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Fusion of Lysosomes with Secretory organelles leads to Uncontrolled Exocytosis in the Lysosomal Storage disease Mucopolidosis Type IV

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THE EMBO JOURNAL

1st Editorial Decision

18 December 2014

Dear Shmuel,

Thank you for submitting your manuscript entitled 'Lysosomes and Secretory organelles fusion in mucopolidosis type 4 causes Uncontrolled Exocytosis'. I have read it carefully, and I discussed your work within our editorial team. Additionally, I also sought advice from an expert in the field.

You analyze TRPML1 knock-out mice as a model for Mucopolidosis Type IV (MLIV). You examine secretory cells, and, interestingly, observe that secretion is increased upon loss of TRPML1. The observed glutamate secretion from neurons could cause the disease-associated neurodegeneration by triggering excitotoxicity. You further show that lysosomal content is secreted, because the lysosomes fuse with secretory granules to give rise to enlarged vesicles. The observed effects are specific to lysosomal storage disorder caused by loss of function of TRPML1, since *Npc1*^{-/-} cells do not display enhanced secretion.

We appreciate your findings and find them potentially very interesting. However, we also noted that it remains rather unclear at this stage how TRPML1 prevents fusion of specialized granules with lysosomes and how the absence of TRPML1 increases secretion in secretory cells. Therefore,

we thought that the insight provided, while interesting, might a bit too limited for further consideration here. In light of this, I consulted as mentioned above an expert in the field. I am afraid to say that the expert thinks that additional insight is required for peer-review here, given the rather descriptive nature of the study at this point. However, I would like to point out that the expert also thinks that the observations are intriguing. Therefore, while I am very sorry to say that we cannot offer publication at this stage, I would like to encourage you to add more mechanistic insight, and to resubmit your work to us at a later time point. Please let me know in case you want to discuss this further.

I am very sorry that I have to disappoint you on this occasion.

Appeal

22 December 2014

Thanks you for your message and for evaluation of our manuscript. I would like to bring one particular point that may have been overlooked in case it will help in sending the manuscript for review.

In your message you raise two points. The first how TRPML1 deletion leads to fusion with secretory granules and the second is the mechanism by which deletion of TRPML1 results in augmented exocytosis. It is not obvious how the first question can be fully addressed in that deletion of TRPML1 changes practically all lysosomal functions from inhibition of lysosomal Ca²⁺ release, over acidification of the lysosomes, inhibition of lipid and protein hydrolysis, just to name a few. However, as we and others have already showed, deletion of TRPML1 causes mistargeting of the lysosomes within the cell. Our studies show that in polarized cells, the lysosomes ARE SPECIFICALLY mistargeted to the granular area at the apical pole (Figure 2). This specific mistargeting increases the chance of lysosomal-granules interaction and perhaps fusion. Although this does not provide concrete mechanism and whether the deletion of TRPML1 resulted only in mistargeting of the lysosomes to the granules area, it does provide some evidence for why the fusion take place.

We can make a stronger point for why exocytosis is augmented. In the discussion we indicates that the dramatic increase in granules size changes the surface/volume ratio of the granules that must result in increased exocytosis. In addition, we show that in the hybrid organelles become more fusogenic by demonstrating enhanced v-SNARE/t-SNARE interaction (Fig. 6). The two effects provide a mechanistic evidence for the enhanced granules fusion and thus exocytosis.

We thank you for considering these points.

2nd Editorial Decision

23 January 2015

Thank you for submitting your manuscript entitled 'Lysosomes and Secretory organelles fusion in mucopolipidosis type 4 causes Uncontrolled Exocytosis'. I have now received reports of all three referees, which are enclosed below.

As you will see, while the referees consider that your study is potentially interesting, they also conclude that the work is too preliminary at this stage and that many of your conclusions are not sufficiently supported by the data provided. I won't list all concerns here, but essentially more substantive controls, rescue experiments and statistically significant analyses are required. The referees also note that the study is currently not well developed at the mechanistic level and referee #1 offers input on how to extend your analysis towards a better mechanistic understanding of your observations. We don't expect you to solve the mechanism underlying increased lysosome-secretory granule fusion and secretion in TRPML1 mutant cells in full, but anything that you can add would be appreciated.

Given the very constructive comments provided by the referees, I would like to invite you to provide me with a revised version of your manuscript, should you be able to substantiate your work along the lines suggested by the referees. This clearly demands a lot of work, and I can extend the revision time to 6 months, should that be helpful.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

This is an important and interesting paper that identifies both a major role for an intracellular ion channel in controlling fusion between different organelles and ii) provides a molecular basis for neurodegeneration that arises from the lysosomal storage disease mucopolipidosis type IV. The observation that lysosomes deficient in TRPML fuse with secretory vesicles in excitable and non-excitable cells suggests that the authors have discovered a basic mechanism, of considerable pathophysiological relevance.

The experiments have been well executed, and are carefully controlled with appropriate statistical analyses. The paper reads well and the conclusions drawn are well supported by the evidence. I have the following comments.

It is striking that all forms of exocytosis are enhanced, regardless of tissue or nature of the second messenger that triggers secretion (Ca vs cAMP). This suggests that fusion of lysosomes with secretory organelles somehow promotes the interaction of V- and T-SNARE proteins, providing a possible molecular basis for the enhanced exocytosis. Perhaps the authors could consider this possibility further.

It is not entirely clear, at least to me, why the Ca oscillations to CCK are reduced in frequency in TRPML^{-/-} cells. CCK recruits both the IP3 and NAADP pathways, neither of which are thought to act on TRPML. Perhaps the Ca content of the lysosome has changed, indirectly affecting the NAADP response. Regardless, the authors should clarify in the text what the mechanism is.

10 pM CCK evokes Ca oscillations in TRPML^{-/-} cells that are identical to those elicited by 3 pM CCK in WT acinar cells. Yet only the former leads to fusion between lysosomes and secretory granules. If both doses trigger oscillations through the same mechanism, this might suggest that the fusion mechanism is not critically dependent on calcium i.e. provides indirect evidence that the pH of the lysosome is more important here.

How is the overlap between the lysosomes and secretory granules measured in Figure 2C? This should be described and the extent of co-localisation quantified e.g. with a Pearson's correlation coefficient.

The data from cerebral cortical neurons are very nice. It looks as if the basal secretion is increased much more than the evoked in TRPML^{-/-} deficient cells. The evoked response increases only moderately compared with WT cells. What happens if pulses of KCl are applied? It would be very interesting to see if facilitation occurs exclusively in the TRPML^{-/-} cells.

In terms of mechanism, can the enhanced secretory response in neurons or acinar cells be reversed or at least reduced by altering the pH of the lysosome (e.g. acetate/NH₄Cl pulse)?

A control that should be shown is the Ca signal evoked by KCl in WT and TRPML^{-/-} cells.

Referee #2:

The manuscript by Park et al. describes a new analysis of TRP channel TRPML1 null mutant mice. TRPML1 mutations are responsible for Lysosomal Storage disease Mucopolipidosis Type IV. As the authors argue in the Introduction, clear mismatches existed until now in the mutant mouse phenotype and other cellular studies on the one hand and the patient symptoms on the other. This study contributes data to resolve this and presents a variety of evidence to suggest that regulated exocytosis in pancreas, salivary gland and neurons is affected by TRPML1 loss.

This study presents several intriguing novel ideas on how dysfunction of this channel might cause

disease and opens a new direction of research to establish that more firmly. These findings will also trigger interest in TRPML1 in relation to the regulation of exocytosis in different tissues. The novel insights presented here may also help to treat Mucopolidosis patients eventually. However, the current manuscript is not yet convincing. Some assays are not state of the art (all secretion assays), the current study does not yet provide clues on the molecular mechanism how TRPML1 loss or mutations produce secretion defects (and it may be a long way doing that) and several aspects of the data in this study remain unexplained. Moreover, many conclusions are flawed by serious misconceptions in the application of statistical tests or by omission of the relevant information to be able to judge the validity of conclusions. Furthermore, the absence of rescue experiments make the current conclusions suggestive rather than convincing. These omissions must be corrected with (many) additional experiments before the main conclusions are justified. In addition, several conclusions appear not supported by sufficient data. Finally, several data sets in the paper are incompletely analysed: raw data is not quantified, or only one parameter, and group differences not tested.

Hence, at least many additional experiments are required and preferably the authors should also team up with experts in the secretion field to obtain data with state of the art methods. Especially a good patch clamp analysis of synaptic transmission in mutant, wt and rescued neurons would make this study a lot more convincing and important. Also, a better documentation/analysis of lysosomal acid phosphatase exocytosis, potentially one of the most informative findings and one of the biggest effects, would strengthen the paper substantially. With such additions/improvement and provided that the main conclusions still stand after that, this study can have a large impact on both the field of lysosomal storage diseases and the secretion field.

Major points:

1- Statistics

Statistical significance of differences between wt and mutant tissue should be computed using the number of INDEPENDENT observations, not ALL observations in one animal. The conclusions in several aspects of this study do not obey this rule and conclusions are not justified. For instance in Fig 2A the data are obtained from only 1-3 animals. The differences in granule size can easily be the result of some variation among pups (which is large, as judged from panel D). The plotted p-values in Fig 2A and 5B are ridiculous. More independent observations are required for the experiments in Fig 2A and probably for most of Fig 1, certainly for Fig 4d-f and Fig 5b, and probably for Fig 6A-E.

In several cases, the number of independent observations is not even mentioned (Fig 2B, Fig 5a-d). The generic statement in the Methods section "All experiments were repeated at least three times" should be omitted and the exact number of total and independent observations (n and N) should be listed for every experiment and the number of independent observations used to compute p-values.

2- Rescue

The main phenotypes of the null mutant must be tested for reversal by rescue experiments. Without rescue (and given major point 1), the data are suggestive but not convincing. Since additional observations are required anyway (see point 1), a rescue group can be included, at least in all cellular assays (acini, cultured neurons).

3- Conclusions not supported by sufficient data

The claims that pancreatitis "results from" high Ca^{2+} - and cAMP-stimulated exocytosis, that synaptic vesicles and secretory granules are enlarged "due to" fusion with lysosomes and that enhanced of glutamate exocytosis is "due to" enlarged synaptic vesicles all suggest causal relations that are not demonstrated in the current study. The whole manuscript should be carefully re-examined for such over-interpretations.

4- Incomplete analysis of available data

In some cases data are not quantified and tested for group differences between wt and mutants. This should be performed for the data in Fig 1A and 4A.

In other cases only one parameter is quantified, as in Fig 2A, Fig 5A,B and Fig 6A-C. Please provide also the average number of vesicles/granules per cross section or synapse, their average distance to membrane, active zone length. These are important parameters instrumental to specify the ultrastructural phenotype.

Minor issues:

Fig 2a: Mal should be Male

Referee #3:

In this manuscript, Park et al. propose that TRPML1-deficiency leads to uncontrolled exocytosis due to fusion of lysosomes with secretory vesicles. Although this is an interesting hypothesis, the data to support this hypothesis is not of high quality, the presentation is far from optimal and the discussion vague.

Specific comments:

1) Much of the presented data are presented such that the quality of the data cannot be assessed:

-In Figure 1 + corresponding text, it is claimed that the ML1^{-/-} cells have a normal biphasic Ca²⁺ response but more secretion. However, there are no data or statistics provided to substantiate the point that the Ca²⁺ signal is unaltered. As a consequence, the validity of the conclusions based on this figure cannot be appreciated.

-Figure 1d a significant increase in amylase release is shown for the ML1^{-/-}, but the text states that no difference was observed.

-In Figure 2, the resolution and magnification of the multicolor figures does not allow to see what the authors claim. For instance, higher resolution images for the individual colors should be shown along with the overlay for Figs. 2b,c. Now it seems that there is much more amylase signal in ML1^{-/-}, and it is unclear (photoshop) how co-localization was actually determined. In my version, I could not see the "large vesicles" and arrows pointing at them in Fig. 2c, and how the analysis was done here (ImageJ) is again unclear.

-In Figure 4a, the reason for the large peak at 300 s is unclear; why a different protocol for WT and ML1^{-/-}?

-Fig. 5d again has too low resolution to see what the authors describe. What is the definition of an "enlarged lysosome"?

-Fig. 6c: the resolution of the figure does not allow appreciating the difference in PSD.

-Fig. E2. The quality of some of the gels, e.g. panel C - VAMP2, is quite poor.

2) The evidence for the main claim, namely that lack of ML1 causes fusion of lysosomes with secretory vesicles, is mainly circumstantial. The larger vesicles may also arise from fusion of secretory vesicles with each other or problems in their biogenesis. Is there a reduction in the number of lysosomes and in the density of the content of the secretory vesicles, as one would predict?

3) The method to measure glutamate release does not allow determining the actual synaptic glutamate release in an accurate manner. It would be important to show actual synaptic communication, and the size of mini's using electrophysiology.

4) There is no clear hypothesis or mechanism for the main observations. Why would loss of ML1 cause more exocytosis of e.g. glutamate-containing vesicles? Why does loss of ML1 cause reduced Ca-signals at low CCK concentrations? Why does loss of ML1 cause fusion of lysosomes with other organelles?

5) There is no clear discussion of how the findings relate to the human disease. Do patients exhibit signs of pancreatitis or altered salivary gland function? Is there any evidence of glutamate neurotoxicity in MLIV patients?

6) The manuscript is very sloppy. Just some examples: the 4th and 5th sentence of the abstract are missing words or unclear, "supperfamily", "Carulein", "Mal", "MOP".

1st Revision

13 July 2015

Thank you for your interest in our work and the opportunity to submit this revised manuscript. In this revision, we have addressed all reviewers' comments according to your instructions. We have

performed several new experiments and expanded the analysis. This required the addition of several members to the authors list.

The most important experiment is the recording of the mini Evoked Post Synaptic Currents (EPSC) in brain splices, which represent single exocytotic events. The analysis reveals that deletion of TRPML1 markedly increases the frequency of exocytosis. Moreover, the number of larger EPSCs is increased in the *Trpml1*^{-/-} neurons. These findings provide further molecular evidence and strong support for the conclusion of enhanced fusogenicity of secretory vesicles and thus of exocytosis in the *Trpml1*^{-/-} mice. As suggested by reviewer 1, we tested the effect of collapsing the lysosomal pH gradient on both Ca²⁺ signaling and on regulated exocytosis and present the results in Figure E2. In addition, as required by reviewer 2 we performed rescue experiments showing that expression of TRPML1 in *Trpml1*^{-/-} neurons restored the normal rate of both basal and stimulated exocytosis. Finally, we analyzed images from additional wild type and *Trpml1*^{-/-} mice to strengthen the statistical analysis. Below we provide a point-by-point response to the comments.

Referee #1:

This is an important and interesting paper that identifies both a major role for an intracellular ion channel in controlling fusion between different organelles and ii) provides a molecular basis for neurodegeneration that arises from the lysosomal storage disease mucopolipidosis type IV. The observation that lysosomes deficient in TRPML fuse with secretory vesicles in excitable and non-excitable cells suggests that the authors have discovered a basic mechanism, of considerable pathophysiological relevance.

The experiments have been well executed, and are carefully controlled with appropriate statistical analyses. The paper reads well and the conclusions drawn are well supported by the evidence. I have the following comments.

[Response: Thank you very much for the positive and supporting comments.](#)

It is striking that all forms of exocytosis are enhanced, regardless of tissue or nature of the second messenger that triggers secretion (Ca vs cAMP). This suggests that fusion of lysosomes with secretory organelles somehow promotes the interaction of V- and T-SNARE proteins, providing a possible molecular basis for the enhanced exocytosis. Perhaps the authors could consider this possibility further.

It is not entirely clear, at least to me, why the Ca oscillations to CCK are reduced in frequency in TRPML^{-/-} cells. CCK recruits both the IP3 and NAADP pathways, neither of which are thought to act on TRPML. Perhaps the Ca content of the lysosome has changed, indirectly affecting the NAADP response. Regardless, the authors should clarify in the text what the mechanism is.

[Response: The NAADP response is mediated at least in part by TPC2 which resides at the lysosomes together with TRPML1 \(see J Biol Chem. 2011 Jul 1;286\(26\):22934-42\) and pancreatic acini do express *trc2* which mediates that NAADP response \(see Cell Calcium. 2015 Jun 10. pii: S0143-4160\(15\)00095-0.\). It is likely that the CCK response releases Ca²⁺ from the lysosomal compartment that requires the function of TRPML1. This is now indicated in the text.](#)

10 pM CCK evokes Ca oscillations in TRPML^{-/-} cells that are identical to those elicited by 3 pM CCK in WT acinar cells. Yet only the former leads to fusion between lysosomes and secretory granules. If both doses trigger oscillations through the same mechanism, this might suggest that the fusion mechanism is not critically dependent on calcium i.e. provides indirect evidence that the pH of the lysosome is more important here.

[Response: Fusion likely occurred prior to the stimulation with CCK since co-localization of lysosomal and granule markers is observed in unstimulated *Trpml1*^{-/-} cells. However, as to your point, only exocytosis in the first 3-5 minutes of stimulation with CCK is strictly dependent of Ca²⁺. When this form of exocytosis is compared, 10 pM CCK efficiently stimulates exocytosis in *Trpml1*^{-/-} cells. Please see Figure 1c](#)

How is the overlap between the lysosomes and secretory granules measured in Figure 2C? This should be described and the extent of co-localisation quantified e.g. with a Pearson's correlation coefficient.

Response: The overlap was analyzed by ImageJ, which provides quantitative measure of the overlap. Further detailed are provided in the methods section under "immunofluorescence".

The data from cerebral cortical neurons are very nice. It looks as if the basal secretion is increased much more than the evoked in TRPML^{-/-} deficient cells. The evoked response increases only moderately compared with WT cells. What happens if pulses of KCl are applied? It would be very interesting to see if facilitation occurs exclusively in the TRPML^{-/-} cells.

Response: Unfortunately, we were not able to do these experiments. When measuring mEPSCs, repeated stimulation with KCl resulted with runaway currents, merging of mEPSCs and loss of the whole cell recording. However, the new results with mEPSCs confirm the large increase in exocytosis in the Trpml1^{-/-} neurons. Moreover, they reveal marked increased mEPSCs frequency in Trpml1^{-/-} neurons, indicating increased fusogenicity of synaptic vesicles.

In terms of mechanism, can the enhanced secretory response in neurons or acinar cells be reversed or at least reduced by altering the pH of the lysosome (e.g. acetate/NH₄Cl pulse)?

Response: Thank you for the suggestion. The results of these experiments are shown in Extended Fig. 2. Collapsing the lysosomal (and likely all) pH gradients had minimal effect on Ca²⁺ oscillations, and although reduced the extent of exocytosis it did not reduce the increased exocytosis in either pancreatic or parotid Trpml1^{-/-} acini.

A control that should be shown is the Ca signal evoked by KCl in WT and TRPML^{-/-} cells.

Response: This is now shown in Figure 6.

Referee #2:

The manuscript by Park et al. describes a new analysis of TRP channel TRPML1 null mutant mice. TRPML1 mutations are responsible for Lysosomal Storage disease Mucopolysaccharidosis Type IV. As the authors argue in the Introduction, clear mismatches existed until now in the mutant mouse phenotype and other cellular studies on the one hand and the patient symptoms on the other. This study contributes data to resolve this and presents a variety of evidence to suggest that regulated exocytosis in pancreas, salivary gland and neurons is affected by TRPML1 loss.

This study presents several intriguing novel ideas on how dysfunction of this channel might cause disease and opens a new direction of research to establish that more firmly. These findings will also trigger interest in TRPML1 in relation to the regulation of exocytosis in different tissues. The novel insights presented here may also help to treat Mucopolysaccharidosis patients eventually.

Response: Thank you for the positive and supporting comments.

However, the current manuscript is not yet convincing. Some assays are not state of the art (all secretion assays),

Response: In the case of pancreatic and parotid acini and saliva secretion, the techniques used are the standard in the field. Measurements of capacitance in these cells are not reliable due to the minimal expansion of the luminal membrane during exocytosis and counting secretory profiles in individual acini is not sufficiently quantitative.

To enhance the neuronal studies we measured mEPSCs in brain slices and the new results are included in the new Figure 6.

the current study does not yet provide clues on the molecular mechanism how TRPML1 loss or mutations produce secretion defects (and it may be a long way doing that) and several aspects of the data in this study remain unexplained. Moreover, many conclusions are flawed by serious misconceptions in the application of statistical tests or by omission of the relevant information to be able to judge the validity of conclusions.

[Response: Thanks you. To address the statistical aspect of the comments we analyzed additional mice and images and re-analyzed the entire set of the data, which strengthen the statistical analysis and the conclusions.](#)

Furthermore, the absence of rescue experiments makes the current conclusions suggestive rather than convincing. These omissions must be corrected with (many) additional experiments before the main conclusions are justified. In addition, several conclusions appear not supported by sufficient data.

[Response: We have now performed the rescue experiments and the results are shown in the Figures 7f and E4c,d. It can be seen that expression of TRPML1 completely rescued both the basal and stimulated exocytosis in *Trpml1*^{-/-} neurons.](#)

Finally, several data sets in the paper are incompletely analysed: raw data is not quantified, or only one parameter, and group differences not tested. Hence, at least many additional experiments are required and preferably the authors should also team up with experts in the secretion field to obtain data with state of the art methods. Especially a good patch clamp analysis of synaptic transmission in mutant, wt and rescued neurons would make this study a lot more convincing and important.

[Response: Please see response above concerning exocytosis in secretory cells and mEPSCs.](#)

Also, a better documentation/analysis of lysosomal acid phosphatase exocytosis, potentially one of the most informative findings and one of the biggest effects, would strengthen the paper substantially.

[Response: It is now clear to us how can this be analyzed beyond measuring the parallel secretion of digestive enzymes and AP in both isolated pancreatic and parotid acini AND secretion of AP to the saliva.](#)

With such additions/improvement and provided that the main conclusions still stand after that, this study can have a large impact on both the field of lysosomal storage diseases and the secretion field.

[Response: We hope that you will find the above response satisfactory and the manuscript suitable for publication.](#)

Major points:

1- Statistics Statistical significance of differences between wt and mutant tissue should be computed using the number of INDEPENDENT observations, not ALL observations in one animal. The conclusions in several aspects of this study do not obey this rule and conclusions are not justified. For instance in Fig 2A the data are obtained from only 1-3 animals. The differences in granule size can easily be the result of some variation among pups (which is large, as judged from panel D). The plotted p-values in Fig 2A and 5B are ridiculous. More independent observations are required for the experiments in Fig 2A and probably for most of Fig 1, certainly for Fig 4d-f and Fig 5b, and probably for Fig 6A-E. In several cases, the number of independent observations is not even mentioned (Fig 2B, Fig 5a-d). The generic statement in the Methods section "All experiments were repeated at least three times" should be omitted and the exact number of total and independent observations (n and N) should be listed for every experiment and the number of independent observations used to compute p-values.

Response: We analyzed additional mice and use only independent observations. Results were obtained from 3-4 mice of each phenotype. Moreover, multiple images were analyzed from each mouse. This is now indicated for all figures. The p values given originally are the actual calculated values. However, now we only indicate that $p < 0.001$.

The statement "All experiments were repeated at least three times" is omitted and the exact number of total and independent observations is listed.

2- Rescue The main phenotypes of the null mutant must be tested for reversal by rescue experiments. Without rescue (and given major point 1), the data are suggestive but not convincing. Since additional observations are required anyway (see point 1), a rescue group can be included, at least in all cellular assays (acini, cultured neurons).

Response: Please see new Figures 7f and E4c,d for the results of the rescue experiments.

3- Conclusions not supported by sufficient data. The claims that pancreatitis "results from" high Ca^{2+} - and cAMP-stimulated exocytosis, that synaptic vesicles and secretory granules are enlarged "due to" fusion with lysosomes and that enhanced of glutamate exocytosis is "due to" enlarged synaptic vesicles all suggest causal relations that are not demonstrated in the current study. The whole manuscript should be carefully re-examined for such over-interpretations.

Response: The manuscript was revised to tone down these statements.

4- Incomplete analysis of available data. In some cases data are not quantified and tested for group differences between wt and mutants. This should be performed for the data in Fig 1A and 4A.

Response: The averages for Fig. 1A are given in the columns under the traces and the traces in Fig. 4A are the actual averages with their mean \pm s.e.m.

In other cases only one parameter is quantified, as in Fig 2A, Fig 5A,B and Fig 6A-C. Please provide also the average number of vesicles/granules per cross section or synapse, their average distance to membrane, active zone length. These are important parameters instrumental to specify the ultrastructural phenotype.

Response: The results of these analyses are now provided in the new Extended Figure E3 and in the figure legends.

Minor issues:

Fig 2a: Mal should be Male

Response: Thank you. This was corrected.

Referee #3:

In this manuscript, Park et al. propose that TRPML1-deficiency leads to uncontrolled exocytosis due to fusion of lysosomes with secretory vesicles. Although this is an interesting hypothesis, the data to support this hypothesis is not of high quality, the presentation is far from optimal and the discussion vague.

Specific comments:

1) Much of the presented data are presented such that the quality of the data cannot be assessed: -In Figure 1 + corresponding text, it is claimed that the ML1-/- cells have a normal biphasic Ca^{2+} response but more secretion. However, there are no data or statistics provided to substantiate the point that the Ca^{2+} signal is unaltered. As a consequence, the validity of the conclusions based on this figure cannot be appreciated.

Response: Example Ca^{2+} traces at 100 pM CCK and the average Ca^{2+} responses at high CCK concentrations are provided in the new Fig. 1b. It can be seen that the Ca^{2+} signals to high agonist concentrations are similar.

-Figure 1d a significant increase in amylase release is shown for the ML1^{-/-}, but the text states that no difference was observed.

Response: Thank you. This was corrected.

-In Figure 2, the resolution and magnification of the multicolor figures does not allow to see what the authors claim. For instance, higher resolution images for the individual colors should be shown along with the overlay for Figs. 2b,c. Now it seems that there is much more amylase signal in ML1^{-/-}, and it is unclear (photoshop) how co-localization was actually determined. In my version, I could not see the "large vesicles" and arrows pointing at them in Fig. 2c, and how the analysis was done here (ImageJ) is again unclear.

Response: Higher magnification images are now provided as inserts showing the increased size of the acidic vesicles. All images are submitted at high resolution. However the files (in particular the EM) are very large and have to be converted to PDF for submission. The resolution is likely reduced when the files are converted to the PDF format. All images were analyzed by ImageJ using the particle counting procedure provided by the program to determine overlap. This is now described in more details. This procedure can be found in the software instructions.

-In Figure 4a, the reason for the large peak at 300 s is unclear; why a different protocol for WT and ML1^{-/-}?

Response: The larger peak is due to Ca^{2+} influx that is very large in salivary gland cells. This has been studied in details before by us (for example see J Biol Chem. 2004 May 14;279(20):21511-9) and many others. The agonist rapidly depletes the stores in both cell types. External Ca^{2+} is re-added at any time after $[\text{Ca}^{2+}]_i$ returns to basal level.

-Fig. 5d again has too low resolution to see what the authors describe. What is the definition of an "enlarged lysosome"?

Response: Higher magnification images are now provided as inserts and see above comments about resolution. Enlarged granules are considered granules with diameter larger than those in wild-type cells.

-Fig. 6c: the resolution of the figure does not allow appreciating the difference in PSD.

Response: Portions of the Figures with higher magnification images are now provided.

-Fig. E2. The quality of some of the gels, e.g. panel C - VAMP2, is quite poor.

Response: The blot was replaced with a new blot using different antibodies.

2) The evidence for the main claim, namely that lack of ML1 causes fusion of lysosomes with secretory vesicles, is mainly circumstantial. The larger vesicles may also arise from fusion of secretory vesicles with each other or problems in their biogenesis. Is there a reduction in the number of lysosomes and in the density of the content of the secretory vesicles, as one would predict?

Response: The later is difficult to assay since the lysosomal and granule markers co-localize, preventing the possibility of using markers to distinguish the organelles in acini of *Trpm1*^{-/-} mice. However, analysis of synaptosomes in Extended Fig. E3b suggest reduction in the number of secretory organelles in the *Trpm1*^{-/-} mice. Our conclusion of lysosomes-granules fusion is based on several independent and complimentary observations that together support fusion between the organelles: a) marked increase in the size of organelles; b) increased LAMP1-Amylase colocalization; c) increase lysotracker at the apical pole of acinar cells; d) increased Co-IP of granular and lysosomal markers; e) Stimulated secretion of acid phosphatase that coincides with

stimulated secretion of amylase; f) probably the strongest evidence is development of pancreatitis in *Trpm1*^{-/-} mice that includes intracellular activation of trypsin, which is amply established to be due cleavage of the pro-trypsin by lysosomal cathepsin B. We believe that together, these findings do lead to the conclusion of fusion between lysosomes and secretory granules. In addition, we do not exclude the possibility that fusion between secretory organelles contribute to the increase in granules size. However, the simplest explanation for all other observations is the fusion of lysosomes with the secretory organelles, which is more likely to cause the pathology.

3) The method to measure glutamate release does not allow determining the actual synaptic glutamate release in an accurate manner. It would be important to show actual synaptic communication, and the size of mini's using electrophysiology.

Response: This is now shown in the new Figure 6, in which we measured mEPSCs in acutely isolated brain slices. It can be seen that both the frequency and the number of large exocytotic events are higher in *Trpm1*^{-/-} neurons. In response to reviewer 2 comments, we further show that expression of TRPML1 in the *Trpm1*^{-/-} neurons rescues normal basal and stimulated exocytosis.

4) There is no clear hypothesis or mechanism for the main observations. Why would loss of ML1 cause more exocytosis of e.g. glutamate-containing vesicles? Why does loss of ML1 cause reduced Ca-signals at low CCK concentrations? Why does loss of ML1 cause fusion of lysosomes with other organelles?

Response: These are all excellent questions but we believe they are beyond the scope of this manuscript. We can only suggest/speculate that TRPML1 either directly or indirectly prevents fusion of lysosomes with other organelles. In the absence of TRPML1 the lysosomes accumulate undigested proteins and lipids and fuse with other organelles, including autophagosomes. Our study suggests that these lysosomes also fuse with other fusogenic organelles like secretory granules. The presence of the fusogenic lysosomes in the granules seems to increase their fusogenicity to increase exocytosis.

As to the response to CCK, it is known that CCK stimulation releases Ca²⁺ stored in acidic compartments by generation of NAADP that releases Ca²⁺ from the lysosomes. TRPML1 is a lysosomal Ca²⁺ release channel and thus deletion of TRPML1 is expected to impair the CCK-evoked Ca²⁺ oscillations. At high CCK concentrations generation of large amount of IP₃ and massive Ca²⁺ release from the ER overwhelms the NAADP response. The effect of high CCK concentrations is now shown in Figure 1b is response to reviewer 2 comments. This is now clarified in the manuscript.

5) There is no clear discussion of how the findings relate to the human disease. Do patients exhibit signs of pancreatitis or altered salivary gland function? Is there any evidence of glutamate neurotoxicity in MLIV patients?

Response: Unfortunately, neurodegeneration and muscle weakness are by far the most debilitating in the disease and thus for the most part patients are not diagnosed for problems with other organs. However, as indicated and discussed in the manuscript, the patients do have achlorhydia, which is due to aberrant function of gastric parietal cells, another epithelial tissue.

6) The manuscript is very sloppy. Just some examples: the 4th and 5th sentence of the abstract are missing words or unclear, "supperfamily", "Carulein", "Mal", "MOP".

Response: We apologize for these and attempted to correct all errors.

3rd Editorial Decision

4 September 2015

Thank you for submitting the revised version of your manuscript entitled 'Lysosomes and Secretory organelles fusion in mucopolidosis type 4 causes Uncontrolled Exocytosis' to us. I have now received reports of all three original referees, which are enclosed below. I am very sorry for the delay in getting back to you, but not all referees were immediately available when you submitted

the revised version, and I wanted to avoid including new referees at this stage.

As you will see, referee #1 now supports publication. Referee #2 and #3, however, think that your manuscript is unsuitable for publication in *The EMBO Journal*. Three main deficiencies that were already outlined in the first round remain in their view unsatisfactorily addressed, and as you will notice this has caused substantial irritation with both referees. Referee #2 still thinks that the neuronal phenotype needs further support from patch-clamp analyses, and referee #3 notes that the current manuscript still suffers from overstatements and poor statistical analyses. The conclusions are therefore in the view of both referees not sufficiently supported by the data provided.

As you know, we only allow one major round of revision. The additional work required to substantiate your claims clearly go beyond a minor revision. Therefore, we see no other choice but to reject your work at this stage. Please note that this decision was not taken lightly. I have extensively discussed your manuscript within the team and with our chief editor, Bernd Pulverer, and I also engaged the referees into cross-commenting on the reports. Based on this, we concluded that we cannot offer further consideration at this stage. I am very sorry to disappoint you on this occasion, and I hope that you will nevertheless find the referees' comments useful.

REFEREE REPORTS

Referee #1:

The authors have carried out new experiments that nicely address the issues raised in my review, and have revised the text accordingly. The paper is now very convincing; it is an important and timely contribution and I support publication in *EMBO Journal*.

Referee #2:

The revised manuscript contains several major improvements. However, two of the main issues I raised earlier are not addressed and therefore I cannot support publication of this manuscript. First, this manuscript is still full of over-interpretation, up to the abstract and title. Causal relations are still claimed that are not justified by the data. This would have been easy to address. What does it say about the authors if they do not address this seriously? Second, one of the major new findings in this study is the potential presynaptic effect. I previously stated that if that observation would be substantiated with state of the art methods in an optimal experimental design, incl. rescue, that could be an important asset for publication. I also mentioned that this would probably be solved best by teaming up with a lab that are real experts in the area. However, the authors have decided not to do all this, but instead present some data from brain slices, reporting only spontaneous events. This is all far from convincing. Changes in spontaneous events can be explained by many different things, including metabolic changes, excitability changes and differences in synaptogenesis. Rescue is not possible. The data do not connect to mainstream analysis of presynaptic defects.

Referee #3:

Whereas the presented data are interesting and potentially important, and additional experiments have been included, the manuscript is still unconvincing and the revision very disappointing. The most important problem of the manuscript lies in the statistics, which are flawed in many instances, making it difficult to appreciate the real value of the findings. This issue was raised in the first round of reviews by two referees. The most problematic issue is that the authors used only one statistical test, namely non-paired student's t-test. For the large majority of the data in this manuscript, this test is simply unfit because 1) multiple comparisons were made, and 2) normality of the data is not granted. The authors should be advised to adhere to the author guidelines in this respect (<http://emboj.embopress.org/authorguide#figureformat>), which state that: "In particular:

- When using statistical methods based on the normal distribution authors should explain how they tested their data for normality. If the data do not meet the assumptions of the test, then a non-parametric alternative should be used.
- When making multiple statistical comparisons on a single data set, authors should explain how they

adjusted the alpha level to avoid an inflated Type I error rate, or they should select statistical tests appropriate for multiple groups.

•In cases where n is small, appropriate statistical tests should be employed and justified in the text." In many instances, ANOVA and non-parametric tests should be used instead of Student's t-test. In some cases, no statistics are provided, despite the fact that the authors claim that there is a difference (e.g. the size of the minis). In any case, in the absence of solid statistics, this paper should not be published.

A second important and annoying point is the sloppiness and multiple typos/grammatical errors, starting in the abstract. This point was already raised in my earlier review. Below are some important examples.

Abstract: "However, this cannot explain impaired neuromuscular or secretory cells functions that their cardinal function is regulated exocytosis."

Page 2: "Whether... affect" should read "affects"

Page 2: last sentence should read "These findings have significant implications"

Page 3: "...to the area of membrane damaged" should read "...to the area of damaged membrane" or similar.

Figure 1b: "pick Fura ratio" should read "peak Fura ratio"

Figure 2a. Individual points are not visible. Histograms would be a much clearer way of visualization. Nevertheless, already from the provided data, it is clear that the assumptions of a Student's t-test (normality, equal variance) are unlikely to be met.

Page 4: "chatapsins" should probably read "cathepsins"

Figure 4a: I still don't understand why two different protocols were used for wt and KO, and therefore wonder what kind of comparison can be made. The statement in the text that the calcium signal is "nearly normal" is unscientific. Please include a comparison using the same protocol for both genotypes and an adequate statistical test. There is also no statistics provided for Figure 4d.

Figure 5: There still is no real definition of "enlarged" lysosomes. Now the authors state in their letter that these are "considered granules with diameter larger than those in wild-type cells", which seems a very arbitrary definition, and makes one wonder how the count for wild-type can be higher than zero.

Page 5: "...while inhibition action potentials with TTX and GABAA receptors with Bicuculline." Please reword.

Page 5: "Notably"

Figure 6c: It is unclear how this histogram was constructed. There seems to be a pattern of clusters of ~6 wild type and ~6 knockout bars, but that did not make sense to me. In any case, this presentation could not convince me that there are more large mEPSCs in the knockout, and there is no statistics to support this statement. There is also no statistics for the data in Figure 6e.

Page 6: "secret" should read "secrete"

Page 6: "The lysosomes participates..."

Page 6: "Impaired lysosomal function, as occur..."

Page 7: "Additionally, we observed that trypsin activation of unstimulated TRPML1-/- mice pancreatic acini, which can never occur in immature granules."

Page 7: "Activation of trypsin from chemotrypsin requires proteolysis than within the acini that is mediated by lysosomal proteases."

Page 7: "Hence, the phenotype reported hear..."

Page 7: "At this time it is not known how TRPML1 carryout this role".

The generic statement that "all experiments were repeated at least three times" is still there, although in their letter the authors state that they removed it.

Figures are often sloppy, with inconsistent size of lettering, different styles of bars within one figure, no indication of the zero level in calcium imaging figures.

EMBO REPORTS

1st Editorial Decision

20 October 2015

Thank you for your patience while we have waited for the assessment of your statistical analyses by our external advisor, which I am attaching to this email for your information.

I am therefore happy to accept your manuscript for publication in EMBO reports provided a few minor issues will be addressed:

1. our statistics expert felt that in two cases additional tests have to be performed (his/her comment #3) and that in a few instances, re-wording is required.

However, this seems all rather minor, so I do not anticipate any major problems at this stage.

In addition, there are a few more issues that I would like you to address:

2. At the moment there are materials & methods in the supplementary section of the manuscript, which is not allowed for a full article. Please move the M&M section to the main body in their entirety.

3. Please reduce the number of Expanded View Figures down to five (probably easiest to combine some of them), as this is the maximum number we can process. Also, the Expanded View Figures should be labeled and referenced as Figure EV1, Figure EV2 etc. in the main text of the manuscript. The legends for the EV figures should be incorporated in the main body of the text after the legends for the main figures. Please modify your additional figures accordingly.

4. We are also using a number-based reference style and I am attaching the end note file to this email. Please modify your references accordingly

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

6. Please also provide a short, two-sentence summary of the manuscript, two to three bullet points highlighting the main findings of the study, and a schematic figure in tiff format (with the exact width of 550 pixels and an approximate height of 400 pixels) that can be used as part of a visual synopsis on our website

7. Please send us a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide#revision>)

Once you have made these minor revisions, please submit the final version of your study through our website.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

NOTES ON THE STATISTICS SECTION

"Differences between the groups were analyzed for statistical significance either by arc-sin transformation for percentage and overlap results and calculated t-test assuming equal variances"

* try to rephrase this sentence, there is a second end missing in the "either by" part of the sentence. In general, please make sure that people know which transformation has been applied to which data in which figure.

"Comparisons between more than two groups (Fig. 3) were analyzed by Holm Bonferroni statistical t-test ..."

* try to rephrase like so:
... were analyzed by the t-test and the resulting p-values were then corrected for multiple testing using the Holm-Bonferroni method. (Since the Holm method is not a statistical test)

"Comparisons between more than two groups (Fig. 3)..."

* Simultaneous comparisons between more than two groups are also performed in all time series experiments, so the Holm method has to be applied to p-values from the the data shown in Fig. 1d, 1e, 4e and 4f as well. Here multiple significance tests relative to the time zero condition are performed, so these are also comparisons between multiple groups.

1st Revision - authors' response

27 October 2015

Thank you so much for the positive response considering of manuscript. Please find the revised manuscript titled "Fusion of Lysosomes with Secretory organelles leads to Uncontrolled Exocytosis in the Lysosomal Storage disease Mucopolipidosis Type IV". We have made the following changes:

1. We have performed the additional statistical analysis and list the actual p values in the figures.
2. We moved all the methods to the main text.
3. We reduced the EV figures to 5 by combining figures as you suggested and cite the figures as EV1-5 in the text.
4. References are cited numerically using the appropriate EndNote stile.
5. We uploaded a file with the original uncut blots.
6. We provide a short, two-sentence summary of the manuscript and three bullet points highlighting the main findings.
7. We unloaded a complete author checklist.

2nd Editorial Decision

04 November 2015

Thanks a lot for submitting the final version of your manuscript to EMBO reports. I am happy to accept it in its current form and you will find the official acceptance letter below.