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HSP110 dependent HSP70 disaggregase generates toxic spreading-competent α-synuclein species

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Dr. Carmen Nussbaum-Krammer
Universität Heidelberg
Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH)
Im Neuenheimer Feld 282
Heidelberg, Baden-Württemberg 69120
Germany

10th Dec 2019

Re: EMBOJ-2019-103954
HSP110 dependent disaggregation machinery generates toxic spreading-competent α-synuclein species

Dear Dr. Nussbaum-Krammer,

Thank you for submitting your manuscript on the role of Hsp110 in alpha-synuclein toxicity for consideration by The EMBO Journal. We have now received three referee reports on your study, which are included below for your information.

As you will see, the referees are overall positive and acknowledge the interest and quality of the study. Nonetheless they still raise some issues that would need to be experimentally addressed or discussed in a revised version of the manuscript. In particular, the concerns both referee #1 and #2-1 raise regarding the K69M SSE1 mutant should be addressed, as appropriate through analysis of alternative mutant(s) in key experiments (ref#1-6, ref#2-1's major concern). In addition, as referee #2-2 and #3 point out, it will be important to further clarify the proposed role of Hsp110 in disaggregation as well as cell-to-cell transmission of alpha-synuclein aggregates. Should you be able to adequately address these issues in addition to responding to the more specific concerns raised by each of the referees, we would be happy to consider this study further for publication. Therefore I would like to invite you to prepare and submit a revised manuscript. Please note that it is our policy to allow only a single round of major revision and that it is therefore important to clarify all key concerns raised at this stage.

Please feel free to contact me should you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.

Kind regards,

Stefanie Boehm

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Editor
The EMBO Journal

Referee #1:

This manuscript is very interesting overall and there is some room for improvement. I don't really understand why it is written from a yeast prion perspective. Hsp110 is not equivalent to Hsp104 and it isn't a disaggregase, per se. It would seem more appropriate to frame it within the perspective of the role of Hsp70 in my opinion. I don't think it needs to be entirely reframed but more reference to the role of the Hsp70 machine and NEF activity should be added. The data are interesting, but provide little advance to mechanistic understanding in the field. Nonetheless, it is a solid body of work that contributes to our understanding of how co-chaperones and regulators impact protein aggregation.

1. In figure 2E, it would be good to show Q35 foci data for longer (up to 12 days) for both HP1 and HP2. It is unclear as to why the difference is only with HP1 and not with HP2, and this should be discussed.
2. Fig 2C: shows data for day 3 but no data on #foci for day 3.
3. Figure 3A, for alpha-syn; HP1; FES1 replace the panel with a more visible one as nothing can be seen.
4. Figure 3B, the author claims that the toxicity is increased, however, they need to check the comparison of the statistics for; alpha syn(HP1) from day 4-7 and also with Sse1-WT and Sse1-K69M from days 4-7. Most importantly, they need to show statistically the difference between alpha-syn(HP1) vs. Alpha-syn (HP1) + SSE1 WT and alpha-syn(HP1) vs. Alpha-syn (HP1) + SSE1 K69M. Moreover, it would interesting to see the effect of SSe1-K69M in a non-HP1 set up with only alpha-syn.
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7. Which data corresponds to the method section about Real-TIME PCR?
8. Figures 4D and 4E: the statistical data for comparison of FLUCSM(HP1) vs FLUCSM(HP1) for all the days and similarly for FLUCSM(HP2) is missing.
9. Fig 5 seems unnecessary and really doesn't add much. There really is nothing mechanistic about it and doesn't pertain to the model they have shown in their experiments. I think a better Fig 5 would show the differences between KD of Hsp110 and how they are explaining its effect in dissagregation.
10. It will great to actually see if by inducing heat shock, there is any global change in the heat shock factors and other chaperones in this case with HP1 and HP2 induced and non-induced background.
11. Overall, the claim that the protein folding homeostasis is disturbed is speculation. The language of this should be altered as such. No experiments address the functionality of the Ub-proteasome system, the UPR, or autophagy.

Minor points:

Fig 2A: Typo on image labeling, add "asyn::YFP"

It would be helpful to outline the H & M tissues in all images throughout the manuscript, similar to 2A and 3A.

Fig 4E, Fig 4F: different age ranges, no explanation.

Referee #2:

This paper "HSP110 dependent disaggregation machinery generates toxic spreading-competent α-synuclein species" by Tittelmeier et al., is a natural and much-appreciated continuation of earlier

work on the role of HSP110 in the proteostasis and aging of C. elegans, by members of the Bukau laboratory.
Hence, Rampelt et al., in 2012, first showed that RNAi knock-down of C. elegans HSP110 compromises the dissolution of heat-induced protein aggregates and severely shortens lifespan after heat shock. This implied that Hsp70-Hsp40, powered by Hsp110 nucleotide exchange is a crucial disaggregation machinery restoring protein homeostasis and counteracting cytotoxic protein misfolding and aggregation in the cytosol of metazoan cells. Using already than, the least K69M mutant of SSE1, they reached the same conclusion as presently, that ATP hydrolysis by HSP110s, is unlikely to be necessary for its action as a disaggregating co-chaperone of HSP70. In Kisten et al., 2017 they further used an RNAi knockdown approach, as in the present paper, to describe how class A and B of J-proteins cooperate to form an interactive network that targets preferentially HSC70 onto heat-stress-induced aggregates and polyQ aggregates that form during aging.
Here, using C. elegans models with pathological protein folding phenotypes of α-synuclein and polyQ diseases, such as the formation of intracellular amorphic and fibrillar aggregates, and their intercellular spreading and toxicity, Tittelmeier et al., inhibited the cytosolic HSP70-HSP40-HSP110 dependent disaggregase machinery by depleting HSP110 with RNAi. They monitored the effect on α-synuclein related phenotypes and found that, expectedly, HSP110 knock down, impaired HSC70-mediated disaggregation activity, prevented the solubilisation of amorphous aggregates and compromised proteostasis. Surprisingly however, HSP110 depletion was also found to reduce α-synuclein foci formation, cell-to-cell transmission and toxicity. These data convincingly demonstrated that in the cytosol of animals that lack Hsp104 disaggregases, the solubilisation of compact, least toxic aggregates by HSP70-HSP40-HSP110 can be dangerous, as this may transiently generate highly toxic, less compact and more active aggregates that can overflow the capacity of the cellular proteostasis machinery, and lead to the accumulation of toxic amyloids, and cause cell death rather than curing.
This is an excellent, well-performed and very well-written paper. The experiments were perfectly designed. With the exception of their statement based on the K69M SSE1 mutant, about the ATPase activity of HSP110 being unimportant for its role in disaggregation (see below), the other conclusions are important, well-founded and not overstated.

Minor points:
1) The need of information from quantitative proteomics: This paper is about the cytosolic chaperone machinery mediating active disaggregation of toxic protein aggregates in C. elegans muscle cells. For discussing the mechanism of action, it is therefore essential that the authors will discuss existing data or generate their own proteomic quantitative data from mass spectrometry, about the true cellular amounts (and stoichiometric ratios) in C. elegans (muscle) cells, of HSC70, HSP110, of other cytosolic HSP70s, of cytosolic DNAJAs, DNABs and of Bag1.
2) The need of western blots to estimate relative HSP amounts: Fig 2S addresses the sound possibility that the expression of SSE1, K69M SSE1 and FES1 may have caused a compensatory activation of the heat shock response. At basal growth temperature, without heat-shock, the HSP mRNA levels were apparently not significantly different in the transformed animals. Yet, in all eukaryotes, the heat-shock response is a transient response in which the HSP mRNA level initially increases, and then decreases, despite the ongoing heat stress. It is therefore possible that the proteostasis-stressed HSP110-depleated animals at low temperature, while still expressing higher levels of HSPs would already have low steady-state levels of the corresponding HSP mRNAs. The measures of Fluorescently-labelled HSP16.2 should therefore be complemented, if possible, by western blots of HSC70, HSP90, HSP16.2.

Regarding the experimental setup of Fig 3:
3) A Blast search shows that, oddly, there is no Fes1 "armadillo" type of HSP70 NEF in C. elegans,

although FES1 is present in yeast, fungi, plants and possibly also in the ER of cordates. The question arises why yeast FES1 was used in this experiment and not, for example, human Bag 1, as previously used in Rampelt et al 2012? More importantly, there is a Bag1 homologue in C. elegans (gene GeneID:172373), which the authors would have been well inspired to knock down by RNAi. Such an experiment would have potentially revealed the role of an endogenous NEF, other then HSP110, in the suppression of toxic protein aggregation by HSC70 and HSP110 in C. elegans muscles.
One Major point:
4) My major concern comes from the use of the SSE1 K69M mutant to reach the claim on page 11, that "the intrinsic ATPase activity of HSP110 is likely to be of no importance for its role in aggregate disaggregation". My problem is that in our hand, the Sse1-K69M mutant is not an ATPase-null mutant. Raviol et al., 2005 initially showed that Sse1-K69M may have an ATPase activity, which although being at least 10 folds lower than wild-type SSE1, is not nil. Recently, Kumar V. et al., 2019, showed that when WT SSE1 is truncated of its SBD, the remaining NBDs+linker has a dramatically higher ATPase activity (6.671{plus minus}0.953 min-1) compared to the full-length protein (0.013{plus minus}0.007 min-1) (https://doi.org/10.1111/febs.15045)(see Fig 9d). At this point, we find it important to graciously inform the authors of an unpublished minor result from our laboratory: We have generated and purified a Sse1-K69M mutant that was similarly truncated of its C-terminal SBD. This K69M NBD+linker 40 KDa protein showed a dramatically higher ATPase activity (0.320{plus minus}0.025 min-1) than full length K69M SSE1 (0.009{plus minus}0.004 min-1). Although the ATPase activity of the K69M NBD+linker was 20 times lower than that of the WT NBD+linker, it clearly indicated that the K69M mutation did not completely destroy the intirinsic ATPase activity of SSE1. It is thus possible that in vivo, the 20 times slower, albeit still strictly necessary ATPase activity of HSP110 would be needed for its role in protein disaggregation. Thus, Tittelmeier et al., cannot interpret their observation as evidence that the intrinsic ATPase activity of HSP110 is of no importance for its role in protein disaggregation.
The authors should either remove this part from the manuscript or repeat their experiment using an SSE1 mutant more severely impaired in its ATPase, for example double mutations: K69MD8A, K69MD174A or K69MD203A.
Aside from this specific important point, the other remarks are mainly text cosmetics. Once addressed, especially the point about the K69M mutant, I highly recommend this paper for publication.

Comments from the other agreed upon co-expert:

"This is a very elegant work, very carefully done. As far as I can see, no controls are missing and they are careful about the limitations of their system. They used all the proper controls, including two different strain for HP and several assays for each point.
I highly recommend for publication.
The only comment I have, is that given the impact of hsp-110 on refolding by overflowing the system, the statement that it is involved in disaggregation because aggregates are not cleared, although most likely correct, could be a little too strong. The spreading data lands support for this conclusion, so I am not sure if there is any point of asking them to tone down a little early in paper: "Thus, the HSP-110 KD led to persistence of heat-induced amorphous FLUCSM aggregates, indicating that disaggregation activity was indeed impaired (Rampelt et al., 2012)."
I think if they can either rephrase this, or use a direct measure of aggregation, X-34 staining, SDS agarose gel or other methods looking at the aggregate size with and without HSP-110 is required. Again, this is small text issue the data is great and the work is very interesting."

Referee #3:

This manuscript by Tittelmeier and co-workers explores the consequences of knocking down the function of the Hsp70-Hsp110 disaggregation system on protein aggregation and the spreading of protein aggregates from cell to cell by seeding in an organismal model. The authors find some expected results; for example, knocking down Hsp110 caused an increase in aggregation of a firefly luciferase-based reporter construct. The authors also find some unexpected results: that knocking down Hsp110 actually diminishes the rate of cell-to-cell transmission of alpha-synuclein aggrgates. I found this work to be interesting, well executed, and well communicated and I think it would be appropriate for publication in The EMBO Journal. I have only one major comment that the authors may wish to address: is it possible that knocking down Hsp110 impairs a system responsible for the release of alpha-synuclein aggregates from cells in which the aggregates originate, or the endocytosis of alpha-synuclein aggregates into target cells, thereby mitigating the cell-to-cell transmission of toxic aggregates? I ask because it is known that the Hsp70 system has a role in endocytosis.

Referee #1:

This manuscript is very interesting overall and there is some room for improvement. I don't really understand why it is written from a yeast prion perspective. Hsp110 is not equivalent to Hsp104 and it isn't a disaggregase, per se. It would seem more appropriate to frame it within the perspective of the role of Hsp70 in my opinion. I don't think it needs to be entirely reframed but more reference to the role of the Hsp70 machine and NEF activity should be added. The data are interesting, but provide little advance to mechanistic understanding in the field. Nonetheless, it is a solid body of work that contributes to our understanding of how co-chaperones and regulators impact protein aggregation.

We thank the reviewer for the overall positive feedback. We wrote the manuscript in a yeast prion perspective because the general phenomenon and phenotype of the Hsp-110 dependent Hsp70 disaggregation machinery with respect to α-Syn propagation in *C. elegans* is similar to that of Hsp104 with respect to [PSI^+] prion propagation in yeast. The primary aim of this study was not to gain new mechanistic insights into the Hsp70/Hsp110 disaggregation system but to investigate the physiological role of the disaggregase in disease progression. The central question of our study was whether the Hsp70 system is required for the propagation and toxicity of α-Syn. Therefore we refer to the yeast prion replication system, for which a crucial role for the Hsp104/Hsp70 disaggregation system is established. While the two disaggregation systems differ in some mechanistic aspects, they fulfill the same function.

Therefore, we have added the following sentence on page 5 to stress the difference between the two chaperone machines: “Although the HSP70 disaggregation machinery is mechanistically different from HSP104/HSP70-mediated disaggregation, the resulting phenotype could still be similar.”. Following the suggestion of the reviewer, we also put more emphasis on the central Hsp70 function in disaggregation and changed the manuscript title to: “HSP110 dependent HSP70 disaggregase generates toxic spreading-competent α-synuclein species”.

1. In figure 2E, it would be good to show Q35 foci data for longer (up to 12 days) for both HP1 and HP2. It is unclear as to why the difference is only with HP1 and not with HP2, and this should be discussed.

We thank the reviewer for pointing this out. We have repeated this experiment with freshly thawed strains and assessed #foci and motility defects up to 12 days. The new dataset clearly shows that both HPI and HPII follow the same trend in reducing Q35 foci and toxicity in young animals, which is lost in old animals, confirming our overall conclusion from the experiment. We believe that the HPII animals probably had acquired a suppressor mutation in the previous experiment, because we could solve this discrepancy by using freshly thawed strains.

We added the data until day 7 to Figure 2E and 2F. The data from day 7 to 12 were added to the Figures 4G, EV5E and EV5F, because these later time points are discussed in the last section of the manuscript that reports on the effects of aging.

2. Fig 2C: shows data for day 3 but no data on #foci for day 3.

We have now added the #foci for day 3 to Figure 2B. α-Syn already forms foci at that day in WT but not HPI and HPII animals. Initial α-Syn foci formation does not immediately affect muscle function and motility, but with a delay, suggesting that the misfolded α-Syn must accumulate to a certain level before muscle function is affected.

3. Figure 3A, for alpha-syn; HP1; FES1 replace the panel with a more visible one as nothing can be seen.
We have replaced the relevant panel (now Figure EV2A) and apologize for the bad quality of the original one.

4. Figure 3B, the author claims that the toxicity is increased, however, they need to check the comparison of the statistics for; alpha syn(HP1) from day 4-7 and also with Sse1-WT and Sse1-K69M from days 4-7. Most importantly, they need to show statistically the difference between alpha-syn(HP1) vs. Alpha-syn (HP1) + SSE1 WT and alpha-syn(HP1) vs. Alpha-syn (HP1) + SSE1 K69M. Moreover, it would interesting to see the effect of SSe1-K69M in a non-HP1 set up with only alpha-syn.
We now show the statistics comparing α-Syn WT vs. α-Syn;HPI, α-Syn;HPI vs. α-Syn;HPI + SSE1 WT and α-Syn;HPI vs. α-Syn;HPI + SSE1-K69M in the relevant Figures (new Figure 3B and Figure EV2B). Adding the overall trend during aging and the statistical differences of all possible combinations to the respective figures would be very overwhelming. Therefore, we have now added all details about the statistics as data source file to each figure so that the interested reader can find all relevant information.
We did not assess the effect of SSE1-K69M on only α-Syn because it can fully replace HSP-110 function (HPI set up). Therefore one does not expect an effect of SSE1-K69M in α-Syn WT animals expressing endogenous HSP-110.

5. Fig 3C: Data from Day 4 in this panel looks different from 2H. Also, no images are shown.
The data from day 4 in the previous Figure 3C (now Figure EV2D) deviate only slightly from the data in Figure 2H (approx. 5% transmission for α-Syn and α-Syn;HPI in Fig. EV2D compared to approx. 10% transmission for α-Syn and α-Syn;HPI in Fig. 2H). Since both experiments were performed independently by different persons with different animals, this minor variation is to be expected.
As requested, we have added the images corresponding to the spreading assays, which are now shown in Figure 3C and EV2C.

6. It is unclear why the authors use a K69M variant which has a deficient ATPase activity and is dispensable, instead of using an SSE1 variant which disrupts the "disaggregation" activity of Sse1.
An SSE1 variant, which specifically affects disaggregation activity, has not yet been reported to the best of our knowledge. SSE1 function in disaggregation requires Hsp70 interaction *in vitro* and we therefore followed the suggestion of the reviewer and tested $SSE1^{N572Y\ E575A}$ activity upon HSP-110 knockdown. This SSE1 mutant harbors two point mutations in its (putative) substrate binding site and is specifically impaired in Hsp70 interaction and therefore lacks NEF activity (Polier et al. 2008; Rampelt et al. 2012). We show that $SSE1^{N572Y\ E575A}$ does not restore α-Syn foci formation, spreading and toxicity. Accordingly, this mutant does not support disaggregation of heat-aggregated Luciferase. These findings are shown in the new Figure 3 and demonstrate that Hsp70 binding is essential for Hsp110 function in protein disaggregation.

7. Which data corresponds to the method section about Real-TIME PCR?
The data shown in Figure EV1A correspond to this method section.

8. Figures 4D and 4E: the statistical data for comparison of FLUCSM(HP1) vs FLUCSM(HP1) for all the days and similarly for FLUCSM(HP2) is missing.
We feel that it will be very overwhelming to add all possible comparisons to each graph. Therefore, we have added all relevant data about the statistics as a data source file.

9. Fig 5 seems unnecessary and really doesn't add much. There really is nothing mechanistic about it and doesn't pertain to the model they have shown in their experiments. I think a better Fig 5 would show the differences between KD of Hsp110 and how they are explaining its effect in dissagregation.
We have edited and improved the Figure according to the reviewer's suggestion.

10. It will great to actually see if by inducing heat shock, there is any global change in the heat shock factors and other chaperones in this case with HP1 and HP2 induced and non-induced background.
We would like to kindly refer to Figure EV1, in which we assessed the expression of HSF-1 and Daf-16 dependent heat shock genes in WT vs. HPI and HPII animals with and without heat shock. We found that the canonical heat shock genes are not induced under normal growth conditions, but can be still induced by increased temperatures.

11. Overall, the claim that the protein folding homeostasis is disturbed is speculation. The language of this should be altered as such. No experiments address the functionality of the Ub-proteasome system, the UPR, or autophagy.
We would like to differentiate between the more specific term "protein folding homeostasis" and the term "protein quality control / proteostasis". "Protein folding homeostasis" refers to the folding state of proteins and the activity of molecular chaperones and does not include protein degradation via the UPS or autophagy. We show that the folding of folding sensors (firefly luciferase, endogenous ts mutant proteins) is disturbed upon HSP-110 knockdown (Figures 4A and 4D), demonstrating that protein folding homeostasis is disturbed. We agree with the reviewer that HSP-110 does not necessarily affect functionality of the degradative system and added a respective statement to the revised manuscript on page 17: "The degradative pathways, such as the ubiquitin-proteasome system or autophagy, which are also interconnected with the HSP70 system, might further contribute to α-Syn spreading and our folding sensors only monitor the cellular protein folding capacity and do not address the functionality of these degradation pathways. Therefore, future experiments should investigate whether low levels of HSP-110 also impact the capacity of degradation systems that might influence α-Syn propagation.".

Minor points:
Fig 2A: Typo on image labeling, add "asyn::YFP"
It would be helpful to outline the H & M tissues in all images throughout the manuscript, similar to 2A and 3A.
We have updated the Figures accordingly.

Fig 4E, Fig 4F: different age ranges, no explanation.
We have chosen these age ranges so that they overlap one day with the corresponding data sets in Fig 1B/C (for FLUCSM) and 2C (for α-Syn) because these experiments were performed at later time points and do not originate from a single experiment. We wanted to make sure that at

least one time point between these data sets overlaps and shows the same results. The age range varies slightly between the different strains because the onset of aggregation or the experimental set-up is slightly different and depends on the intrinsic features of the particular model protein. We have clarified this point in the revised manuscript and added the following information to the materials and methods section on page 20: “Since the age of onset of transgene aggregation or toxicity depends on the intrinsic properties of the respective model protein or experimental design, slightly different age ranges were chosen in each experiment. Where experiments on younger and older animals were conducted separately and did not originate from a single experiment, the age ranges were chosen so that they overlapped with the corresponding data sets on at least one day. Nevertheless, each model protein was examined on several days during aging to verify the results and ensure experimental reproducibility.”

Referee #2:

This paper "HSP110 dependent disaggregation machinery generates toxic spreading-competent α-synuclein species" by Tittelmeier et al., is a natural and much-appreciated continuation of earlier work on the role of HSP110 in the proteostasis and aging of C. elegans, by members of the Bukau laboratory.
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Here, using C. elegans models with pathological protein folding phenotypes of α-synuclein and polyQ diseases, such as the formation of intracellular amorphic and fibrillar aggregates, and their intercellular spreading and toxicity, Tittelmeier et al., inhibited the cytosolic HSP70-HSP40-HSP110 dependent disaggregase machinery by depleting HSP110 with RNAi. They monitored the effect on α-synuclein related phenotypes and found that, expectedly, HSP110 knock down, impaired HSC70-mediated disaggregation activity, prevented the solubilisation of amorphous aggregates and compromised proteostasis. Surprisingly however, HSP110 depletion was also found to reduce α-synuclein foci formation, cell-to-cell transmission and toxicity. These data convincingly demonstrated that in the cytosol of animals that lack Hsp104 disaggregases, the solubilisation of compact, least toxic aggregates by HSP70-HSP40-HSP110 can be dangerous, as this may transiently generate highly toxic, less compact and more active aggregates that can overflow the capacity of the cellular proteostasis machinery, and lead to the accumulation of toxic amyloids, and cause cell death rather than curing.
This is an excellent, well-performed and very well-written paper. The experiments were perfectly designed. With the exception of their statement based on the K69M SSE1 mutant, about the ATPase activity of HSP110 being unimportant for its role in disaggregation (see below), the other conclusions are important, well-founded and not overstated.
We thank the reviewer for this detailed summary of our previous work on the function and mechanic details of the HSP70-HSP40-HSP110 disaggregase and for the very positive feedback on the specific contribution of this study.

Minor points:
1) The need of information from quantitative proteomics: This paper is about the cytosolic chaperone machinery mediating active disaggregation of toxic protein aggregates in C. elegans muscle cells. For discussing the mechanism of action, it is therefore essential that the authors will discuss existing data or generate their own proteomic quantitative data from mass spectrometry, about the true cellular amounts (and stoichiometric ratios) in C. elegans (muscle) cells, of HSC70, HSP110, of other cytosolic HSP70s, of cytosolic DNAJAs, DNABs and of Bag1.
We agree with the reviewer that it would be interesting to discuss our findings in the context of proteomics data that would reveal the exact cellular amounts and stoichiometric rations of the involved chaperones in *C. elegans* muscle cells. Unfortunately, it is not easily feasible to detect

chaperone levels in specific tissues. One can dissect the gonad or the intestine from *C. elegans* animals, but not muscle cells, which are relevant to our work. Therefore most studies are using whole animal samples in proteomics experiments, which lack tissue specific resolution. Since chaperone levels can vary dramatically between tissues such data do not provide relevant information for our study because we specifically manipulate chaperone levels only in muscle cells.

2) The need of western blots to estimate relative HSP amounts: Fig 2S addresses the sound possibility that the expression of SSE1, K69M SSE1 and FES1 may have caused a compensatory activation of the heat shock response. At basal growth temperature, without heat-shock, the HSP mRNA levels were apparently not significantly different in the transformed animals. Yet, in all eukaryotes, the heat-shock response is a transient response in which the HSP mRNA level initially increases, and then decreases, despite the ongoing heat stress. It is therefore possible that the proteostasis-stressed HSP110-depleated animals at low temperature, while still expressing higher levels of HSPs would already have low steady-state levels of the corresponding HSP mRNAs. The measures of Fluorescently-labelled HSP16.2 should therefore be complemented, if possible, by western blots of HSC70, HSP90, HSP16.2.
We agree that the heat shock response is a transient response and that HSP mRNA levels might decrease after a while. However, we used GFP as reporter protein that was expressed from an Hsf-1-dependent promoter (Fig. EV1B). GFP is very stable, and protein levels remain high even several days after heat shock (see also original reference of this reporter: https://www.ncbi.nlm.nih.gov/pubmed/10590837 (Link et al. 1999)). Therefore, we believe that this reporter would enable us to detect a transient induction of the heat shock response, which is however not observed.
In addition, we fear that we would not be able see a significant difference using western blots if the chaperone levels were to change only in muscle cells, because the levels of Hsc70 and Hsp90 in *C. elegans* are generally relatively high. To illustrate this problem, we took “overview” images of whole animals that we constructed using CRISPR/Cas9 to harbor endogenously GFP tagged HSP-110 in the WT or HPI and HPII background (see Figure for Reviewers). In these animals, the GFP fluorescence corresponds to the amount of HSP-110::GFP protein. The tissue-specific depletion of HSP-110 only in muscle cells by additional expression of the hairpin constructs is hardly visible in these images, since the signal from the remaining tissues is very high. Only zooming into the respective areas shows the tissue-specific depletion in muscle cells as depicted in Figure 1A. In western blots, the muscle-specific depletion of HSP-110 is undetectable (data not shown). Therefore, we believe that the use of western blots would not be appropriate to monitor potential tissue-specific changes in heat shock protein levels. Instead, we think that the use of RT-PCR (more sensitive to subtle changes) (Fig. EV1A) and a HSP16.2 promoter fusion (allowing tissue-specific resolution of potential gene induction as previously shown (Guisbert et al. 2013)) (Fig. EV1B) is more suitable.

Regarding the experimental setup of Fig 3:
3) A Blast search shows that, oddly, there is no Fes1 "armadillo" type of HSP70 NEF in C. elegans, although FES1 is present in yeast, fungi, plants and possibly also in the ER of cordates. The question arises why yeast FES1 was used in this experiment and not, for example, human Bag 1, as previously used in Rampelt et al 2012? More importantly, there is a Bag1 homologue in C. elegans (gene GeneID:172373), which the authors would have been well inspired to knock down by RNAi. Such an experiment would have potentially revealed the role of an endogenous NEF,

other then HSP110, in the suppression of toxic protein aggregation by HSC70 and HSP110 in C. elegans muscles.

The reviewer is correct, *C. elegans* expresses *bag-1* (the homolog of human Bag1) as alternative NEF. However, expression of this NEF cannot compensate for the loss of HSP-110 in the HP animals, directly demonstrating that BAG-1 does not play a role in α-Syn propagation and toxicity. This is consistent with former data showing that human Bag1 did not support HSP70 mediated disaggregation *in vitro* (Rampelt et al. 2012). We therefore considered it very unlikely that additional expression of human Bag1 shows an impact on α-Syn toxicity.

The reviewer is also right that there is no endogenous Fes1 homolog in *C. elegans*. Therefore, we considered it possible that providing this type of NEF could compensate for the loss of endogenous HSP-110. This was not the case. However, as we have also mentioned in the manuscript, we only have negative data and therefore we cannot exclude that the failure to rescue disaggregation activity could be due to an inability of Fes1 to interact with the *C. elegans* Hsp70 machinery.

One Major point:

4) My major concern comes from the use of the SSE1 K69M mutant to reach the claim on page 11, that "the intrinsic ATPase activity of HSP110 is likely to be of no importance for its role in aggregate disaggregation". My problem is that in our hand, the Sse1-K69M mutant is not an ATPase-null mutant. Raviol et al., 2005 initially showed that Sse1-K69M may have an ATPase activity, which although being at least 10 folds lower than wild-type SSE1, is not nil. Recently, Kumar V. et al., 2019, showed that when WT SSE1 is truncated of its SBD, the remaining NBDs+linker has a dramatically higher ATPase activity (6.671{plus minus}0.953 min-1) compared to the full-length protein (0.013{plus minus}0.007 min-1) (https://doi.org/10.1111/febs.15045)(see Fig 9d). At this point, we find it important to graciously inform the authors of an unpublished minor result from our laboratory: We have generated and purified a Sse1-K69M mutant that was similarly truncated of its C-terminal SBD. This K69M NBD+linker 40 KDa protein showed a dramatically higher ATPase activity (0.320{plus minus}0.025 min-1) than full length K69M SSE1 (0.009{plus minus}0.004 min-1). Although the ATPase activity of the K69M NBD+linker was 20 times lower than that of the WT NBD+linker, it clearly indicated that the K69M mutation did not completely destroy the intirinsic ATPase activity of SSE1. It is thus possible that in vivo, the 20 times slower, albeit still strictly necessary ATPase activity of HSP110 would be needed for its role in protein disaggregation. Thus, Tittelmeier et al., cannot interpret their observation as evidence that the intrinsic ATPase activity of HSP110 is of no importance for its role in protein disaggregation.

The authors should either remove this part from the manuscript or repeat their experiment using an SSE1 mutant more severely impaired in its ATPase, for example double mutations: K69MD8A, K69MD174A or K69MD203A.

Aside from this specific important point, the other remarks are mainly text cosmetics. Once addressed, especially the point about the K69M mutant, I highly recommend this paper for publication.

We thank the reviewer for pointing this out. We have refined our conclusion, taking into account the reviewer's concern that the residual activity of the ATPase may be sufficient and necessary to confer disaggregation activity. We also thank the reviewer for his suggestions to construct ATPase dead SSE1 mutants, however, those mutations have not been published so far. We think that an *in vivo* use of such variants presupposes a careful *in vitro* characterization and this would go far beyond the scope of this work.

Alternatively, as also mentioned in our answer to specific point 6 of reviewer 1, we have now added data from a new SSE1 variant, which is unable to interact with the core Hsp70 machinery (new Figure 3). SSE1 harboring the point mutations N572Y and E575A that specifically impair the interaction with Hsp70 lacks NEF activity (Polier et al. 2008; Rampelt et al. 2012). Our previous data *in vitro* showed that these mutations inhibit disaggregation activity, suggesting that SSE1 NEF activity is needed for substrate disaggregation (Rampelt et al. 2012). We now show that this also applies to the disaggregation activity *in vivo* in *C. elegans*. SSE1$^{N572Y\ E575A}$ was unable to rescue the loss of HSP-110 in contrast to SSE1 WT and SSE1^{K69M}, indicating that Hsp70 binding and likely NEF activity are crucial for HSP-110 function.

Comments from the other agreed upon co-expert:

"This is a very elegant work, very carefully done. As far as I can see, no controls are missing and they are careful about the limitations of their system. They used all the proper controls, including two different strain for HP and several assays for each point.
I highly recommend for publication.
The only comment I have, is that given the impact of hsp-110 on refolding by overflowing the system, the statement that it is involved in disaggregation because aggregates are not cleared, although most likely correct, could be a little too strong. The spreading data lands support for this conclusion, so I am not sure if there is any point of asking them to tone down a little early in paper: "Thus, the HSP-110 KD led to persistence of heat-induced amorphous FLUCSM aggregates, indicating that disaggregation activity was indeed impaired (Rampelt et al., 2012)."
I think if they can either rephrase this, or use a direct measure of aggregation, X-34 staining, SDS agarose gel or other methods looking at the aggregate size with and without HSP-110 is required.
Again, this is small text issue the data is great and the work is very interesting."
We thank the reviewer for this kind and encouraging feedback. As suggested, we have rephrased the sentence and avoid calling the FLUCSM foci "aggregates" because we have not thoroughly biochemically proven that they are indeed aggregates. The sentence now reads as follows: "Thus, the HSP-110 KD led to persistence of heat-induced amorphous FLUCSM foci, suggesting that disaggregation activity is impaired (Rampelt et al., 2012)."

Referee #3:

This manuscript by Tittelmeier and co-workers explores the consequences of knocking down the function of the Hsp70-Hsp110 disaggregation system on protein aggregation and the spreading of protein aggregates from cell to cell by seeding in an organismal model. The authors find some expected results; for example, knocking down Hsp110 caused an increase in aggregation of a firefly luciferase-based reporter construct. The authors also find some unexpected results: that knocking down Hsp110 actually diminishes the rate of cell-to-cell transmission of alpha-synuclein aggrgates.

I found this work to be interesting, well executed, and well communicated and I think it would be appropriate for publication in The EMBO Journal. I have only one major comment that the authors may wish to address: is it possible that knocking down Hsp110 impairs a system responsible for the release of alpha-synuclein aggregates from cells in which the aggregates originate, or the endocytosis of alpha-synuclein aggregates into target cells, thereby mitigating the cell-to-cell transmission of toxic aggregates? I ask because it is known that the Hsp70 system has a role in endocytosis.

We are pleased that the reviewer shares our enthusiasm about this work. The reviewer raises an interesting point. We can rule out that knocking down HSP-110 impairs a system that is responsible for the endocytosis of α-Syn into target cells because in our experimental set-up, we specifically knock down HSP-110 only in the "donor" muscle cells. Therefore HSP-110 is still expressed in every other tissue of the worms (see Figure for reviewers), including the "receiving" hypodermal cells, which take up α-Syn. In consequence, the effect we see on α-Syn transmission cannot be explained by HSP70's role in endocytosis.

Regarding a potential role in the secretion of α-Syn: both, α-Syn and the hairpin construct are expressed in the "donor" muscle cells, so a potential effect on α-Syn secretion is in theory possible. We consider this scenario unlikely, because we see a reduction of α-Syn foci upon HSP-110 KD. If the KD of HSP-110 would block the release of α-Syn, we would expect α-Syn to accumulate in the "donor" muscle cells and eventually to form more foci. Instead, we observe the opposite, namely less foci. We therefore consider it unlikely that HSP-110 could affect the secretion of α-Syn. We have now discussed this aspect in the discussion section of the revised manuscript on page 17: "The HSP70 system has several functions besides substrate disaggregation, including the uncoating of clathrin vesicles during endocytosis (Sousa & Lafer, 2015), that could influence the prion-like propagation of α-Syn. Nevertheless, an influence on α-Syn transmission due to HSP70's role in endocytosis can be excluded, since in our experimental set-up we specifically knock down HSP-110 only in the "donor" muscle cells and not in the "receiving" tissue. Moreover, recent publications suggest that the HSP70 machinery may be involved in the unconventional secretion of misfolded cytosolic proteins (Fontaine, Zheng et al., 2016, Jung, Kim et al., 2016), and since both α-Syn and the hairpin construct are co-expressed in the "donor" muscle cells, an effect on α-Syn secretion is theoretically possible. However, if the KD of HSP-110 would block the release of misfolded α-Syn, we would expect α-Syn to accumulate in the "donor" muscle cells and eventually form more foci. Instead, we observe the opposite, namely a reduction of α-Syn foci upon HSP-110 KD. We therefore consider it unlikely that HSP-110 affects α-Syn secretion. The degradative pathways, such as the ubiquitin-proteasome system or autophagy, which are also interconnected with the HSP70 system, might further contribute to α-Syn spreading and our folding sensors only monitor the cellular protein folding capacity and do not address the functionality of these degradation pathways. Therefore, future experiments should investigate whether low levels of HSP-110 also impact the capacity of degradation systems that might influence α-Syn propagation.".

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Link CD, Cypser JR, Johnson CJ, Johnson TE. 1999. Direct observation of stress response in Caenorhabditis elegans using a reporter transgene. Cell Stress Chaperones 4: 235-242.

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1st Revision - Editorial Decision 9th Apr 2020

Dr. Carmen Nussbaum-Krammer
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9th Apr 2020

Re: EMBOJ-2019-103954R
HSP110 dependent HSP70 disaggregase generates toxic spreading-competent α-synuclein species

Dear Carmen,

Thank you for submitting your revised manuscript for our consideration. Please apologize the delay in communicating this decision to you, which was due to delayed referee reports on account of the current Covid-19 pandemic. We now have the reports from two of the original referees (see comments below). I am pleased to say that the referees find that their comments have been satisfactorily addressed. I would therefore now like to ask you to address a number of editorial issues that are listed in detail below in a final revised version. Please make any changes to the manuscript text in the attached document only using the "track changes" option. Once these remaining issues are resolved, we will be happy to formally accept the manuscript for publication.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving your final revision. Please feel free to contact me if you have further questions regarding the revision or any of the specific points listed below.

Kind regards,

Stefanie

Stefanie Boehm
Editor
The EMBO Journal

Referee #2:

Having examined the author's reply to my comments, and to the comments of other referees , I am fully satisfied and recommend publication.

Referee #3:

The authors have addressed my concern. I believe this manuscript is acceptable as is.

The Authors have made the requested editorial changes.

2nd Revision - Editorial Decision 23rd Apr 2020

Dr. Carmen Nussbaum-Krammer
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23rd Apr 2020

Re: EMBOJ-2019-103954R1
HSP110 dependent HSP70 disaggregase generates toxic spreading-competent α-synuclein species

Dear Carmen,

Thank you again for submitting the final revised version of your manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley, who will contact you with further information regarding production/publication procedures and license requirements.

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

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Congratulations on your successful publication, and thank you again for this contribution to The EMBO Journal! Please continue to consider EMBO Journal for your work in the future.

Kind regards,

Stefanie

Stefanie Boehm
Editor
The EMBO Journal

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Carmen Nussbaum-Krammer
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2019-103954

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
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2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We chose to use 15 animals in 3 independent biological replicates to ensure adequate power and to avoid oversampling.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We chose to use 15 animals in 3 independent biological replicates to ensure adequate power and to avoid oversampling.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Experiments were performed by different persons, if possible blinded, to ensure reproducibility.
For animal studies, include a statement about randomization even if no randomization was used.	Experiments were performed by different persons, if possible blinded, to ensure reproducibility.
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5. For every figure, are statistical tests justified as appropriate?	Every figure legend contains the information about the statistical test used, all additional information is provided in the respective source data files.
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C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Information about antibodies used is detailed in the material and methods section.
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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	We have used multiple C. elegans strains. Detailed information about the strains is provided in a separate table. The age of animals is indicated in each figure. Growth conditions are described in the materials and methods section.
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G- Dual use research of concern

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