aster-C KO affects the guanine nucleotide loading state of Rag GTPases.

Re: We thank the reviewer for the insightful comments. We agree that it is possible that Aster-C deficiency might affect guanine nucleotide loading state of Rag GTPases, but this type of experiment is beyond our technical capability. As alternative approaches to answering this question, we carried out additional experiments to determine whether Aster-C interacts with TSC2 and RagA or RagC by Co-IP analysis. The results show that Aster-C neither interacted with TSC2 (Figure EV4D-E in the revised manuscript), nor with RagA or RagC (Figure 2F in the revised manuscript). Additionally, we further analyzed the effect of Aster-C depletion on TSC2 phosphorylation which triggers its dissociation from lysosomes. As shown in Fig R3, Aster-C deficiency did not significantly change the phosphorylation levels of TSC2 and Akt during serum starvation, nor their sensitivity to stimulation by insulin. Together, these new data suggest that the effect of Aster-C on lysosomal dissociation of TSC2 is likely caused by feedback response from constitute activation of mTORC1.

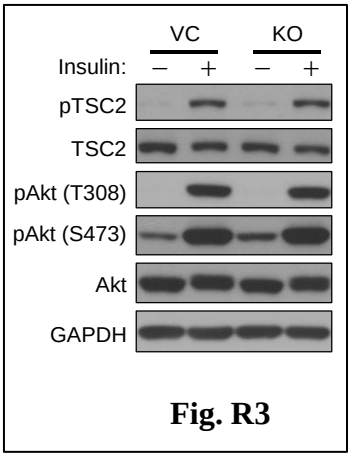

Fig. R3

4) Figure EV1 shows that Aster-C KO increases Akt phosphorylation on T308, the activation loop site whose phosphorylation activates Akt. This result puts into question the mechanism proposed by that authors that Aster-C KO increases mTORC1 signaling. Active Akt

phosphorylates Tsc2 to induce Tsc2 dissociation off lysosomal membranes (Menon et al 2014). Thus, Aster-C KO could increase mTORC1 signaling by this known mechanism and not by loss of mTOR sequestration on a non-lysosomal subcellular site.

Re: We thank the reviewer for this comment. However, we believe this scenario is not supported by our data. First, Aster-C did not interact with TSC2 (Figure EV4D-E in the revised manuscript). In addition, as shown in Fig. R3, both Akt and Tsc2 phosphorylation levels were inhibited in Aster-C KO cells by serum starvation when mTORC1 was activated under the same condition. Moreover, Akt phosphorylation levels in Aster-C KO cells were equally stimulated by insulin as in the vector controls.

5) The immunofluorescence (IF) microscopy experiments examining the subcellular localization of mTOR, Aster-C, and CopA and the biochemistry experiments examining how Aster-C KO affects mTORC1 signaling were only performed in one cell type, C12C12 myoblasts, which limits potential impact of this work. Moreover, all the interesting, AA sensitive interactions between Aster-C, mTOR, and GATOR2 were examined in a different cell type, HEK293T cells. Impact would be strengthened if the key experiments regarding mTOR subcellular localization, activity, and interactions were assessed in the same cell type and then extended to at least one other cell type. For example, Does Aster-C KO in HEK293T cells (or other cell types) increase mTORC1 signaling in a way consistent with how Aster-C interacts with mTOR and GATOR2 depending an AA levels? Does Aster-C interact with mTOR and GATOR2 in an amino acid repressible manner in C2C12 myoblasts?

Re: We thank the reviewer for the comments. Although most of our experiments were carried out in C2C12 cells, some of our key findings were also repeated in other cell lines, including the confocal imaging analysis of the subcellular localization of Aster-C (Figure 2A and Appendix Figure S1 in the revised manuscript), Co-IP experiments (Figure 3B-C in the revised manuscript), and the subcellular fractionation analysis (Fig. R1, and Figure 2B-C in the revised manuscript). These results indicated that our findings on Aster-C and mTORC1 are common phenomenon in all cell types tested.

6) To extend the point above, Figure 6 makes the interesting observation that Aster-C interacts with non-muscle myosin (MYH10) in HEK293T cells, KO of MYH10 increases mTORC1 signaling in MEFs (similar to KO of Aster-C in C2C12 cells), and inhibition of MYH10 function with BBS (blebbistatin) induces co-localization of mTOR with COPA in C2C12 cells. These experimental results need to be corroborated in the same cell type, and preferably confirmed in a different cell type. For example, it would be simple to determine whether KO of MYH10 in MEFs also induces co-localization of mTOR with COPA to phenocopy the effect of BBS in C2C12 cells. Moreover, Figure 7 makes the interesting observation that actin polymerization is required for mTOR to form puncta on actin cables during AA stimulation. For reasons that remain unclear, this experiment was done in Cos-7

cells. Why not examine C2C12 cells? Also, does actin de-polymerization reduce mTOR puncta formation on lysosomes (co-stain with Lamp1/2)?

Re: We agree with the reviewer's comments. As mentioned above, the Co-IP experiment, which showed the interaction of Aster-C and MYH10, was carried out in 293T cell due to lack of a good commercial anti-Aster-C antibody and high plasmid transfection efficiency in 293T cells. As suggested, we carried out additional confocal analysis to determine the co-localization of mTOR and COPA in WT and MYH10 KO MEF cells. In support of our finding from C2C12 cells, BBS treatment caused the co-localization of mTOR and COPA (Fig. R4, arrows indicated the co-localization of mTOR and COPA). In contrast, mTOR co-localized with COPA in MYH10 KO MEF cells under AA starvation without any BBS treatment (Fig.R4, lower panel).

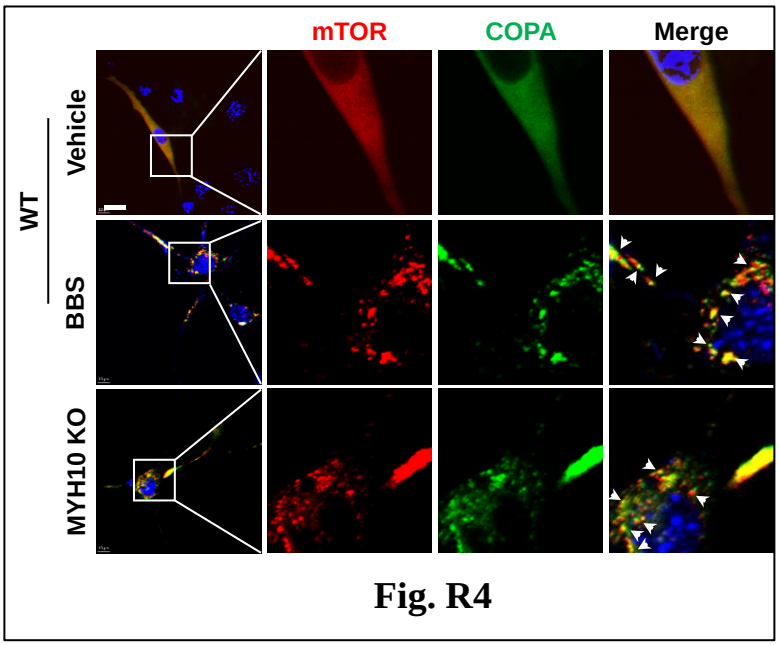

Fig. R4

The reason we chose COS-7 cells for actin staining is because COS-7 cells are bigger with less actin filaments than C2C12 cells, which make it easier for confocal analysis. However, we also repeated the actin staining in C2C12 cells. As shown in Fig.R5A, AA stimulation activated the mTOR, leading to co-localization with actin (As indicated by

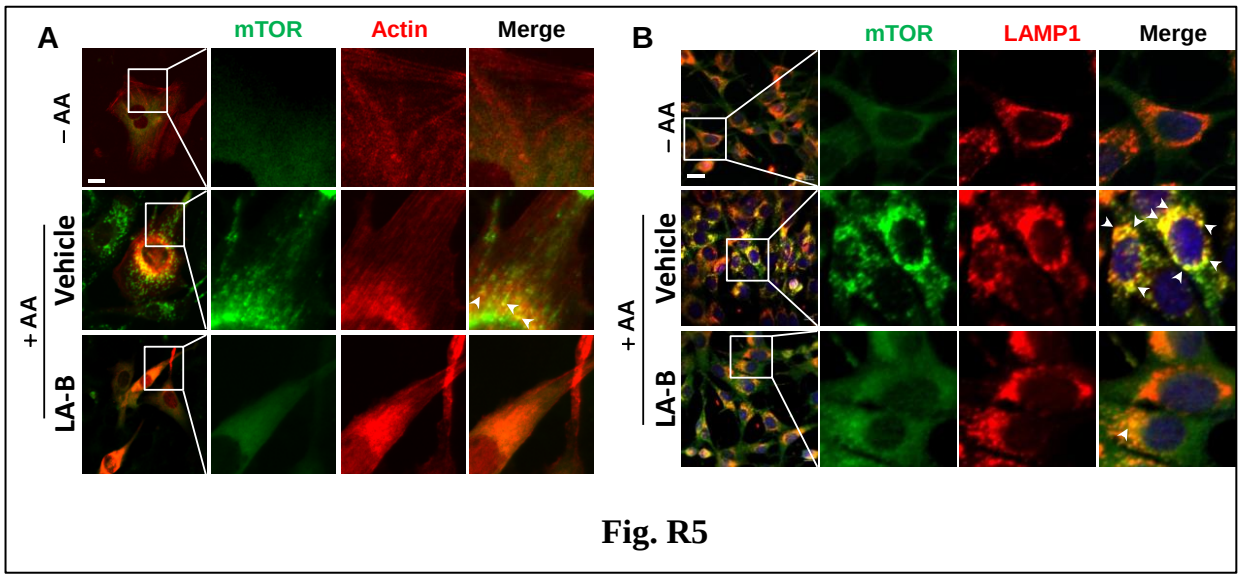

Fig. R5

arrows). In contrast latrunculin b (LA-B) treatment inhibited mTORC1 activation and co-localization with actin in response to AA stimulation (Fig.R5A, lower panel). This result is consistent with data in COS-7 cells (Figure 7A in the revised manuscript). Also, in response to the reviewer's suggestion, we carried out additional confocal imaging analysis of co-localization of endogenous mTOR with lysosomes under actin de-polymerization condition in C2C12 cells. The results show that LA-B treatment prevented AA-induced co-localization of mTOR with LAMP1 when compared with vehicle control. (Fig.R5B, also see Figure 7B in the revised manuscript). This result is consistent with the western blot data, in which LA-B treatment inhibited the phosphorylation of S6K and 4E-BP1 (Figure 7C in the revised manuscript).

7) Insufficient data support the conclusion that Aster-C restricts mTORC1 activation by sequestering mTORC1 on ER membranes in the absence of AA. While mTOR co-IPed with Aster-C, raptor did not. Either Aster-C interacts with unassembled mTOR, Aster-C interacts with an unknown mTOR complex, or the raptor interaction was below the level of detection for some technical reason.

Re: We agree with the reviewer that our model is not fully supported by our data. In response to the comments, we determined the possibility whether the raptor interaction was below the level of detection by our previous Co-IP experiment. To resolve this issue, we doubled the amount of protein lysate used for the Co-IP experiment. Indeed, this significantly increased the sensitivity of the detection. As shown in Fig. R6 (Also see Figure 2E in the revised manuscript), Aster-C interacted with Raptor, but not Rictor, under AA starvation, whereas AA stimulation significantly decreased the interaction of Aster-C both WDR24 and Raptor. These results provided further support to our hypothesis that Aster-C sequestered mTORC1, but not mTORC2 complex, during AA starvation, at the RER.

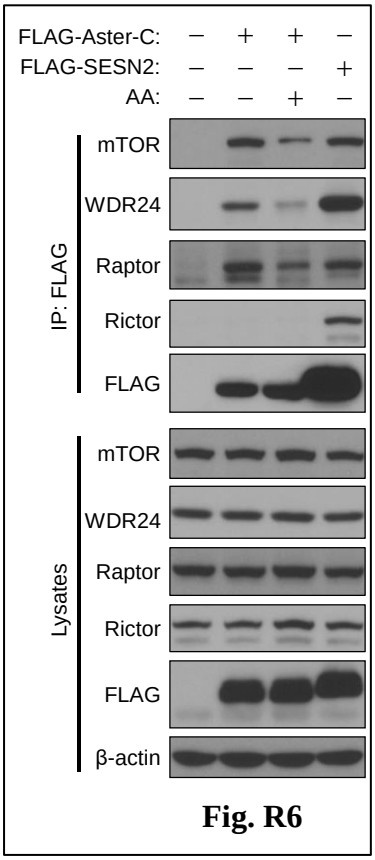

Fig. R6

8) The data shown in Figure EV1 are quite surprising. EV1 shows that amino acid starvation potently reduced Akt phosphorylation on T308 and S473 while AA stimulation robustly increased these phosphorylation events. Note that Pdk1 phosphorylates Akt T308 while mTORC2 phosphorylates S473; in addition, Akt S473 phosphorylation can boost Pdk1-mediated Akt T308 phosphorylation in many cellular contexts. Abundant evidence in the literature (as well as data from this reviewer's lab) indicates that AAs do not modulate Akt phosphorylation on T308 and S473. Interestingly, published work indicates that acidic pH of cell culture media inhibits mTORC1 and mTORC2. In addition, work form this reviewer's lab

indicates that feeding cells with media at basic pH (pH 9-10) is sufficient to robustly increase Akt T308 and S473 phosphorylation. Of concern, addition of MEM amino acids to buffers such as KRPH or D-PBS increases the pH to 9-10. Thus, KRPH + MEM amino acid solution must be pHed back down to 7.4 and if it is not, then KRPH + MEM AAs will increase Akt phosphorylation. While the Materials and Method sections states that the KRPH buffer -/+ amino acid solutions were indeed pHed back to 7.4, this reviewer wonders whether this step was missed.

Re: We thank reviewer for this comment. We do adjust the buffer pH back to 7.4 after we add the AA to the KRPH buffer. We searched the literature, and found that the regulatoryrole of AA on Akt phosphorylation is controversial. Guang Yang, *etc.* reported that AA inhibited the Akt T308 phosphorylation in HEK293E cells [1], while in another study, AA induced both Akt T308 and S473 phosphorylation in HeLa, HEK293, MCF7, Huh7, C2C12, and mouse embryonic fibroblasts [2]. Jonathan M. Cooper, *etc.* also reported that essential AA induced the both Akt T308 and S473 phosphorylation in mouse embryonic fibroblasts [3]. We noticed that the starvation methods used in the three reports mentioned above are different. Thus, we analyzed the effect of the three experimental conditions on Akt T308 and S473 phosphorylation in C2C12-vector and Aster-C KO cells. In the first study [1], cells were incubated 2 hrs in KRPH buffer containing dialyzed serum, and then treated with a mixture of amino acids corresponding to the concentrations present in DMEM. In the second study [2], cells were deprived of serum overnight, and then incubated for an additional 2 hrs with DPBS (Dulbecco's phosphate-buffered saline containing 1 g/L D-glucose and 36 mg/L sodium pyruvate, calcium, and magnesium). In the third study [3], cells were incubated 3 hrs in Earle's Balanced Salt Solution (EBSS). During the final hour of EBSS treatment, cells were "primed" by 2 mM L-glutamine in EBSS. We followed the three methods, and all the buffers were pHed back to 7.4 after adding the AA. As shown in Fig. R7, AA induced Akt T308 and S473 phosphorylation at 10 min in all three conditions, while Akt T308 and S473 phosphorylation was inhibited by AA at 30 min in the second and third conditions. The Akt phosphorylation under the second and third conditions was very similar, and both resulted

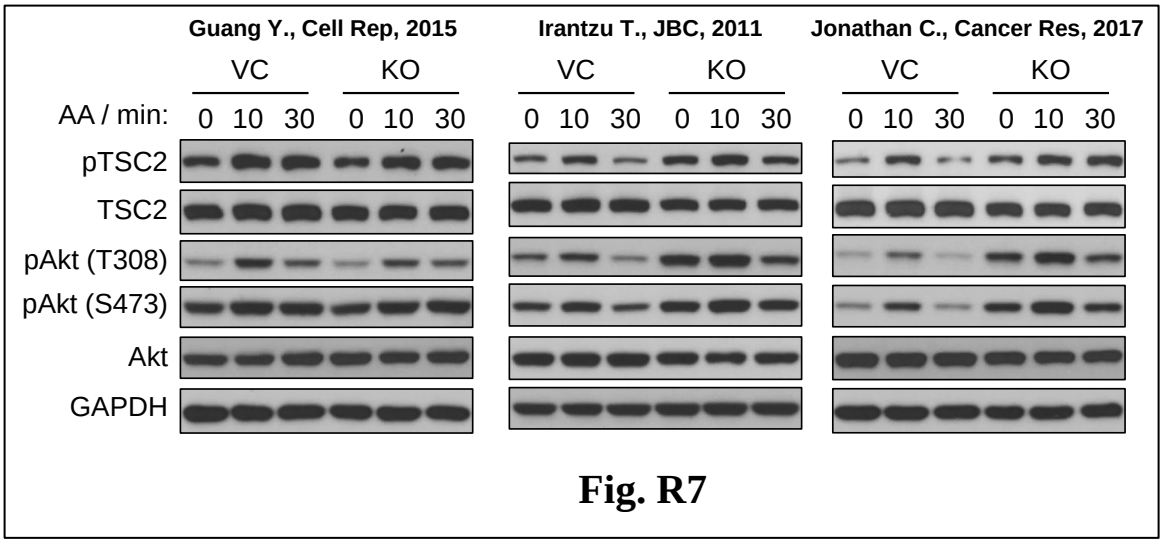

Fig. R7

in higher basal Akt T308 and S473 phosphorylation in Aster-C KO cells than the vector cells. Our findings suggest that the starvation methods may play an important role in Akt phosphorylation in response to AA stimulation.

Referee #2:

Zhang et al. report a novel function fort the lipid binding protein Aster-C in regulating mTORC1 activation. Aster-C interacts with mTORC1 and its deletion increases mTORC1 signaling while preventing mTORC1 inactivation under starvation. The authors identify COPA and COPG as Aster-C binding proteins and present evidence that COP proteins mediate lysosomal translocation of mTORC1 upon amino acid stimulation in an actin-dependent process.

It has been unclear where mTORC1 localizes upon release from lysosomal membranes during amino acid starvation. Zhang et al. propose that mTORC1 in amino acid-starved cells is sequestered at lysosomal membranes in a process that depends on the ER-resident protein Aster-C. This is an interesting study that sheds light on one of the central questions regarding mTORC1 signaling. The data are of overall high quality and phenotypes are often confirmed by biochemical and cell biological approaches and with both pharmacological and genetic manipulations. The results are clearly presented in a well-organized manuscript. A few comments that would strengthen the author's conclusions are detailed below.

1. To conclusively show that mTORC1 localizes to the ER during amino acid starvation, the authors should demonstrate co-localization or co-fractionation of mTORC1 with ER markers specifically under this condition.

Re: We thank the reviewer for this very good suggestion. As suggested, we carried out subcellular fractionation analysis of mTORC1 and ER markers in 293T cells transiently expressing the FLAG-Aster-C, Aster-C KO cells, and the C2C12-vector cells under AA starvation and re-simulation. The results show that Aster-C co-localized well with the rough ER marker during AA starvation, and dissociated in response to AA stimulation, as detailed in our detailed response to Reviewer #1's comment #1 (Fig. R1, also see Figure 2B-C in the revised manuscript).

2. As the authors point out, COP I vesicles are small and not normally visible with conventional microscopy. By contrast, they identify COP I containing structures of a size similar to that of lysosomes. The authors should discuss the possible nature of these structures (e.g. COP I vesicles vs. COP I containing organelles or membrane domains).

Re: We agree with the reviewer's comment. COPI vesicles are below the resolution of the light microscope, which is supported by our observations that GFP-COPA exhibited a diffused pattern in the cytoplasm in C2C12 cells transiently expressing GFP-COPA during amino acids starvation (Figure EV5B in the revised manuscript). COPA became visible only after AA stimulation due to its co-localization with the lysosomes (Figure EV5B).

3. It is surprising that Aster-C co-IPs with mTOR but not Raptor or Rictor (Figure EV2C). How do the authors explain a pool of mTOR that is neither part of mTORC1 nor mTORC2?

Re: This is a great question. In response to the reviewer's comment, we repeated the Co-IP experiments in 293T cells by doubling the total amount of protein used for the Co-IP experiments. Indeed, this has enabled us to detect the association of Aster-C with Raptor, but not Rictor, under AA starvation, whereas AA stimulation significantly decreased the interaction with Raptor, as detailed in our response to Reviewer #1' comment #7, and in Fig. R6 (Also see Figure 2E in the revised manuscript). The new data further support our conclusion that Aster-C selectively interreacted with mTORC1, but not mTORC2, at the rough ER under AA starvation.

4. The autophagy phenotypes are not very convincing (Figure EV2A,B). Lysosomal lipid droplets and LC3-II are increased in Aster-C KO cells. This suggests increased induction of autophagy or decreased degradation of autophagic cargo. However, Aster-C KO cells have increased mTORC1 activity, which blocks autophagy induction. How do the authors explain this?

Re: We thank the reviewer for this insightful comment. We completely agree that mTORC1 activation inhibits the induction of autophagy. Some studies also showed that mTORC1 negatively regulates the fusion of the autophagosome with the lysosome [4,5]. We believe that the accumulation of lysosomal lipid droplets and LC3-II were mainly due to the decreased degradation of autophagic cargo in Aster-C KO cells. To verify this concept, we carried out a lysosomal proteolytic degradation analysis of DQ-red-BSA using a method as previously described previously [6]. Indeed, the result showed that Aster-C deficiency significantly impaired proteolytic degradation activity of the lysosomes when compared with the vector cells under AA starvation (Fig. R8, also see Figure EV2B in the revised manuscript). We believe this defect also caused defective lysosomal degradation of autophagic cargo, leading to accumulation of lysosomal lipid droplets and LC3-II in Aster-C KO cells.

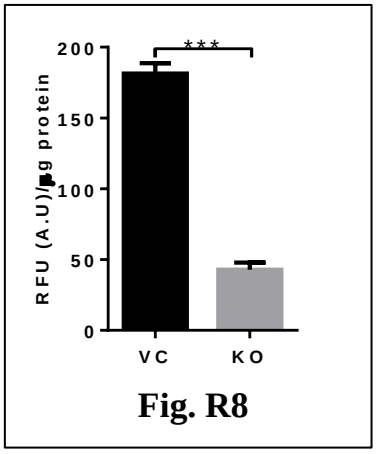

Fig. R8

5. Related to the above point, Ulk1 phosphorylation is completely lost in Aster-C KO (Figure EV2B). The authors do not specify the phospho-site. Is this the the mTORC1 phospho-site on Ulk1? If yes, why is phosphorylation not increased in the KO? To functionally probe for ULk1 activity in the KO, an LC3-based flux assay would be informative.

Re: We are sorry for not specifying the phosphorylation site of ULK1 in the method part, and thank the reviewer for pointing out this. The phosphorylation site of ULK1 we analyzed was S555 which is a phosphorylation site of AMPK [7], but not mTOR. Phosphorylation of S555 is believed to activate of ULK1, leading to activation of autophagic initiation. Since AMPK and mTORC1 have opposing effects on autophagy [8], we believe the decreased S555 phosphorylation is likely caused by hyperactivation of mTORC1 in Aster-C KO cells during starvation. Though ULK1 S555 phosphorylation negatively regulates the autophagic initiation step, we believe that the accumulation of LC3-II was mainly due to the decreased degradation of autophagic cargo in Aster-C KO cells. Please refer to our explanation above, as well as the result of lysosomal proteolytic degradation analysis in C2C12-vector and Aster-C KO cells under starvation (Fig R8). However, the underlying molecular mechanisms on how Aster-C regulates autophagy and lysosomal function is quite interesting and remains to be elucidated in future studies.

6. Figure EV7: It would be nice to show redistribution of COPA from ER to lysosomes in the same cell type and experiment.

Re: We thank the reviewer for the suggestion. In response to the comment, we carried out additional confocal analysis to determine the localization of COPA with ER in C2C12 cells transiently transfected with GFP-COPA and DsRed-ER. Consistent with the results in COS-7 cells, COPA exhibited a diffused pattern in C2C12 cells under AA starvation, and became punctated in response to AA stimulation (Fig. R9, also see Figure EV5A in the revised manuscript).

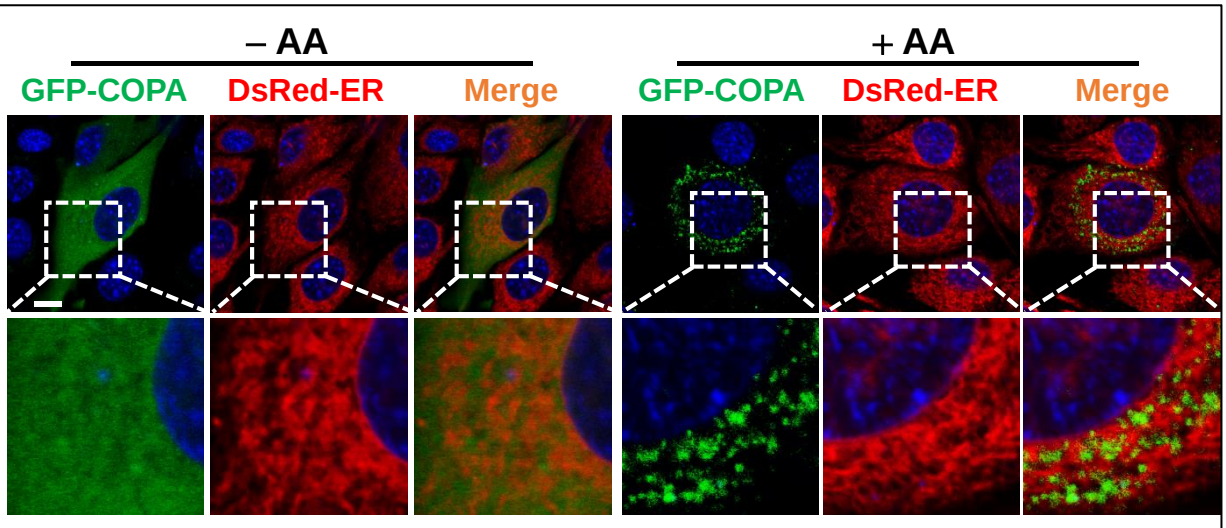

Fig. R9

7. Methods and figure legends lack some experimental details (specific antibody phosphosites, concentrations for several inhibitors, qPCR primer sequences).

Re: We thank the reviewer for the comments. We have added the experimental details in the Methods and figure legends. Please refer to the revised manuscript.

8. In the discussion of ARF1 and mTORC1 signaling, the authors should comment on Jewell et al., 2015.

Re: Yes, we have cited this beautiful Science paper by Jewell, *et al*. on differential regulation of mTORC1 by leucine and glutamine in the results and discussion parts of the revised manuscript. Please refer to pages 13 and 18 of the revised manuscript (reference 19 in the revised manuscript).

9. On p. 14, the authors misleadingly relate their findings on the role of actin in amino acid-mediated mTORC1 activation to Jacinto et al., who study functions of mTORC2 in regulating actin dynamics.

Re: We thank the reviewer for pointing out this error. We have cited a different reference in the revised manuscript. Please refer to page 15 of the revised manuscript.

Referee #3:

The manuscript by the Shi group reports several upstream regulators of mTORC1 localization upon amino acid starvation and stimulation. They propose that Aster C sequesters mTORC1 in the ER during starvation conditions while COP I vesicles and actin dynamics participates in delivering mTORC1 to the lysosomal membranes upon stimulation. The manuscript contains several interesting observations suitable for publication in Embo reports. However, the attempt in identifying the whole pathway from Aster C to COP I and actomyosin contractility is ambitious and not fully supported by the experimental data. One suggestion may be to strengthen the Aster C-ER-TSC2-mTORC1 part and leave out the COP I vesicle-actomyosin part for future in depth analysis.

1) The claim that mTOR interacts with Aster C in the ER is based on co-IP experiments using overexpressed Aster C and mTOR (Fig. 2). This should be confirmed using endogenous proteins and immunofluorescence studies. In addition, mTOR is also present in mTORC2 complexes that have been reported in different subcellular compartments, including ER. The authors should perform immunofluorescence detection of mTORC1-specific localization in the ER, together with Aster C. Tag-versions of the proteins should be expressed at levels comparable to endogenous by retroviral transduction.

Re: We thank the reviewer for these comments and good suggestions. First of all, the reason we used overexpressed Aster-C in Co-IP experiments is because of the lack of a

good commercial anti-Aster-C antibody. As suggested, instead of using the retroviral transduction, we engineered a lentiviral expression system for FLAG-tagged Aster-C. The recombinant lentivirus was successfully packaged in HEK293T cells by co-transfecting cells with pLJM1-FLAG-Aster-C, psPAX2 and pVSVG plasmids. The culture medium which contained the lentivirus was collected and used to infect the C2C12 cells, which was then selected by puromycin (2 μg/mL). Unfortunately, this work was recently disrupted by the COVID-19 pandemic.

As an alternative to the suggested experiments, we carried out subcellular fractionation analysis of Aster-C, endogenous mTOR, and components of the GATOR2 complex (WDR24 and Mios) to determine whether Aster-C co-localizes with the endogenous mTOR and the GATOR2 complex at the ER under AA starvation, as suggested by both reviewer 1 and 2. HEK293T cells transiently transfected with FLAG-tagged Aster-C were fractionated into the rough ER (RER), lysosomal, and microsomal fractions by differential centrifugation, followed by western blot analysis of each of the proteins. The results showed that Aster-C predominantly co-localized with the endogenous mTOR and the GATOR2 complex at the rough ER (RER) during amino acids starvation. In response to AA stimulation, both mTOR and GATOR2 complex, but not Aster-C, translocated from the RER to lysosomes (Fig. R1A, also see Figure 2B in our revised manuscript). In contrast, Aster-C deficiency caused a significant shift of mTOR from the RER fraction to the lysosomal fraction during AA starvation, and is no longer responsive to AA stimulation (Fig. R1B, also see Figure 2C in our revised manuscript). Together, these new data provide further support of our findings from co-IP and confocal imaging analysis that Aster-C sequesters mTOR at the ER during AA starvation.

We also repeated the Co-IP experiment by doubling the total protein used in immunoprecipitation analysis. Please refer to Fig. R6 and Figure 2E in the revised manuscript, as well as the details described in response to Reviewer #1's comment #7. The data showed that Aster-C interacted with Raptor, but not Rictor, indicating that Aster-C sequestered mTORC1, but not mTORC2 complex, under starvation.

2) Again, the co-localization of COPA and mTOR is based on massive overexpression of ectopic proteins by transient transfection of C2C12 cells (Fig. 3D). The authors should switch to retroviral vectors in order to achieve stable expression similar to endogenous.

Re: As suggested, we engineered pLJM1-EGFP-COPA lentiviral expression plasmid for generating EGFP-COPA stable expression cell line. As explained above, our effort in generating recombinant lentiviral expression for EGFP-COPA was also delayed by the coronavirus outbreak. However, we believe that overexpression of GFP-COPA should not affect our conclusion for the following reasons. First, overexpression of the GFP tagged COPA did not affect its biological function, as previously reported [9,10]. Second, our data

show that overexpressed GFP-COPA not only specifically interacted with the endogenous mTOR, but also with the endogenous COPG, another component of COPI vesicles (Figure 3B in the revised manuscript). Moreover, the interaction between GFP-COPA and mTOR is significantly enhanced in response to AA stimulation (Figure 3B in the revised manuscript). These findings were further corroborated by the results from our Co-IP experiment which showed that the endogenous COPA interacted with mTOR, and the interaction was enhanced by AA stimulation (Figure 3C in the revised manuscript).

3) The immunofluorescence localization of mTORC1 should be analyzed in ARF1 knockout cells, and not only after the pharmacological inhibition that may be unspecific (Fig. 5A). The effect on mTORC1 activation is minor in ARF1 knockout cells as compared to pharmacological agents (compare Fig. 5D with 5B and C).

Re: We thank the reviewer for the suggestion. In response to the comments, we carried out the immunofluorescence localization analysis of mTOR on lysosomes in the vector and Arf1 KO cells as suggested. The results show that AA induced mTOR activation and localized with lysosomes in vector cells, while much less mTOR was activated and localized with lysosomes in Arf1 KO cells (Fig R10). These results were consistent with the immunoblot data, and further confirmed that Arf1 deficiency inhibited the mTORC1 activation.

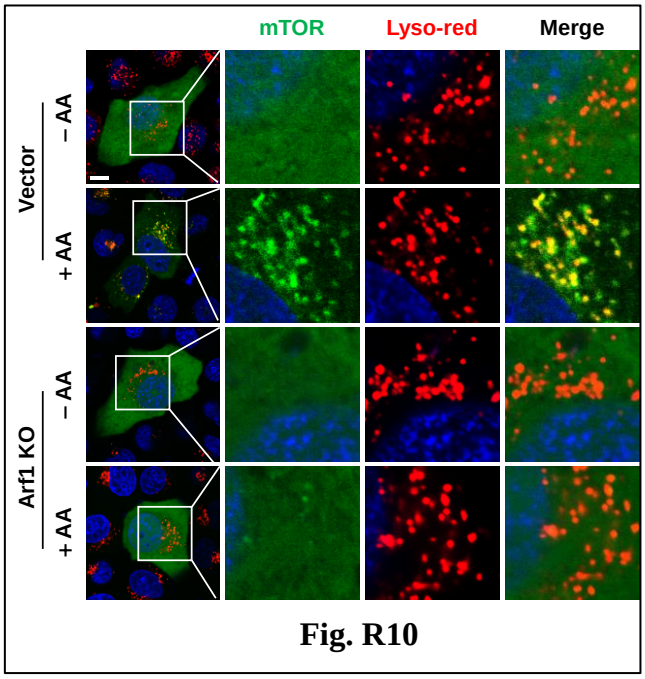

Fig. R10

4) MYH10 knock-out appears to induce the up-regulation of mTORC1 independent on nutrient availability (Fig. 6B). No resistance to starvation is detected. This is different to the Aster C knockout studies shown in Fig. 1A, for instance. My interpretation would be that MYH10 has a general effect on mTORC1 activity, independent to nutrient sensing and Aster C function.

Re: This is certainly a possibility, since MYH10 regulates diverse cellular functions, including cytokinesis, regulation of cell shape, adhesion and migration. In support of this notion, a previous study showed that inhibition of MYH10 by blebbistatin activated mTORC1 through induction the activity of phosphoinositide 3-kinase [11]. However, our interpretation was based on the findings that Aster-C interacted with MYH10, and the interaction was blocked by blebbistatin treatment (Figure 6A in the revised manuscript).

Reference

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1st Revision - Editorial Decision 26th May 2020

Dear Prof. Shi,

Thank you for submitting the revised version of your manuscript. It has now been seen by all of the original referees.

As you can see, the referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address some minor points below:

• Please address the remaining concerns of referees 1 and 3 textually.
• As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database.
• Movies need to be ZIPped with their legends. The legends should be removed from the Article file.
• We noted that scale bars of Figure 1D and G are either missing or not visible enough.
• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

This manuscript by Zheng et al. interrogates important questions into how amino acid sufficiency controls the subcellular localization and trafficking of mTORC1. The work centers on the interesting finding that knockout (KO) of Aster-C, an ER resident protein, increases mTORC1 signaling in cells cultured in complete media as well as those starved or stimulated with amino acids. It fills important gaps in knowledge regarding where in the cell mTOR localizes in the absence of amino acids, and how mTOR traffics to lysosomal membranes during amino acid stimulation. The authors have

addressed my comments in a mostly satisfactory manner by including new, convincing experimental data and revising the text appropriately. Note that point 2 below needs to be addressed, as the authors' rebuttal still concerns me. Below find additional comments.

1. Text on pg. 5 explains that Aster-C knockout cells, which display elevated mTORC1 signaling, also display reduced phosphorylation of the AMPK site on ULK1 (S555). A recent paper reported that mTORC1 directly inhibits AMPK (Ling et al, 2020, Nature Metabolism 2(1): 41). This finding may explain why Aster-C knockout cells display reduced P-ULK1-S555, and the authors should consider citing this paper.

2. In Point 8 (Rev1), I noted my concern with the finding that amino acid stimulation of starved cells increased P-Akt S473 and T308. I'm worried that amino acid stimulation apparently Akt phosphorylation due to high pH shock rather than by amino acids themselves. We have found that commercial amino acid mixtures (at 50X and when diluted to a working stock of 5X) possess a pH of ~10, and if the amino acid mixture is not pH'ed from 10 to ~7.4 prior to amino acid stimulation, the high pH amino acids indeed increase P-Akt S473 and T308 and do so rapidly (2-10 min). In addressing my comment, the authors explain that they added back amino acids to starved cells, and then pH'ed the media to 7.4. Thus, they likely added pH 10 amino acids to the cells, which likely rapidly increased Akt phosphorylation. The authors should pH the amino acid mixture to 7.4 first before adding it to cells. I bet amino acids at pH 7.4 will fail to increase P-Akt in their hands when added this way, across the three different methods for amino acid starvation and stimulation.

The authors should consider removing this data, as it may not be correct, or repeat the experiment as proposed above (I acknowledge that this may not be possible to do in a timely manner, however, due Covid-19 shutdowns).

Referee #2:

Zhang et al submit a substantially revised manuscript that addresses my concerns to my satisfaction.

Referee #3:

The authors addressed some concerns. Unfortunately, they state that they could not do the lentiviral transduction experiments due to Covid. This is understandable, but still a pity as the intracellular localization experiments really need to be controlled for expression of the tag protein. This caveat should be added to the discussion.

Response to Reviewers' comments:

First of all, we would like to thank editor and all the reviewers for their comments and suggestions, and consideration for publishing our study on *EMBO Reports*. Below are our response to editor and reviewers' comments.

Editor's comments:

As you can see, the referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address some minor points below:

• Please address the remaining concerns of referees 1 and 3 textually.

Re: We have replied the reviewers' comments. Please see the point-to-point response below.

• As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database.

• Movies need to be ZIPped with their legends. The legends should be removed from the Article file.

• We noted that scale bars of Figure 1D and G are either missing or not visible enough.

• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.

• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Re: We have addressed all the comments and suggestions of the editor and the production/data editors in our revised manuscript.

Referee #1:

This manuscript by Zheng et al. interrogates important questions into how amino acid sufficiency controls the subcellular localization and trafficking of mTORC1. The work centers on the interesting finding that knockout (KO) of Aster-C, an ER resident protein, increases mTORC1 signaling in cells cultured in complete media as well as those starved or stimulated with amino acids. It fills important gaps in knowledge regarding where in the cell mTOR localizes in the absence of amino acids, and how mTOR traffics to lysosomal membranes during amino acid stimulation. The authors have addressed my comments in a mostly satisfactory manner by including new, convincing experimental data and revising the text appropriately. Note that point 2 below needs to be addressed, as the authors' rebuttal still concerns me. Below find additional comments.

1. Text on pg. 5 explains that Aster-C knockout cells, which display elevated mTORC1 signaling, also display reduced phosphorylation of the AMPK site on ULK1 (S555). A recent paper reported that mTORC1 directly inhibits AMPK (Ling et al, 2020, Nature Metabolism 2(1): 41). This finding may explain why Aster-C knockout cells display reduced P-ULK1-S555, and the authors should consider citing this paper.

Re: We would like to thank the reviewer for the suggestion. We have cited the report mentioned in our revised manuscript (Reference 8). Please refer to page 5 of the revised manuscript.

2. In Point 8 (Rev1), I noted my concern with the finding that amino acid stimulation of starved cells increased P-Akt S473 and T308. I'm worried that amino acid stimulation apparently Akt phosphorylation due to high pH shock rather than by amino acids themselves. We have found that commercial amino acid mixtures (at 50X and when diluted to a working stock of 5X) possess a pH of ~10, and if the amino acid mixture is not pH'ed from 10 to ~7.4 prior to amino acid stimulation, the high pH amino acids indeed increase P-Akt S473 and T308 and do so rapidly (2-10 min). In addressing my comment, the authors explain that they added back amino acids to starved cells, and then pH'ed the media to 7.4. Thus, they likely added pH 10 amino acids to the cells, which likely rapidly increased Akt phosphorylation. The authors should pH the amino acid mixture to 7.4 first before adding it to cells. I bet amino acids at pH 7.4 will fail to increase P-Akt in their hands when added this way, across the three different methods for amino acid starvation and stimulation.

The authors should consider removing this data, as it may not be correct, or repeat the experiment as proposed above (I acknowledge that this may not be possible to do in a timely manner, however, due Covid-19 shutdowns).

Re: We would like to thank the reviewer for the comment. As we discussed in the previous rebuttal, we did adjusted the amino acids buffer pH back to 7.4 before we added to cells, as the way the reviewer suggested, in all the experiments of this study. Additionally, we used 2X amino acids working concentration in our experiments. When the 50X MEM amino acids stock solution (Invitrogen, Cat#11130051) was diluted to 2X in KRPH buffer, the pH was about 3.6. As shown in our results (see previous rebuttal Fig R7), amino acids induced phosphorylation of Akt T308 and S473 at 10 min stimulation, while decreased the Akt T308 and S473 phosphorylation by 30 min. Based on the literature, the regulatory role of amino acids on Akt phosphorylation is controversial, and the differences may due to different cell type, starvation and amino acids re-stimulation methods, as well as the amino acids stimulation time. However, as suggested, we have removed the amino acids related Akt phosphorylation data from our manuscript, since these data do not affect the conclusion of this study.

Referee #2:

Zhang et al submit a substantially revised manuscript that addresses my concerns to my satisfaction.

Referee #3:

The authors addressed some concerns. Unfortunately, they state that they could not do the lentiviral transduction experiments due to Covid. This is understandable, but still a pity as the intracellular localization experiments really need to be controlled for expression of the tag protein. This caveat should be added to the discussion.

Re: We thank reviewer for the comment, and we completely agree with the reviewer. Based on the results from our experiments, including the subcellular fractionation analysis, the Co-IP analysis and the confocal imaging analysis, the transiently expressed tag proteins (FLAG-Aster-C, GFP-Aster-C and GFP-COPA) maintained biological function in cells, and did not affect our conclusions in this study. However, we have discussed this deficiency in the results part of our revised manuscript. Please refer to page 8 of the revised manuscript.

2nd Revision - Editorial Decision 12th Jun 2020

Dear Dr. Shi,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Before we can transfer your manuscript to our production team, we need to sort out the following. We note that Figure 1D+G rows 2,3,4 are missing a scale bar. You can send the updated figure per email. We are looking forward to receiving it.

Kind regards,

Deniz Senyilmaz Tiebe
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Yuguang Shi
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-49898V1

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?	Sample size selection and statistical analysis were carried out by following standard of practice. Triplicate samples were used for real-time qPCR analysis, whereas more than 100 cells were used for the measurement of cellular size. Western blot data are representative of at least two independent experiments. Confocal analysis were done in three independent experiments, and 15-30 cells were selected for Pearson's correlation coefficient analysis. Statistical analysis were carried out by Student t-test or one-way ANOVA. Data were presented as mean +/- SD.
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?	No samples were excluded from the analyses of the data reported in this mansucript.
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. All the experiments in this project were done in cell lines and were not necessary to be allocated to groups.
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.	Yes. When the data was needed to be confirmed or cross verified by different investigators involved in the studies, the investigator was blinded to the cell genotype.
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. Statistical test were justified appropriate. Students t-test was used for two group comparison. For multiple comparisons, statistical significance was determined using one-way ANOVA.
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?	NA

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

http://www.ebi.ac.uk/ega

http://biomodels.net/

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

Is the variance similar between the groups that are being statistically compared?	No significant difference in variance was observed between the groups that were statistically compared.

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).	All the antibodies used for the studies in this manuscript were selected based on published papers or our previous experience, and were further validated for their authenticity in our pilot test before they were used for the experiments detailed in this manuscript . The supplier name and catalog number of the antibodies are detailed in the methods section. Validation statements and relevant references can be found for each antibody on the manufacturer's website.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	C2C12, COS-7, and HEK293T cell lines were obtained from one of our collaborators' lab who initially purchased the cell lines from ATCC. are routinely tested for mycoplasma contamination. The wild type mouse embryonic fibroblasts (MEFs) and MEFs from myosin IIb knockout mice were obtained from Drs. Robert Adelstein and Xuefei Ma at NIH/NHLBI .All cells were maintained under sterile conditions with multiple aliquots frozen down from initial expansions. When stable cell lines were engineered, they were maintained at low passage number for all the studies reported in this manuscript. Multiple individual cell clones were selected, characterized, and expanded before being frozen in liquid nitrogen tank for long-term usage. We routinely assess for mycoplasma contamination on a quarterly basis or when new lines are introduced to the laboratory. The parental C2C12 cell lines stably transfected with Aster-C CRISPR/HDR plasmids or empty vector plasmid were selected and maintained antibiotic resistance to puromycin, and routinely screened for Aster-C expression levels by qPCR analysis, since no antibodies were available.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

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