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Opposing roles of Ubp3-dependent deubiqutination regulates replicative life span and heat resistance

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Submission date:	06 September 2013
Preliminary Decision:	15 October 2013
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Editor: Hartmut Vodermaier

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.)

Preliminary Decision 15 October 2013

Thank you for submitting your manuscript on Ubp3 in life spans and heat resistance regulation for our editorial consideration, and please excuse the delay in getting back to you with an editorial decision. Three expert referees had agreed to review your study, but so far we have - despite multiple email reminders and phone calls - only received the reports from two of them. Since these two reports are overall in fair agreement regarding this work's principle interest and potential suitability for The EMBO Journal, I decided - in the interest of time - to contact you now with a preliminary decision and invitation to revise the manuscript based on the comments of the first two referees. As the specific points raised by the referees appear generally well-taken and self-explanatory, I will not go through them in detail here, but still need to point out that it is EMBO Journal policy to allow a single round of revision only, and that it is thus essential that you completely answer the points raised if you wish the manuscript ultimately to be accepted. I should furthermore stress that this remains a preliminary decision and thus still subject to change should the outstanding third report still come in and bring up serious additional concerns. In case we should still receive this last report, I will forward it to you and finalize/confirm my decision.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

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REFEREE REPORTS:

Referee #1 :

In this interesting study Oeling et al. investigated the function of the ubiquitin hydrolase Ubp3 in Hsp70 deficient yeast cells and in replicative aging. They confirm results from the Craig laboratory that overexpression of Ubp3 suppresses the ts growth defect of ssa1 /ssa2 cells. In a series of elegant experiments, using proteasome impaired and proteasome activated strains, they conclude that Ubp3 functions by rescuing proteins from (premature) degradation, thereby increasing their functional activity. Interestingly, this differs from the role of Ubp3 in the removal of juxtanuclear protein aggregates and in extending replicative life span where Ubp3 enhances protein degradation via the proteasome pathway.

At this point this well executed study describes a series of interesting phenomena but presents only limited insight into the underlying mechanisms, although certain likely scenarios are proposed. Nevertheless, the paper provides valuable information on the organization of the proteostasis system and should be of interest to the readers of EMBO J after adequate revisions to address the following questions:

1. The action of Ubp3 overexpression in ssa1 /ssa2 cells should be clarified. How are unfolded/misfolded proteins stabilized in these cells? The authors excluded that Ssa4 is induced upon Ubp3 overproduction, but a possible role of Ssa3 should also be addressed.
2. The mechanistic basis for how Ubp3 determines different fates of misfolded proteins (degradation or refolding) remains unclear. Apparently, these different effects depend on the ubiquitin hydrolase activity of Ubp3? How is the ubiquitin status of proteins like ssCPY and Ubc9 ts modulated by Ubp3?
3. Does overexpression of other Ubp proteins have a similar effect or are the observations specific for Ubp3?

Minor points:
1. Error bars are missing in Fig.1 H.
2. Details of protein identification in 2D gel electrophoresis should be included in methods.
3. Error bars are missing in Fig. 5D.
6. Reuse of RLS data from Fig.3 in Fig. 7 should be indicated in figure legend.

Referee #2 :

This manuscript by Öling et al concerns the role of the deubiquitinating enzyme Ubp3 in cellular function, and the affect a reduction or increase in its activity has when other cellular processes are disrupted. How deubiquitination plays into the overall cellular "proteostasis" is a complex question; there are many pieces of data in the literature that are on the surface contradictory. This is a careful analysis and adds significantly to our understanding of the role(s) of deubiquitination.

Points:

1. As noted by the authors, there has been confusion in the literature concerning whether Ubp3 action results in stabilization or enhanced degradation of proteasome substrates. A strength of this manuscript is the analysis of two substrates that are affected in opposite directions (ubc9ts and a CPY derivative). However, the manuscript would be further strengthened by the testing of a couple of other proteins in the same assays that reported previously to be affected by deletion of UBP3 (as mentioned in the discussion).

2. The Ssa2-GFP fusion is used extensively. What evidence do the authors have that this fusion is fully functional? For example, can it rescue the phenotype of the ssa1 ssa2 strain (or even better the ssa1 ssa2 ssa4 strain)?

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3. Some mention about the distribution of replicative lifespan between experiments in figure 1 and those in 2. It appears as if the population (before heat stress) in Fig 1b has 1% or less cells with Ssa2-GFP aggregates, but in Fig 2b, after 6-10 generations ~ 15% of cells have aggregates, with more at later generations. What is the average generation # in the mixed population in figure 1?

4. Can the authors comment on the possible characteristics that may be the cause of the differential affect of changing Ubp3 expression on stabilization/destabilization of proteins?

5. Since this work spans more that the field of proteolysis, this reviewer suggests that the authors think about the abstract and spend a bit of time trying to put forth the main ideas in a way that non experts in the field can better appreciate.

1st Editorial Decision 25 October 2013

We have now received the third and final report on your recent submission, copied below for your information. As you will see, referee 3 also considers your findings of interest in principle, but at the same time raises some well-taken major concerns that in my view would appear important to be experimentally addressed. In light of these comments, I would like to finalize my preliminary earlier decision, and confirm that we would be able to consider a revised manuscript further for publication, in case you should be able to satisfactorily address the key points raised by all three referees. Once more, please be reminded that we only permit a single round of major revision, but we would be open to extending the revision deadline in case this should be needed for adequately addressing the referees' comments. Should you have any questions related to this decision or the referee reports, please do not hesitate to contact me.

Referee 3

This is a very interesting manuscript exploring previous data indicating that overexpression of Ubp3, a DUB, can suppress the loss of function of the major cytoplasmic Hsp70s, SSA1-4. The manuscript uses two reporter proteins Ubc9ts and CPY* to conclude that Ubp3 can promote the degradation of some proteins and antagonize the degradation of others. These observations had already been partially reported in the literature, but this study has a more global profound perspective, as it attempts to place these findings into the context of aging and stress.

While overall, the study asks the right questions, in its present form, it is not appropriate for EMBO Journal, as it is very descriptive and doesn't convincingly demonstrate its major conclusions.

-It is unclear if the effects observed for Ubp3 are direct or indirect. One egregious absence is the demonstration that Ubp3 affects ubiquitination of proteins in general and most importantly, the ubiquitination of the reported substrates employed here.
Thus, a key piece in the argument in this story is that Ubp3 deubiquitinates proteins misfolded in Hsp70s absence, but this is not shown anywhere in the manuscript.

- The finding that the same DUB could mediate proteasomal degradation of one protein while stabilizing another is counter-intuitive. An issue with the study is that these two conclusions are arrived by testing degradation in two different ways: for Ubc9 by following GFP fluorescence and for CPY* by western blot and a viability assay. Are the same conclusions maintained if both proteins are analysed through the same assays?

I do not recommend publication in EMBO until a more mechanistic and solid backing supports the study's conclusions.

Additional comments:

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• It is implied that Ubp3 can reduce toxicity due to heat stress by removing ubiquitin chains from proteins targeted for degradation to allow them an opportunity to fold. Is this mostly true in the short term? Is Ubp3 equally protective during long term stress or repeated stress? At some point, shunting protein quality control away from proteasomal degradation may have deleterious effects to the cell.
• First paragraph, first sentence. "We hypothesized that Ubp3 might be required for efficient management of misfolded proteins accumulating in aggregates/inclusions". What specific roles were hypothesized that Ubp3 could play in aggregate formation?
• Figure 1. It would be nice to see the stability of the substrate by Western pulse- chase analysis. Although number of aggregates can be counted over time, the aggregates appear to change in morphology and it is not possible to gauge total levels of substrate over time by the imaging shown.
• Ssa2p-GFP: the C-terminus of Hsp70 is functionally important: does the GFP C-terminal fusion complement the null?
• In previous studies it was shown that Ubp3 overexpression promotes aggregation of Ubc9ts: how does this fit into the story presented here?
• Can number of aggregates and altered biogenesis of aggregates in general correlate with shortened lifespan or could this observation be substrate-specific?
• Figure 3E: y-axis should read "% cells containing GFP-Ubc9ts in JUNQ"
• Figure 4A: An interesting parameter seems to be missing...did the authors try overexpressing UBP3 in delta rpn4 cells?
• In the absence of Hsp70s, which pathways may mediate re-folding of substrates when Ubp3 is overexpressed? Do substrates re-gain solubility or function? Perhaps the authors could try a few additional substrates such as luciferase.
• The aging and heat stress stories should be interwoven more. Perhaps more experiments could be shown or more literature could be reviewed to draw connections between the state of the cell during heat shock and that during aging.

1st Revision - authors' response 12 January 2014

Referee #1:

In this interesting study Oeling et al. investigated the function of the ubiquitin hydrolase Ubp3 in Hsp70 deficient yeast cells and in replicative aging. They confirm results from the Craig laboratory that overexpression of Ubp3 suppresses the ts growth defect of ssa1Δ/ssa2Δ cells. In a series of elegant experiments, using proteasome impaired and proteasome activated strains, they conclude that Ubp3 functions by rescuing proteins from (premature) degradation, thereby increasing their functional activity. Interestingly, this differs from the role of Ubp3 in the removal of juxtanuclear protein aggregates and in extending replicative life span where Ubp3 enhances protein degradation via the proteasome pathway.

At this point this well executed study describes a series of interesting phenomena but presents only limited insight into the underlying mechanisms, although certain likely scenarios are proposed. Nevertheless, the paper provides valuable information on the organization of the proteostasis system and should be of interest to the readers of EMBO J after adequate revisions to address the following questions:

1. The action of Ubp3 overexpression in ssa1Δ/ssa2Δ cells should be clarified. How are unfolded/misfolded proteins stabilized in these cells? The authors excluded that Ssa4 is induced upon Ubp3 overproduction, but a possible role of Ssa3 should also be addressed.

We have now included data on Ssa3 as requested. We show that Ubp3 overproduction is effectively suppressing heat sensitivity also of a triple ssa1/ssa2/ssa3 deletion mutant (see below; figure added as figure 4G) demonstrating that Ssa3 is not required for Ubp3-dependent suppression of Hsp70 deficiency. In addition, we used Q-PCR to test if the expression of Ssa3 is elevated by Ubp3 overproduction and found that this was not the case (data included as figure 4H). We have also

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performed IP experiments and show that the stabilization of unfolded ΔssCPY protein by Ubp3 overproduction is accompanied by a reduction in CPY* ubiquitination, which support the notion that this protein is diverted away from proteolysis by the removal of ubiquitin (data added in figure 6, see also point 2 below).

2. The mechanistic basis for how Ubp3 determines different fates of misfolded proteins (degradation or refolding) remains unclear. Apparently, these different effects depend on the ubiquitin hydrolase activity of Ubp3? How is the ubiquitin status of proteins like ΔssCPY and Ubc9 ts modulated by Ubp3?

In view of previous reports (Kvint et al, 2008; Mao & Smerdon, 2010) and the data presented herein about DssCPY* species and Ubc9^{ts}, we hypothesized that Ubp3-dependent de-ubiquitination of substrates may act at different stages towards destruction by the proteasome. If de-ubiquitination occurs prior to a stage of final "commitment" to destruction, the protein is saved from proteolysis ('rescue pathway') whereas de-ubiquitination at the "committed" stage promotes destruction, e.g. through facilitating entry into the proteasomal cavity ('destruction pathway'; (Verma et al, 2002; Yao & Cohen, 2002); Fig. 6A). The predictions of this model are that in the rescue pathway, substrates (like DssCPY* species) would display lower ubiquitin levels in cells (e.g. *ssa1*Δ *ssa2*Δ cells) overproducing Ubp3, while being rapidly committed and degraded in cells lacking Ubp3. In the destruction pathway however, substrates (like Ubc9^{ts}) would become more ubiquitinated in *ubp3D* cells and thus saved from proteolysis, while being rapidly degraded in cells overexpressing Ubp3 and becoming undetectable.
To test these predictions, we purified DssCG* and Ubc9^{ts} by immunoprecipitation and analyzed their ubiquitin status in the different genetic backgrounds used. This analysis demonstrated that DssCG* displayed a reduced ubiquitin status in the Ubp3-overproducing strain, which is consistent with markedly higher levels of the protein in this strain background. In the *ubp3*Δ mutant, there was very little DssCG* compared to the wild type strain, most likely due to the fact the ubiquinated DssCG* protein was rapidly destroyed (Fig. 5B and 6B). In contrast, the ubiquitin status of Ubc9^{ts} was markedly elevated in a *ubp3*Δ mutant (which counteracted degradation) while no significant difference could be observed in the Ubp3-overproding strain, which facilitated degradation (Fig. 6C). These data are consistent with Ubp3 being involved in the de-ubiquitination of both DssCG* and Ubc9^{ts} but that the outcome of this is dependent on the substrate and its stage towards destruction. See new paragraph in the Result section. Also, these observations are discussed in the Discussion section.

3. Does overexpression of other Ubp proteins have a similar effect or are the observations specific for Ubp3?

We agree that this is an interesting question but find it unrealistic to overproduce all the Ubp proteins in the same way as Ubp3 in wild type and *ssa1D ssa2D* double deletion mutants and re-check all the phenotypes displayed by Ubp3. Nevertheless, we have examined the effect of *ubp* deletions on the clearance of Ubc9 aggregates/JUNQ and found that *ubp3*Δ mutants are worse-of than any other *ubp* mutants. However, *ubp4/5* and *6* deletion mutants display a modest but significant reduction in their ability to handle Ubc9^{ts} aggregates. This data is now included in figure S1C. In addition, we tested if other *ubpD* mutants than *ubp3*Δ was required to stabilize ΔssCPY using the ΔssCPY-LEU2 growth test assay. This analysis demonstrated that Ubp2, 6, 8, 14 and 15, like Ubp3 affected ΔssCPY-LEU2 function. The data is presented in figure S2D.

Minor points:
1. Error bars are missing in Fig.1 H.
We thank reviewer for pointing this out. We have now added error bars.

2. Details of protein identification in 2D gel electrophoresis should be included in methods.
This has now been added in MS.

3. Error bars are missing in Fig. 5D.

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We have now performed the stability measurements with both flow cytometry and Western protocols, which yielded the same results. We have added the stability measurements from cytometry (as also requested by Reviewer #3) and added the statistics as requested.

6. Reuse of RLS data from Fig.3 in Fig. 7 should be indicated in figure legend.
This is now corrected.

Referee #2:

This manuscript by Oling et al concerns the role of the deubiquitinating enzyme Ubp3 in cellular function, and the affect a reduction or increase in its activity has when other cellular processes are disrupted. How deubiquitination plays into the overall cellular "proteostasis" is a complex question; there are many pieces of data in the literature that are on the surface contradictory. This is a careful analysis and adds significantly to our understanding of the role(s) of deubiquitination.

Points:

1. As noted by the authors, there has been confusion in the literature concerning whether Ubp3 action results in stabilization or enhanced degradation of proteasome substrates. A strength of this manuscript is the analysis of two substrates that are affected in opposite directions (ubc9ts and a CPY derivative). However, the manuscript would be further strengthened by the testing of a couple of other proteins in the same assays that reported previously to be affected by deletion of UBP3 (as mentioned in the discussion).

We believe that the literature has clearly shown that Ubp3 can have opposite effects on the stability of different proteins so we assume that the confusion referred to relates to how Ubp3 is accomplishing this, rather than that the literature includes conflicting data. In view of previous reports (Kvint et al, 2008; Mao & Smerdon, 2010) and the data presented herein about DssCPY* species and Ubc9^{ts}, we hypothesized that Ubp3-dependent de-ubiquitination of substrates may act at different stages towards destruction by the proteasome. If de-ubiquitination occurs prior to a stage of final “commitment” to destruction, the protein is saved from proteolysis (‘rescue pathway’) whereas de-ubiquitination at the “committed” stage promotes destruction, e.g. through facilitating entry into the proteasomal cavity (‘destruction pathway’; (Verma et al, 2002; Yao & Cohen, 2002); Fig. 6A). The predictions of this model are that in the rescue pathway, substrates (like DssCPY* species) would display lower ubiquitin levels in cells (e.g. *ssa1*Δ *ssa2*Δ cells) overproducing Ubp3, while being rapidly committed and degraded in cells lacking Ubp3. In the destruction pathway however, substrates (like Ubc9^{ts}) would become more ubiquitinated in *ubp3D* cells and thus saved from proteolysis, while being rapidly degraded in cells overexpressing Ubp3 and becoming undetectable.
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We are not fully convinced that adding experiments on the other substrates mentioned in the literature would clarify matters because these studies deals with different conditions than the ones used herein, i.e. heat shock in Hsp70-deficient cells. Nevertheless, we have tested two other substrates, Rpb1 and Rad4 previously reported to be affected by Ubp3. We found that Rpb1,

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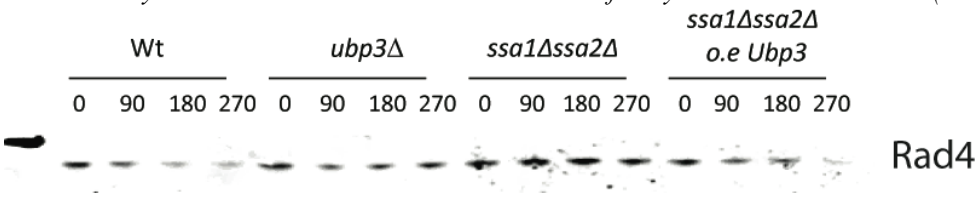
previously shown to be stabilized by Ubp3 during UV stress and transcriptional pausing, was stable during a heat shock in both wild type and *ssa1/2* mutant cells (with and without Ubp3 overproduction; see figure below). Rad4 on the other hand behaved like Ubpc9^{ts}; i.e. the protein was stabilized in the absence of *UBP3* (see figure below). We are not certain that these experiments/data significantly improves the manuscript and would therefore need editorial advice as to whether the data set should be included or not in the MS.

Rpb1-stability in indicated strains, temperatures and minutes after cycloheximide was added (min 0).

Rad4-stability at 30 C in indicated strains and minutes after cycloheximide was added (min 0).



2. The Ssa2-GFP fusion is used extensively. What evidence do the authors have that this fusion is fully functional? For example, can it rescue the phenotype of the ssa1 ssa2 strain (or even better the ssa1 ssa2 ssa4 strain)?

This is a vital question and we should have added this information from the beginning – thanks for bringing it up! As now shown in supplementary Figure S1A, an *ssa1*Δ*/ssa4*Δ*/ssa2-GFP* is fully viable whereas *ssa1/2/4* deletion strains are dead (Werner-Washburne et al, 1987). Thus, Ssa2-GFP rescues the phenotype. In addition, an *ssa1*Δ*/ssa2*Δ*/ubp3*Δ strain is also dead and, again, Ssa2-GFP rescues this phenotype.

3. Some mention about the distribution of replicative lifespan between experiments in figure 1 and those in 2. It appears as if the population (before heat stress) in Fig 1b has 1% or less cells with Ssa2-GFP aggregates, but in Fig 2b, after 6-10 generations ~ 15% of cells have aggregates, with more at later generations. What is the average generation # in the mixed population in figure 1?

The mixed population is growing exponentially so 50% of the cells are new-born daughter cells (zero generation), 25% are 1-generation mothers, 12.5% are 2-generation mothers and so on. We have added info on this in the figure legend as requested.

4. Can the authors comment on the possible characteristics that may be the cause of the differential affect of changing Ubp3 expression on stabilization/destabilization of proteins?

Yes, see response to point 1.

5. Since this work spans more that the field of proteolysis, this reviewer suggests that the authors think about the abstract and spend a bit of time trying to put forth the main ideas in a way that non experts in the field can better appreciate.

We tried to clarify this as suggested.

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Referee #3:
This is a very interesting manuscript exploring previous data indicating that overexpression of Ubp3, a DUB, can suppress the loss of function of the major cytoplasmic Hsp70s, SSA1-4. The manuscript uses two reporter proteins Ubc9ts and CPY to conclude that Ubp3 can promote the degradation of some proteins and antagonize the degradation of others. These observations had already been partially reported in the literature, but this study has a more global profound perspective, as it attempts to place these findings into the context of aging and stress.*

While overall, the study asks the right questions, in its present form, it is not appropriate for EMBO Journal, as it is very descriptive and doesn't convincingly demonstrate its major conclusions.

-It is unclear if the effects observed for Ubp3 are direct or indirect. One egregious absence is the demonstration that Ubp3 affects ubiquitination of proteins in general and most importantly, the ubiquitination of the reported substrates employed here.
Thus, a key piece in the argument in this story is that Ubp3 deubiquitinates proteins misfolded in Hsp70s absence, but this is not shown anywhere in the manuscript.

In view of previous reports (Kvint et al, 2008; Mao & Smerdon, 2010) and the data presented herein about DssCPY* species and Ubc9^{ts}, we hypothesized that Ubp3-dependent de-ubiquitination of substrates may act at different stages towards destruction by the proteasome. If de-ubiquitination occurs prior to a stage of final "commitment" to destruction, the protein is saved from proteolysis ('rescue pathway') whereas de-ubiquitination at the "committed" stage promotes destruction, e.g. through facilitating entry into the proteasomal cavity ('destruction pathway'; (Verma et al, 2002; Yao & Cohen, 2002); Fig. 6A). The predictions of this model are that in the rescue pathway, substrates (like DssCPY* species) would display lower ubiquitin levels in cells (e.g. *ssa1*Δ *ssa2*Δ cells) overproducing Ubp3, while being rapidly committed and degraded in cells lacking Ubp3. In the destruction pathway however, substrates (like Ubc9^{ts}) would become more ubiquitinated in *ubp3D* cells and thus saved from proteolysis, while being rapidly degraded in cells overexpressing Ubp3 and becoming undetectable.
To test these predictions, we purified DssCG* and Ubc9^{ts} by immunoprecipitation and analyzed their ubiquitin status in the different genetic backgrounds used. This analysis demonstrated that DssCG* displayed a reduced ubiquitin status in the Ubp3-overproducing strain, which is consistent with markedly higher levels of the protein in this strain background. In the *ubp3*Δ mutant, there was very little DssCG* compared to the wild type strain, most likely due to the fact the ubiquinated DssCG* protein was rapidly destroyed (Fig. 5B and 6B). In contrast, the ubiquitin status of Ubc9^{ts} was markedly elevated in a *ubp3*Δ mutant (which counteracted degradation) while no significant difference could be observed in the Ubp3-overproding strain, which facilitated degradation (Fig. 6C). These data are consistent with Ubp3 being involved in the de-ubiquitination of both DssCG* and Ubc9^{ts} but that the outcome of this is dependent on the substrate and its stage towards destruction. See new paragraph in the Result section. Also, these observations are discussed in the Discussion section.

- The finding that the same DUB could mediate proteasomal degradation of one protein while stabilizing another is counter-intuitive. An issue with the study is that these two conclusions are arrived by testing degradation in two different ways: for Ubc9 by following GFP fluorescence and for CPY by western blot and a viability assay. Are the same conclusions maintained if both proteins are analysed through the same assays?*

We agree with the reviewer that the same method should be used and we have now performed the stability measurements with both flow cytometry and Western protocols, which yielded the same results. We have added the stability measurements from cytometry in the main paper (now in figure 5D) and added the statistics as requested.

Additional comments:

• It is implied that Ubp3 can reduce toxicity due to heat stress by removing ubiquitin chains from proteins targeted for degradation to allow them an opportunity to fold. Is this mostly true in the

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short term? Is Ubp3 equally protective during long term stress or repeated stress? At some point, shunting protein quality control away from proteasomal degradation may have deleterious effects to the cell.

This is an excellent point and we believe this might be part of the reason for why there is a difference in the demands of protein quality control between heat stress and aging. The former would exemplify a sudden stress while protein aberrancies, presumably, are generated in a more progressive fashion during aging, which, in fact, can be retarded by elevating proteasome activity. Also, as shown in the MS, the lack of Ubp3 accelerates the formation of protein inclusions and the subsequent accumulation also of small peripheral aggregates. We have now included a discussion on this topic in the Discussion section.

• *First paragraph, first sentence. "We hypothesized that Ubp3 might be required for efficient management of misfolded proteins accumulating in aggregates/inclusions". What specific roles were hypothesized that Ubp3 could play in aggregate formation?*

Since Ubp3 is a known ubiquitin protease and ubiqutination has been shown to be required for directing misfolded proteins to JUNQ inclusion bodies rather than IPODs, we hypothesized that Ubp3 might determine the ratio between JUNQ and IPODs during misfolding stress. We have added this note in the text.

• *Figure 1. It would be nice to see the stability of the substrate by Western pulse-chase analysis. Although number of aggregates can be counted over time, the aggregates appear to change in morphology and it is not possible to gauge total levels of substrate over time by the imaging shown.*

We have now included data on total GFP-Ubc9ts levels over time in wt and *ubp3D* null measured by flow cytometry (Fig. S2C).

• *Ssa2p-GFP : the C-terminus of Hsp70 is functionally important: does the GFP C-terminal fusion complement the null?*

Yes, we have added data that shows such complementation. As now shown in supplementary Figure S1A, an *ssa1Δ/ssa4Δ/ssa2-GFP* is fully viable whereas *ssa1/2/4* deletion strains are dead (Werner-Washburne et al, 1987). In addition, a *ssa1Δ* Ssa2-GFP strain is not heat sensitive, nor is it slow growing at 30 C. Thus, Ssa2-GFP rescues the phenotype. In addition, an *ssa1Δ/ssa2Δ/ubp3Δ* strain is also dead and, again, Ssa2-GFP rescues this phenotype.

• *In previous studies it was shown that Ubp3 overexpression promotes aggregation of Ubc9ts: how does this fit into the story presented here?*

We believe this is a misunderstanding. It has been shown that Ubp4 promotes Ubc9ts aggregation (Kaganovich et al, 2008) but we cannot find any such data for Ubp3 overproduction in the literature.

• *Can number of aggregates and altered biogenesis of aggregates in general correlate with shortened lifespan or could this observation be substrate-specific?*

This is an interesting question but we do not have an answer to this yet. We believe that there is a correlation between lifespan and the occurrence of smaller aggregates but more data is needed to make a firm connection between the two. Also, the smaller age-related aggregates observed in this study are detected using Hsp70-GFP reporters of indigenously aggregating proteins making it difficult to say anything about substrate specificity.

• *Figure 3E: y-axis should read "% cells containing GFP-Ubc9ts in JUNQ"*

Yes, we agree and have corrected this.

• *Figure 4A: An interesting parameter seems to be missing...did the authors try overexpressing UBP3 in delta rpn4 cells?*

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We have now done the experiment requested and added the data in figure 4A. Overexpression of Ubp3 did not alter proteasome levels in the *rpn4D* mutant. Also, overexpression of Ubp3 did not alter proteasome levels in *RPN4*$^{+}$ cells (figure 4A).

• *In the absence of Hsp70s, which pathways may mediate re-folding of substrates when Ubp3 is overexpressed? Do substrates re-gain solubility or function? Perhaps the authors could try a few additional substrates such as luciferase.*

In the absence of Ssa1/Ssa2 there is still residual Hsp70 activity from Ssa4/Ssa3 and even though the levels of these proteins are not elevated by Ubp3 overproduction we assume that they provide residual folding activity. Thus, as in the Craig model, when ubiquitin is removed and the protein stabilized, this allows time for this residual Hsp70 activity to fold the protein. Thus, we do not believe that any non-canonical folding pathway needs to be hypothesized to explain these results. In this context, using luciferase in a folding assay is tricky because of its severe instability and that protease inhibitors need to be added in the folding assay. Thus, the assay we tell us if cells with elevated Ubp3 levels display an elevated folding activity, which we do not expect since Ssa4 and Ssa3 levels were not elevated. This is indeed what we found when performing this assay, i.e. heat-denatured luciferase is refolded at the same rate in *ssa1D ssa2D* with and without Ubp3 overexpression (the data is included in the section dealing with whether Ssa4 and Ssa3 overproduction could explain Ubp3 suppression).

• *The aging and heat stress stories should be interwoven more. Perhaps more experiments could be shown or more literature could be reviewed to draw connections between the state of the cell during heat shock and that during aging.*

This is a great idea and we have extended the discussion on this as suggested.

Kaganovich D, Kopito R, Frydman J (2008) Misfolded proteins partition between two distinct quality control compartments. *Nature* **454:** 1088-1095

Kvint K, Uhler JP, Taschner MJ, Sigurdsson S, Erdjument-Bromage H, Tempst P, Svejstrup JQ (2008) Reversal of RNA polymerase II ubiquitylation by the ubiquitin protease Ubp3. *Mol Cell* **30:** 498-506

Mao P, Smerdon MJ (2010) Yeast deubiquitinase Ubp3 interacts with the 26 S proteasome to facilitate Rad4 degradation. *J Biol Chem* **285:** 37542-37550

Verma R, Aravind L, Oania R, McDonald WH, Yates JR, 3rd, Koonin EV, Deshaies RJ (2002) Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* **298:** 611-615

Werner-Washburne M, Stone DE, Craig EA (1987) Complex interactions among members of an essential subfamily of hsp70 genes in Saccharomyces cerevisiae. *Mol Cell Biol* **7:** 2568-2577

Yao T, Cohen RE (2002) A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature* **419:** 403-407

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Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Referee #1
The authors have satisfactorily addressed the points raised by this reviewer.

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