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Metazoan Hsp70 machines use Hsp110 to power protein disaggregation

Heike Rampelt, Janine Kirstein-Miles, Nadinath B. Nillegoda, Kang Chi, Sebastian R. Scholz, Richard I. Morimoto, Bernd Bukau

Corresponding author: Bernd Bukau, Heidelberg University

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 22 December 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. As you will see, while referees 2 and 3 consider the study as interesting in principle, none of the referees offers strong support for publication of the study here. I will not repeat all their individual points of criticism here, but referees 1 and 3 think that deeper mechanistic insight into how Hsp110 acts to help Hsp70 solubilise aggregates would be required. Referee 3 also thinks that a possible role of small Hsps would need to be analysed in more depth or taken into account. Furthermore, concerns about the conclusiveness of the C. elegans data are raised. Another point brought up by referee 1 is the paper published by J. Shorter in October (PMID: 22022600). This paper clearly puts forward the general concept, a role for Hsp110 in dissolving aggregates together with Hdp70 in the mammalian system, already, and does also provides some initial mechanistic insight into its mode of action. While we can see that your present submission takes this idea further into cells and into in vivo experiments in C. elegans, this earlier publication still needs to be taken into account. In the light of this point together with the concerns with the dataset as it stands and the limited support by the referees, we have now come to the conclusion that we cannot offer publication of the manuscript.

Thank you in any case for the opportunity to consider this manuscript. I am very sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

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Yours sincerely,
Editor
The EMBO Journal

Referee reports:

Referee #1 (Remarks to the Author):

This study presents evidence for an important role of animal Hsp110 in protein disaggregation. Removal of aggregated proteins is critical for survival of cells upon recovery from stress. In bacteria, fungi and plants, AAA+ disaggregases of the Hsp100 family cooperate with the Hsp70 system in resolving protein aggregates upon recovery from stress. Curiously, the cytosol of animal cells does not contain such disaggregases. However, large protein aggregates are also found in many human diseases, and healthy cells appear to deal with aggregated protein adequately. The essential Hsp110 proteins are remote homologs of Hsp70; they act as Hsp70 nucleotide exchange factors and passive holdases for stress-denatured proteins in eukaryotes. Hsp110 proteins themselves do not seem to actively undergo a nucleotide-dependent conformational cycle as described for proper Hsp70 molecular chaperones.
Using purified proteins the authors show that mammalian Hsp110, but not its yeast ortholog Sse1, together with its cognate Hsp70/Hsp40 is active in recovering aggregated firefly luciferase and MDH, while Hsp70/Hsp40 alone is not. The mammalian nucleotide exchange factor BAG-1 is inactive. Addition of exogenous Hsp110/Hsp70/Hsp40 to heat-treated mammalian cell lysates resulted eventually in reduced turbidity and partial recovery of luciferase activity. Again, Sse1/Ssa1/Ydj1 had no effect. RNAi-mediated down-regulation of the C. elegans ortholog of Hsp110 had a negative effect on the breakdown of GFP-luciferase aggregates and reduced worm lifespan after heat shock, especially if Hsp70 was also down-regulated. However, these effects might also be a result of a strong reduction of the essential NEF function of Hsp110 and thereby of Hsp70 function.
These are intriguing observations. However, the manuscript provides little insight into how animal Hsp110 might actually assist Hsp70-Hsp40 in processing aggregates. Although Hsp110 sequences are less conserved than canonical Hsp70s and type I J-domain proteins, it seems puzzling that yeast Sse1 and mammalian Hsp110 (Apg2) differ so dramatically in their function. Previously described Hsp110 mutants should be used to dissect the critical functions of these molecular chaperones: Is ATP binding to Hsp110 necessary for disaggregation activity? Is ATP hydrolysis by Hsp110 necessary? Is Hsp70 binding / NEF function essential? Which role, if any, does the beta-sandwich domain, the putative substrate binding domain of Hsp110, play?
A recent study by Shorter and colleagues in PLoS-One reported that Hsp110 (Apg2) functions in disaggregation of luciferase in the mammalian system. The combination of yeast Sse1-Ssa1-Sis1 (the latter is the type II Hsp40 in S. cerevisiae) was also found to be active. Moreover, the Shorter study investigated functional mutations in the yeast chaperones. This has been overlooked in the present study and should be properly discussed.

Referee #2 (Remarks to the Author):

In their manuscript ÑHsp110 enables the Hsp70 machine to solubilize aggregates in animal cells" the authors propose a new function of Hsp110 proteins in the resolubilization and reactivation process of aggregated proteins. They demonstrate this function in vitro for the human chaperone proteins and find related activities in human cell lines and in nematodes. Moreover they speculate that this function of Hsp110 proteins may be a substitute for the activity of the AAA-ATPase Hsp104, which is the major disaggregase in the bacterial and yeast system but is not found in the genome of higher eukaryotes. While these results seem interesting, some experiments lack the necessary controls and some experiments comparing four different systems are rather confusing.
Specific points:
1. Initially the authors compare the activities of the Hsp70-Hsp40 system towards chemically denatured luciferase (figure 1). The proteins concentrations are missing in the figure legend. In methods section only ranges of concentrations are given for the NEFs. The description of experimental conditions needs to be improved throughout the manuscript (in particular also in figure 2 and 3). The importance of this issue can also be seen in supporting figure 1C, where apparently 0.2

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and 0.4 µM Hsp105 lead to strong reactivation, while 0.3 µM does not.
2. What is the evidence for defining the substrates in the experiments depicted in figure 1 and 2 as "aggregates"? SEC-HPLC or another suitable method could be employed to prove that indeed aggregates and not soluble oligomers are present at the start of the experiment. How long was the incubation in the refolding buffer before chaperones were added?
3. In figure 3, traces and data for "hs + Hsc70, Hdj1" are missing. Also the controls "hs + Hsc70, Hdj1, Bag-1" and in figure 3B "hs 70/40" are important. Which Hsp110 protein was used in figure 3B? Without these controls it is not possible to judge the results. The same is true for figure 3C. It cannot be assumed that Hsc70/Hsp40 is inactive in the cell lysates just because it was inactive in the in vitro refolding assays.
4. Worms were heat-shocked and then aggregation was investigated in the absence or presence of Hsp110 RNAi. Is the worm shown in figure 4A at 12h and then at 24 h the same worm or just another worm from the same experiment. The experiment would be more conclusive if it were the same individual. Does the available Hsp110 knock-out line give the same result?
5. In the life span assays, the authors apparently started the RNAi treatment on day one. This is known to induce a strong phenotype for HSP-1 leading to arrest at early larval stages (Kampath et al., Wormbase), including the inability to reach the adult and fertile state. The authors do not mention how they dealt with this issue in their analysis or whether this phenotype was also altered by the double RNAi experiment with C30C11.4. It is hard to imagine a life span analysis on these severely affected worms.
6. It would be interesting to include a figure in the supplement which shows the homology between Apg1, apg2, hsp110, c30c11 and sse1.
7. Why do the authors mention that urea concentration was "4 or 6 M urea" before dilution, as there is only one data set in the manuscript?
8. Why is the final turbidity in the cell extract system at the end of the assay including Hpg2, hsp110 and Hsp70 identical to the chaperone free hs-trace? The way it is presented (absence of controls, no quantification of the "aggregated proteins" at least at the endpoint) makes it hard to follow the conclusions.
9. Information on the newly created worm strain is lacking. Was it integrated or is it a stable non-integrated line?
10. It is difficult to rationalize why RNAi against Hsp70 and Hsp110 exhibits a so much stronger effect than RNAi against Hsp70, as the presence of Hsc70 is required for Hsp110 function. The authors may want to discuss this in more detail. How does the Hsc70 knockdown influence the Hsp110 knock-out strain?
11. The authors need to define "severe" and "permissive" aggregation conditions.
12.The origin of the antibody against luciferase is not stated in the text.
13. Scale bars are missing in figure 4A which is important for estimating the age of the nematode. According to the procedures it should be 24 hours + hs + 12/24 hours old.
14. Instead of the bacterial system in figure 3A, it would be better to include the controls for the human system.
15. The inactive yeast system is included in figure 3C. To make the point that this system is inactive in the absence of Hsp104, an experiment in which Hsp104 was added back should be included.

Referee #3 (Remarks to the Author):

In their MS, Rampelt et al propose that mammalian cells -that lack the disaggregation activity of the yeast Hsp104 AAA protease- can utilise the HSPH/APG/Hsp110 proteins in conjunction with the HSP70-HSP40 machine for disaggregation. In a set of elegant in vitro experiments, it is shown that the HSP110-HSP70-HSP40 chaperone complex can reactivate proteins from chemically- or thermally induced aggregates. HSP110 can act as nucleotide exchange factor (NEF) for HSP70, but it is show that this action (alone) is not sufficient for disaggregation as other NEFs were inactive in disaggregation. This implies that maybe the ability of HSP110 to bind substrates is required, but precise insights in the putative mechanisms or the actual HSP110 domains required for the disaggregation activity were not provided.
The authors furthermore show some evidence with mammalian cell systems that support that also in living cells the HSP110-HSP70-HSP40 may indeed exert disaggregation activity, but these data are less conclusive and require some additional experiments as well as some reconsiderations in terms of interpretation (see below).
Finally, data in C. elegans are provided that show that siRNA-mediated knockdown of Ce-Hsp110

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results in delayed disappearance of heat-induced protein aggregates and that this knockdown has substantial effects on lifespan especially when the animals were treated with heat shock at young age and when also Ce-Hsp-1 (one of the worm Hsp70s) was also downregulated. Again, the data are in support of an important role of HSP110 in disaggregating activity, but also here a few additional experiments seem required.

Overall, the findings are novel and potentially very interesting. The paper is generally well-written and good to follow for a specialist in the field. However, for the more general readership of the EMBO J, it may require some rewriting. In particular, the use of equivalent chaperone complexes from different sources in the various experiments and the nomenclature (Hsp100, Hsp110/HSPH/Apg2 versus ClpB/Hsp104) may be confusing to non-experts: at least it would be helpful to use something like 'NEF-Hsp110' versus the 'AAA-protease Hsp104'. Also, the explanation of use of small HSP in the heat denaturation experiments and the interpretation of some of the outcomes of these data requires attention.

<B>Major Comments</B>
Figure 1 and 2:
Although I am aware that a functional Hsp70 folding machine usually seems to require at least both Hsp70 and a DnaJ, I would pose the question whether for this unprecedented novel disaggregation complex either Hsc70 or Hdj2 could be dispensable. Especially, could it be that Hsp110 together with Hjd1 is sufficient for the disaggregation activity?
The authors nicely show that the NEF Bag-1 cannot substitute the Hsp110 in the disaggregation activity. Given the suggested role of Bag-1 in protein degradation and also in relation to the data in figure 3 with the bacterial KJEB complex, it seems relevant to know what the effect is of adding Bag-1 to the HSP110-HSP70-HSP40 complex for the disaggregation activity.

Figure 2:
The difference between the panels C, B versus D is the ratio of luciferase:Hsp26 used during heating. The presentation of these data both in the graphs (e.g order of labelling in panel D) and text however is somewhat confusing. After quit some puzzling, I think the conclusions drawn from these experiments are all correct, but clarity must be improved. I suggest at least to split this set of data into 4 panels, 2 for the human and 2 for the yeast chaperones. Also, all panels should contain the same combinations and color coding for the orthologous HSPs to make a comparison easier.
Also, the authors should emphasize more clearly that at the lower luciferase:Hsp26 ratio, Hsc70-Hdj1 without the need of Hsp110 already have some disaggregation activity. The same is true for MDH (panel E) where they do mention this feature 'en passant' in their text. Whilst this does not undermine their conclusion that Hsp110 provides the cell with (extra) disaggregation power, this finding suggest that this may only be required if aggregates have formed that were not kept competent for Hsp70-Hsj1 disaggregation by the small Hsp. This is even more so relevant as most mammalian cells generally do express small Hsps constitutively (see also comments on figure 3, S3)

Figure 3:
Panel A in this figure is not very informative/conclusive and in my view could go the supplementary figure. Panels B and C are again labelled in a confusing manner and lack a number of controls/experimental conditions.
panel B: the use of the bacterial KJEB system here is confusing as it was not used in the in vitro studies (figures 1, 2). In stead, the Ssa1-Ydj1-Sse1 complex is missing in this panel as well as the Ssa1-Ydj1-Hsp104 (also lacks in panel C)
panel B, C: as stated above, these U2OS cells in which luciferase was heat denatured likely express small HSP. It is therefore essential to show whether the sole addition of Hsp70-Hsj1 suffices to reactivate the luciferase under the conditions used here. In addition, like in the in vitro experiment also the combination of Hsp70-Hsj1 and Bag1 should be included to demonstrate that NEF activity is insuficient also in this situation. To be really complete, also effects of Hsp70 and Hsj1 alone or Hsp70+ Hsp110 and Hsj1+Hsp110 could be included.
Panel C: it is string to see that the KJEB is very efficient here, whilst the Ssa1-Ydj1-Sse1 is ineffecient. To be consistent with figure 2, it would therefore be informative if one would add data on combining Hsp104 with the Ssa1-Ydj1-Sse1 complex in this reaction.

Figure 3S:
The images are nice could provide important additional insights. Such data should probably be

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incorporated as a main figure; however, the data are yet incomplete.
In order to relate to the functional disaggregation complex, co-staining with Hsp70 and Hsj-1 is required.
Also, given that for the heat denaturation experiments in vitro always small Hsp were used, it would be informative (maybe not required) to also stain these aggregates for the presence of small Hsp .
It is striking to see that Apg2/Hsp110 remains associated with the aggregates after 1 hour recovery. The authors do not discuss these data. Also, several luciferase foci seem not to be decorated with Apg2/Hsp110. In fact, most of these seem to have disappeared after 1 hour of recovery, whereas the ones that remain still are decorated with Apg2/Hsp110. Is this a correct and is this also consistently found in other images? If so, how would the authors interpret such findings? Again, it might be relevant to see how such patterns overlap with stainings for the other Hsps mentioned.
It is also striking to see that a few large nuclear luciferase (non-reversible) foci are not staining positive for Apg2/Hsp110. Authors' comment?

Figures 4 and 5:
Obviously these experiments cannot include all the variables used in the in vitro and cellular experiments, but at least it would be nice to test (if possible) whether a knock-down of another ce-NEF besides Hsp110 would have similar effects.
The authors should furthermore provide more information on the number of Hsp70 and Hsp110 genes in C. elegans the actual specificity and level of knock-down achieved

Figures 4:
Here, the authors should at least also show aggregation data with the same Hsp70 siRNA and Hsp110/Hsp70 siRNA lines they have used in their lifespan analysis.

<B>Minor comments:</B>
Whilst I agree that these findings are the first to show disaggregation promoting activity of the NEF-Hsp110 members, I am not sure whether one can state that this generates a novel disaggregase activity in metazoa (abstract) given the findings both in vitro and in cells that the Hsp70/40 machine can recover proteins from an aggregated state in which small Hsp are present. The addition of Hsp110 either boost this activity or may be required if small Hsps are not in the aggregate (although the latter has not been formally proven). I would therefore also suggest to slightly revise the title to e.g 'Hsp110 <B>empowers</B> the Hsp70 machine to solubilize protein aggregates in animal cells'
Page 7, line 6 from bottom: for clarity, please specifically state here that the chaperones were added to the aggregate-containing cell lysates.
In the experiments in figure 2, the authors used the yeast Hsp26 as small Hsp. Why did they not (also) use a mammalian small Hsp? It seems theoretically possible that the interaction between a human small Hsp and the human Hsp70-Hsj1 machine would be even better in collaborating in disaggregating/reactivating heat denatured proteins which would imply a reduced requirement of Hsp110 assistance in these systems.

Re-submission 29 May 2012

Point-to-point response to the reviewers' comments

Referee #1

1. *This study presents evidence for an important role of animal Hsp110 in protein disaggregation. (...) These are intriguing observations. However, the manuscript provides little insight into how animal Hsp110 might actually assist Hsp70-Hsp40 in processing aggregates. Although Hsp110 sequences are less conserved than canonical Hsp70s and type I J-domain proteins, it seems puzzling that yeast Sse1 and mammalian Hsp110 (Apg2) differ so dramatically in their function.*

We appreciate the reviewer's positive assessment. We have included additional experiments into the manuscript which address the mechanism by which human Hsp110s enable or stimulate disaggregation by human Hsp70 and Hsp40 (Fig. 4, see next paragraph). Since we find that their NEF activity is the primary activity responsible for stimulating disaggregation, and human and yeast Hsp110s promote nucleotide exchange by the same mechanism, and since we observe that Sse1 can

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substitute for Apg2 in the human Hsp70 system, the species difference likely does not result from the Hsp110s. Also, we show that alternative NEFs can in principle support a less efficient disaggregation in the presence of a type II Hsp40 (Fig. 5). These observations suggest that the gain in disaggregation capacity may instead either stem from another member of the Hsp70 system or from differences in the interplay of the sets of (co-)chaperones.

2. Previously described Hsp110 mutants should be used to dissect the critical functions of these molecular chaperones: Is ATP binding to Hsp110 necessary for disaggregation activity? Is ATP hydrolysis by Hsp110 necessary? Is Hsp70 binding / NEF function essential? Which role, if any, does the beta-sandwich domain, the putative substrate binding domain of Hsp110, play?

We have included additional data addressing most of these questions (Fig. 4). Our results suggest that interaction with Hsp70 / NEF activity of Apg2 is essential for supporting disaggregation. In contrast, ATPase activity of Apg2 and thus putative nucleotide-regulated substrate interactions are not required for stimulation of disaggregation. We conclude that nucleotide exchange on Hsp70 is the predominant mechanism by which Apg2 promotes efficient disaggregation. We also tested Apg2 mutants with mutations in the C-terminal putative substrate binding domain. However, interestingly these mutations did not affect substrate interactions of Apg2 (in aggregation prevention assays) and therefore were inconclusive. The substrate binding properties of Hsp110 chaperones are obviously not easy to dissect and demand an entire new approach as part of an independent study.

3. A recent study by Shorter and colleagues in PLoS-One reported that Hsp110 (Apg2) functions in disaggregation of luciferase in the mammalian system. The combination of yeast Sse1-Ssa1-Sis1 (the latter is the type II Hsp40 in S. cerevisiae) was also found to be active.

We have performed this experiment as well but obtained quite different results. Sis1 supported reactivation by Ssa1 and Sse1 better than Ydj1, to a final yield of about 20% reactivated luciferase, Supp. Fig. 2E). This yeast system however, was still marginal in comparison to the human chaperones (Hsc70, Hdj1, Apg2) which reactivated luciferase to about 70%. Thus, in contrast to the study published by Shorter who reports (rather slow, inefficient) disaggregation for Ssa1-Sse1 with either Sis1 or Ydj1, we do not observe efficient disaggregation by either combination of yeast chaperones.

4. Moreover, the Shorter study investigated functional mutations in the yeast chaperones. This has been overlooked in the present study and should be properly discussed.

Our study was conducted in parallel to and independently of the recently published Shorter study and therefore we did not discuss the results from that study in our submitted manuscript. Importantly, our results show strong differences to those obtained by Shorter, e.g. regarding the requirement for ATP hydrolysis. In this revised manuscript, we have included additional experiments using functional Apg2 and Sse1 mutations as well as a discussion of similarities and differences between ours and the Shorter study (Fig. 4 and Discussion).

5. RNAi-mediated down-regulation of the C. elegans ortholog of Hsp110 had a negative effect on the breakdown of GFP-luciferase aggregates and reduced worm lifespan after heat shock, especially if Hsp70 was also down-regulated. However, these effects might also be a result of a strong reduction of the essential NEF function of Hsp110 and thereby of Hsp70 function.

We addressed this concern by an additional experiment. To control for an unspecific effect caused merely by reduction of cytosolic NEF activity, we have now performed RNAi against the *bag-1* homolog (F57B10.11), as well, and did not observe a detrimental effect on aggregate removal (Fig. 6). We furthermore noticed that Hsp110 depletion in the RNAi-treated worms did not lead to a strong general phenotype at non-heat shock temperatures.

Referee #2

While these results seem interesting, some experiments lack the necessary controls and some experiments comparing four different systems are rather confusing.

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We are grateful for the appreciation and hope that our newly included experiments and extensive changes to the manuscript will satisfy the reviewer.

Specific points:
1. Initially the authors compare the activities of the Hsp70-Hsp40 system towards chemically denatured luciferase (figure 1). The proteins concentrations are missing in the figure legend. In methods section only ranges of concentrations are given for the NEFs. The description of experimental conditions needs to be improved throughout the manuscript (in particular also in figure 2 and 3). The importance of this issue can also be seen in supporting figure 1C, where apparently 0.2 and 0.4 µM Hsp105 lead to strong reactivation, while 0.3 µM does not.

We have introduced the requested changes to figure legends and the methods section to include more details regarding the experimental conditions. Furthermore, we would like to thank the reviewer for pointing out an aspect in which the figure layout is misleading: 0.3 µM Hsp105 (Supp. Fig. 1C) did not fail to support disaggregation, but this concentration was not tested. We have changed the figure legend to clearly state this fact.

2. What is the evidence for defining the substrates in the experiments depicted in figure 1 and 2 as "aggregates"? SEC-HPLC or another suitable method could be employed to prove that indeed aggregates and not soluble oligomers are present at the start of the experiment. How long was the incubation in the refolding buffer before chaperones were added?

The pelleting experiments shown in Figs. 1A and 2A demonstrate that the material is insoluble, qualifying it as aggregated as opposed to misfolded. The period between dilution of the aggregated material and addition of chaperones was in the order of a few minutes.

3. In figure 3, traces and data for "hs + Hsc70, Hdj1" are missing. Also the controls "hs + Hsc70, Hdj1, Bag-1" and in figure 3B "hs 70/40" are important. Which Hsp110 protein was used in figure 3B? Without these controls it is not possible to judge the results. The same is true for figure 3C. It cannot be assumed that Hsc70/Hsp40 is inactive in the cell lysates just because it was inactive in the in vitro refolding assays.

As requested, we have included the control "hs + Hsc70, Hdj1, Bag-1" in Supp. Fig. 3D. We did not determine disaggregation by Hsc70 and Hdj1 without a NEF in this experiment, because previous experiments have demonstrated already that Apg2 strongly stimulates disaggregation by Hsc70 and Hdj1. The goal of the experiments shown in Fig. 3 was to test whether the significant capacity of the complete human Hsp70-Hsp40-Hsp110 system extends to aggregates formed in a cell lysate. These experiments demonstrate that the combination of Hsc70, Hdj1 and Apg2 supports almost quantitative resolubilization of endogenously expressed EGFP-luciferase and that about 50% of the luciferase are reactivated, suggesting a significant disaggregation capacity of this chaperone system. The experiments shown in Fig. 3B (now Fig. 3A) were performed with the same chaperone combinations as the ones in the rest of Fig. 3, i.e. Apg2 was the Hsp110 used. We have stated this more clearly in the figure legend. Due to the reviewers' comments, the light scattering experiments originally included as Fig. 3A were omitted from the revised manuscript (see also comment no. 8).

8. Why is the final turbidity in the cell extract system at the end of the assay including Hpg2, hsp110 and Hsp70 identical to the chaperone free hs-trace? The way it is presented (absence of controls, no quantification of the "aggregated proteins" at least at the endpoint) makes it hard to follow the conclusions.

We apologize for not making clear that the pelleting experiment presented in Fig. 3B employs the same chaperone combinations as the light scattering experiment in Fig. 3A. This control demonstrates that the great majority of luciferase present in the aggregates is solubilized by the action of Hsc70, Hdj1 and Apg2. In addition, we have observed that this chaperone combination causes significant light scattering which is independent of substrate, but depends on the presence of ATP. Therefore we suggest that the high level of light scattering observed after completion of this reaction reflects a macromolecular complex formed by the human chaperones. However, due to the apparent ambiguity of these results, we have omitted this panel from the revised manuscript.

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14. Instead of the bacterial system in figure 3A, it would be better to include the controls for the human system.
15. The inactive yeast system is included in figure 3C. To make the point that this system is inactive in the absence of Hsp104, an experiment in which Hsp104 was added back should be included.

As requested, we have included the control "hs + Hsc70, Hdj1, Bag-1" for the human system (see comment no. 3). We included the yeast Hsp70 system without Hsp104 as a control because it is one of the main points of our study that the human Hsp70 system has gained disaggregation activity beyond the capabilities of the yeast complement of chaperones. We did not include the yeast bichaperone system (with Hsp104) because it was less potent than the human Hsp70 system. Therefore, we employed the powerful bacterial bichaperone system as a positive control.

4. Worms were heat-shocked and then aggregation was investigated in the absence or presence of Hsp110 RNAi. Is the worm shown in figure 4A at 12h and then at 24 h the same worm or just another worm from the same experiment. The experiment would be more conclusive if it were the same individual.

The nematodes shown in these images are not the same. However, they are representative images, as indicated in the figure legend. The results of the Hsp110 knock-down on the disaggregation activity of Luciferase-YFP aggregates are highly reproducible with little variation between individual animals. This is shown in the quantitation with error bars in Fig. 6C (previously Fig. 4C).

Does the available Hsp110 knock-out line give the same result?

We chose to study the effect of Hsp110 after the formation and appearance of protein aggregates. Only by using an RNAi mediated knock-down after the first appearance of Luciferase-YFP aggregates is it possible to analyze the effect of Hsp110 on disaggregation as opposed to a prevention of aggregation. Moreover, the only available Hsp110 knock-out strain (VC1157) is sterile and only viable when maintained as heterozygote. This strong phenotype supports our findings of a vital role for Hsp110 in maintaining proteostasis.

5. In the life span assays, the authors apparently started the RNAi treatment on day one. This is known to induce a strong phenotype for HSP-1 leading to arrest at early larval stages (Kampath et al., Wormbase), including the inability to reach the adult and fertile state. The authors do not mention how they dealt with this issue in their analysis or whether this phenotype was also altered by the double RNAi experiment with C30C11.4. It is hard to imagine a life span analysis on these severely affected worms.

While it is true that a knock-down of *hsp-1* results in a delay in development, the nematodes reached adulthood and were fertile. We did not observe a larval arrest. The knock-down of *hsp-1* resulted in a reduced lifespan compared to wild type with a medium lifespan of 11.5 and 14 days, respectively. The double knock-down of *hsp-1* and *C30C11.4* lead to a more severe reduction in lifespan, which is especially apparent after heat shock. The nematodes displayed a developmental delay comparable to the single knock-down of *hsp-1*, but all animals reached adulthood and were fertile.

6. It would be interesting to include a figure in the supplement which shows the homology between Apg1, apg2, hsp110, c30c11 and sse1.

A sequence alignment of various Hsp110 homologs including Sse1, Hsp105 and C30C11.4 has been published by Polier et al (Cell 2008). Since we find that the NEF activity of Apg2 is the primary activity responsible for stimulating disaggregation and we also observe that Sse1 can substitute for Apg2 in the human Hsp70 system (Fig. 4F), we feel that an alignment of the Hsp110s would not add to the understanding of disaggregation by the human Hsp70 system.

7. Why do the authors mention that urea concentration was "4 or 6 M urea" before dilution, as there is only one data set in the manuscript?

We have clarified this issue in the methods section of the manuscript.

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9. Information on the newly created worm strain is lacking. Was it integrated or is it a stable non-integrated line?

We provided more specific information on this strain in the method section of the manuscript.

10. It is difficult to rationalize why RNAi against Hsp70 and Hsp110 exhibits a so much stronger effect than RNAi against Hsp70, as the presence of Hsc70 is required for Hsp110 function. The authors may want to discuss this in more detail. How does the Hsc70 knockdown influence the Hsp110 knock-out strain?

The weaker phenotypes of the single knock-downs of hsp-1 and C30C11.4 suggest that alternative NEFs and Hsp70 functionally cooperate with HSP-1 and C30C11.4, respectively. The reviewer raised an important point and we included a discussion on this aspect in the manuscript (p. 19): The stronger effect of the Hsp110-Hsp70 double knock-down (Fig. 7) suggests that alternative NEFs and Hsp70 functionally cooperate with HSP-1 and C30C11.4, respectively. The *C. elegans* genome encodes for 10 Hsp70 genes, of which 7 are localized in the cytosol (*hsp-1, K09C4, F54C9, C12C8.1, F44E5.4, F44E5.5* and *F11F1.1*) and two cytosolic non-Hsp110 NEFs: *F57B10.11* (*bag-1*) and the hardly characterized *H14N18.1a* (*unc-23*) (Nikolaides & Nei, 2004). The high number of the Hsp70 chaperones and its co-factors in higher metazoans suggests the possibility of a range of functional cooperations of the various Hsp70 members with their NEFs and thus could explain the observed weaker phenotype in the single knock-downs of Hsp70 and Hsp110.

11. The authors need to define "severe" and "permissive" aggregation conditions.

The conditions we employed for stringent and permissive thermal aggregation of luciferase differ by a factor of 100 in the substrate concentration during the heat treatment (2 μM vs. 20 nM luciferase). The fact that concentration during the aggregation step influences the amenability of the resulting aggregates is evidenced for example by the study of Cashikar *et al*., 2005, and by our own observation that co-aggregation of Hsp26 is essential for reactivation of luciferase from the stringently obtained aggregates, but not from the ones obtained under permissive conditions. We have included these data into the Supplementary Material (Supp. Fig. 2A-C) and have discussed these aspects in detail on p. 7-8 of the manuscript. A more functional criterium may be applied to differentiate disaggregation of chemically vs. thermally aggregated luciferase: Since urea-aggregated luciferase is partially amenable to the yeast Hsp70 system with any NEF (Ssa1, Ydj1, Sse1 or Snl1ΔN, without Hsp104), whereas thermally aggregated luciferase is not, one may assume that the former aggregation method is more permissive than the latter. We have included this definition on p. 7 of the manuscript.

12.The origin of the antibody against luciferase is not stated in the text.

We apologize for this lack of information. The origin of the antibody is now mentioned in the methods section.

13. Scale bars are missing in figure 4A which is important for estimating the age of the nematode. According to the procedures it should be 24 hours + hs + 12/24 hours old.

We included scale bars in the images of figure 4A (now Fig. 6A). The time indicated in the images refer to the recovery time.

Referee #3 (Remarks to the Author):

1. Overall, the findings are novel and potentially very interesting. The paper is generally well-written and good to follow for a specialist in the field. However, for the more general readership of the EMBO J, it may require some rewriting. In particular, the use of equivalent chaperone complexes from different sources in the various experiments and the nomenclature (Hsp100, Hsp110/HSPH/Apg2 versus ClpB/Hsp104) may be confusing to non-experts: at least it would be helpful to use something like 'NEF-Hsp110' versus the 'AAA-protease Hsp104'. Also, the explanation of use of small HSP in the heat denaturation experiments and the interpretation of some of the outcomes of these data requires attention.

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We thank the reviewer for this positive evaluation and for pointing out measures with which we can improve the text to make it more comprehensible also to a general audience. We have followed the reviewer's advice by defining the members of different chaperone classes more clearly throughout the manuscript. We have also included additional experiments addressing the role of small Hsps and discussed the implications of our findings (see below).

2. Major Comments, Figure 1 and 2:
Although I am aware that a functional Hsp70 folding machine usually seems to require at least both Hsp70 and a DnaJ, I would pose the question whether for this unprecedented novel disaggregation complex either Hsc70 or Hdj2 could be dispensable. Especially, could it be that Hsp110 together with Hjd1 is sufficient for the disaggregation activity?

We have done this experiment and did not observe any luciferase reactivation by Apg2 with Hdj1. Generally, our data (see newly included experiments in Figs. 4 and 5) suggest that the NEF activity of Hsp110 proteins is central to their stimulatory activity in disaggregation. Thus the disaggregation reaction appears to be centered around the well-studied ATPase cycle of Hsp70 chaperones where co-chaperones may stimulate as well as modify Hsp70 functions.

The authors nicely show that the NEF Bag-1 cannot substitute the Hsp110 in the disaggregation activity. Given the suggested role of Bag-1 in protein degradation and also in relation to the data in figure 3 with the bacterial KJEB complex, it seems relevant to know what the effect is of adding Bag-1 to the HSP110-HSP70-HSP40 complex for the disaggregation activity.

We did not test the combination of Hsp70-Hsp40-Hsp110 with Bag-1 in the context of *in vitro* disaggregation because we expect that Bag-1 would compete with Hsp110 for interacting with Hsp70, which would result in less efficient disaggregation as compared to Hsp70-Hsp40-Hsp110. However, the reviewer raises an important point here, the question whether certain Hsp70 co-chaperones specifically feed Hsp70 substrates into distinct pathways of proteasomal or autophagic degradation or disaggregation or even aggregation. To investigate this question conclusively, a minimal system for the assessment of such triage decisions should be reconstituted from purified components, which however is an entire new study. We have, however, included a discussion of this aspect on p. 18/19 of our manuscript.

3. Major Comments, Figure 2:
The difference between the panels C, B versus D is the ratio of luciferase:Hsp26 used during heating. The presentation of these data both in the graphs (e.g order of labelling in panel D) and text however is somewhat confusing. After quit some puzzling, I think the conclusions drawn from these experiments are all correct, but clarity must be improved. I suggest at least to split this set of data into 4 panels, 2 for the human and 2 for the yeast chaperones. Also, all panels should contain the same combinations and color coding for the orthologous HSPs to make a comparison easier. Also, the authors should emphasize more clearly that at the lower luciferase:Hsp26 ratio, Hsc70-Hdj1 without the need of Hsp110 already have some disaggregation activity. The same is true for MDH (panel E) where they do mention this feature 'en passant' in their text. Whilst this does not undermine their conclusion that Hsp110 provides the cell with (extra) disaggregation power, this finding suggest that this may only be required if aggregates have formed that were not kept competent for Hsp70-Hsj1 disaggregation by the small Hsp. This is even more so relevant as most mammalian cells generally do express small Hsps constitutively (see also comments on figure 3, S3)

We apologize for the lack of clarity concerning the difference between Fig. 2B,C and Fig. 2D,E which we have now improved thoroughly in the manuscript. The aggregation conditions do not differ in the ratio of luciferase to Hsp26, but in the absolute substrate concentrations during the thermal aggregation. The luciferase as well as Hsp26 concentrations during aggregation were 100-fold higher for Fig. 2B,C than for Fig. 2D,E, while the final concentrations during the reactivation assays were identical. High substrate concentration during aggregation is expected to generate a high density of exposed hydrophobic residues during the aggregation step (Hartl 2011). We also provide new evidence demonstrating that the two conditions generate aggregates with different amenability (Supp. Fig. 2A-C): When aggregated at a high concentration, luciferase depends on the co-aggregation of Hsp26 for disaggregation, while aggregates obtained at low substrate concentration can be efficiently

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reactivated in the absence of Hsp26. Thus, the latter represents a less stringent aggregation method, which likely explains the fact that Hsc70-Hdj1 alone can partially reactivate these aggregates.
In addition to the newly added experiments, we have included a detailed explanation as well as discussion of the experiments addressing disaggregation of heat-induced aggregates in the absence or presence of Hsp26. Also, we have followed the reviewer's advice and placed more emphasis on the finding that Hsc70-Hdj1 has significant activity towards aggregates obtained under rather permissive conditions. Finally, we have separated Fig. 2D into two panels (Fig. 2D,E) with the same colour coding as in the remainder of Fig. 2, as suggested by the reviewer.

Figure 3:
Panel A in this figure is not very informative/conclusive and in my view could go the supplementary figure. Panels B and C are again labelled in a confusing manner and lack a number of controls/experimental conditions. panel B: the use of the bacterial KJEB system here is confusing as it was not used in the in vitro studies (figures 1, 2). In stead, the Ssa1-Ydj1-Sse1 complex is missing in this panel as well as the Ssa1-Ydj1-Hsp104 (also lacks in panel C) panel B, C: as stated above, these U2OS cells in which luciferase was heat denatured likely express small HSP. It is therefore essential to show whether the sole addition of Hsp70-Hsj1 suffices to reactivate the luciferase under the conditions used here. In addition, like in the in vitro experiment also the combination of Hsp70-Hsj1 and Bag1 should be included to demonstrate that NEF activity is insuficient also in this situation. To be really complete, also effects of Hsp70 and Hsj1 alone or Hsp70+ Hsp110 and Hsj1+Hsp110 could be included.
Panel C: it is string to see that the KJEB is very efficient here, whilst the Ssa1-Ydj1-Sse1 is ineffecient. To be consistent with figure 2, it would therefore be informative if one would add data on combining Hsp104 with the Ssa1-Ydj1-Sse1 complex in this reaction.

We have omitted the panel 3A from the revised manuscript.
We agree with the reviewer that the use of the bacterial bichaperone system in these experiments is confusing. We resorted to this measure since the bacterial chaperones proved to be more potent in resolubilizing aggregates than the yeast bichaperone system, so KJEB resulted as the better positive control for these assays. As requested, we have included the control "hs + Hsc70, Hdj1, Bag-1" in Supp. Fig. 3D. We did not determine disaggregation by Hsc70 and Hdj1 without a NEF in this experiment, because previous experiments have demonstrated already that Apg2 strongly stimulates disaggregation by Hsc70 and Hdj1. The goal of the experiments shown in Fig. 3 was to test whether the significant capacity of the complete human Hsp70-Hsp40-Hsp110 system extends to mixed aggregates formed in a cell lysate. These experiments demonstrate that the combination of Hsc70, Hdj1 and Apg2 supports almost quantitative resolubilization of endogenously expressed EGFP-luciferase and that about 50% of the luciferase are reactivated, suggesting a significant disaggregation capacity of this chaperone system.

Figure 3S:
The images are nice could provide important additional insights. Such data should probably be incorporated as a main figure; however, the data are yet incomplete.
In order to relate to the functional disaggregation complex, co-staining with Hsp70 and Hsj-1 is required.
Also, given that for the heat denaturation experiments in vitro always small Hsp were used, it would be informative (maybe not required) to also stain these aggregates for the presence of small Hsp .
It is striking to see that Apg2/Hsp110 remains associated with the aggregates after 1 hour recovery. The authors do not discuss these data. Also, several luciferase foci seem not to be decorated with Apg2/Hsp110. In fact, most of these seem to have disappeared after 1 hour of recovery, whereas the ones that remain still are decorated with Apg2/Hsp110. Is this a correct and is this also consistently found in other images? If so, how would the authors interpret such findings? Again, it might be relevant to see how such patterns overlap with stainings for the other Hsps mentioned.

We heartily agree that there are multiple open questions regarding the details of how the aggregates are dealt with *in vivo*, such as identifying other interacting chaperones and following the fate of individual aggregates over time, as suggested by the reviewer. However, we feel that addressing these questions in satisfactory depth, ideally employing more than one cell line, is beyond the scope of this study and merits a separate study.
Since we did not follow luciferase activity during recovery of the heat-shocked cells, we cannot draw firm conclusions regarding the question whether luciferase is reactivated under these conditions.

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Also, we did not perform a time lapse experiment and thus cannot follow the fate of individual aggregates in the absence or presence of Apg2. However, the finding that aggregates persist and appear to cluster with time indicates that they are successively remodeled rather than solubilized. Therefore, the observation of this reviewer that Apg2-free aggregates are present at early timepoints, but less so at later timepoints, may indicate that Apg2 colocalization with aggregates increases progressively. We have included a more detailed examination of these experiments in the manuscript (see p. 13).

It is also striking to see that a few large nuclear luciferase (non-reversible) foci are not staining positive for Apg2/Hsp110. Authors' comment?

After heat shock, we have frequently observed one or two foci in the GFP channel that are located in the nucleus or in its immediate vicinity and that never colocalize with Apg2. As yet, we do not know what these structures might be, however we have observed them occasionally also in cells that had lost EGFP-luciferase expression, suggesting that these structures display autofluorescence.
In general, colocalization of EGFP-luciferase and Apg2 was not 100%. In fact, Apg2 accumulated in foci also in cells without EGFP-luciferase signal, indicating that it might colocalize with aggregated endogenous proteins. We have made mention of these aspects on p. 13 of the manuscript.

Figures 4 and 5:
Obviously these experiments cannot include all the variables used in the in vitro and cellular experiments, but at least it would be nice to test (if possible) whether a knock-down of another ce-NEF besides Hsp110 would have similar effects.

The *C. elegans* genome encodes for two cytosolic non-Hsp110 NEFs: *F57B10.11* (*bag-1*) and *H14N18.1a* (*unc-23*). To compare the findings of the Hsp110 NEF to an Hsp70-unrelated NEF, we decided to test the effect of the *bag-1* homolog (F57B10.11) as *unc-23* is not well characterized. We have included these additional data in Fig. 6 (previously Fig. 5) of the revised manuscript.

The authors should furthermore provide more information on the number of Hsp70 and Hsp110 genes in C. elegans the actual specificity and level of knock-down achieved.

The *C. elegans* Hsp70, Hsp110 and Grp170 chaperone families were previously analyzed (Heschl & Baillie, 1990; Nikolaidis & Nei 2003; www.wormbase.org). 13 genes belong to the Hsp70 superfamily, of which 10 are Hsp70 genes, two are Grp170 genes and one is an Hsp110. The two Grp170 gene products are ER localized. The only Hsp110 gene product, *C30C11.4*, is cytosolic. We included this information in the manuscript for more clarity. We used the well-established Ahringer RNAi library for the gene specific knock-down. The reliability of the Ahringer library was recently analyzed and its accuracy confirmed (Qu, W et. al., 2011 BMC Genomics). The lack of antibodies for *C. elegans* Hsp70 and Hsp110 does not allow for an analysis of a reduction of the protein level upon RNAi-mediated knock-down. However, the observed strong phenotypes in response to dsRNA feeding suggest a suficient knock-down for both Hsp70 and Hsp110.

Minor comments:
Whilst I agree that these findings are the first to show disaggregation promoting activity of the NEF-Hsp110 members, I am not sure whether one can state that this generates a novel disaggregase activity in metazoa (abstract) given the findings both in vitro and in cells that the Hsp70/40 machine can recover proteins from an aggregated state in which small Hsp are present. The addition of Hsp110 either boost this activity or may be required if small Hsps are not in the aggregate (although the latter has not been formally proven). I would therefore also suggest to slightly revise the title to e.g 'Hsp110 <B>empowers</B> the Hsp70 machine to solubilize protein aggregates in animal cells'.

We agree with the reviewer that under some conditions, Hsp110 stimulates an intrinsic activity of Hsp70-Hsp40 rather than enabling a completely novel activity. We have changed the title to "Metazoan Hsp70 machines use Hsp110 to power protein disaggregation" and reworded passages in the abstract as suggested.

Page 7, line 6 from bottom: for clarity, please specifically state here that the chaperones were added to the aggregate-containing cell lysates.

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We have included this statement into the description of the experiment (now on p. 9).

In the experiments in figure 2, the authors used the yeast Hsp26 as small Hsp. Why did they not (also) use a mammalian small Hsp? It seems theoretically possible that the interaction between a human small Hsp and the human Hsp70-Hsj1 machine would be even better in collaborating in disaggregating/reactivating heat denatured proteins which would imply a reduced requirement of Hsp110 assistance in these systems.

We absolutely agree with the reviewer that disaggregation in the metazoan system may well be even more efficient if aggregates contain a more appropriate sHsp or if additional factors are present. We discuss this possibility in our manuscript. However, we chose to limit our use of sHsps to yeast Hsp26 because it is conveniently activated by heat shock, whereas the best studied mammalian sHsp, Hsp27, is activated by dynamic cycles of phosphorylation and dephosphorylation, resulting in a much more complex, not well controllable system.

2nd Editorial Decision 13 July 2012

Thank you for sending us a new version of your manuscript (previously EMBOJ-2011-80153) as a new submission. In the meantime, and after some delay due to difficulties with the availability of our original referees at the time of resubmission, the referees have seen the manuscript again and are now more positive (please see below). Still, they raise a number of points that should be addressed or responded to in an amended version of the manuscript.

In addition, there two editorial issues that need further attention:

* Please add statistical details, including the number of independent repeats, to the legends of figures 3B, 4, 5, 6, 7, S1, S2, S3.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,
Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The revised manuscript is very much improved and now a very nice work.
Now compelling evidence is presented that it is mostly the NEF activity of metazoan Hsp110s that greatly enhances the disaggregation activity of the Hsp70 system. Together with mammalian Hsc70 and Hdj1, the yeast Hsp110 Sse1 can functionally replace Apg2. All components of the yeast and mammalian systems (Hsp110s, Hsp40s) have been systematically explored, and a differentiated picture is presented.
The competing work by Shorter in PLoS-One is adequately discussed.
Minor points:
Abstract, second line: Comma missing after bacteria.
Introduction, first line: 'perturbs' instead of 'perturb'

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Page 3, middle: 'yeast and bacterial Hsp70-Hsp40-systems directly interact' instead of 'yeast and bacterial Hsp70-Hsp40-system directly interacts'
Citations: Umlauts in authors Schrˆder and G‰ssler missing
Page 11, bottom: Functional differences Bag-1, Snl1, Apg2 explained by different kinetics of the Hsp70 cycle. Here, ATP expenditure could be recorded.
Page 13, middle: Delete 'the' in front of 'Hdj1'
Page 16, middle: Heat shock on day 1 of worm life. Is this not day 1 of their adult life, i.e. after development is completed?

Referee #2 (Remarks to the Author):

In their MS, Rampelt et al propose that mammalian cells - that lack the disaggregation activity of the yeast Hsp104 AAA protease - can utilize the HSPH/APG/Hsp110 proteins in conjunction with the Hsp70-Hsp40 machine for disaggregation. In a set of elegant in vitro experiments, it is shown that the Hsp110-Hsp70-Hsp40 chaperone complex can reactivate proteins from chemically- or thermally induced aggregates. This action requires the nucleotide exchange factor (NEF) activity and not the ATPase activity of Hsp110. Whether the substrate binding domain of Hsp110 is involved remains unclear. However, Hsp110 is not exclusively required as other NEFs were also active in disaggregation if combined with the proper DnaJ protein. Albeit true that Hsp110 seems to be more "powerful" or "general", it must be stated that only 2 of the many DNAJ proteins were tested and that combinations of the many DNAJs as expressed in mammalian cells might be as effective as Hsp110. So, what the actual prerequisites are for the Hsp70 machines to provide it with (maximal) disaggregation activity remains somewhat elusive.
The authors also show some evidence with mammalian cell systems that support that also in living cells the HSP110-HSP70-HSP40 may indeed exert disaggregation activity, but these data remain less conclusive (see below).
Finally, data in C. elegans are provided that show that siRNA-mediated knockdown of Ce-Hsp110 after heat shock results in delayed disappearance of heat-induced protein aggregates. These elegant data do support the importance and the likely more general and powerful role of Hsp110 in disaggregation, although it must be noted that in mammals more DNAJ proteins exist that at least support similar activities. The finding that Hsp110 knockdown also has substantial effects on lifespan especially when the animals were treated with heat shock at young age and when also Ce-Hsp-1 (one of the worm Hsp70s) was also down-regulated further support its general and vital importance in surviving heat damage, likely by empowering disaggregating activity of the Hsp70 machines.

Main comments

- Whilst the authors have excluded that the Hsp110 effects on disaggregation require its ATPase dependent chaperoning activity, they cannot exclude that a Hsp110 binding to substrates plays no role. The data, including those where the authors have combined DNAJA2 with Hsc70 and Bag1 suggest the possibility that a dual client binding of hsc70 with either Hsp110 or DNAJ proteins combined with a NEF activity provided either by Bag-1 or Hsp110 drives the disaggregation activity. Different DNAJ proteins may bind to different sites along the substrates. If the DNAJ binds close enough to the Hsc70 binding on the substrate, this may lead to proficient disaggregase activity (driven by any NEF). If the DNAJ does not bind close enough, a secondary client binding may be provided by Hsp110. Since Hsp110, like Hsp70, may have a more promiscuous peptide recognition motif, this may explain why Hsp110 may be more potent and general and thus more important for such an activity (in vivo).

- Figure 3 is actually somewhat incomplete. panel A not only lacks the Ssa1/Ydj1/Sse1 combination present in panel B, but both panels also lack the combination of Hsc70/DNAJA2/Bag1 that was found to be (partially) effective in the cell free experiments.

- The data on the localization of Hsp110 to EGFP-luciferase foci is incomplete, rather vague and adds not much to the current paper. The fact that they cluster into larger clusters is not necessarily indicative of structural remodeling but also could indicate their transport to e.g. aggresomes without the aggregates undergoing actual remodeling. In any case, they provide no insight whatsoever on what Hsp110 may do even though there is more at later timepoints in the larger clusters of foci.

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- In the discussion the authors start to state that 'Hsp110 CONVERTS the Hsp70-Hsp40 system into a disaggregase and is a critical component REQUIRED for efficient protein aggregation'. I think this is an overstatement. First DNAJA2 with BAG-1 can do the same things (albeit less efficiently). Moreover, the suggestion that it changes the machinery from one activity to another activity lacks evidence. In general, the authors seem to slightly over-emphasize the special role of Hsp110: the principle seems to apply to any NEF activity (depending on the DNAJ that are use/available), the special effect of Hsp110s being that their effects seem independent on which DNAJ is used (with the restriction that only 2 DNAJs are tested so far).

Minor comments

- The authors repeatedly in their MS refer to disaggregation activity, when measuring protein activity. Although, the interpretation in the end may turn out to be correct, it is suggested to first describe the actual measurements before interpreting them.

- p2 abstract: comma's are need between Bacteria, fungi, and plants....

- p3, 2nd para: .. components of the protein quality control system that prevent aggregates and promotes refolding....<2x delete s>

- p5, last 3 lines. There are errors in the categorization of the DNAJ proteins here. Hdj1 (or DNAJB1) and Sis-1 are type II Hsp40s, whilst DNAJA2 and Ydj-1 are type I Hsp40s. In the rest of the MS this is annotated correctly.

- p12, lines 10-12: I do not understand why the authors conclude that specifically in this case activity recovery only comes from a soluble fraction. I feel this is unwarranted speculation.

- p16, 2nd para: I wonder how the authors want to link the findings of the intracellular Hsp110 activity to the accumulation of extracellular insoluble Abeta?

Referee #3 (Remarks to the Author):

The authors present a revised version of their study on the function of Hsp110 proteins. Most of our technical concerns are sufficiently addressed in this revision. However, a number of important critiques have been ignored, including:
1. Given the focus of the manuscript, a clear understanding of the input aggregate is mandatory. At present, little attention has been paid to characterizing the aggregates. The authors themselves point out that there are at least two types of aggregates in their assays. As the requirement for Hsp110 seems to critically depend on the treatment of the substrate protein sample, it is essential to understand how this is manifested in differences in the physical nature of the aggregate. A clear evaluation of the two different aggregate species would provide insights into questions such as whether the Hsp70/Hdj1/Hsp110 system is sufficient to handle all types of aggregates or whether the inclusion of Hsp26 shifts the state of the input material from a true aggregate to a large (pelletable) oligomer, which is responsive to their system.

2. The manuscript aims to demonstrate the effect of Hsp110 as a disaggregase, which together with Hsp70 and Hdj1 refolds the non-native proteins. This is shown using purified components and also with an extract-based assay. In figure 3B, the effects of Hdj1 and Hsp70 need to be shown for comparison. As it is known that Hsp40 and Hsp70 alone have some refolding activity, demonstrating the gain by adding Hsp110 is of importance to support the title of the manuscript. Also, as previously mentioned, the Hsp104 add back experiment is still missing. It is an important control and should be included.
3. The authors state that they included a more detailed description of urea unfolding (4-6 M urea) in the methods section; however, the point is not any clearer than it was before.
4. The authors state that the RNAi did result in a delay of development in some cases. The statistics on these effects should be presented including the total number of animals counted and percent showing the phenotype. The authors should clearly describe how they performed the RNAi experiments including synchronization and hatching conditions. They also should express the time of the heat shock on day 1 in hours, as "day 1" is too general to allow reproducibility. The authors also

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should clearly state the phenotypes, which were observed in addition to the aggregates to allow an estimation of the strength of the RNAi effect. If no additional phenotypes were observed, this also should be stated to allow comparison to previous RNAi experiments listed in the respective Wormbase sections. "NMG plates" should probably read "NGM plates". Also, it should be stated that different animals are shown for the two different time points.

1st Revision - authors' response 01 August 2012

Response to the Reviewers' Comments

Referee #1 (Remarks to the Author):

The revised manuscript is very much improved and now a very nice work. Now compelling evidence is presented that it is mostly the NEF activity of metazoan Hsp110s that greatly enhances the disaggregation activity of the Hsp70 system. Together with mammalian Hsc70 and Hdj1, the yeast Hsp110 Sse1 can functionally replace Apg2. All components of the yeast and mammalian systems (Hsp110s, Hsp40s) have been systematically explored, and a differentiated picture is presented. The competing work by Shorter in PLoS-One is adequately discussed.

We appreciate this very positive assessment of our manuscript and are grateful for the suggested corrections.

Minor points:
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Introduction, first line: 'perturbs' instead of 'perturb'
Page 3, middle: 'yeast and bacterial Hsp70-Hsp40-systems directly interact' instead of 'yeast and bacterial Hsp70-Hsp40-system directly interacts'
Citations: Umlauts in authors Schr
Page 13, middle: Delete 'the' in front of 'Hdj1'

We have corrected these mistakes.

Page 11, bottom: Functional differences Bag-1, Snl1, Apg2 explained by different kinetics of the Hsp70 cycle. Here, ATP expenditure could be recorded.

We agree that determination of ATP expenditure of the various chaperone combinations should be informative with regard to the question whether disaggregation efficiency is correlated with the kinetics of the Hsp70 cycle. Much of the requested information is already included in the present manuscript. We determined the rates by which the different NEFs (Apg2, Bag-1 and Snl1) accelerate nucleotide exchange by Hsp70 (Supp. Fig. 1), and adjusted the NEF concentrations in our disaggregation experiments such that comparable exchange rates are operative. This approach allows us to suggest that the observed differences in disaggregation activity are not solely due to differences in NEF activity.
A highly informative additional information would be to know the ATP expenditure during an ongoing disaggregation reaction. The required experiments, however, are very difficult to perform and require a study on its own. For example, it is not sufficient to simply determine the steady state ATPase during the disaggregation process, since this activity is composed of ATP hydrolysis during (i) the ground state of Hsp70/cochaperones, (ii) the ATP consumption during disaggregation, and (iii) the substrate-stimulated ATP consumption during subsequent refolding of solubilized proteins. Obtaining interpretable data would therefore require to experimentally assign and distinguish the different steps of the disaggregation process, then to measure the ATP expenditure of each step, and to further place these values in relation to the kinetic features of the chaperone-substrate interactions.

Page 16, middle: Heat shock on day 1 of worm life. Is this not day 1 of their adult life, i.e. after development is completed?

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It is day one of life, just as stated in the text.

Referee #2 (Remarks to the Author):

In their MS, Rampelt et al propose that mammalian cells - that lack the disaggregation activity of the yeast Hsp104 AAA protease - can utilize the HSPH/APG/Hsp110 proteins in conjunction with the Hsp70-Hsp40 machine for disaggregation. In a set of elegant in vitro experiments, it is shown that the Hsp110-Hsp70-Hsp40 chaperone complex can reactivate proteins from chemically- or thermally induced aggregates. This action requires the nucleotide exchange factor (NEF) activity and not the ATPase activity of Hsp110. Whether the substrate binding domain of Hsp110 is involved remains unclear. However, Hsp110 is not exclusively required as other NEFs were also active in disaggregation if combined with the proper DnaJ protein. Albeit true that Hsp110 seems to be more "powerful" or "general", it must be stated that only 2 of the many DNAJ proteins were tested and that combinations of the many DNAJs as expressed in mammalian cells might be as effective as Hsp110. So, what the actual prerequisites are for the Hsp70 machines to provide it with (maximal) disaggregation activity remains somewhat elusive. The authors also show some evidence with mammalian cell systems that support that also in living cells the HSP110-HSP70-HSP40 may indeed exert disaggregation activity, but these data remain less conclusive (see below).
Finally, data in C. elegans are provided that show that siRNA-mediated knockdown of Ce-Hsp110 after heat shock results in delayed disappearance of heat-induced protein aggregates. These elegant data do support the importance and the likely more general and powerful role of Hsp110 in disaggregation, although it must be noted that in mammals more DNAJ proteins exist that at least support similar activities. The finding that Hsp110 knockdown also has substantial effects on lifespan especially when the animals were treated with heat shock at young age and when also Ce-Hsp-1 (one of the worm Hsp70s) was also down-regulated further support its general and vital importance in surviving heat damage, likely by empowering disaggregating activity of the Hsp70 machines.

We appreciate the reviewer's positive evaluation of our study.

Main comments

1. Whilst the authors have excluded that the Hsp110 effects on disaggregation require its ATPase dependent chaperoning activity, they cannot exclude that a Hsp110 binding to substrates plays no role. The data, including those where the authors have combined DNAJA2 with Hsc70 and Bag1 suggest the possibility that a dual client binding of hsc70 with either Hsp110 or DNAJ proteins combined with a NEF activity provided either by Bag-1 or Hsp110 drives the disaggregation activity. Different DNAJ proteins may bind to different sites along the substrates. If the DNAJ binds close enough to the Hsc70 binding on the substrate, this may lead to proficient disaggregase activity (driven by any NEF). If the DNAJ does not bind close enough, a secondary client binding may be provided by Hsp110. Since Hsp110, like Hsp70, may have a more promiscuous peptide recognition motif, this may explain why Hsp110 may be more potent and general and thus more important for such an activity (in vivo).

- p12, lines 10-12: I do not understand why the authors conclude that specifically in this case activity recovery only comes from a soluble fraction. I feel this is unwarranted speculation.

We agree with the reviewer that Hsp110-substrate interactions may participate in disaggregation. Indeed, we observe that Apg2 can partially prevent luciferase aggregation, and we have included these data into Supp. Fig. 3B. We would like to point out that a functional Hsp110-substrate interaction would provide the Hsp70-Hsp40 system with a third site for substrate interactions. In line with the suggestion by this reviewer and our new data we have included a discussion of this possibility in the revised manuscript (p. 18/19). As to the mechanism of disaggregation employing different (co-) chaperone combinations, we offer several possible explanations in our discussion, including differential substrate interactions by different J-proteins or by Hsp110.
Regarding the efficiencies of Hdj1 vs. DNAJA2 in disaggregation, it is our understanding that DNAJA2-mediated reactivation is strictly dependent on aggregation conditions. While non-stringently obtained aggregates are solubilized efficiently in the presence of DNAJA2, from

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stringently obtained aggregates, only 10% of luciferase can be reactivated using DNAJA2, a very low level which might indeed be background (please also refer to Fig. 2A). Thus, we conclude that the combinations Hsc70-DNAJA2-NEF as well as Hsc70-Hdj1-Bag-1 are deficient in an activity required for stringent disaggregation. We have made this point clearer in the manuscript.

2. In the discussion the authors start to state that 'Hsp110 CONVERTS the Hsp70-Hsp40 system into a disaggregase and is a critical component REQUIRED for efficient protein aggregation'. I think this is an overstatement. First DNAJA2 with BAG-1 can do the same things (albeit less efficiently). Moreover, the suggestion that it changes the machinery from one activity to another activity lacks evidence. In general, the authors seem to slightly over-emphasize the special role of Hsp110: the principle seems to apply to any NEF activity (depending on the DNAJ that are use/available), the special effect of Hsp110s being that their effects seem independent on which DNAJ is used (with the restriction that only 2 DNAJs are tested so far).

We agree with the reviewer and have worded the contribution of Hsp110 more carefully to read "Hsp110 empowers the Hsp70-Hsp40 system to efficiently solubilize and reactivate substrates *in vitro* from various, including stringently obtained, protein aggregates.". We wish to point out though that our RNAi experiments in *C. elegans* demonstrate that Hsp110 is uniquely required for aggregate solubilization. The finding that Hsp110s support disaggregation irrespective of the J-protein used suggests that they provide the Hsp70 system with an additional activity or an enhanced integration of the individual components. This capacity likely explains the irreplaceable function of Hsp110 in aggregate solubilization *in vivo*.

3. Figure 3 is actually somewhat incomplete. panel A not only lacks the Ssa1/Ydj1/Sse1 combination present in panel B, but both panels also lack the combination of Hsc70/DNAJA2/Bag1 that was found to be (partially) effective in the cell free experiments.

We did not perform these experiments because they did not seem particularly informative. The Ssa1-Ydj1-Sse1 combination did not reactivate any luciferase (Fig. 3B) nor did it reduce light scattering of heat-treated lysate at all. Further, since we already demonstrated that the combination Hsc70-Hdj1-Bag-1 is partially active (Supp. Fig. 3E), we expect the combination Hsc70-DNAJA2-Bag-1 to exhibit some activity, as well. Thus testing also the latter combination would not gain new insights and was therefore omitted.

4. The data on the localization of Hsp110 to EGFP-luciferase foci is incomplete, rather vague and adds not much to the current paper. The fact that they cluster into larger clusters is not necessarily indicative of structural remodeling but also could indicate their transport to e.g. aggresomes without the aggregates undergoing actual remodeling. In any case, they provide no insight whatsoever on what Hsp110 may do even though there is more at later timepoints in the larger clusters of foci.

We agree that solely based on colocalization data, we cannot judge whether aggregates are being remodeled. For this reason, we have removed the insinuation of potential remodeling. This over-interpretation having been removed, we prefer to show these data (in the Supplementary Data) because to the best of our knowledge, a co-localization of human Hsp110 with non-amyloid aggregates has not been demonstrated previously. In light of our findings regarding the role of Hsp110 *in vitro* and in *C. elegans*, the observed partial co-localization is relevant although we have not addressed the actions of Hsp110 at these aggregates in human cells in further detail.

Minor comments

- The authors repeatedly in their MS refer to disaggregation activity, when measuring protein activity. Although, the interpretation in the end may turn out to be correct, it is suggested to first describe the actual measurements before interpreting them.

We have amended our wording in several cases.

- p2 abstract: comma's are need between Bacteria, fungi, and plants....
- p3, 2nd para: .. components of the protein quality control system that prevent aggregates and promotes refolding....

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We have corrected these mistakes.

- p5, last 3 lines. There are errors in the categorization of the DNAJ proteins here. Hdj1 (or DNAJB1) and Sis-1 are type II Hsp40s, whilst DNAJA2 and Ydj-1 are type I Hsp40s. In the rest of the MS this is annotated correctly.

We are grateful to the reviewer for bringing these errors to our attention, we have corrected them.

- p16, 2nd para: I wonder how the authors want to link the findings of the intracellular Hsp110 activity to the accumulation of extracellular insoluble Abeta?

We apologize for the imprecise wording in our reference of the publication from Eroglu *et al*., 2010. We now state instead that deletion of Hsp105 "causes accumulation of hyperphosphorylated tau and neurofibrillary tangles in mice". While the authors demonstrate a direct interaction of Hsp105 with tau, the mechanism of increased Aβ accumulation observed in that publication in *hsp105*$^{-/-}$ mice is unclear.

Referee #3 (Remarks to the Author):

The authors present a revised version of their study on the function of Hsp110 proteins. Most of our technical concerns are sufficiently addressed in this revision. However, a number of important critiques have been ignored, including:
1. Given the focus of the manuscript, a clear understanding of the input aggregate is mandatory. At present, little attention has been paid to characterizing the aggregates. The authors themselves point out that there are at least two types of aggregates in their assays. As the requirement for Hsp110 seems to critically depend on the treatment of the substrate protein sample, it is essential to understand how this is manifested in differences in the physical nature of the aggregate. A clear evaluation of the two different aggregate species would provide insights into questions such as whether the Hsp70/Hdj1/Hsp110 system is sufficient to handle all types of aggregates or whether the inclusion of Hsp26 shifts the state of the input material from a true aggregate to a large (pelletable) oligomer, which is responsive to their system.

Actually, we demonstrate that the Hsc70-Hdj1-Hsp110 system is sufficient to handle all types of aggregates tested in this study. In the presence of Hsp110, we observe significant, and for the respective Hsp70-Hsp40 combination maximal, disaggregation for chemically aggregated luciferase, thermally aggregated luciferase at two aggregation concentrations, thermally aggregated MDH and thermally aggregated luciferase within human cell lysate. Also, we demonstrate that Hsp26-containing luciferase aggregates are insoluble and require the disaggregase Hsp104 for reactivation when using the yeast chaperones. In fact, inclusion of Hsp26 is essential for luciferase reactivation by the yeast Hsp70 + disaggregase system when aggregation is performed at a high substrate concentration. This agrees very well with the publication from Cashikar *et al.*, 2005, who show that Hsp26 is required for disaggregation by the yeast bichaperone system *in vitro*. While inclusion of Hsp26 clearly decreases the aggregate size (see Cashikar *et al.*, 2005, and Haslbeck *et al.*, 2005) and enhances their amenability to chaperones, the question whether luciferase-Hsp26 aggregates are true aggregates or large pelletable oligomers seems rather semantic given the insolubility and the requirement for a disaggregase in the yeast or bacterial systems.
We have followed the reviewer's suggestion and investigated potential structural differences between the aggregates by comparing them by gradient centrifugation. We find that the more chaperone-resistant aggregates (luciferase aggregated at 2 μM) enter more deeply into the gradient, suggesting they are either larger or denser than the more chaperone-amenable aggregates obtained at 20 nM luciferase (Supp. Fig. 2A).

2. The manuscript aims to demonstrate the effect of Hsp110 as a disaggregase, which together with Hsp70 and Hdj1 refolds the non-native proteins. This is shown using purified components and also with an extract-based assay. In figure 3B, the effects of Hdj1 and Hsp70 need to be shown for comparison. As it is known that Hsp40 and Hsp70 alone have some refolding activity, demonstrating the gain by adding Hsp110 is of importance to support the title of the manuscript. Also, as

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previously mentioned, the Hsp104 add back experiment is still missing. It is an important control and should be included.

We have performed the requested controls and included them in Fig. 3B.

3. The authors state that they included a more detailed description of urea unfolding (4-6 M urea) in the methods section; however, the point is not any clearer than it was before.

The averages shown in Fig. 1B were calculated from a set of experiments performed upon unfolding with either 4M or 6M urea. We have articulated this fact clearly now.

4. The authors state that the RNAi did result in a delay of development in some cases. The statistics on these effects should be presented including the total number of animals counted and percent showing the phenotype.

We included that information in the Result and Materials & Methods sections. We could observe a delay in development upon RNAi of *hsp-1* and the double RNAi of *hsp-1* and *C30C11.4*. This delay became evident as the nematodes reached adulthood (onset of fecundity) about 12-24 hours later compared to control animals. This developmental delay affected about 80% of the nematodes grown on *hsp-1* and *hsp-1/C30C11.4* RNAi plates. We added this information in the Result section: "The knock-down of *hsp-1* lead to a developmental delay of about one day in 80% of the analyzed animals. However, all animals grown on RNAi against *hsp-1* and *hsp-1/C30C11.4* did reach adulthood and did not display any other obvious phenotypes."

The authors should clearly describe how they performed the RNAi experiments including synchronization and hatching conditions. They also should express the time of the heat shock on day 1 in hours, as "day 1" is too general to allow reproducibility. The authors also should clearly state the phenotypes, which were observed in addition to the aggregates to allow an estimation of the strength of the RNAi effect. If no additional phenotypes were observed, this also should be stated to allow comparison to previous RNAi experiments listed in the respective Wormbase sections. "NMG plates" should probably read "NGM plates". Also, it should be stated that different animals are shown for the two different time points.

We thank the reviewer for pointing out the missed details and added the information in the Materials & Methods section, accordingly:
"For synchronization, gravid adults from one 10 cm NGM plate were collected in a canonical tube and treated with 20% alkaline hypochlorite solution under vigorous agitation for 4 min. The eggs were then washed 3 times with cold 0.1 M NaCl solution. The eggs were allowed to hatch in M9 medium at 20°C for 22 h. The arrested L1 larvae were subsequently used for RNAi or lifespan experiments."

"Nematodes were transferred as L1s to IPTG (1 mM) containing NGM plates seeded with the respective dsRNA expressing E. coli strains (J. Ahringer, University of Cambridge, UK). 20 nematodes were used per 3 cm plate and five replica were used for each condition totalling 100 nematodes altogether. Nematodes were passaged regularly to fresh RNAi plates to separate the animals from their progeny. One parallel was heat shocked on day 1 (12 h post hatching) for 1 h at 35°C and subsequently shifted back to 20°C for the remaining time course of the experiment. The number of living animals was scored each day. Animals were scored as dead if they did not respond to gentle prodding on the head and subsequently removed from the plate."

3rd Editorial Decision 17 August 2012

Thank you for sending us your revised manuscript. Referee 3 has now seen it again, and you will be pleased to learn that in his/her view you have now addressed all criticisms in a satisfactory manner.

Prior to formal acceptance, a number of statistical issues need further attention:

* Based on the figure legends, a number of panels are based on two independent experiments (n=2):

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part of figures 3B, 5, S1, S2, S3

In the light of this low sample number, we need to ask you to either show one representative experiment and to indicate this in the figure legend or to use an alternative presentation for the data that shows the two individual values rather than the average.

* In figure 4D, last column, there is no error bar.

* Please use a comparable presentation in supplementary figure S1C: one representative experiment should be shown for all samples and indicated in the legend. That there are repeats for Apg1, Apg2 and Hsp105 could also be mentioned in the legend.

* Figure S2: It is not fully clear which panels are based on n=2 and n=3 (please see inconsistencies in the legend)

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your kind cooperation.

Yours sincerely

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #3 (Remarks to the Author):

The authors have addressed my queries in an adequate manner.

2nd Revision - authors' response 26 August 2012

Thank you for the very positive news on our revised manuscript (EMBOJ-2012-82177R) entitled: "Metazoan Hsp70 machines use Hsp110 to power protein disaggregation". We have made all changes to figures that you requested and feel that the manuscript is now in excellent shape.

Specifically, we now show one representative dataset in those instances where experiments were performed only twice, and indicate this fact accordingly in the legends. Figure 4D which was suboptimal in terms of repeats has been replaced by a more representative dataset that focuses on higher, inhibitory concentrations of Apg2. Because we were recently able to overcome a technical issue that previously caused us to lose information about higher-density fractions in the aggregate characterization (Supp. Fig. 2A), we have included a panel that characterizes the aggregates more accurately, includes additional information about the distribution of Hsp26 in the gradient, and therefore substantially improves the quality of the information obtained from this experiment. The general conclusion that luciferase aggregates formed at 2 μM migrate more deeply into the gradient remains the same. Also, we have corrected the inconsistencies in the legend of Supp. Fig. 2.
We would like to opt out of the source data initiative.

With these changes to our revised manuscript, we have complied with the reviewersí suggestions as well as with EMBO Journal standards and therefore hope that our manuscript will soon be accepted for publication.

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