

Rapid activation of p62 body-mediated autophagy in human cells under hyperosmotic stress

Corresponding Author: Dr Naoki Tamura

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this study, Tamura and Waguri investigated the role of hyperosmotic stress on the degradation of p62 bodies by autophagy. For this purpose, the authors used human cell lines and organoids as experimental models and immunofluorescence, live-cell imaging, western blot and correlative light electron microscopy (CLEM) as techniques. They found that p62 body formation occurs very rapidly upon osmolarity of the cell medium increases (sucrose) and that their formation is independent of stress granules biogenesis. Moreover, they found that the hypertonic-dependent formation of p62 bodies leads to their degradation by autophagy and not by the proteasomal pathway. This mechanism is conveyed by K63-linked Ubiquitin, which is a signal typically associated with autophagy. On the contrary, K48-linked Ub chains (typically associated with proteasomal degradation) were not associated with the autophagy markers with which p62 bodies were stained, supporting their degradation via autophagy. Degradation of p62 occurs only at lower sucrose concentrations. They also characterized the morphological differences between p62 bodies and stress granules upon hyperosmotic stress induction by using CLEM. Finally, they have investigated that p62 body degradation also occurs in epithelial organoids via autophagy.

Overall, the data (including immunofluorescence, time-lapse imaging, western blot and CLEM) are of good quality. I particularly appreciated the quality of TEM and CLEM images, they look very nice and generally the study supports that p62 bodies are specifically degraded by autophagy. However, novelty is missing, and several shortcomings made my support of this study very difficult to sustain for the following reasons: 1. A certain number of conclusions are not supported by the data presented in the manuscript, 2. The description of how the quantifications were conducted is totally absent leading to several doubts, 3. Several scale bars in the images are clearly wrong, leading to questioning quantification analysis. Below, I provide the authors a point-by-point list of my concerns.

Major points:

1. Line 267: "Taken together, our findings demonstrate for the first time that p62 body formation under hyperosmotic stress mediates the rapid elimination of cytoplasmic proteins by autophagy."

While the authors state that p62 is degraded by autophagy under hyperosmotic stress for the first time, this phenomenon has been previously described also by themselves and is therefore not entirely novel. The authors previously demonstrated, to a less extent, that p62 is degraded by autophagy upon hyperosmotic stress (sucrose) (Tamura et al 2019, DOI: 10.1128/MCB.00024-19, Figure 1 and 4). Other studies have shown similar conclusions: Pena-Oyarzun et al 2017 (doi: 10.18632/oncotarget.18995), Dafre et al 2019 (doi: 10.1155/2019/2715810).

2. Methods about how the authors quantified the number of puncta, the size and the colocalization are totally lacking. They only provided the name of the software used for quantification (Photoshop) without describing if they manually/automatically counted the puncta, how colocalization was measured (Pearson, Manders or other coefficients?). Concerned Figures: Figure 1B, 1C, 2B, 2F, 3C, 3D, 3E, 7C, 7D.

a. "Size of p62-positive puncta (pixels)" in Figure 1C does not provide any information about the size of these puncta if the "pixel size" value (in nm) is not provided.

3. Concerns about scale bars in several Figures.

a. Figure 4A. Scale bar of 500 nm in (iii), (iv) and (v). One or more of these scale bars are visibly wrong.
b. Doubts about scale bars in Figure 7A, 7B, 7E.

- c. Doubts about scale bars in several immunofluorescence figures.
- d. Figure 7F. Scale bar in (iii) and (iv) cannot be 500 nm. Mitochondria are visible in the micrographs and their size would be around 250 nm, which is not possible (in this context).
- e. If these scale bars are wrong and Photoshop was used for most of the images, without considering that photoshop does not automatically calibrate the figures, my concerns about the scale bars extend to all images of the manuscript.
- f. I strongly suggest using Fiji/ImageJ, which automatically recognizes the metadata containing the information about the pixel size. For this purpose, the original image files are needed. Please, double check all scale bars.
4. Line 125: We also observed that the number of p62 bodies decreased over time (Fig. 2A, B), suggesting their fusion.” Decrease in p62 number does not necessarily mean they fused. This is not supported by data shown in Figure 2A and 2B.
5. Lined 126: “Indeed, live-cell imaging confirmed the fusion of small p62 bodies (Fig. 2C, Supplementary Fig. 2B and Supplementary Movie 1). The live-cell movie does not support fusion events. The Supplementary Movie 1 shows a massive formation of p62 puncta over time, but most of these puncta are static and do not show fusion. The fusion events shown in Figure 2C represent a very limited number of fusion events that cannot be interpreted as a general mechanism.
6. Line 151: “Double IF staining for LC3 and p62 or G3BP1 at 0.4 M sucrose, a condition that induces obvious stress granule formation, showed that 94.5% of LC3-positive puncta colocalized with p62 bodies, which were rarely (< 1%) associated with G3BP1-positive puncta (Fig. 3A, B, E, and Supplementary Fig. 3A, B).” The authors say that 94% of LC3 puncta colocalize with p62 upon 0.4M incubation with sucrose (Figure 3E) but they previously observed and stated that p62 colocalization with LC3 is reduced to 30% when using the same sucrose concentration (0.4M, Figure 3D). This is contradictory.
7. The discussion part is not well written. I had difficulties to understand to flow, most of the conclusions overinterpret results. I strongly suggest the authors re-write this part based on what they found and improve it. A detailed description of the discussion point I am referring is into the “minor points” section.
- Minor points:
8. Figure 1B: Number of puncta per cell. Do the authors see differences if normalized the number of puncta per cell to the cell area? I suggest to systematically divide the number of puncta per cell by the cell area to avoid big differences.
9. Line 120: “These results suggest that p62 bodies are formed differently and more sensitively than stress granules”. The results show an increase in the formation of puncta but it does not show that puncta are formed in different ways. What does “sensitively than stress granules” means? This is not supported by data.
10. Figure 2B: There is a strong discrepancy in the number of puncta per cell at 20 minutes (0.4M sucrose) in Figure 2B when compared to the same condition in Figure 1B. Figure 1B shows around 170 p62 puncta per cell (T24) and around 250 p62 puncta per cell in U2OS, while in Figure 2B the number of p62 puncta in U2OS is reduced to around 100 puncta per cell. How do the authors explain such a difference in the same conditions? Did they change the parameters to analyze the 2 sets of experiments? Information about how they analyzed are missing.
11. Line 152: “LC3-positive puncta colocalized with p62 bodies”, referring to Figure 3A, 3B and 3E while the graphs show p62 colocalization with LC3 and not the opposite. If Mander coefficient, for example, was used to calculate the colocalization, this can change everything. I strongly suggest to be more precise and add how did they calculate the colocalization.
12. Line 161: “Western blot (WB) analysis showed that, while p62 protein levels significantly decreased in medium containing 0.1–0.4 M sucrose, with the lowest levels observed at 0.2 M sucrose, the levels of stress granule markers G3BP1 and YB1 remained unchanged in T24 and U2OS cells (Fig. 3G, H).” The authors see a decrease in p62 levels upon 0.1-0.4M sucrose incubation. They first normalized to “no me” and then to “0” condition. I do not find this normalization method correct. I suggest, first, to normalize each p62 (and other markers) time point to the same corresponding GAPDH time point and, secondly, normalize this value to the “0” point and plot it in the graph. The “0” point is the real control of the experiment, rather than “no me”.
13. Lined 184: “Taken together with the biochemical and IF data, these findings support the conclusion that hyperosmotic stress-induced autophagy specifically targets and degrades p62 bodies.”. The data shown in this result section supports the fact that p62 fluorescence correlates with autophagic structures and G3BP1 fluorescence correlates with fibrillar structures. Biochemical and immunofluorescence data should not be even mentioned here. This is more a conclusion to add into the discussion part.
14. Figure 5A and 5B: Images and quantification from WB results. Same problem of normalization as in point 12, in this list. The control to use to normalize should be “0 mM sucrose” and not “no me”.
15. Discussion part to be improved.
- a. Line 241: “These p62 bodies undergo fusion events until sequestration by autophagosomes”. This sentence is not supported by results. Time-laps video does not support fusion events as it actually appears to be a very rare event when carefully looking at all puncta.
- b. Line 252: “This difference may explain why most of the p62 bodies induced by hyperosmotic stress were relatively small in size, allowing entire bodies to be enclosed by the isolation membrane”. What does “relatively small in size” means? The authors do not measure the size of the p62 over time but only under different concentrations of sucrose (Figure 1C). Additionally, it is not because p62 bodies are little that they can be enclosed by the isolation membrane while if bigger they cannot.
- c. Line 255: “In contrast, under conditions that promote the formation of larger p62 bodies, a piecemeal type of autophagy is likely to occur”. This would mean that little p62 are degraded by bulk autophagy and larger p62 by selective autophagy?. This part is not clear.
- d. Line 256: “It should be noted that p62 body-mediated autophagy is distinct from starvation-induced autophagy, in which LC3-positive p62 puncta are also detectable at the light microscopic level 30-32”. This sentence does not make a lot of sense. The authors also saw LC3-positive p62 puncta in their analysis (Figure 3A-3B-3C-3D-3E). How do they state that the 2 mechanisms are different, when they also actually detected?
- e. Line 256: “light microscopic level” does not sound appropriate do describe immunofluorescence images.
- f. Line 261-265: “Intriguingly, the optimal osmolality of 0.2 M sucrose (500 mOsm/L) promoted efficient degradation in T24

and U2OS cells (Fig. 3D), with p62 bodies appearing largest under light microscopy (Fig. 1C) and showing the highest rate of LC3 colocalization (Fig. 3D), supporting enhanced p62 degradation.” The authors state that the osmolarity of 0.2M is the optimal condition for p62 degradation, but in the graphs also 0.1M looks to be as much as efficient than 0.2M (Figure 1C, 3D).

Reviewer #2

(Remarks to the Author)

The manuscript shows that p62 bodies form within 1 minute of hyperosmotic stress, and increase with stress severity, in T24 human bladder carcinoma cell line and U2OS human osteosarcoma cells. They form faster and with greater sensitivity than stress granules, form by phase separation and exhibit liquid-like properties. Unlike stress granules, p62 bodies frequently colocalize with the autophagosome markers LC3 and WIPI-2, and are degraded via autophagy. Correlative light-electron microscopy revealed that these p62 bodies are more compact than stress granules, and often associate with the autophagic isolation membrane (also called the phagophore). Autophagy receptors NBR1 and TAX1BP1, but not OPTN1 or NDP52, are components of the p62 bodies and are specifically degraded by autophagy under hyperosmotic stress. p62 bodies preferentially contain K63-linked ubiquitin chains. The authors conclude that hyperosmotic stress-induced autophagy specifically targets and degrades p62 bodies. Finally, p62 body-mediated autophagy was also observed in epithelial organoids.

The main finding is the identification of p62 bodies as a new type of cytoplasmic degradation platform that is activated under hyperosmotic stress. The manuscript reads extremely well and the Figures are excellent, including the correlative light-electron microscopy images. Some details are missing on image analysis, as detailed below.

1. It would be helpful for the readers if there would be discussion on the physiological relevance of the findings. What kind of conditions could cause hyperosmotic stress in multicellular organisms? How would the observed degradation mechanism help the organism to manage the stress?
2. Would it be possible to quantify the colocalization of p62, PSMD4, K48Ub and K63Ub in Figure 6C and 6D? Please also include details on how the colocalization is analyzed.
3. Would it be possible to quantify the colocalization of p62 and WIPI2, NBR1 and p62, and TAX1BP1 and p62, in Figure 5E and 5F?
4. Please give details on how the “p62 bodies co-localizing with LC3” was analyzed and defined in Figure 3D. What exactly was analyzed in Figure 3E?
5. In Methods (line 359) only Photoshop is mentioned for image analysis. However, in the legend of Figure 2, ImageJ is mentioned for panel D. Please add ImageJ (with appropriate Reference) to the Methods section as well.
6. Legend to Supplementary Figure 2: “(B) Low magnification image of U2OS cells at 11 min after medium exchange with DMEM containing 0.5 M sucrose. The boxed region is shown as sequential still images (Fig. 2B) and Supplementary Movie 1.” The U2OS cells must be expressing a fluorescent-tagged version of p62. This should be mentioned in the legend.
7. Legend to Supplementary Figure 3, line 754: LC should read LC3.
8. Legend to Supplementary Figure 5: “(C) Immunostaining of U2OS cells transfected with GFP-tagged receptors,” The annotation in the panel C reads ‘HeLa’, not U2OS.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Reviewer #1

I appreciate the authors’ efforts in revising the manuscript and addressing several of the concerns raised during the initial review. The revised version shows improvement, and the data presented now more clearly support the main hypothesis of the study.

However, several important issues remain either unresolved or insufficiently addressed. In particular, concerns regarding data quantification, methodological clarity, and the use of appropriate and unbiased analysis tools still persist. I want to stress that if the analysis had been done more rigorously, and if the authors had properly explained or corrected the remaining issues, then I would have fully supported this work. Nonetheless, scientific integrity relies on transparent, reproducible, and unbiased data handling, which remains a critical expectation for publication in any journal. Below, I answered to authors replies on the previous round of reviewing and outlined specific comments and suggestions in bold for further clarification and revision.

Overall, the data (including immunofluorescence, time-lapse imaging, western blot and CLEM) are of good quality. I particularly appreciated the quality of TEM and CLEM images, they look very nice and generally the study supports that p62 bodies are specifically degraded by autophagy. However, novelty is missing, and several shortcomings made my support of this study very difficult to sustain for the following reasons: 1. A certain number of conclusions are not supported by the data presented in the manuscript, 2. The description of how the quantifications were conducted is totally absent leading to several doubts, 3. Several scale bars in the images are clearly wrong, leading to questioning quantification analysis. Below, I provide the authors a point-by-point list of my concerns.

Reply

Thank you for your constructive comments and for recognizing the value of our manuscript. The novelty of this study lies in the finding that, under hyperosmotic stress, autophagic degradation is mediated by the formation of p62, rather than by direct incorporation of individual or oligomeric p62 molecules into autophagosomes. (1) Inappropriate conclusions have been revised, and additional explanatory texts have been included to clarify our interpretations. (2) Detailed methods for quantification of puncta in fluorescent images have been added, and (3) the scale bars in both immunofluorescence and EM images have been corrected. Please refer to the following responses for further details.

Major points:

1. Line 267: "Taken together, our findings demonstrate for the first time that p62 body formation under hyperosmotic stress mediates the rapid elimination of cytoplasmic proteins by autophagy."

While the authors state that p62 is degraded by autophagy under hyperosmotic stress for the first time, this phenomenon has been previously described also by itself and is therefore not entirely novel. The authors previously demonstrated, to a less extent, that p62 is degraded by autophagy upon hyperosmotic stress (sucrose) (Tamura et al 2019, DOI: 10.1128/MCB.00024-19, Figure 1 and 4). Other studies have shown similar conclusions: Pena-Oyarzun et al 2017 (doi: 10.18632/oncotarget.18995), Dafre et al 2019 (doi: 10.1155/2019/2715810).

Reply

We appreciate the reviewer's comment and agree that it is already known that p62 is degraded by autophagy under hyperosmotic stress. We have acknowledged this point at the beginning of the relevant paragraph in Discussion (lines 249–250). Importantly, the novelty of our study lies in the demonstration that this degradation is mediated by p62-condensate formation. To clarify this, we have revised the text as follows to better highlight this mechanistic insight:

(lines 274–276)

"Taken together, our findings demonstrate for the first time that it is p62 body formation that mediates the rapid autophagic elimination of cytoplasmic proteins under hyperosmotic stress."

In addition, we have now properly cited Dafre et al 2019, which was omitted in the original manuscript.

Line 267: The phrase "at the resolution of light microscopy" is somewhat awkwardly phrased. I do not think that the sentence refers to a resolution matter but simply to a fluorescence microscopy technique. Consider rewording to "by light microscopy" or "within the limits of light microscopy resolution." If the authors wanted to highlight a resolution matter.

Line 269: The expression "p62 localizes on the restricted parts of autophagosome membranes" is stylistically unusual. A more precise and grammatically natural phrasing might be: "p62 localizes to specific subdomains of the autophagosome membrane."

Line 271: The construction "rather than the typical selective autophagy of p62 bodies" is vague and potentially misleading. I suggest rephrasing as: "rather than a canonical selective autophagic degradation of p62-positive bodies."

Line 272: The sentence "observed in both previous¹¹ and present studies" can be improved. For clarity, consider rewording to: "as previously observed¹¹ and in the present study."

Line 274: The expression "that it is p62 body formation that mediates the rapid autophagic..." could be more clearly stated. A suggested revision would be: "that the formation of p62 bodies mediates the rapid autophagic clearance..."

2. Methods about how the authors quantified the number of puncta, the size and the colocalization are totally lacking. They only provided the name of the software was used for quantification (Photoshop) without describing if they manually/automatically counted the puncta, how colocalization was measured (Pearson, Manders or other coefficients?). Concerned Figures: Figure 1B, 1C, 2B, 2F, 3C, 3D, 3E, 7C, 7D.

Reply

We apologize for not providing a detailed description of the quantification method. In our analysis, the number of puncta and those showing colocalization were manually counted using the counting tool in Photoshop, based on either single channel or merged images. In this revision, we initially attempted to quantify colocalization using Pearson's and Manders'

coefficients. However, we found that acquired images were not readily suitable for these analyses, as such methods require stringent optimization of immunostaining conditions and image acquisition parameters. Therefore, we opted for manually counting, which we believe yields more reliable and accurate data under our current experimental setup. We have accordingly revised the relevant section in the Methods (lines 384–390).

The authors argue that quantitative colocalization analysis could not be performed due to suboptimal immunostaining. However, Pearson's and Manders' coefficients are robust metrics that do not require highly stringent staining conditions, and can still provide valuable insights even when image resolution is not optimal. Relying on manual particle counting using Photoshop is not an appropriate alternative, as this approach is inherently subjective and lacks reproducibility. Unbiased analysis is a critical matter in research. As previously suggested but ignored, I strongly recommend again the use of standardized, unbiased image analysis tools such as Fiji/ImageJ for quantification.

a. "Size of p62-positive puncta (pixels)" in Figure 1C does not provide any information about the size of these puncta if the "pixel size" value (in nm) is not provided.

Reply

Thank you for this comment. We converted the puncta size from "pixels" to "µm" in the revised Fig. 1C. **The new analysis is now correct.**

3. Concerns about scale bars in several Figures.

a. Figure 4A. Scale bar of 500 nm in (iii), (iv) and (v). One or more of these scale bars are visibly wrong.

The scale bar in Figure 4A(v) still appears to be incorrectly labeled. Given that panels (iii) and (iv) are labeled as 1 µm, and the scale bar in panel (v) is approximately four times longer than that in panel (iii), it cannot correspond to 200 nm. A more accurate value in (v) would be 4 times the scale bar in (iii) and not 5 times as is in the current figure. I would like to note that concerns regarding scale bar accuracy were already raised during the first round of review, and I had expected such issues to be carefully addressed in the revised version. It is therefore disappointing to find that at least one figure still contains a scale bar inconsistency. I strongly urge the authors to carefully review and verify all scale bars throughout the manuscript to avoid further misrepresentation of the data.

b. Doubts about scale bars in Figure 7A, 7B, 7E. **It looks now correct.**

c. Doubts about scale bars in several immunofluorescence figures.

d. Figure 7F. Scale bar in (iii) and (iv) cannot be 500 nm. Mitochondria are visible in the micrographs and their size would be around 250 nm, which is not possible (in this context). **It looks now correct.**

e. If these scale bars are wrong and Photoshop was used for most of the images, without considering that photoshop does not automatically calibrate the figures, my concerns about the scale bars extend to all images of the manuscript.

f. I strongly suggest using Fiji/ImageJ, which automatically recognizes the metadata containing the information about the pixel size. For this purpose, the original image files are needed. Please, double check all scale bars. **This recommendation addresses a fundamental point in the analysis and should have been carefully considered, which is not the case.**

Reply

Thank you for these comments and deeply apologize for putting several incorrect scale bars. We have checked all scale bars in relevant Figures by comparing with original figures.

4. Line 125: We also observed that the number of p62 bodies decreased over time (Fig. 2A, B), suggesting their fusion." Decrease in p62 number does not necessarily mean they fused. This is not supported by data shown in Figure 2A and 2B.

5. Lined 126: "Indeed, live-cell imaging confirmed the fusion of small p62 bodies (Fig. 2C, Supplementary Fig. 2B and Supplementary Movie 1). The live-cell movie does not support fusion events. The Supplementary Movie 1 shows a massive formation of p62 puncta over time, but most of these puncta are static and do not show fusion. The fusion events shown in Figure 2C represent a very limited number of fusion events that cannot be interpreted as a general mechanism.

Reply (to Number 4 and 5)

As this reviewer pointed out, the original descriptions are inappropriate and not enough. We revised the sentences as follows:

(lines 126–132)

"We also observed that the number of p62 bodies decreased over time (Fig. 2B). Live-cell imaging demonstrated that p62 bodies simultaneously emerged across the cytoplasm immediately after changing hypertonic medium and progressively grew larger and more distinct (Supplementary Fig. 2B and Supplementary Movie 1). Moreover, careful observations revealed that a subset of p62 bodies occasionally undergo fusion with each other (Fig. 2C and supplementary Fig. 2B), which may account for the observed decrease in the number of p62 bodies (Fig. 2B)."

This paragraph sounds now ok. Please revise the English phrasing in the sentence for clarity and readability. The expression "immediately after changing hypertonic medium" is awkward and would be better phrased as "upon addition of hypertonic solution". Similarly, "and progressively grew larger and more distinct" could be reworded to "and their size increased over time" for clarity. Lastly, the phrase "with each other" is unnecessary and should be removed.

Regarding the fusion events, we included additional regions in Supplementary Fig. 2C, each showing 2-3 fusion events over a 150 sec interval. These observations suggest the existence of at least two subsets of p62 bodies: one that undergo fusion and another that remain static, either recruiting surrounding p62 molecules or being rapidly engulfed by isolation membranes. We believe the fusion events occurs occasionally rather than rarely, based on the following observation: for example, Figure 2C shows two fusion events occurring within 150 seconds in approximately one-seventh of the total cytoplasmic area. Therefore, it can be estimated that roughly 50 fusion events (2 events x 4 per 10 min x 7 region) may occur per cell over a 10 min, which could account for the decrease in p62 body number shown in Fig. 2B.

6. Line 151: "Double IF staining for LC3 and p62 or G3BP1 at 0.4 M sucrose, a condition that induces obvious stress granule formation, showed that 94.5% of LC3-positive puncta colocalized with p62 bodies, which were rarely (< 1%) associated with G3BP1-positive puncta (Fig. 3A, B, E, and Supplementary Fig. 3A, B)." The authors say that 94% of LC3 puncta colocalize with p62 upon 0.4M incubation with sucrose (Figure 3E) but they previously observed and stated that p62 colocalization with LC3 is reduced to 30% when using the same sucrose concentration (0.4M, Figure 3D). This is contradictory.

Reply

We apologize for the confusion caused by the complex descriptions for Fig. 3D and E. To improve clarity, we have revised this section as follows, including an additional explanatory sentence:

(lines 153–160)

"Double immunofluorescence microscopy for LC3 and p62 showed that ~70% of p62 bodies contained LC3 at 0.1 M sucrose, and this fraction decreased to ~30% at 0.4 M sucrose (Fig. 3A, D Supplementary Fig. 3A). We then examined to what extent LC3-positive puncta colocalize with p62 bodies or G3BP1. Double IF staining for LC3 and p62 or G3BP1 at 0.4 M sucrose, a condition that induces obvious stress granule formation, showed that 94.5% of LC3-positive puncta colocalized with p62 bodies, which were rarely (< 1%) associated with G3BP1-positive puncta (Fig. 3A, B, E, and Supplementary Fig. 3A, B)."

In addition, we have corrected the y-axis labels of Fig. 3D and Fig. 3E to read "Percentage of p62 with LC3" and "Percentage of LC3 with p62 or G3BP1", respectively.

The revised sentence is now clearer; however, the use of "contained" is not appropriate in this context. I recommend replacing it with "overlapped with LC3", as this more accurately reflects the observation. Additionally, since no quantitative colocalization analysis was performed, the term "overlap" is more suitable than "colocalization", which implies a degree of quantification.

7. The discussion part is not well written. I had difficulties to understand to flow, most of the conclusions overinterpret results. I strongly suggest the authors re-write this part based on what they found and improve it. A detailed description of the discussion point I am referring is into the "minor points" section.

Reply

Thank you for the detailed comments (in the minor points) for the Discussion section. Based on your suggestion, along those from another reviewer, we have substantially revised the Discussion section by reorganizing the contents, adding further explanation and including a new paragraph highlighting the significance of this study for higher organisms. Detailed responses to each comment are provided below.

Minor points:

8. Figure 1B: Number of puncta per cell. Do the authors see differences if normalized the number of puncta per cell to the cell area? I suggest to systematically divide the number of puncta per cell by the cell area to avoid big differences.

Reply

Thank you for the critical comment. We re-analyzed the data by quantifying the number in 40 cells and comparing the variation between "count per cell" and "count per area." As shown in the figure below, the reduction in standard deviation observed in the "count per area" was minimal. This is likely due to the presence of cells with highly extended cytoplasm, which disproportionately decreases the values in the "count per area" compared to the "count per cell." Based on this observation, we have chosen to adopt "count per cell" in the revised manuscript. **thank you for comparing the analysis. There no meaningful difference between the two analysis presented. As a result, the authors' choice and explication of this comparison is pointless.**

9. Line 120: "These results suggest that p62 bodies are formed differently and more sensitively than stress granules". The results show an increase in the formation of puncta but it does not show that puncta are formed in different ways. What does "sensitively than stress granules" means? This is not supported by data.

Reply

We have revised the text as follows to ensure that it more accurately reflects our data:

(lines 120–122)

"These results suggest that p62 bodies and stress granules form as distinct condensates under hyperosmotic stress, and that formation of p62 bodies is more sensitive to stress intensity than that of stress granules."

The statement "and that formation of p62 bodies is more sensitive to stress intensity than that of stress granules" is

unclear and potentially misleading. The use of the term “sensitive” is not well-defined in this context. Based on the data, the authors are observing an increase in puncta formation, but this does not necessarily imply greater sensitivity. I suggest rephrasing the sentence to more accurately reflect the observed changes in puncta number, without implying mechanistic differences in stress responsiveness unless further evidence is provided.

10. Figure 2B: There is a strong discrepancy in the number of puncta per cell at 20 minutes (0.4M sucrose) in Figure 2B when compared to the same condition in Figure 1B. Figure 1B shows around 170 p62 puncta per cell (T24) and around 250 p62 puncta per cell in U2OS, while in Figure 2B the number of p62 puncta in U2OS is reduced to around 100 puncta per cell. How do the authors explain such a difference in the same conditions? Did they change the parameters to analyze the 2 sets of experiments? Information about how they analyzed are missing.

Reply

Thank you for pointing out this important mistake. We deeply apologize for the oversight. Upon review, we realized that experiments for Fig. 2A and B were performed under 0.3M sucrose condition, not 0.4M as previously described. This choice was made to clearly detect both p62 bodies and stress granules in the time-course experiments. This correction explains the observed reduction in the number of p62 bodies compared to Fig. 1B.

This is a critical issue, and the explanation provided by the authors does not sufficiently clarify the inconsistencies. In the previous version of the manuscript, the concentration was indicated as 0.4 M in Figures 2A and 2B, and 0.5 M in the legend of Figure 2C. In the revised version, this concentration was changed to 0.3 M due to an initial mistake, and now Figure 2C (legend) refers to 0.38 M. The rationale behind these adjustments is not convincingly justified. Given that both p62 and G3BP1 puncta formation are clearly visible at 0.4 M, the authors’ reasoning for choosing 0.3M and 0.38 M over the originally reported concentrations is unconvincing. In fact, using 0.4 M would have likely yielded clearer observations. Therefore, the explanation for the changes in experimental conditions remains insufficient, and the mistake (together with biased analysis and scale bars) raises concerns about the consistency and reproducibility of the experimental design.

11. Line 152: “LC3-positive puncta colocalized with p62 bodies”, referring to Figure 3A, 3B and 3E while the graphs show p62 colocalization with LC3 and not the opposite. If Mander coefficient, for example, was used to calculate the colocalization, this can change everything. I strongly suggest to be more precise and add how did they calculate the colocalization.

Reply

As mentioned in our response to the Comment No. 6, we revised both the text and the figure accordingly.

12. Line 161: “Western blot (WB) analysis showed that, while p62 protein levels significantly decreased in medium containing 0.1–0.4 M sucrose, with the lowest levels observed at 0.2 M sucrose, the levels of stress granule markers G3BP1 and YB1 remained unchanged in T24 and U2OS cells (Fig. 3G, H).” The authors see a decrease in p62 levels upon 0.1–0.4M sucrose incubation. They first normalized to “no me” and then to “0” condition. I do not find this normalization method correct. I suggest, first, to normalize each p62 (and other markers) time point to the same corresponding GAPDH time point and, secondly, normalize this value to the “0” point and plot it in the graph. The “0” point is the real control of the experiment, rather than “no me”.

Reply

Thank you for this comment. As suggested by the reviewer, the values were normalized to the “0” time point instead of “no me”. However, we did not normalize to GAPDH, as it was not used as an internal control, but rather represents a cytosolic protein that can be incorporated into autophagosomes through bulk macroautophagy. We have added a text to clarify this intention, as follows:

(lines 169–171)

Western blot (WB) analysis showed that, while p62 protein levels significantly decreased in medium containing 0.1–0.4 M sucrose, with the lowest levels observed at 0.2 M sucrose. In contrast, the levels of stress granule markers G3BP1 and YB1, as well as the cytosolic protein GAPDH, remained unchanged in T24 and U2OS cells (Fig. 3G, H).

GAPDH levels appear unchanged under the conditions tested and should therefore serve as a suitable internal loading control. While GAPDH has been reported to undergo degradation via chaperone-mediated autophagy under conditions of nutrient deprivation, there is no evidence to date supporting its degradation through bulk macroautophagy. Moreover, this study focuses on osmotic stress rather than nutrient depletion, making GAPDH degradation via CMA unlikely in this context.

13. Lined 184: “Taken together with the biochemical and IF data, these findings support the conclusion that hyperosmotic stress-induced autophagy specifically targets and degrades p62 bodies.”. The data shown in this result section supports the fact that p62 fluorescence correlates with autophagic structures and G3BP1 fluorescence correlates with fibrillar structures. Biochemical and immunofluorescence data should not be even mentioned here. This is more a conclusion to add into the discussion part.

Reply

We agree this comment. Therefore, this sentence has been deleted.

14. Figure 5A and 5B: Images and quantification from WB results. Same problem of normalization as in point 12, in this list.

The control to use to normalize should be “0 mM sucrose” and not “no me”.

Reply

As mentioned in the response to Comment No. 12, the values were normalized to the “0” time point instead of “no me”.

15. Discussion part to be improved.

a. Line 241: “These p62 bodies undergo fusion events until sequestration by autophagosomes”. This sentence is not supported by results. Time-laps video does not support fusion events as it actually appears to be a very rare event when carefully looking at all puncta.

Reply

As described above (reply to Number 5), a certain fraction of p62 bodies undergo fusion while others are not. We revised this sentence as follows.

(lines 253–256)

“The p62 body formation occurs very rapidly within 1 min throughout the cytoplasm, followed by gradual fusion events and/or the recruitment of surrounding p62 molecules, leading to their sequestration by autophagosomes.”

b. Line 252: “This difference may explain why most of the p62 bodies induced by hyperosmotic stress were relatively small in size, allowing entire bodies to be enclosed by the isolation membrane”. What does “relatively small in size” means? The authors do not measure the size of the p62 over time but only under different concentrations of sucrose (Figure 1C). Additionally, it is not because p62 bodies are little that they can be enclosed by the isolation membrane while if bigger they cannot.

c. Line 255: “In contrast, under conditions that promote the formation of larger p62 bodies, a piecemeal type of autophagy is likely to occur”. This would mean that little p62 are degraded by bulk autophagy and larger p62 by selective autophagy?. This part is not clear.

Reply (to b and c)

We acknowledge that these statements were speculative, as we do not have information on the size of p62 bodies, and therefore have removed them from the revised manuscript.

d. Line 256: “It should be noted that p62 body-mediated autophagy is distinct from starvation-induced autophagy, in which LC3-positive p62 puncta are also detectable at the light microscopic level 30-32”. This sentence does not make a lot of sense. The authors also saw LC3-positive p62 puncta in their analysis (Figure 3A-3B-3C-3D-3E). How do they state that the 2 mechanisms are different, when they also actually detected?

e. Line 256: “light microscopic level” does not sound appropriate do describe immunofluorescence images.

Reply (to d and e)

To clarify this issue, we substantially revised this section by providing more detailed explanations and by moderating our previous argument that p62-body mediated autophagy and starvation induced autophagy are entirely distinct processes.

(Lines 266–276)

“It should be noted that LC3-positive p62 puncta are also detectable during starvation-induced autophagy at the resolution of light microscopy 30,31. However, our previous study using structured illumination microscopy and immuno-electron microscopy revealed that p62 localizes on the restricted parts of autophagosome membranes, occasionally forming aggregate-like structures 31. These findings support a bulk degradation mechanism rather than the typical selective autophagy of p62 bodies observed in both previous 11 and present studies. Nevertheless, whether p62 body formation also plays a functional role in the starvation-induced autophagy remains to be elucidated. Taken together, our findings demonstrate for the first time that it is p62 body formation that mediates the rapid autophagic elimination of cytoplasmic proteins under hyperosmotic stress.”

f. Line 261-265: “Intriguingly, the optimal osmolality of 0.2 M sucrose (500 mOsm/L) promoted efficient degradation in T24 and U2OS cells (Fig. 3D), with p62 bodies appearing largest under light microscopy (Fig. 1C) and showing the highest rate of LC3 colocalization (Fig. 3D), supporting enhanced p62 degradation.” The authors state that the osmolality of 0.2M is the optimal condition for p62 degradation, but in the graphs also 0.1M looks to be as much as efficient than 0.2M (Figure 1C, 3D).

Reply

We first apologize for the incorrect figure citation. The efficient degradation observed at the optimal osmolality of 0.2 M sucrose is shown in Fig. 3H, not Fig. 3D. Regarding the reviewer’s comment on Fig. 1C (size) and Fig. 3D (colocalization with LC3), we agree with the concerns raised. We have avoided the use of ‘largest’ or ‘highest’. The relevant sentences were moved to the final paragraph of the Discussion (lines 316–337), where we discuss the significance of this study in the context of higher organisms.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

Point by point responses to Reviewers' comments

Reviewer #1

Overall, the data (including immunofluorescence, time-lapse imaging, western blot and CLEM) are of good quality. I particularly appreciated the quality of TEM and CLEM images, they look very nice and generally the study supports that p62 bodies are specifically degraded by autophagy. However, novelty is missing, and several shortcomings made my support of this study very difficult to sustain for the following reasons: 1. A certain number of conclusions are not supported by the data presented in the manuscript, 2. The description of how the quantifications were conducted is totally absent leading to several doubts, 3. Several scale bars in the images are clearly wrong, leading to questioning quantification analysis. Below, I provide the authors a point-by-point list of my concerns.

Reply

Thank you for your constructive comments and for recognizing the value of our manuscript. The novelty of this study lies in the finding that, under hyperosmotic stress, autophagic degradation is mediated by the formation of p62, rather than by direct incorporation of individual or oligomeric p62 molecules into autophagosomes. (1) Inappropriate conclusions have been revised, and additional explanatory texts have been included to clarify our interpretations. (2) Detailed methods for quantification of puncta in fluorescent images have been added, and (3) the scale bars in both immunofluorescence and EM images have been corrected. Please refer to the following responses for further details.

Major points:

1. Line 267: "Taken together, our findings demonstrate for the first time that p62 body formation under hyperosmotic stress mediates the rapid elimination of cytoplasmic proteins by autophagy."

While the authors state that p62 is degraded by autophagy under hyperosmotic stress for the first time, this phenomenon has been previously described also by themselves and is therefore not entirely novel. The authors previously demonstrated, to a less extent, that p62 is degraded by autophagy upon hyperosmotic stress (sucrose) (Tamura et al 2019, DOI: 10.1128/MCB.00024-19, Figure 1 and 4). Other studies have shown similar conclusions: Pena-Oyarzun et al 2017 (doi: 10.18632/oncotarget.18995), Dafre et al 2019 (doi: 10.1155/2019/2715810).

Reply

We appreciate the reviewer's comment and agree that it is already known that p62 is degraded by autophagy under hyperosmotic stress. We have acknowledged this point at the beginning of the relevant paragraph in Discussion (lines 249–250). Importantly, the novelty of our study lies in the demonstration that this degradation is mediated by p62-

condensate formation. To clarify this, we have revised the text as follows to better highlight this mechanistic insight:

(lines 274–276)

“Taken together, our findings demonstrate for the first time that it is p62 body formation that mediates the rapid autophagic elimination of cytoplasmic proteins under hyperosmotic stress.”

In addition, we have now properly cited Dafre et al 2019, which was omitted in the original manuscript.

2. Methods about how the authors quantified the number of puncta, the size and the colocalization are totally lacking. They only provided the name of the software was used for quantification (Photoshop) without describing if they manually/automatically counted the puncta, how colocalization was measured (Pearson, Manders or other coefficients?). Concerned Figures: Figure 1B, 1C, 2B, 2F, 3C, 3D, 3E, 7C, 7D.

Reply

We apologize for not providing a detailed description of the quantification method. In our analysis, the number of puncta and those showing colocalization were manually counted using the counting tool in Photoshop, based on either single channel or merged images. In this revision, we initially attempted to quantify colocalization using Pearson's and Manders' coefficients. However, we found that acquired images were not readily suitable for these analyses, as such methods require stringent optimization of immunostaining conditions and image acquisition parameters. Therefore, we opted for manually counting, which we believe yields more reliable and accurate data under our current experimental setup. We have accordingly revised the relevant section in the Methods (lines 384–390).

a. “Size of p62-positive puncta (pixels)” in Figure 1C does not provide any information about the size of these puncta if the “pixel size” value (in nm) is not provided.

Reply

Thank you for this comment. We converted the puncta size from “pixels” to “ μm ” in the revised Fig. 1C.

3. Concerns about scale bars in several Figures.

a. Figure 4A. Scale bar of 500 nm in (iii), (iv) and (v). One or more of these scale bars are visibly wrong.

b. Doubts about scale bars in Figure 7A, 7B, 7E.

c. Doubts about scale bars in several immunofluorescence figures.

d. Figure 7F. Scale bar in (iii) and (iv) cannot be 500 nm. Mitochondria are visible in the micrographs and their size would be around 250 nm, which is not possible (in this context).

e. If these scale bars are wrong and Photoshop was used for most of the images, without

considering that photoshop does not automatically calibrate the figures, my concerns about the scale bars extend to all images of the manuscript.

f. I strongly suggest using Fiji/ImageJ, which automatically recognizes the metadata containing the information about the pixel size. For this purpose, the original image files are needed. Please, double check all scale bars.

Reply

Thank you for these comments and deeply apologize for putting several incorrect scale bars. We have checked all scale bars in relevant Figures by comparing with original figures.

4. Line 125: We also observed that the number of p62 bodies decreased over time (Fig. 2A, B), suggesting their fusion.” Decrease in p62 number does not necessarily mean they fused. This is not supported by data shown in Figure 2A and 2B.

5. Lined 126: “Indeed, live-cell imaging confirmed the fusion of small p62 bodies (Fig. 2C, Supplementary Fig. 2B and Supplementary Movie 1). The live-cell movie does not support fusion events. The Supplementary Movie 1 shows a massive formation of p62 puncta over time, but most of these puncta are static and do not show fusion. The fusion events shown in Figure 2C represent a very limited number of fusion events that cannot be interpreted as a general mechanism.

Reply (to Number 4 and 5)

As this reviewer pointed out, the original descriptions are inappropriate and not enough. We revised the sentences as follows:

(lines 126–132)

“We also observed that the number of p62 bodies decreased over time (Fig. 2B). Live-cell imaging demonstrated that p62 bodies simultaneously emerged across the cytoplasm immediately after changing hypertonic medium and progressively grew larger and more distinct (Supplementary Fig. 2B and Supplementary Movie 1). Moreover, careful observations revealed that a subset of p62 bodies occasionally undergo fusion with each other (Fig. 2C and supplementary Fig. 2B), which may account for the observed decrease in the number of p62 bodies (Fig. 2B).”

Regarding the fusion events, we included additional regions in Supplementary Fig. 2C, each showing 2-3 fusion events over a 150 sec interval. These observations suggest the existence of at least two subsets of p62 bodies: one that undergo fusion and another that remain static, either recruiting surrounding p62 molecules or being rapidly engulfed by isolation membranes. We believe the fusion events occurs occasionally rather than rarely, based on the following observation: for example, Figure 2C shows two fusion events occurring within 150 seconds in approximately one-seventh of the total cytoplasmic area. Therefore, it can be estimated that roughly 50 fusion events (2 events x 4 per 10 min x 7 region) may occur per cell over a 10 min, which could account for the decrease in p62 body number shown in Fig. 2B.

6. Line 151: “Double IF staining for LC3 and p62 or G3BP1 at 0.4 M sucrose, a condition that induces obvious stress granule formation, showed that 94.5% of LC3-positive puncta colocalized with p62 bodies, which were rarely (< 1%) associated with G3BP1-positive puncta (Fig. 3A, B, E, and Supplementary Fig. 3A, B).” The authors say that 94% of LC3 puncta colocalize with p62 upon 0.4M incubation with sucrose (Figure 3E) but they previously observed and stated that p62 colocalization with LC3 is reduced to 30% when using the same sucrose concentration (0.4M, Figure 3D). This is contradictory.

Reply

We apologize for the confusion caused by the complex descriptions for Fig. 3D and E. To improve clarity, we have revised this section as follows, including an additional explanatory sentence:

(lines 153–160)

“Double immunofluorescence microscopy for LC3 and p62 showed that ~70% of p62 bodies contained LC3 at 0.1 M sucrose, and this fraction decreased to ~30% at 0.4 M sucrose (Fig. 3A, D Supplementary Fig. 3A). We then examined to what extent LC3-positive puncta colocalize with p62 bodies or G3BP1. Double IF staining for LC3 and p62 or G3BP1 at 0.4 M sucrose, a condition that induces obvious stress granule formation, showed that 94.5% of LC3-positive puncta colocalized with p62 bodies, which were rarely (< 1%) associated with G3BP1-positive puncta (Fig. 3A, B, E, and Supplementary Fig. 3A, B).”

In addition, we have corrected the y-axis labels of Fig. 3D and Fig. 3E to read “Percentage of p62 with LC3” and “Percentage of LC3 with p62 or G3BP1”, respectively.

7. The discussion part is not well written. I had difficulties to understand to flow, most of the conclusions overinterpret results. I strongly suggest the authors re-write this part based on what they found and improve it. A detailed description of the discussion point I am referring is into the “minor points” section.

Reply

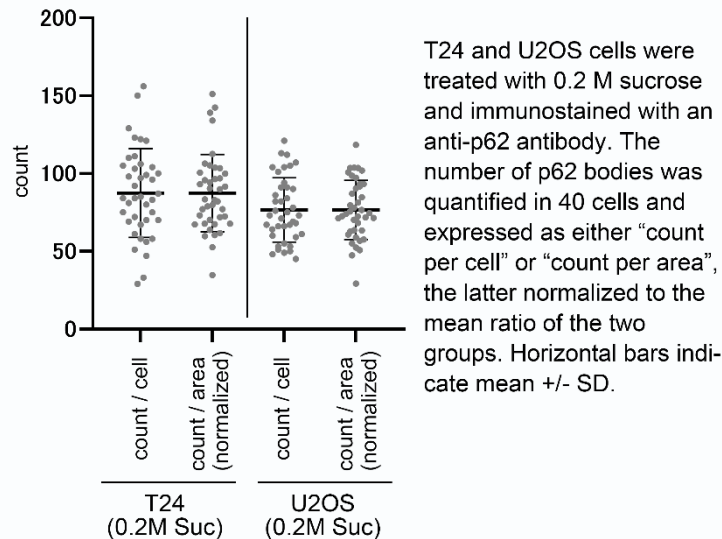
Thank you for the detailed comments (in the minor points) for the Discussion section. Based on your suggestion, along those from another reviewer, we have substantially revised the Discussion section by reorganizing the contents, adding further explanation and including a new paragraph highlighting the significance of this study for higher organisms. Detailed responses to each comment are provided below.

Minor points:

8. Figure 1B: Number of puncta per cell. Do the authors see differences if normalized the number of puncta per cell to the cell area? I suggest to systematically divide the number of puncta per cell by the cell area to avoid big differences.

Reply

Thank you for the critical comment. We re-analyzed the data by quantifying the number in 40 cells and comparing the variation between “count per cell” and “count per area.”



As shown in the figure below, the reduction in standard deviation observed in the “count per area” was minimal. This is likely due to the presence of cells with highly extended cytoplasm, which disproportionately decreases the values in the “count per area” compared to the “count per cell.” Based on this observation, we have chosen to adopt “count per cell” in the revised manuscript.

9. Line 120: “These results suggest that p62 bodies are formed differently and more sensitively than stress granules”. The results show an increase in the formation of puncta but it does not show that puncta are formed in different ways. What does “sensitively than stress granules” means? This is not supported by data.

Reply

We have revised the text as follows to ensure that it more accurately reflects our data:

(lines 120–122)

“These results suggest that p62 bodies and stress granules form as distinct condensates under hyperosmotic stress, and that formation of p62 bodies is more sensitive to stress intensity than that of stress granules.”

10. Figure 2B: There is a strong discrepancy in the number of puncta per cell at 20 minutes (0.4M sucrose) in Figure 2B when compared to the same condition in Figure 1B. Figure 1B shows around 170 p62 puncta per cell (T24) and around 250 p62 puncta per cell in U2OS, while in Figure 2B the number of p62 puncta in U2OS is reduced to around 100 puncta per cell. How do the authors explain such a difference in the same

conditions? Did they change the parameters to analyze the 2 sets of experiments? Information about how they analyzed are missing.

Reply

Thank you for pointing out this important mistake. We deeply apologize for the oversight. Upon review, we realized that experiments for Fig. 2A and B were performed under 0.3M sucrose condition, not 0.4M as previously described. This choice was made to clearly detect both p62 bodies and stress granules in the time-course experiments. This correction explains the observed reduction in the number of p62 bodies compared to Fig. 1B.

11. Line 152: “LC3-positive puncta colocalized with p62 bodies”, referring to Figure 3A, 3B and 3E while the graphs show p62 colocalization with LC3 and not the opposite. If Mander coefficient, for example, was used to calculate the colocalization, this can change everything. I strongly suggest to be more precise and add how did they calculate the colocalization.

Reply

As mentioned in our response to the Comment No. 6, we revised both the text and the figure accordingly.

12. Line 161: “Western blot (WB) analysis showed that, while p62 protein levels significantly decreased in medium containing 0.1–0.4 M sucrose, with the lowest levels observed at 0.2 M sucrose, the levels of stress granule markers G3BP1 and YB1 remained unchanged in T24 and U2OS cells (Fig. 3G, H).” The authors see a decrease in p62 levels upon 0.1-0.4M sucrose incubation. They first normalized to “no me” and then to “0” condition. I do not find this normalization method correct. I suggest, first, to normalize each p62 (and other markers) time point to the same corresponding GAPDH time point and, secondly, normalize this value to the “0” point and plot it in the graph. The “0” point is the real control of the experiment, rather than “no me”.

Reply

Thank you for this comment. As suggested by the reviewer, the values were normalized to the “0” time point instead of “no me”. However, we did not normalize to GAPDH, as it was not used as an internal control, but rather represents a cytosolic protein that can be incorporated into autophagosomes through bulk macroautophagy. We have added a text to clarify this intention, as follows:

(lines 169–171)

Western blot (WB) analysis showed that, while p62 protein levels significantly decreased in medium containing 0.1–0.4 M sucrose, with the lowest levels observed at 0.2 M sucrose. In contrast, the levels of stress granule markers G3BP1 and YB1, as well as the cytosolic protein GAPDH, remained unchanged in T24 and U2OS cells (Fig. 3G, H).

13. Lined 184: *“Taken together with the biochemical and IF data, these findings support the conclusion that hyperosmotic stress-induced autophagy specifically targets and degrades p62 bodies.”. The data shown in this result section supports the fact that p62 fluorescence correlates with autophagic structures and G3BP1 fluorescence correlates with fibrillar structures. Biochemical and immunofluorescence data should not be even mentioned here. This is more a conclusion to add into the discussion part.*

Reply

We agree this comment. Therefore, this sentence has been deleted.

14. Figure 5A and 5B: *Images and quantification from WB results. Same problem of normalization as in point 12, in this list. The control to use to normalize should be “0 mM sucrose” and not “no me”.*

Reply

As mentioned in the response to Comment No. 12, the values were normalized to the “0” time point instead of “no me”.

15. Discussion part to be improved.

a. Line 241: *“These p62 bodies undergo fusion events until sequestration by autophagosomes”. This sentence is not supported by results. Time-laps video does not support fusion events as it actually appears to be a very rare event when carefully looking at all puncta.*

Reply

As described above (reply to Number 5), a certain fraction of p62 bodies undergo fusion while others are not. We revised this sentence as follows.

(lines 253–256)

“The p62 body formation occurs very rapidly within 1 min throughout the cytoplasm, followed by gradual fusion events and/or the recruitment of surrounding p62 molecules, leading to their sequestration by autophagosomes.”

b. Line 252: *“This difference may explain why most of the p62 bodies induced by hyperosmotic stress were relatively small in size, allowing entire bodies to be enclosed by the isolation membrane”. What does “relatively small in size” means? The authors do not measure the size of the p62 over time but only under different concentrations of sucrose (Figure 1C). Additionally, it is not because p62 bodies are little that they can be enclosed by the isolation membrane while if bigger they cannot.*

c. Line 255: *“In contrast, under conditions that promote the formation of larger p62 bodies, a piecemeal type of autophagy is likely to occur”. This would mean that little p62 are degraded by bulk autophagy and larger p62 by selective autophagy?. This part is not clear.*

Reply (to b and c)

We acknowledge that these statements were speculative, as we do not have information on the size of p62 bodies, and therefore have removed them from the revised manuscript.

d. Line 256: “It should be noted that p62 body-mediated autophagy is distinct from starvation-induced autophagy, in which LC3-positive p62 puncta are also detectable at the light microscopic level 30-32”. This sentence does not make a lot of sense. The authors also saw LC3-positive p62 puncta in their analysis (Figure 3A-3B-3C-3D-3E). How do they state that the 2 mechanisms are different, when they also actually detected?

e. Line 256: “light microscopic level” does not sound appropriate to describe immunofluorescence images.

Reply (to d and e)

To clarify this issue, we substantially revised this section by providing more detailed explanations and by moderating our previous argument that p62-body mediated autophagy and starvation induced autophagy are entirely distinct processes.

(Lines 266–276)

“It should be noted that LC3-positive p62 puncta are also detectable during starvation-induced autophagy at the resolution of light microscopy^{30,31}. However, our previous study using structured illumination microscopy and immuno-electron microscopy revealed that p62 localizes on the restricted parts of autophagosome membranes, occasionally forming aggregate-like structures³¹. These findings support a bulk degradation mechanism rather than the typical selective autophagy of p62 bodies observed in both previous¹¹ and present studies. Nevertheless, whether p62 body formation also plays a functional role in the starvation-induced autophagy remains to be elucidated. Taken together, our findings demonstrate for the first time that it is p62 body formation that mediates the rapid autophagic elimination of cytoplasmic proteins under hyperosmotic stress.”

f. Line 261-265: “Intriguingly, the optimal osmolality of 0.2 M sucrose (500 mOsmol/L) promoted efficient degradation in T24 and U2OS cells (Fig. 3D), with p62 bodies appearing largest under light microscopy (Fig. 1C) and showing the highest rate of LC3 colocalization (Fig. 3D), supporting enhanced p62 degradation.” The authors state that the osmolarity of 0.2M is the optimal condition for p62 degradation, but in the graphs also 0.1M looks to be as much as efficient than 0.2M (Figure 1C, 3D).

Reply

We first apologize for the incorrect figure citation. The efficient degradation observed at the optimal osmolarity of 0.2 M sucrose is shown in Fig. 3H, not Fig. 3D. Regarding the reviewer’s comment on Fig. 1C (size) and Fig. 3D (colocalization with LC3), we agree with the concerns raised. We have avoided the use of ‘largest’ or ‘highest’. The relevant sentences were moved to the final paragraph of the Discussion (lines 316–337), where we discuss the significance of this study in the context of higher organisms.

Reviewer #2

The main finding is the identification of p62 bodies as a new type of cytoplasmic degradation platform that is activated under hyperosmotic stress. The manuscript reads extremely well and the Figures are excellent, including the correlative light-electron microscopy images. Some details are missing on image analysis, as detailed below.

Reply

Thank you for your constructive comments and recognition of the significance of our manuscript.

1. It would be helpful for the readers if there would be discussion on the physiological relevance of the findings. What kind of conditions could cause hyperosmotic stress in multicellular organisms? How would the observed degradation mechanism help the organism to manage the stress?

Reply

In response to this comment, we added a paragraph (lines 316–337) that discusses pathophysiological relevance in higher organisms by citing specific values of osmolality.

2. Would it be possible to quantify the colocalization of p62, PSMD4, K48Ub and K63Ub in Figure 6C and 6D? Please also include details on how the colocalization is analyzed.

Reply

We have quantified the colocalizations by manually counting the puncta. The data was added to Fig. 6C and D. In our analysis, the number of puncta showing colocalization, which is indicated by merged signals, were manually counted using the counting tool in Photoshop. We have accordingly revised the relevant section in the Methods (lines 384–390).

3. Would it be possible to quantify the colocalization of p62 and WIPI2, NBR1 and p62, and TAX1BP1 and p62, in Figure 5E and 5F?

Reply

We have quantified the colocalizations, which was added to Figs. 5G and 5H.

4. Please give details on how the “p62 bodies co-localizing with LC3” was analyzed and defined in Figure 3D. What exactly was analyzed in Figure 3E?

Reply

We apologize for the confusion caused by the complex descriptions for Fig. 3D and E. To improve clarity, we have revised this section as follows, including an additional explanatory sentence:

(lines 153–160)

“Double immunofluorescence microscopy for LC3 and p62 showed that ~70% of p62 bodies contained LC3 at 0.1 M sucrose, and this fraction decreased to ~30% at 0.4 M sucrose (Fig. 3A, D Supplementary Fig. 3A). We then examined to what extent LC3-positive puncta colocalize with p62 bodies or G3BP1. Double IF staining for LC3 and p62 or G3BP1 at 0.4 M sucrose, a condition that induces obvious stress granule formation, showed that 94.5% of LC3-positive puncta colocalized with p62 bodies, which were rarely (< 1%) associated with G3BP1-positive puncta (Fig. 3A, B, E, and Supplementary Fig. 3A, B).”

In addition, we have corrected the y-axis labels of Fig. 3D and Fig. 3E to read “Percentage of p62 with LC3” and “Percentage of LC3 with p62 or G3BP1”, respectively.

5. In Methods (line 359) only Photoshop is mentioned for image analysis. However, in the legend of Figure 2, ImageJ is mentioned for panel D. Please add ImageJ (with appropriate Reference) to the Methods section as well.

Reply

We apologize for not providing the information. Image J was added with Reference to Methods section in the revised manuscript (line 401).

6. Legend to Supplementary Figure 2: “(B) Low magnification image of U2OS cells at 11 min after medium exchange with DMEM containing 0.5 M sucrose. The boxed region is shown as sequential still images (Fig. 2B) and Supplementary Movie 1.” The U2OS cells must be expressing a fluorescent-tagged version of p62. This should be mentioned in the legend.

Reply

Thank you for this comment. We have corrected “U2OS cells” to “U2OS cells expressing mCherry-p62.”

7. Legend to Supplementary Figure 3, line 754: LC should read LC3.

Reply

“LC” was corrected to “LC3.”

8. Legend to Supplementary Figure 5: “(C) Immunostaining of U2OS cells transfected

with GFP-tagged receptors,” The annotation in the panel C reads ‘HeLa’, not U2OS.

Reply

We used U2OS cells. The label was corrected.

Point-by-point response to comments of Reviewer #1

(2nd review comments)

I appreciate the authors' efforts in revising the manuscript and addressing several of the concerns raised during the initial review. The revised version shows improvement, and the data presented now more clearly support the main hypothesis of the study. However, several important issues remain either unresolved or insufficiently addressed. In particular, concerns regarding data quantification, methodological clarity, and the use of appropriate and unbiased analysis tools still persist. I want to stress that if the analysis had been done more rigorously, and if the authors had properly explained or corrected the remaining issues, then I would have fully supported this work. Nonetheless, scientific integrity relies on transparent, reproducible, and unbiased data handling, which remains a critical expectation for publication in any journal. Below, I answered to authors replies on the previous round of reviewing and outlined specific comments and suggestions in bold for further clarification and revision.

<REPLY>

We would like to thank the reviewer once again for the careful reading of our manuscript and to the valuable suggestions, including English editing in some part. In this second round of revision, we have extensively reanalyzed the original images for particle counting and colocalization using Manders' coefficients in Fiji ImageJ software. Although some degree of subjectivity remains in the threshold setting, we believe that this semi-unbiased data processing has greatly improved the reliability of our results. The issue regarding the scale bars appears to have been arisen from inappropriate descriptions in the figure legends. Other minor comments have also been addressed accordingly. In this "point-by-point response", we reorganize the points to improve readability.

(1) Quantification of particle number and colocalization

(1st review comments)

2. Methods about how the authors quantified the number of puncta, the size and the colocalization are totally lacking. They only provided the name of the software was used for quantification (Photoshop) without describing if they manually/automatically counted the puncta, how colocalization was measured (Pearson, Manders or other coefficients?). Concerned Figures: Figure 1B, 1C, 2B, 2F, 3C, 3D, 3E, 7C, 7D.

(1st reply)

We apologize for not providing a detailed description of the quantification method. In our analysis, the number of puncta and those showing colocalization were manually counted using the counting tool in Photoshop, based on either single channel or merged images. In this revision, we initially attempted to quantify colocalization using Pearson's and Manders' coefficients. However, we found that acquired images were not readily suitable for these analyses, as such methods require stringent optimization of immunostaining conditions and image acquisition parameters. Therefore, we opted for manually counting, which we believe yields more reliable and accurate data under our current experimental setup. We have accordingly revised the relevant section in the Methods (lines 384–390).

(2nd review comments)

The authors argue that quantitative colocalization analysis could not be performed due to suboptimal immunostaining. However, Pearson's and Manders' coefficients are robust metrics that do not require highly stringent staining conditions, and can still provide valuable insights even when image resolution is not optimal. Relying on manual particle counting using Photoshop is not an appropriate alternative, as this approach is inherently subjective and lacks reproducibility. Unbiased analysis is a critical matter in research. As previously suggested but ignored, I strongly recommend again the use of standardized, unbiased image analysis tools such as Fiji/ImageJ for quantification.

<2nd REPLY >

We have extensively reanalyzed the original images for particle counting and colocalization using Manders' coefficients in Fiji ImageJ software. The detailed methods have been added to the "Materials and Methods" section. Although the threshold value had to be set individually for each experiment, which inevitably introduces some subjectivity, we believe that this semi-unbiased data processing has greatly improved the reliability of our results. As a result, graphs in Figs 1B, 2B, 2F, 3C, 3D, 3E, 5F, S5C, 6C, 6F, 7C, and 7D have been updated or newly added. Previous Fig. 3D, which showed colocalization based on manual counting has been moved to Supplementary Fig. S3D to demonstrate actual overlap as particles rather than as pixels. The new data do not affect our main conclusions;

however, the text in the Results section has been revised accordingly to reflect these updates.

(2) Concerns about scale bars in Fig. 4.

(2nd review comments)

The scale bar in Figure 4A(v) still appears to be incorrectly labeled. Given that panels (iii) and (iv) are labeled as 1 μm , and the scale bar in panel (v) is approximately four times longer than that in panel (iii), it cannot correspond to 200 nm. A more accurate value in (v) would be 4 times the scale bar in (iii) and not 5 times as is in the current figure. I would like to note that concerns regarding scale bar accuracy were already raised during the first round of review, and I had expected such issues to be carefully addressed in the revised version. It is therefore disappointing to find that at least one figure still contains a scale bar inconsistency. I strongly urge the authors to carefully review and verify all scale bars throughout the manuscript to avoid further misrepresentation of the data.

<2nd REPLY>

Thank you again for pointing out the issue regarding the scale bars. The scale bars in the figures are correct. We realized that the misunderstanding arose from the difference in the scale bar size between Fig. 4A (2 μm for panel [iii] and [iv], and 500 nm for [v]) and 4B (1 μm for [iii and iv], and 200 nm for [v]). To avoid confusion, we have now unified the size in Fig. 4A and 4B, which we believe will resolve the reviewer's concern.

(2) Number of puncta per cell or per cell area

(1st review comments)

8. Figure 1B: Number of puncta per cell. Do the authors see differences if normalized the number of puncta per cell to the cell area? I suggest to systematically divide the number of puncta per cell by the cell area to avoid big differences.

(1st reply)

Thank you for the critical comment. We re-analyzed the data by quantifying the number in 40 cells and comparing the variation between “count per cell” and “count per area.” As shown in the figure below, the reduction in standard deviation observed in the

“count per area” was minimal. This is likely due to the presence of cells with highly extended cytoplasm, which disproportionately decreases the values in the “count per area” compared to the “count per cell.” Based on this observation, we have chosen to adopt “count per cell” in the revised manuscript.

(2nd review comments)

thank you for comparing the analysis. There no meaningful difference between the two analysis presented. As a result, the authors' choice and explication of this comparison is pointless.

<2nd REPLY>

In this revision, we used the particle counting function in Fiji with the multiple ROI setting, which allowed us to measure cell area. Accordingly, the values are expressed as counts per 100 μm^2

(3) The issue of adopting 0.3M or 0.38M sucrose for time course observation

(1st review comments)

10. Figure 2B: There is a strong discrepancy in the number of puncta per cell at 20 minutes (0.4M sucrose) in Figure 2B when compared to the same condition in Figure 1B. Figure 1B shows around 170 p62 puncta per cell (T24) and around 250 p62 puncta per cell in U2OS, while in Figure 2B the number of p62 puncta in U2OS is reduced to around 100 puncta per cell. How do the authors explain such a difference in the same conditions? Did they change the parameters to analyze the 2 sets of experiments? Information about how they analyzed are missing.

(1st reply)

Thank you for pointing out this important mistake. We deeply apologize for the oversight. Upon review, we realized that experiments for Fig. 2A and B were performed under 0.3M sucrose condition, not 0.4M as previously described. This choice was made to clearly detect both p62 bodies and stress granules in the time-course experiments. This correction explains the observed reduction in the number of p62 bodies compared to Fig. 1B.

(2nd review comments)

This is a critical issue, and the explanation provided by the authors does not sufficiently

clarify the inconsistencies. In the previous version of the manuscript, the concentration was indicated as 0.4 M in Figures 2A and 2B, and 0.5 M in the legend of Figure 2C. In the revised version, this concentration was changed to 0.3 M due to an initial mistake, and now Figure 2C (legend) refers to 0.38 M. The rationale behind these adjustments is not convincingly justified. Given that both p62 and G3BP1 puncta formation are clearly visible at 0.4 M, the authors' reasoning for choosing 0.3M and 0.38 M over the originally reported concentrations is unconvincing. In fact, using 0.4 M would have likely yielded clearer observations. Therefore, the explanation for the changes in experimental conditions remains insufficient, and the mistake (together with biased analysis and scale bars) raises concerns about the consistency and reproducibility of the experimental design.

<2nd REPLY>

To address this comment, we included following statement at the beginning of the paragraph,

(lines 124-126) "For time-course experiments in T24 and U2OS cells, we employed a hyperosmotic condition of 0.3 M sucrose, which was considered to be of sufficiently low stress to allow the detection of both p62 bodies and stress granules."

For the live-cell imaging, it was technically difficult to smoothly exchange the medium without causing a substantial shift in focal plane. Therefore, DMEM containing 0.5 M sucrose was applied and exchanged twice at half volume each. This resulted in a final concentration of about 0.38 M sucrose. This method is included in the "Materials and Methods" section (lines 413-415) and referred to in the legends of Fig 2 and Fig S2.

(4) Normalization to GAPDH in Western blotting

(1st review comments)

12. Line 161: "Western blot (WB) analysis showed that, while p62 protein levels significantly decreased in medium containing 0.1–0.4 M sucrose, with the lowest levels observed at 0.2 M sucrose, the levels of stress granule markers G3BP1 and YB1 remained unchanged in T24 and U2OS cells (Fig. 3G, H)." The authors see a decrease in p62 levels upon 0.1-0.4M sucrose incubation. They first normalized to "no me" and then to "0" condition. I do not find this normalization method correct. I suggest, first, to

normalize each p62 (and other markers) time point to the same corresponding GAPDH time point and, secondly, normalize this value to the “0” point and plot it in the graph. The “0” point is the real control of the experiment, rather than “no me”.

(1st Reply)

Thank you for this comment. As suggested by the reviewer, the values were normalized to the “0” time point instead of “no me”. However, we did not normalize to GAPDH, as it was not used as an internal control, but rather represents a cytosolic protein that can be incorporated into autophagosomes through bulk macroautophagy. We have added a text to clarify this intention, as follows:

(lines 169–171)

Western blot (WB) analysis showed that, while p62 protein levels significantly decreased in medium containing 0.1–0.4 M sucrose, with the lowest levels observed at 0.2 M sucrose. In contrast, the levels of stress granule markers G3BP1 and YB1, as well as the cytosolic protein GAPDH, remained unchanged in T24 and U2OS cells (Fig. 3G, H).

(2nd review comments)

GAPDH levels appear unchanged under the conditions tested and should therefore serve as a suitable internal loading control. While GAPDH has been reported to undergo degradation via chaperone-mediated autophagy under conditions of nutrient deprivation, there is no evidence to date supporting its degradation through bulk macroautophagy. Moreover, this study focuses on osmotic stress rather than nutrient depletion, making GAPDH degradation via CMA unlikely in this context.

<2nd REPLY>

According to the reviewer’s suggestion, the data were normalized to GAPDH, followed by calculation of the ratio.

(5) Corrections of inappropriate words and expressions

(2nd review comments)

Line 267: The phrase “at the resolution of light microscopy” is somewhat awkwardly phrased. I do not think that the sentence refers to a resolution matter but simply to a fluorescence microscopy technique. Consider rewording to “by light microscopy” or “within the limits of light microscopy resolution.” If the authors wanted to highlight a

resolution matter.

Line 269: The expression “p62 localizes on the restricted parts of autophagosome membranes” is stylistically unusual. A more precise and grammatically natural phrasing might be: “p62 localizes to specific subdomains of the autophagosome membrane.”

Line 271: The construction “rather than the typical selective autophagy of p62 bodies” is vague and potentially misleading. I suggest rephrasing as: “rather than a canonical selective autophagic degradation of p62-positive bodies.”

Line 272: The sentence “observed in both previous¹¹ and present studies” can be improved. For clarity, consider rewording to: “as previously observed¹¹ and in the present study.”

Line 274: The expression “that it is p62 body formation that mediates the rapid autophagic...” could be more clearly stated. A suggested revision would be: “that the formation of p62 bodies mediates the rapid autophagic clearance...”

<2nd REPLY>

Thank you very much for your helpful suggestions on improving several sentences. We have revised them accordingly.

(1st reply)

(lines 126–132)

“We also observed that the number of p62 bodies decreased over time (Fig. 2B). Live-cell imaging demonstrated that p62 bodies simultaneously emerged across the cytoplasm immediately after changing hypertonic medium and progressively grew larger and more distinct (Supplementary Fig. 2B and Supplementary Movie 1). Moreover, careful observations revealed that a subset of p62 bodies occasionally undergo fusion with each other (Fig. 2C and supplementary Fig. 2B), which may account for the observed decrease in the number of p62 bodies (Fig. 2B).”

(2nd review comments)

This paragraph sounds now ok. Please revise the English phrasing in the sentence for clarity and readability. The expression “immediately after changing hypertonic medium” is awkward and would be better phrased as “upon addition of hypertonic solution”. Similarly, “and progressively grew larger and more distinct” could be reworded to “and

their size increased over time” for clarity. Lastly, the phrase “with each other” is unnecessary and should be removed.

<2nd REPLY>

Thank you again for the suggestions on the text edits. We have revised them accordingly.

(1st reply)

(lines 153–160)

“Double immunofluorescence microscopy for LC3 and p62 showed that ~70% of p62 bodies contained LC3 at 0.1 M sucrose, and this fraction decreased to ~30% at 0.4 M sucrose (Fig. 3A, D Supplementary Fig. 3A). We then examined to what extent LC3-positive puncta colocalize with p62 bodies or G3BP1. Double IF staining for LC3 and p62 or G3BP1 at 0.4 M sucrose, a condition that induces obvious stress granule formation, showed that 94.5% of LC3-positive puncta colocalized with p62 bodies, which were rarely (< 1%) associated with G3BP1-positive puncta (Fig. 3A, B, E, and Supplementary Fig. 3A, B).”

(2nd review comments)

The revised sentence is now clearer; however, the use of “contained” is not appropriate in this context. I recommend replacing it with “overlapped with LC3”, as this more accurately reflects the observation. Additionally, since no quantitative colocalization analysis was performed, the term “overlap” is more suitable than “colocalization”, which implies a degree of quantification.

<2nd REPLY>

We used the term “overlapped with” instead of “contained” in lines 157 and 182.

(1st reply)

(lines 120–122)

“These results suggest that p62 bodies and stress granules form as distinct condensates under hyperosmotic stress, and that formation of p62 bodies is more sensitive to stress intensity than that of stress granules.”

(2nd Review comments)

The statement “and that formation of p62 bodies is more sensitive to stress intensity

than that of stress granules” is unclear and potentially misleading. The use of the term “sensitive” is not well-defined in this context. Based on the data, the authors are observing an increase in puncta formation, but this does not necessarily imply greater sensitivity. I suggest rephrasing the sentence to more accurately reflect the observed changes in puncta number, without implying mechanistic differences in stress responsiveness unless further evidence is provided.

<2nd REPLY>

According to this suggestion, we corrected the text as follows,

(line 120-123) “These results suggest that p62 bodies and stress granules form as distinct condensates under hyperosmotic stress, with p62 bodies forming at lower levels of hyperosmotic stress than stress granules.”

(line 147-149) “Taken together, these results indicate that p62 bodies form via phase separation under hyperosmotic stress, independently of stress granules, and form more rapidly and at lower stress intensities than stress granules.”

In addition, we avoided using the term “sensitive” in the “Abstract” and other parts of the “Results” section, but remained it in one paragraph of the “Discussion”.