Journal of Cell Science

Oligopeptide transporter Slc15A modulates macropinocytosis in *Dictyostelium* by maintaining intracellular nutrient status

Yiwei Zhang, Hui Tu, Yazhou Hao, Dong Li, Yihong Yang, Ye Yuan, Zhonglong Guo, Lei Li, Haibin Wang and Huaqing Cai
DOI: 10.1242/jcs.259450

Editor: Mahak Sharma

Review timeline
Original submission: 9 October 2021
Editorial decision: 22 November 2021
First revision received: 11 February 2022
Accepted: 2 March 2022

Original submission

First decision letter

MS ID#: JOCES/2021/259450

MS TITLE: Oligopeptide transporter Slc15A modulates macropinocytosis in Dictyostelium by maintaining intracellular nutrient status

AUTHORS: Huaqing Cai, Yiwei Zhang, Haibin Wang, Yazhou Hao, Hui Tu, Dong Li, Zhonglong Guo, Yihong Yang, Ye Yuan, and Lei Li
ARTICLE TYPE: Short Report

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

Both reviewers found the study to be interesting and well executed in parts. As you will find, the reviewers, especially reviewer 1 has raised substantial concerns that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. In particular, reviewer 1 has recommended use of non-axenic Dictyostelium strain and has also pointed to several methodological shortcomings and incomplete analyses. Reviewer 2 also recommends additional experiments to support the hypothesis that 'nutrient availability is specifically detected from early macropinosomes'.
If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Huaqing Cai and coworkers identifies a novel regulatory mechanism of macropinocytosis. Macropinocytosis has long been the least well studied pathway of endocytosis, but recent work in Dictyostelium and other model systems has put it back in center stage. The present study presents mostly convincing findings about the role of a peptide transporter in the nutrient-dependent regulation of the level of pinocytosis in the amoeba Dictyostelium, using a range of molecular and cellular approaches. The authors reasonably speculate about the signalling pathways involved, but do not attempt at investigating whether and how conserved the pathway is in animal phagocytes. The manuscript is concise and well written, and many of the experiments are elegant and solid, especially the excellent use of many fluorescent reporters in live experiments (excellent movies), but some of the experimental designs are relatively weak or inappropriate, and therefore leave room to alternative explanations. In summary, the study is timely and interesting, but would greatly benefit from some stronger experimental demonstration of the mechanisms claimed.

Comments for the author

Major criticisms
1- The potentially new mechanism of regulation of macropinocytosis described here was studied only in axenic laboratory strain(s). First, the differences between macropinocytosis/phagocytosis between wild-type isolates and axenic laboratory strains have to be better described, especially the role of non-sense mutations in NF1, and other "facilitator" mutations that increase macropinocytosis in axenic strains.
Second, now that the non-axenic strains have been opened to routine genetic manipulation, an attempt should be made to KO Slc15A in such a strain, to generalise its involvement in a "natural" setting.
2- p.4, line 89 and Fig 1. The authors mention that they "quantify the rate of macropinocytosis", but they only measure the relative projected surface of the endo-lysosomal compartments filled with a fluid-phase tracer. It is in fact not clear how the total intensity is measured. How many optical sections are recorded?
Do they present the projected sum or average intensity?
3- In addition, p.5, line 105-108, Fig 1. In any case, the authors do not measure a rate of uptake, but only a variable that corresponds to the steady state amount of a fluid-phase tracer. First, intensity of projected surface is not the same as volume, and this introduces a bias in the quantitative interpretation. Second because this is a steady state measurement, it is not the same as a rate, which by definition is a measurement as a function of time. The differences observed in "volume" are interesting, but a decreased volume in the mutants can be due to a lower uptake, or a faster and more efficient exocytosis. Therefore it is crucial that the authors also use standard experimental strategies to measure the rate of uptake, the steady state plateau (the transit time) and the rate of exocytosis, by a combination of pulse and chase regimes of fluorescent tracers. The results from Fig 2B, estimating the size and frequency of macropinocytic events is encouraging, but relies on some subjective criteria. Therefore, it does not replace a more quantitative uptake and transit experiment.
4-The experiments presented in Fig 2A with a series of endocytic markers are welcome, but snapshots do not replace a more dynamic observation of live cells and a more quantitative assessment of the residency time of the various markers as a function of a pulse-chase TD uptake experiment.

5- Similarly, Fig 2B is very interesting, because it allows the authors to visualise the uptake events and the plasma membrane domains involved, but it is VERY surprising to me that they ignore a striking phenomenon. Indeed, the PHcrac-GFP reporter is usually highlighting the plasma membrane domain that is invaginations, as well as the early macropinosome for a few minutes. This is radically different in the slc15A KO mutants, where the reporter leaves the pino some as soon as it pinches off the plasma membrane. This points out to mechanisms that are or not the same as proposed in the discussion. The lead author is a specialists of chemotaxis and cell polarisation and likely saw this phenomenon. Please comment.
6- Because of the above, the authors have to extend the cartography of the PIP species during macropinocytosis in the KO mutant, using not only TAPP1 (as in Fig 4 for wt cells and Slc15A-GFP KI cells), but other standard PIP reporters. And a schematic summary of the working model should include these PIP dynamics.
7- The authors quantitate the number of autophagosomes at steady state and conclude about the dynamic of autophagy in the KO mutant. As for the rate of uptake above, the authors do not use the appropriate experimental design to conclude about the rate of autophagy. Indeed, it is well known that an increase of Atg8 structures can be the result of an increase autophagy initiation or a block in autophagy maturation and digestion. The authors have to perform an autophagy flux assay. Several alternative assays have been established for Dictyostelium and published in recent years.
8- The authors use a relatively crude assay to measure phagocytosis. The clearing of a bacterial suspension offers only a poor proxy for a standard phagocytosis assay, best performed with fluorescent beads and bacteria, usually by FACS. Again, several alternative assays have been established for Dictyostelium and published in recent years.
9- In all the experiments presented, the authors only mention n in their figure legends, but never N. Obviously, it has to be rigorously described how many biological replicates (N) and technical replicates (n) have been performed for each experiment. The Material and Method section mentions some of this, but the best place is the figure legends.
10- The experiments performed on the KO mutant and the "complementation" by a diet of dipeptides is potentially elegant and strong. But I would appreciate that the authors eliminate other causes for a lack of nutrient, which would be that the absence of the Slc15A transporter results in a major decrease of the degradative capacity of the endosomes. For this the pH and the proteolytic activity can be measure precisely as a function of the maturation stage of the macropinosome, using assays that have been established for Dictyostelium and published in recent years. The authors use a mix of FITC and TRITC in Fig S3, but this should be quantified as a function of time. The assay is not described in M&M. Proteolytic activity is best measured by the dequenching of DQ-Green as a function of time. It would also be easy to perform a direct and quantitative bacteria killing assay as published by the Cosson group.
11- Concerning the discussion/speculations, I would like to read more about Slc15A in wild-type non-axenic isolates, because the protein seems to be at the plasma membrane and in early macropinosomes but these are not necessarily environments that have the low pH necessary to its function, and also they probably do not contain significant dipeptide concentrations. I would recommend the authors make really sure Slc15A is not present in post-lysosomes, a compartment where many if not most other transporters are concentrated to extract post-digestion nutrients.
12- Finally, the authors peculate about a possible involvement of mTOR (C1 or C2 ?) but instead of speculations, they could relatively easily monitor the phosphorylation status of known and tested substrates and subunits, such as Raptor and 4E-BP1 etc...

Minor criticisms
1- p.3, line 53, the sentence mentioned "studies", but cites mainly reviews. A greater effort should be spent in citing the relevant primary literature.
2- All Figures, y axes, correct "flourescence" to "fluorescence".
3- Fig 1E-F, the y axis mentions TD fluorescence, whereas the ext indicates that it is normalised to cell size (projected areas). Please, indicate this in the Figures.
4-The Materials and Methods is extremely concise, and some assays are not or imperfectly described.
5- p.7, line 169 and 173, replace the words "prove" and "demonstrate" by "test" and "indicate".
6- p.7, line 186-187. Rephrase, because Slc15A being a transmembrane protein, it cannot dissociate from the endosomes, it can be recycled.
7- p.7, line 188. Please, correct, LmpA is not a lysosomal marker, but is present mainly in post-lysosomes

(Sattler et al, 2018). In addition, the authors use here a fusion protein of LmpA with GFP at its N-terminus whereas the authors of that article used a C-terminal fusion. How sure are the authors that the two proteins are behaving identically?

Reviewer 2

Advance summary and potential significance to field

This is a very nice, clearly written paper describing the identification of a new regulator of macropinocytosis in Dictyostelium. It has previously been shown that these cells regulate their rate of fluid uptake according to nutrient availability. Here the authors identify an oligopeptide transporter that appears to import di- or tri-peptides into the cytosol leading to the upregulation of macropinocytosis.

This is nice work, with good and well controlled experiments that clearly support their main conclusions. The nutrient transporters and how nutrient status is detected and regulates macropinocytosis is very poorly understood in both Dictyostelium and mammalian cells, so this provides an interesting and important advance. The only real criticism I have is the seeming disconnect between acidification and transporter localisation.

Comments for the author

In figure 4E, they show that Slc5A-GFP is on the plasma membrane and gets removed from newly formed macropinosomes approximately 60s after formation (which I estimate as between 30-60secs from their time series. As they state, (L191) these transporters rely on a proton gradient for activity, however both the published literature and their data in Fig S3B, macropinosomes indicate that macropinosomes don't really start to acidify until after this time. I am unconvinced by their argument in the text (L206-208) that acidification is fast enough to significantly overlap with the presence of Slc5A – at best they will overlap for only a few seconds.
They could test this by altering the pH or buffering capacity of the medium, or whether Slc5A coincides with V-ATPase recruitment. Whilst their data clearly support a role for this transporter in oligopeptide transport, it seems more likely Slc5A activity occurs at the plasma membrane in general rather than invoking a specific mechanism on early macropinosomes, as proposed.
If the authors wish to promote their speculative hypothesis that nutrient availability is specifically detected from early macropinosomes they should support this better experimentally, however in my opinion this is not essential prior to publication given a more balanced discussion and adaptation of their model in Fig S5 accordingly.

Minor points:
It would be helpful to have more details on the screen – how many clones did they screen? How many hits were identified – is table 1C all of them? Were any previously-identified macropinocytosis mutants found, and what was the spread of the phenotypes? This is important to understand the sensitivity of this approach and how saturated their screen was.
Did they account for changes in cell size in both the screen and macropinocytosis assays?
Methods are a bit sparse. They should also include more technical details, or at least cite a reference for the inverse PCR used to identify REMI insertion sites.

Reviewer 3

Advance summary and potential significance to field

In this work, via a forward genetic screen, the authors identify Slc15a (homologue of the mammalian SLC15A1 and SLC15A2) as a key player in oligopeptide uptake via micropinocytosis in the Dictyostelium. They also go on to demonstrate that Slc15a is needed for early formation of the macropinosomes, and they connect this to arginine and lysine uptake via the transporter. The combination of identification in the original screen, subsequent knockout directly of the channel, knock-in mutations of the tagged channels and the rescue with amino acids versus peptides are all

done elegantly and carefully. Further, the tagged constructs also suggest that Slc15a is expressed on the plasma membrane and that they are involved in the formation of the early macropinosomes.

Comments for the author

Overall, this is an extremely well done paper and I have no concerns. I support the publication of this work.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
The manuscript by Huaqing Cai and coworkers identifies a novel regulatory mechanism of macropinocytosis. Macropinocytosis has long been the least well studied pathway of endocytosis, but recent work in *Dictyostelium* and other model systems has put it back in center stage. The present study presents mostly convincing findings about the role of a peptide transporter in the nutrient-dependent regulation of the level of pinocytosis in the amoeba *Dictyostelium*, using a range of molecular and cellular approaches. The authors reasonably speculate about the signalling pathways involved, but do not attempt at investigating whether and how conserved the pathway is in animal phagocytes. The manuscript is concise and well written, and many of the experiments are elegant and solid, especially the excellent use of many fluorescent reporters in live experiments (excellent movies), but some of the experimental designs are relatively weak or inappropriate, and therefore leave room to alternative explanations. In summary, the study is timely and interesting, but would greatly benefit from some stronger experimental demonstration of the mechanisms claimed.

Reviewer 1 Comments for the Author:

Major criticisms

1- The potentially new mechanism of regulation of macropinocytosis described here was studied only in axenic laboratory strain(s). First, the differences between macropinocytosis/phagocytosis between wild-type isolates and axenic laboratory strains have to be better described, especially the role of non-sense mutations in NF1, and other "facilitator" mutations that increase macropinocytosis in axenic strains. Second, now that the non-axenic strains have been opened to routine genetic manipulation, an attempt should be made to KO Slc15A in such a strain, to generalise its involvement in a "natural" setting.

RESPONSE: As suggested by the reviewer, we generated *slc15A* KO cell line in the non-axenic strain DdB (new Fig. S2C). Bacterially grown DdB and *slc15A*$^-$ cells were adapted in HL5 medium supplemented with 10% FBS for 24 h before macropinocytosis was measured. TRITC-dextran uptake was considerably lower in DdB than Ax2 cells (new Fig. S2D), and deletion of *slc15A* further decreased this activity (new Fig. 2E-F). We revised the manuscript to incorporate the new data (page 6, lines 126-129). We also added description of the differences between wild-type isolates and axenic laboratory strains (page 3, lines 66-69).

2- p.4, line 89 and Fig 1. The authors mention that they "quantify the rate of macropinocytosis", but they only measure the relative projected surface of the endo-lysosomal compartments filled with a fluid-phase tracer. It is in fact not clear how the total intensity is measured. How many optical sections are recorded? Do they present the projected sum or average intensity?
3- In addition, p.5, line 105-108, Fig 1. In any case, the authors do not measure a rate of uptake, but only a variable that corresponds to the steady state amount of a fluid-phase tracer. First, intensity of projected surface is not the same as volume, and this introduces a bias in the quantitative interpretation. Second, because this is a steady state measurement, it is not the same as a rate, which by definition is a measurement as a function of time. The differences observed in "volume" are interesting, but a decreased volume in the mutants can be due to a lower uptake, or a

faster and more efficient exocytosis. Therefore, it is crucial that the authors also use standard experimental strategies to measure the rate of uptake, the steady state plateau (the transit time) and the rate of exocytosis, by a combination of pulse and chase regimes of fluorescent tracers. The results from Fig 2B, estimating the size and frequency of macropinocytic events is encouraging, but relies on some subjective criteria. Therefore, it does not replace a more quantitative uptake and transit experiment.

RESPONSE: We appreciate the reviewer's suggestions. In most experiments including the screen, macropinocytic activity was quantified by recording the medial optical section of cell and measuring the ratio of total intensity of TD spots to cell area. We found that this method worked well for isolating mutants and estimating their defects, and it allowed us to see variation in a cell population. We didn't fix cells and preform a z-scan to acquire the total intensity because we found that the dextran signal was partially lost after fixation even using lysine-fixable dextran.

However, we agree with the reviewer that this method does not measure the volume or the rate of fluid uptake. Therefore, we performed additional fluorimetric analyses of macropinocytosis and exocytosis. As shown in the newly added Fig. 2C, 2D and S6, deletion of *slc15A* significantly reduced the rate and steady-state plateau of macropinocytosis without affecting exocytosis, and expression of Slc15A-GFP or growing cells in SIH medium rescued the defects. These experiments substantiate our conclusion that Slc15A is required for macropinocytosis regulation. We revised the manuscript to incorporate the new data (page 6, lines 122-126; page 8, lines 187- 188).

4- The experiments presented in Fig 2A with a series of endocytic markers are welcome, but snapshots do not replace a more dynamic observation of live cells and a more quantitative assessment of the residency time of the various markers as a function of a pulse-chase TD uptake experiment.
5- Similarly, Fig 2B is very interesting, because it allows the authors to visualise the uptake events and the plasma membrane domains involved, but it is VERY surprising to me that they ignore a striking phenomenon. Indeed, the PHcrac-GFP reporter is usually highlighting the plasma membrane domain that is invaginations, as well as the early macropinosome for a few minutes. This is radically different in the slc15A KO mutants, where the reporter leaves the pinosome as soon as it pinches off the plasma membrane. This points out to mechanisms that are or not the same as proposed in the discussion. The lead author is a specialists of chemotaxis and cell polarisation and likely saw this phenomenon. Please, comment.
6- Because of the above, the authors have to extend the cartography of the PIP species during macropinocytosis in the KO mutant, using not only TAPP1 (as in Fig 4 for wt cells and Slc15A-GFP KI cells), but other standard PIP reporters. And a schematic summary of the working model should include these PIP dynamics.

RESPONSE: As suggested by the reviewer, we performed live cell imaging on additional PIP markers. The number of macropinosomes successfully generated per cell per minute was significantly reduced in *slc15A*$^-$ cells. In many instances, macropinocytic cups aborted without vesicle formation in *slc15A*$^-$ cells; but in those "productive" events, the conversion of PIP3-to-PI(3,4)P2 and PI(3,4)P2-to-PI(3)P proceeded with similar kinetics. These results are presented in the newly added Fig. 3B and S3. Thus, as concluded in our original manuscript, the overall organization of the macropinocytic pathway appeared undisrupted by *slc15A* deletion. We revised the manuscript to incorporate the new data (page 6, lines 139-149).
The lower panels in Fig. 2B (Fig. 4A in the revised manuscript) likely captured aborted macropinocytosis in *slc15A*$^-$ cells and that was probably why the PHcrac reporter seemed to dissociate rapidly. The whole sequence of events can be better visualized in movie S1.

7- The authors quantitate the number of autophagosomes at steady state and conclude about the dynamic of autophagy in the KO mutant. As for the rate of uptake above, the authors do not use the appropriate experimental design to conclude about the rate of autophagy. Indeed, it is well known that an increase of Atg8 structures can be the result of an increase autophagy initiation or a block in autophagy maturation and digestion. The authors have to perform an autophagy flux assay. Several alternative assays have been established for *Dictyostelium* and published in recent years.

RESPONSE: We analyzed autophagy flux. WT and *slc15A*$^-$ cells expressing GFP-Atg8a were incubated in HL5 medium in the absence or presence of a protease inhibitor cocktail. The number of GFP-Atg8a puncta increased in both cell lines upon the addition of protease inhibitors (new Fig. S5A-B). Expression of Atg8a fused to a tandem RFP-GFP tag also revealed red-only puncta in *slc15A*$^-$ cells (new Fig. S5C). These experiments indicate that autophagy flux was not blocked in the KO cells.

8- The authors use a relatively crude assay to measure phagocytosis. The clearing of a bacterial suspension offers only a poor proxy for a standard phagocytosis assay, best performed with fluorescent beads and bacteria, usually by FACS. Again, several alternative assays have been established for *Dictyostelium* and published in recent years.

RESPONSE: We performed flow cytometry analysis of phagocytosis by mixing WT and *slc15A*$^-$ cells with mCherry-expressing *E.coli*. As shown in the newly added Fig. 4G, *slc15A* deletion did not affect bacterial phagocytosis.

9- In all the experiments presented, the authors only mention n in their figure legends, but never N. Obviously, it has to be rigorously described how many biological replicates (N) and technical replicates (n) have been performed for each experiment. The Material and Method section mentions some of this, but the best place is the figure legends.

RESPONSE: We thank the reviewer for the suggestions. The number of biological and technical replicates are now indicated in the figure legends in the revised manuscript. In addition, we revised the figures to show SuperPlots.

10- The experiments performed on the KO mutant and the "complementation" by a diet of dipeptides is potentially elegant and strong. But I would appreciate that the authors eliminate other causes for a lack of nutrient, which would be that the absence of the Slc15A transporter results in a major decrease of the degradative capacity of the endosomes. For this the pH and the proteolytic activity can be measure precisely as a function of the maturation stage of the macropinosome, using assays that have been established for *Dictyostelium* and published in recent years. The authors use a mix of FITC and TRITC in Fig S3, but this should be quantified as a function of time. The assay is not described in M&M. Proteolytic activity is best measured by the dequenching of DQ-Green as a function of time. It would also be easy to perform a direct and quantitative bacteria killing assay as published by the Cosson group.

RESPONSE: As suggested by the reviewer, we measured macropinosome acidification and degradative capacity of endosomes. Macropinosome acidification was quantified by measuring the ratio of FD to TD as a function of time after cup closure. Degradative activity was measured by the bacteria killing assay. As shown in the newly added Fig. S4B and 4H, *slc15A* deletion did not delay acidification or impair bacteria killing. Therefore, the lack of nutrient in *slc15A*$^-$ cells was unlikely to be caused by a defect in nutrient degradation.

11- Concerning the discussion/speculations, I would like to read more about Slc15A in wild-type non-axenic isolates, because the protein seems to be at the plasma membrane and in early macropinosomes, but these are not necessarily environments that have the low pH necessary to its function, and also they probably do not contain significant dipeptide concentrations. I would recommend the authors make really sure Slc15A is not present in post-lysosomes, a compartment where many if not most other transporters are concentrated to extract post-digestion nutrients.

RESPONSE: We performed additional experiments to determine the localization dynamics of Slc15A and compared it to the kinetics of macropinosome acidification. First, we generated Slc15A-RFP KI cells and imaged Slc15A-RFP together with the PI(3)P marker 2xFYVE. We found that Slc15A remained associated with macropinosomes that pinched off from the plasma membrane. The signal of Slc15A decreased at a later stage, which was accompanied by the gradual accumulation of 2xFYVE (new Fig. 7B). Together with the experiment in the original manuscript showing that Slc15A and TAPP1 colocalized on macropinosomes during initial trafficking (Fig. 7A), these experiments indicate that Slc15A recycles in the early stage of macropinocytic pathway and therefore is less

likely to traffic to more matured structures such as lysosomes and postlysosomes. This finding was confirmed by the lack of colocalization between Slc15A and LmpA (new Fig. S7). Second, we examined whether the presence of Slc15A on macropinosomes overlaps with macropinosome acidification. By incubating Slc15A-RFP KI cells with FITC dextran, we found that new macropinosomes quickly acquired an acidic environment indicated by the decrease of FITC signal before Slc15A-RFP was evidently recycled (new Fig. 7C). Quantification showed that Slc15A associated with newly internalized macropinosome for 115 ±31 sec (from 15 macropinosomes), whereas a significant drop of macropinosomal pH occurred within 60 sec after cup closure (new Fig. S4). Imaging Slc15A-GFP together with VatB also revealed that the recruitment of V-ATPase to macropinosomes started before the recycling of Slc15A (new Fig. 7D). Although these experiments cannot rule out the possibility that Slc15A also functions at the plasma membrane, they suggest that Slc15A at the nascent macropinosomes may have a higher activity for the presence of proton gradients. We added the relevant information in the revised manuscript (page 9, lines 215-241).

12- Finally, the authors peculate about a possible involvement of mTOR (C1 or C2?) but instead of speculations, they could relatively easily monitor the phosphorylation status of known and tested substrates and subunits, such as Raptor and 4E-BP1 etc...

RESPONSE: We tried to analyze mTORC1 activity by Western blot using a phospho-4EBP1 antibody (CST 9459). Although the same antibody was used in a previous study (Chang et al., BMC Biology, 2020), we failed to detect specific signal corresponding to phosphorylated 4EBP1. In our experiment, the antibody recognized a band at approximately 15 kDa, which is about the expected molecular weight of 4EBP1. However, the intensity of this band didn't change upon starvation or rapamycin treatment. At this stage, we don't know the reason for this discrepancy. But as discussed in the manuscript, mTORC1 inhibition does not seem to affect macropinocytosis in *Dictyostelium* (Rosel et al., JCS, 2012; Williams and Kay, JCS, 2018), and thus may not be the cause of macropinocytosis defects seen in *slc15A*$^-$ cells. The exact molecular mechanism underlying Slc15A function remains to be determined, which we hope can be the subject of future studies.

Minor criticisms

1- p.3, line 53, the sentence mentioned "studies", but cites mainly reviews. A greater effort should be spent in citing the relevant primary literature.

RESPONSE: Primary literature was cited in the next two sentences describing in more detail findings made in *Dictyostelium* and mammalian cells on the metabolic function of macropinocytosis (page 3, lines 60-66).

2- All Figures, y axes, correct "flourescence" to "fluorescence".

RESPONSE: We thank the reviewer for pointing out the mistake and have corrected it in the figures.

3- Fig 1E-F, the y axis mentions TD fluorescence, whereas the ext indicates that it is normalised to cell size (projected areas). Please, indicate this in the Figures.

RESPONSE: We corrected this in the figures.

4- The Materials and Methods is extremely concise, and some assays are not or imperfectly described.

RESPONSE: We revised the "Materials and Methods" section to include more technical details (changes are highlighted).

5- p.7, line 169 and 173, replace the words "prove" and "demonstrate" by "test" and "indicate".

RESPONSE: We made the recommended changes in the revised manuscript (page 9, lines 203 and 208).

6- p.7, line 186-187. Rephrase, because Slc15A being a transmembrane protein, it cannot dissociate from the endosomes, it can be recycled.

RESPONSE: We changed "dissociate" to "recycle" in the revised manuscript as recommended (page 9, line 223).

p.7, line 188. Please, correct, LmpA is not a lysosomal marker, but is present mainly in post-lysosomes (Sattler et al, 2018). In addition, the authors use here a fusion protein of LmpA with RFP at its N-terminus, whereas the authors of that article used a C-terminal fusion. How sure are the authors that the two proteins are behaving identically?

RESPONSE: We thank the reviewer for pointing out the inaccuracy. We described LmpA as a lysosomal/postlysosomal marker in the revised manuscript (page 9, line 225). We compared the localization of LmpA with a C-terminal HA tag and LmpA with an N-terminal RFP-tag. As shown in the following immunostaining experiment, LmpA-HA colocalizes with RFP-LmpA on vesicular structures in cells.

***NOTE**: We have removed unpublished data that had been provided for the referees in confidence.*

Reviewer 2 Advance Summary and Potential Significance to Field:
This is a very nice, clearly written paper describing the identification of a new regulator of macropinocytosis in *Dictyostelium*. It has previously been shown that these cells regulate their rate of fluid uptake according to nutrient availability. Here the authors identify an oligopeptide transporter that appears to import di- or tri-peptides into the cytosol leading to the upregulation of macropinocytosis.
This is nice work, with good and well controlled experiments that clearly support their main conclusions. The nutrient transporters and how nutrient status is detected and regulates macropinocytosis is very poorly understood in both *Dictyostelium* and mammalian cells, so this provides an interesting and important advance. The only real criticism I have is the seeming disconnect between acidification and transporter localisation.

Reviewer 2 Comments for the Author:
In figure 4E, they show that Slc5A-GFP is on the plasma membrane and gets removed from newly formed macropinosomes approximately 60s after formation (which I estimate as between 30-60secs from their time series. As they state, (L191) these transporters rely on a proton gradient for activity, however both the published literature and their data in Fig S3B, macropinosomes indicate that macropinosomes don't really start to acidify until after this time. I am unconvinced by their argument in the text (L206-208) that acidification is fast enough to significantly overlap with the presence of Slc5A – at best they will overlap for only a few seconds.
They could test this by altering the pH or buffering capacity of the medium, or whether Slc15A coincides with V-ATPase recruitment. Whilst their data clearly support a role for this transporter in oligopeptide transport, it seems more likely Slc5A activity occurs at the plasma membrane in general rather than invoking a specific mechanism on early macropinosomes, as proposed.
If the authors wish to promote their speculative hypothesis that nutrient availability is specifically detected from early macropinosomes they should support this better experimentally, however in my opinion this is not essential prior to publication given a more balanced discussion and adaptation of their model in Fig S5 accordingly.

RESPONSE: We thank the reviewer for the suggestions and have performed additional experiments to compare the kinetics of macropinosome acidification and Slc15A recycling. First, we generated Slc15A-RFP KI cells and incubated the cells with FITC dextran. We found that nascent macropinosomes quickly acquired an acidic environment indicated by the decrease of FITC signal before Slc15A-RFP was evidently recycled (new Fig. 7C). Quantification revealed that Slc15A associated with newly internalized macropinosome for 115 ±31 sec (from 15 macropinosomes), whereas macropinosome acidification was initiated almost immediately after cup closure with a significant drop in FD/TD ratio occurring within 60 sec (new Fig. S4).
Second, imaging Slc15A-GFP together with VatB showed that the recruitment of V-ATPase to macropinosomes started before the complete removal of Slc15A (new Fig. 7D). These experiments indicate that the presence of Slc15A on newly formed macropinosomes overlaps with the acidification process. However, at this stage we cannot rule out the possibility that Slc15A also

functions at the plasma membrane. As the reviewer suggested, we revised the manuscript to give a more balanced discussion (page 10, lines 229-241).

Minor points:
It would be helpful to have more details on the screen – how many clones did they screen? How many hits were identified – is table 1C all of them? Were any previously-identified macropinocytosis mutants found, and what was the spread of the phenotypes? This is important to understand the sensitivity of this approach and how saturated their screen was.

RESPONSE: The screen is an ongoing project. From the initial screen, ~3,000 independent clones were generated and subjected to high-content imaging analysis. Fifteen mutant clones with macropinocytosis activity below 50% of WT were verified by microscopy imaging and taken forward for gene identification. REMI sites were mapped successfully for 9 mutant clones (table 1C, *srpB* gene identified from three mutant clones). This initial screen did not find any previously-identified macropinocytosis mutants. We are continuingly optimizing the screening condition and hoping to achieve higher sensitivity and coverage.

Did they account for changes in cell size in both the screen and macropinocytosis assays?

RESPONSE: We quantified the ratio of total intensity of TD spots over cell area in the screen and macropinocytosis assays. We apologize for not making this clear before and have corrected it in the revised manuscript.

Methods are a bit sparse. They should also include more technical details, or at least cite a reference for the inverse PCR used to identify REMI insertion sites.

RESPONSE: We revised the "Materials and Methods" section to include more technical details (changes are highlighted) and we added reference for the inverse PCR experiment (page 13, line 313).

Reviewer 3 Advance Summary and Potential Significance to Field:
In this work, via a forward genetic screen, the authors identify Slc15a (homologue of the mammalian SLC15A1 and SLC15A2) as a key player in oligopeptide uptake via micropinocytosis in the *Dictyostelium*. They also go on to demonstrate that Slc15a is needed for early formation of the macropinosomes, and they connect this to arginine and lysine uptake via the transporter. The combination of identification in the original screen, subsequent knockout directly of the channel, knock-in mutations of the tagged channels and the rescue with amino acids versus peptides are all done elegantly and carefully. Further, the tagged constructs also suggest that Slc15a is expressed on the plasma membrane and that they are involved in the formation of the early macropinosomes. Overall, this is an extremely well done paper and I have no concerns. I support the publication of this work.

Second decision letter

MS ID#: JOCES/2021/259450

MS TITLE: Oligopeptide transporter Slc15A modulates macropinocytosis in Dictyostelium by maintaining intracellular nutrient status

AUTHORS: Huaqing Cai, Haibin Wang, Yiwei Zhang, Hui Tu, Yazhou Hao, Dong Li, Yihong Yang, Ye Yuan, Zhonglong Guo, and Lei Li
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

I would like to congratulate the authors for their impeccable revision of the study and manuscript. It is commendable that every single criticism and suggestion has been taken into account and numerous additional experiments performed. Sincerely, this is now one of the best article on mechanisms of macropinocytosis in Dictyostelium I have ever read or evaluated. It is an elegant study with a very strong message.

Comments for the author

No additional comment.

Reviewer 2

Advance summary and potential significance to field

The authors have made impressive efforts to address the comments by the referees.
I think they have done a good job and am happy to recommend publication.

Comments for the author

I have no further comments