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Lysosomal chloride transporter CLH-6 protects lysosome membrane integrity via cathepsin activation

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November 9, 2022

Re: JCB manuscript #202210063

Prof. Xiaochen Wang
Institute of Biophysics, Chinese Academy of Sciences
15 Datun Road, Chaoyang district
Beijing 100101
China

Dear Prof. Wang,

Thank you for submitting your manuscript entitled "Lysosomal chloride transporter CLH-6 protects lysosome membrane integrity via cathepsin activation". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the three reviewers are enthusiastic about your findings; however, they raise a couple of issues that would need to be addressed, with appropriate new data where requested. In particular, reviewer #2 would like that you add the Clc-7 associated protein Ostm1 to the discussion (point 3). Reviewer #3 requests better characterization of the subcellular localization of CLH-6 in hypodermal cells of wild type worms (point B) and would like that you clarify to what extent the intra-lysosomal Cl- levels and cathepsin B and L activity depend on CLH-6 function (point D). We hope that you will be able to address each of reviewers' other points, though.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

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The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-

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When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Li Yu
Monitoring Editor
Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this MS by Zhang et al, the authors used a genetic screen to identify lysosomal genes required for the maintenance of lysosome membrane integrity in vivo. They identified CLH-6, the ortholog of mammalian Cl-/H+ exchanger CLCN7, as a key player in the regulation of lysosome homeostasis. Loss of CLH-6 caused cargo accumulation and lysosomal membrane rupture, which could be restored by blocking cargo delivery or Cathepsin B or L overexpression. Consistently, genetic inactivation of Cathepsin B or L phenocopied the clh-6 mutant. The authors further showed that loss of CLH-6 caused a decrease in the luminal chloride level without affecting lysosomal acidity. More importantly, they obtained in vitro data showing the Cl- dependence of the Cathepsin B and L activities. Collectively, the authors have convincingly demonstrated that CLH-6 maintains the luminal chloride level required for the activities of certain hydrolases, facilitating lysosomal degradation to protect lysosomal membrane integrity. Overall, this is an excellent study with careful experimental design, clear and logical data presentation, and a large amount of high-quality imaging results. The story should be of great interest to the general JCB readership, especially those in lysosomal cell biology and physiology. I only have some relatively minor comments.

1) In Fig. 8F, the Kd for Cathepsin B appeared to be in low mM, yet the Kd in the MST assay was 34 mM. Given the reported lysosomal Cl- concentration (> 60 mM), I am wondering whether the dose-dependence would be shifted to the right (hence more physiological) under acidic pH conditions. It is also recommended if another anion, e.g., NO3- or gluconate- (in sodium salt), can be used to substitute Cl- in the low NaCl solutions in order to maintain constant ionic strength and osmolality in all solutions, in case they might affect the activities of the lysosomal enzymes.
2) As shown in Fig. 6G-J, Cathepsin L and B are as important as CLH-6 in the lysosomal damage assays. Any reason that neither of them was picked up by the genetic screening?
3) I was confused why some NUC::Cherry fluorescence appeared to be localized in the perimeter membrane in Fig. 3G'. I was expecting a primary perimeter localization of the CLH-6:: GFP fluorescence.
4) In Fig. 7H, was the chloride level normalized to the lysosome mass? It would be nicer if another luminal ion could be used as an internal control.
5) Why do Fig. 6B and Fig. 6D look so differently from each other?

Reviewer #2 (Comments to the Authors (Required)):

This is an interesting paper on the role of the lysosmal chloride / proton antiporter Clh-6 for lysosomal integrity in C. elegans. Clh-6's closest homologue in humans is ClC-7, which is mutated in human osteopetrosis.

The results are overall convincing and I have no major objections. A few things need to be corrected and the discussion should include some words on the associated protein Ostm1, as detailed below.

1. Nomenclature: CLCN7 (in italic) denotes the gene that encodes the protein ClC-7.

2. Regarding the assignment of transmembrane domains, the authors need to make reference to CLC-structures, in particular the first structure of a bacterial homologue (Dutzler et al, Nature, 2002), and the more recent structures of ClC-7/Ostm1 (Schrecker et al, elife 2020; Zhang et al, Sci Adv, 2020).

3. The fact that human ClC-7 associates in an obligatory manner with the protein Ostm1 (Lange et al, Nature, 2006) needs to be introduced, and what is known about the C. elegans homologue needs to be discussed (https://www.ncbi.nlm.nih.gov/protein/CAA87781.2?report=genpept&log$=seqview).

4. abstract: "but the mechanism that protects lysosomal membrane stability remains poorly understood." is awkward because it suggests that there is a single mechanism.

5. p. 4: There must be an earlier and more original reference regarding the V-ATPase than Futai et al., 2019.

6. p. 4: The expression "if left unresolved" is awkward.

7. p. 5: In the sentence "It acts as a Cl-/H+ antiporter to mediate coupled movement of Cl- and H+ in opposite directions (Brandt and Jentsch, 1995; Graves et al., 2008)." the references are not pertinent. Leisle at al, Embo J, 2011, were the first to demonstrate that ClC-7 is a Cl- / H+ antiporter.

Reviewer #3 (Comments to the Authors (Required)):

1. In this paper, Zhang et al. characterize an important link between lysosomal function and lysosomal membrane integrity. Controlled lysosomal activity is key to several aspects of cellular homeostasis and depends on the establishment and maintenance of a contained acidic environment. Central to this process is the lysosomal membrane which compartmentalizes hydrolases in the lumen, receives cargo and actively sustain the pH / electric gradients. In this article, Zhang et al. implicate the worm homolog of the Cl- cotransporter CLCN7 (CLH-6) in ensuring lysosomal membrane integrity in C. elegans. CLH-6 localizes to lysosomes where it regulates chloride levels ultimately avoiding leakage of lysosomal content to the cytosol. The authors show that this role of Cl- ions is independent of lumen acidification and instead involves binding of Cl- to cathepsin B (CPR-2) and L (CPL-1) to sustain lysosomal-dependent degradation, which in turn ensures lysosomal integrity. While the link between cathepsin function and chloride levels has been previously described in vitro, the authors expand these finding by characterizing the biological relevance of lysosomal Cl- levels in the context of cargo degradation in vivo. Overall, this is a thoroughly carried out investigation that advances our understanding of lysosomal biology and establishes a relevant in vivo model to further dissect the genetics of lysosomal storage diseases.

2.

A)CLH-6 function is required for membrane integrity.
The authors used an established reporter for endomembrane damage (GFP::Gal3) coupled with expression of a lysosome hydrolase reporter (NUC-1::mCherry) in a genetic screen to identify new mutations affecting lysosomal integrity defects in hypodermal cells. Further characterization of clh-6 mutations and TEM analysis corroborated their conclusions.

B)CLH-6 expression, lysosomal localization.
The authors used a CLH-6::GFP reporter line to demonstrate expression of clh-6 in various tissues in embryo larval stages. The presence of CLH-6 in lysosomes is provided by co-localization with NUC-1::mCherry. While the data support the presence of CLH-6 in lysosomes, considering the disruption in Golgi and ER markers identified clh-6 mutants (FigS3) and previous report of mouse ClC7 accumulating in the Golgi (Maurizi et al., 2019), it may be beneficial for the authors to strengthen the phenotypic characterization of clh-6 mutants by performing a more detailed subcellular localization of CLH-6 in hypodermal cells of wild type worms.

C)Reduction in cargo load suppresses CLH-6 defects.
The authors use a suite of genetic backgrounds and chemical treatment to comprehensively disrupt cargo delivery to lysosomes in clh-6 mutants followed by assessment of GFP::Gal3 distribution. Their results convincingly demonstrate that interfering with cargo delivery to lysosomes alleviates the lysosomal leakage observed in clh-6 worms, implicating cargo processing in the maintenance of membrane integrity.

D)Intra-lysosomal Cl- levels and cathepsin B and L activity depend on CLH-6 function.
The involvement of CPL-1 and CPR-2 in clh-6 phenotypes was demonstrated upon suppression of these defects in worms overexpressing CPL-1 and CPR-2 and the observation of similar phenotypes in cpl-1 and cpr-2 single mutants. Activity of these cathepsins was shown to be reduced in purified lysosome extracts from clh-6 worms. Conversely, chloride was implicated in cathepsin function and lysosomal membrane integrity by detecting a decrease of these ions in the lumen of lysosomes in clh-6

worms and an increase in CPL-1 and CPR-2 function after chloride supplementation. Finally, a direct implication is inferred by establishing that, as previously reported, Cl- ions bind recombinant cathepsins. A few concerns with these experiments:

(a) As mentioned by the authors, the lysosomal integrity defects observed appear to be tissue specific, with other major organs such as the intestine and body wall muscle not being overtly affected by clh-6 mutations. If the LysoIP was performed to isolate lysosomes of Pscav-3SCAV- 3::RFP::3xHA expressing cells, how representative of CLH-6 hypodermal lysosome phenotypes will these conclusions be? It seems this is not a unique case of clh-6. However, if we can accept that the drop in Cl- levels detected in the LysoIP work occurs similarly in cells whose lysosomes are not ultimately disrupted, is it not also possible that Cl- levels themselves do change differently in response to CLH-6 in different cells but that is impossible to extricate from the LysoIP data?

(b) For the LysoIP experiments, worms overexpressing CPL-1 and CPR-2 were used, presumably because purification of clh-6 lysosomes would not be viable. If CPL-1 and CPR-2 naturally bind Cl- ions in the lumen, wouldn't this further reduce free lumen Cl- measurable in the assay? Or cathepsin bound Cl- can also be accounted for? Following the same line of thought, if increasing CPL-1 and CPR-2 loads on lysosomes can, per se (and presumably without increases in intralysosomal Cl- levels), suppress defects in clh-6 worms, wouldn't this argue for a secondary role of chloride instead? Or are the authors arguing that overexpression of cathepsins can functionally bypass insufficient Cl- levels in a lysosome with normal loads of CPL-1 and CPR-2? If so, how would this jive biochemically with a proposed function of Cl- in regulating cathepsin kinetics?

3.

- Along with inset images in 1D-E, add a panel for a WT inset (A) to help comparison with the WT localization of NUC-1::mCherry and GFP::Gal3.

- On page 8:
"The abnormal pattern of endosomes, Golgi, and ER in clh-6 mutants was observed only in the hypodermis at adult stages when lysosomal damage is severe, which suggests that lysosomal rupture and the subsequent leakage of hydrolases may lead to abnormalities of these organelles (Fig. S3M)".

Could this not be instead related directly to CLH-6 function on the collapse of other endomembrane organelles and not the consequence of lysosomal disruption?

- For figure 6 panels (A-E). I would suggest showing separately the images for the GFP::Gal3 channel. The suppression of the defect by overexpressing the two hydrolases is somewhat masked by showing it in the merged image in this figure. This is especially important since the mCherry signals report different transgenes and are not directly comparable.

- In figure 5, what is the significance of the nuclear signal in hypodermal cells expressing Gal3::GFP ? This appears largely absent in WT / untreated, but are pronounced in worms where lysosomal defects were induced.

- In Figure 7H (quantification of chloride levels), shouldn't the WT bar in the X axis also indicate +CLP-1/CRP-2? Alternatively, indicate that in the legend.

- In SupFigS1 - any reason what the image in panel B, but not C,D or E is shown on a grid?

Dear Dr. Morgado-Palacin and Dr. Yu,

Thank you very much for handling our manuscript. We have revised the manuscript to address the concerns raised by the reviewers and to meet the editorial requirements. Now we are submitting the revised manuscript entitled "**Lysosomal chloride transporter CLH-6 protects lysosome membrane integrity via cathepsin activation"** (JCB manuscript #202210063).

We have revised the text and performed additional experiments to address the concerns raised by the reviewers.

The major changes that we made in the revised manuscript are as follows:

1. As suggested by reviewer #1, we included Na-gluconate in the chloride supplement assays to provide sodium and anionic ions in low NaCl conditions. Therefore, ionic strength and osmolarity are maintained at a constant level in all solutions. We also measured the potassium concentration in purified lysosomes as a luminal ion control (Figures 8 and S4 in the revised manuscript).
2. As suggested by reviewer #2, we have revised the text and added appropriate references.
3. To address the concern raised by reviewer #3, we purified lysosomes from *C. elegans* hypodermis and examined chloride concentration and cathepsin activity. Consistent with our data in the original manuscript, chloride levels and the activities of cathepsin L and B were significantly reduced in hypodermal lysosomes in *clh-6(lf)* mutants.

4. The revised text in the manuscript is highlighted in blue.

Our point-by-point responses to the reviewers' comments are listed below:

Review #1

1) In Fig. 8F, the Kd for Cathepsin B appeared to be in low mM, yet the Kd in the MST assay was 34 mM. Given the reported lysosomal Cl- concentration (> 60 mM), I am wondering whether the dose-dependence would be shifted to the right (hence more physiological) under acidic pH conditions. It is also recommended if another anion, e.g., NO3- or gluconate- (in sodium salt), can be used to substitute Cl- in the low NaCl solutions in order to maintain constant ionic strength and osmolality in all solutions, in case they might affect the activities of the lysosomal enzymes.

Response:

In the chloride supplement assays (Fig. 8E-H), cathepsin activity was measured under an acidic condition (pH 5.3). As suggested by the reviewer, we used Na-gluconate to substitute for the sodium and chloride ions in low NaCl conditions, in order to maintain a constant ionic strength and osmolarity in all solutions. Under this condition, we did see better linearity of the dose-dependence curve of Cathepsin B (Fig. 8F).

2) As shown in Fig. 6G-J, Cathepsin L and B are as important as CLH-6 in the lysosomal damage assays. Any reason that neither of them was picked up by the genetic screening?

Response:

We performed a forward genetic screen to isolate mutants defective in lysosome membrane integrity, indicated by accumulation of Gal3-positive structures. The screen, which covered approximately 10,000 haploid genomes, is far from saturation. This is probably why *cpl-1(lf)* and *cpr-2(lf)* mutants were not identified from the screen. In fact, the *cpl-1(qx304)* mutant was identified previously in the lab by a genetic screen

for abnormal lysosomal morphology and that screen covered 30,000 haploid genomes in total.

3) I was confused why some NUC::Cherry fluorescence appeared to be localized in the perimeter membrane in Fig. 3G'. I was expecting a primary perimeter localization of the CLH-6:: GFP fluorescence.

Response:

We apologize for the confusion about the NUC-1::CHERRY image shown in Fig. 3G'. The "perimeter membrane"-like pattern of NUC-1::CHERRY is abnormal and very rarely seen. It is probably due to abnormal accumulation of membrane contents in the lysosomal lumen as we have observed previously in some lipase-defective mutants. In the revised manuscript, we have replaced the image in Fig. 3G' with a new image, which shows a typical pattern of NUC-1::CHERRY.

4) In Fig. 7H, was the chloride level normalized to the lysosome mass? It would be nicer if another luminal ion could be used as an internal control.

Response:

As suggested by the reviewer, we have measured the potassium concentration in lysosomes as a control. These data are included in Fig. S4H-J in the revised manuscript.

5) Why do Fig. 6B and Fig. 6D look so differently from each other?

Response:

GFP::Gal3 is diffuse throughout the cytosol in wild-type worms but accumulates as vesicular and irregular membrane-like structures in lysosomes with membrane damage. The fluorescence intensity of GFP::Gal3 on damaged lysosomes is much stronger than the diffuse cytosolic signal. The image in Fig. 6B was captured with low laser power to avoid overexposure of the vesicular GFP::Gal3. In this case, however, the diffuse signal of GFP::Gal3 in the cytosol was not visible. In the revised manuscript, we re-collected images in which both vesicular and diffuse GFP::Gal3 signals are visible (new Fig. 6B).

We thank reviewer 1 for his/her very helpful and constructive suggestions and comments.

Review #2

1. Nomenclature: CLCN7 (in italic) denotes the gene that encodes the protein ClC-7.

Response:

As suggested by the reviewer, *CLCN7* and ClC-7 are used in the revised manuscript to indicate the gene and the protein it encodes.

2. Regarding the assignment of transmembrane domains, the authors need to make reference to CLC-structures, in particular the first structure of a bacterial homologue (Dutzler et al, Nature, 2002), and the more recent structures of ClC-7/Ostm1 (Schrecker et al, elife 2020; Zhang et al, Sci Adv, 2020).

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pertinent. Leisle at al, Embo J, 2011, were the first to demonstrate that ClC-7 is a Cl- / H+ antiporter.

Response:

We have rephrased the text and added references as suggested in the revised manuscript.

3. The fact that human ClC-7 associates in an obligatory manner with the protein Ostm1 (Lange et al, Nature, 2006) needs to be introduced, and what is known about the C. elegans homologue needs to be discussed (https://www.ncbi.nlm.nih.gov/protein/CAA87781.2?report=genpept&log$=seqview).

Response:

As suggested by the reviewer, we have introduced Ostm1 and its role in stabilizing ClC-7 protein and modulating its activity in the revised manuscript.

It is reported that inactivation of the *C. elegans* homolog of Ostm1 by RNAi causes reduced chloride levels and degradation capacity of lysosomes in coelomocytes, which are scavenger cells that take up macromolecules from the body cavity and degrade them in lysosomes (Chakraborty K. et al., eLife 2017). In addition, Gee K. et al., isolated the *cup-15* mutant from a forward genetic screen and found that it affects *C. elegans* Ostm1 and impairs lysosomal degradation in coelomocytes (Gee K. et al., G3 2017). We examined lysosome morphology and integrity in worms treated by *cup-15* RNAi. The RNAi treatment caused significant reduction in both ectopically expressed CUP-15::GFP and endogenous *cup-15* expression (Figure A-D, below). Consistent with previous reports (Gee K. et al., G3 2017), we found that lysosomes were enlarged and lysosomal degradation of GFP was reduced in coelomocytes of

cup-15 RNAi worms (Figure E-H, below). In hypodermis, however, lysosomal size, morphology, and integrity were unaffected in *cup-15* RNAi worms compared with wild type (Figure I-K, below). Given that CUP-15/Ostm1 is widely expressed in *C. elegans* including hypodermal cells (our preliminary observation), in-depth analyses are needed to determine its role in the maintenance of lysosome degradation activity and integrity. We have discussed this point in the revised manuscript as suggested by the reviewer.

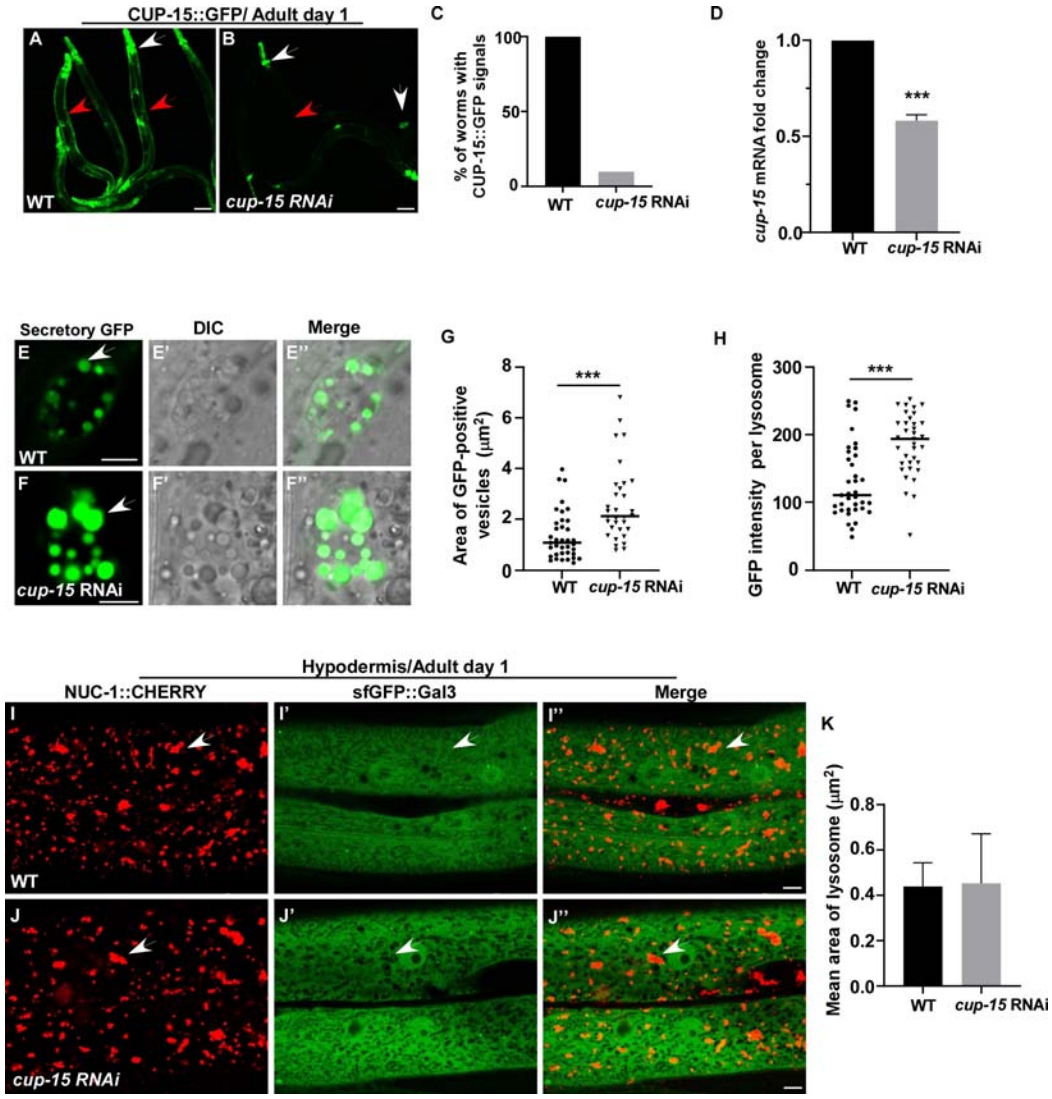

Figure: *cup-15* RNAi affects lysosome size and degradation activity in coelomocytes.
(A, B) Confocal fluorescence images of wild-type and *cup-15* RNAi worms expressing CUP-15::GFP (red arrows). White arrows indicate P_{odr-1}GFP, which serves as a transgenic marker co-injected with the CUP-15::GFP-expressing plasmid. Quantification is shown in (C).
(D) qPCR analyses of *cup-15* expression in wild-type and *cup-15* RNAi worms. At least three independent experiments were performed, and data are shown as mean ± SD.
(E-F'') DIC and confocal fluorescence images of a coelomocyte in wild-type and *cup-15* RNAi worms expressing GFP secreted from body wall muscle cells (Secretory GFP). Arrows indicate lysosomes filled with GFP endocytosed from the body cavity. Quantifications are shown in (G, H). At least 6 worms were scored.
(I-J'') Confocal fluorescence images of the hypodermis in wild-type and *cup-15* RNAi worms expressing NUC-1::CHERRY and sfGFP::Gal3. Lysosomes labeled by NUC-1::CHERRY are negative for sfGFP::Gal3 (arrows). Mean area of lysosomes is quantified in (K). At least 10 animals were scored in each strain and data are shown as mean ± SD.
In (D, G, H, K), unpaired two-tailed Student's *t*-test was performed to compare *cup-15* RNAi datasets with wild type. ***$P<0.0001$; all other points had $P>0.5$.
Scale bars represent 100 μm in (A, B) and 5 μm in (E-F'', I-J'').

We thank the reviewer for his/her very helpful suggestions and comments.

Review #3

2.

B) CLH-6 expression, lysosomal localization.

The authors used a CLH-6::GFP reporter line to demonstrate expression of clh-6 in various tissues in embryo larval stages. The presence of CLH-6 in lysosomes is provided by co-localization with NUC-1::mCherry. While the data support the presence of CLH-6 in lysosomes, considering the disruption in Golgi and ER markers identified clh-6 mutants (FigS3) and previous report of mouse ClC7 accumulating in the Golgi (Maurizi et al., 2019), it may be beneficial for the authors to strengthen the phenotypic characterization of clh-6 mutants by performing a more detailed subcellular localization of CLH-6 in hypodermal cells of wild type worms.

Response:

As suggested by the reviewer, we examined the subcellular localization of CLH-6 by co-expressing CLH-6 and reporters of lysosomes, Golgi, endosomes, and ER. CLH-6 co-localizes with the lysosome marker NUC-1::CHERRY but not reporters of Golgi,

endosomes, or ER (Fig. 3G-K in the revised manuscript).

D) Intra-lysosomal Cl- levels and cathepsin B and L activity depend on CLH-6 function.
The involvement of CPL-1 and CPR-2 in clh-6 phenotypes was demonstrated upon suppression of these defects in worms overexpressing CPL-1 and CPR-2 and the observation of similar phenotypes in cpl-1 and cpr-2 single mutants. Activity of these cathepsins was shown to be reduced in purified lysosome extracts from clh-6 worms. Conversely, chloride was implicated in cathepsin function and lysosomal membrane integrity by detecting a decrease of these ions in the lumen of lysosomes in clh-6 worms and an increase in CPL-1 and CPR-2 function after chloride supplementation. Finally, a direct implication is inferred by establishing that, as previously reported, Cl- ions bind recombinant cathepsins. A few concerns with these experiments:

(a) As mentioned by the authors, the lysosomal integrity defects observed appear to be tissue specific, with other major organs such as the intestine and body wall muscle not being overtly affected by clh-6 mutations. If the LysoIP was performed to isolate lysosomes of Pscav-3SCAV-3::RFP::3xHA expressing cells, how representative of CLH-6 hypodermal lysosome phenotypes will these conclusions be? It seems this is not a unique case of clh-6. However, if we can accept that the drop in Cl- levels detected in the LysoIP work occurs similarly in cells whose lysosomes are not ultimately disrupted, is it not also possible that Cl- levels themselves do change differently in response to CLH-6 in different cells but that is impossible to extricate from the LysoIP data?

Response:

We agree with the reviewer that it is difficult to determine whether lysosomal chloride levels change differently in response to loss of CLH-6 function in different cell types by the current LysoIP approach. In the original manuscript, we utilized the lysosomal membrane protein SCAV-3 as a LysoIP marker. SCAV-3 is expressed in multiple tissues but enriched in the hypodermis when driven by its own promoter (Li et al., JCB 2016). In the revised manuscript, we generated *C. elegans* strains expressing SCAV-3::RFP::3xHA or LMP-1::RFP::3xHA specifically in hypodermal cells where lysosome function and membrane integrity are severely affected by loss of CLH-6. By

LysoIP, we purified lysosomes from the hypodermis and found that chloride levels and the activities of cathepsin B and L were significantly reduced in *clh-6* mutants, consistent with the LysoIP results acquired in P_{scav-3}SCAV-3::RFP::3xHA-expressing cells. We included these new data in Figures 7, 8, and S5 in the revised manuscript.

(b) For the LysoIP experiments, worms overexpressing CPL-1 and CPR-2 were used, presumably because purification of clh-6 lysosomes would not be viable. If CPL-1 and CPR-2 naturally bind Cl- ions in the lumen, wouldn't this further reduce free lumen Cl- measurable in the assay? Or cathepsin bound Cl- can also be accounted for? Following the same line of thought, if increasing CPL-1 and CPR-2 loads on lysosomes can, per se (and presumably without increases in intralysosomal Cl- levels), suppress defects in clh-6 worms, wouldn't this argue for a secondary role of chloride instead? Or are the authors arguing that overexpression of cathepsins can functionally bypass insufficient Cl- levels in a lysosome with normal loads of CPL-1 and CPR-2? If so, how would this jive biochemically with a proposed function of Cl- in regulating cathepsin kinetics?

Response:

We found that chloride levels were unaltered in lysosomes purified from wild type without or with overexpressed CPL-1 or CPR-2 (Fig. 7H in the revised manuscript). This suggests that lysosomal chloride levels are unaffected by overexpression of CPL-1 or CPR-2. Our data show that CPL-1 and CPR-2 bind chloride ions with equilibrium dissociation constants (K_d) of about 7 mM and 34 mM, respectively (Fig. 8I). Given the reported lysosomal chloride concentration (75-108 mM), the chloride supplement assay, and a millimolar range K_d for Cl^- binding, we propose that the activities of CPL-1/Cathepsin L and CPR-2/Cathepsin B are modulated by chloride under high Cl^- conditions such as in the lysosomal lumen. We found that high but not endogenous loading of CPL-1 and CPR-2 suppresses cargo accumulation and restores

membrane integrity of lysosomes in *clh-6* mutants. This suggests that cathepsin activity is reduced but not totally inhibited under low chloride conditions. High loading of CPL-1 or CPR-2 may increase the overall cathepsin L/B activity in *clh-6(lf)* lysosomes, and thus promotes cargo digestion and restores membrane integrity. In-depth biochemical and structural studies are needed to understand how CPL-1 and CPR-2 activity is modulated by Cl^-.

3.

- Along with inset images in 1D-E, add a panel for a WT inset (A) to help comparison with the WT localization of NUC-1::mCherry and GFP::Gal3.

Response:

We have included a panel for a WT inset as suggested by the reviewer.

- On page 8:
"The abnormal pattern of endosomes, Golgi, and ER in clh-6 mutants was observed only in the hypodermis at adult stages when lysosomal damage is severe, which suggests that lysosomal rupture and the subsequent leakage of hydrolases may lead to abnormalities of these organelles (Fig. S3M)".

Could this not be instead related directly to CLH-6 function on the collapse of other endomembrane organelles and not the consequence of lysosomal disruption?

Response:

As suggested by the reviewer, we examined the subcellular localization of CLH-6 and found that CLH-6 localizes to lysosomes but not Golgi, ER or early endosomes (Fig. 3G-K). The abnormal pattern of endosomes, Golgi, and ER in *clh-6* mutants was

observed only in the hypodermis at adult stages when lysosomal damage is severe. Moreover, overexpression of the lysosomal cathepsin CPL-1 suppressed both cargo accumulation and membrane damage of lysosomes in *clh-6* mutants and restored the morphology of endosomes, ER and Golgi (Fig. 2J, K, P, Q, S3J, K, L). Collectively, these data suggest that lysosomal defects may lead to abnormalities of other endomembrane organelles in *clh-6* mutants.

- For figure 6 panels (A-E). I would suggest showing separately the images for the GFP::Gal3 channel. The suppression of the defect by overexpressing the two hydrolases is somewhat masked by showing it in the merged image in this figure. This is especially important since the mCherry signals report different transgenes and are not directly comparable.

- In Figure 7H (quantification of chloride levels), shouldn't the WT bar in the X axis also indicate +CLP-1/CRP-2? Alternatively, indicate that in the legend.

Response:

As suggested by the reviewer, we have included separate images for the GFP::Gal3 channel in Fig. 6A-E" and included data for WT+CPL-1 and WT+CPR-2 in Fig. 7H.

- In figure 5, what is the significance of the nuclear signal in hypodermal cells expressing Gal3::GFP ? This appears largely absent in WT / untreated, but are pronounced in worms where lysosomal defects were induced.

Response:

The nuclear signal of GFP::Gal3 can be observed in wild-type worms but appears to be stronger in lysosome- or autophagy-defective mutants or upon Pitstop 2 treatment. We do not understand why and how the nuclear signal of GFP::Gal3 is altered in these conditions. We have replaced the image in Fig. 5H with a new one which shows the

weak nuclear signal of GFP::Gal3 in wild type.

- In SupFigS1 - any reason what the image in panel B, but not C,D or E is shown on a grid?

Response:

Lysosome damage phenotypes in all *clh-6* mutant alleles shown in Fig. S1B-E were quantified in Fig. S1F.

We thank the reviewer for his/her very helpful suggestions and comments.

We sincerely thank all three reviewers for their very helpful and constructive suggestions and comments. I hope that this revised manuscript is now suitable for publication in *The Journal of Cell Biology*.

Thank you very much for your time and I look forward to hearing from you soon.

Best Regards,

Xiaochen Wang, Ph.D

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March 1, 2023

RE: JCB Manuscript #202210063R

Prof. Xiaochen Wang
Institute of Biophysics, Chinese Academy of Sciences
15 Datun Road, Chaoyang district
Beijing 100101
China

Dear Prof. Wang:

Thank you for submitting your revised manuscript entitled "Lysosomal chloride transporter CLH-6 protects lysosome membrane integrity via cathepsin activation". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully. Please go through all the formatting points paying special attention to those marked with asterisks.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Articles and Tools may have up to 10 main text figures.

Please note that main text figures should be provided as individual, editable files.

3) Figure formatting:
*** Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please, add MW marker to Fig 8B.

Scale bars must be present on all microscopy images, including inset magnifications.

*** Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. As red and green are paired for images in Fig S1B, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

4) Statistical analysis:
Error bars on graphic representations of numerical data must be clearly described in the figure legend.

The number of independent data points (n) represented in a graph must be indicated in the legend. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments).

If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please, see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

Statistical methods should be explained in full in the materials and methods in a separate section.

For figures presenting pooled data the statistical measure should be defined in the figure legends.

Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.).

*** As you used parametric tests in your study (i.e. t-tests), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title:
The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience.

The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

6) Materials and methods:
Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. The text should not refer to methods "...as previously described."

Also, the materials and methods should be included with the main manuscript text and not in the supplementary materials.

7) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate).

Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods.

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The following information must be provided about the acquisition and processing of images:
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b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
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e. Fluorochromes
f. Camera make and model
g. Acquisition software
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There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. There is no limit for supplemental tables.

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A summary of all supplemental material should appear at the end of the Materials and Methods section (please see any recent JCB paper for an example of this summary).

11) Video legends:
Video legends should describe what is being shown, the cell type or tissue being viewed (including relevant cell treatments,

concentration and duration, or transfection), the imaging method (e.g., time-lapse epifluorescence microscopy), what each color represents, how often frames were collected, the frames/second display rate, and the number of any figure that has related video stills or images.

12) eTOC summary:
*** A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

13) Conflict of interest statement:
JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests."

14) Author contribution:
A separate author contribution section is required following the Acknowledgments in all research manuscripts.

All authors should be mentioned and designated by their first and middle initials and full surnames and the CRediT nomenclature is encouraged (https://casrai.org/credit/).

15) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

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All animal and human studies must be conducted in compliance with relevant local guidelines, such as the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals or MRC guidelines, and must be approved by the authors' Institutional Review Board(s). A statement to this effect with the name of the approving IRB(s) must be included in the Materials and Methods section.

*** Journal of Cell Biology now requires a data availability statement for all research article submissions. These statements will be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).

All datasets included in the manuscript must be available from the date of online publication, and the source code for all custom computational methods, apart from commercial software programs, must be made available either in a publicly available database or as supplemental materials hosted on the journal website. Numerous resources exist for data storage and sharing (see Data Deposition: https://rupress.org/jcb/pages/data-deposition), and you should choose the most appropriate venue based on your data type and/or community standard. If no appropriate specific database exists, please deposit your data to an appropriate publicly available database.

17) Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. The Source Data files will be directly linked to specific figures in the published article.

As your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Li Yu
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