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Proteotoxic stress and ageing triggers the loss of redox homeostasis across cellular compartments

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(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.)

Editor: Anne Nielsen

1st Editorial Decision 25 August 2014

Thank you for submitting your manuscript for consideration by The EMBO Journal and my apologies for the slight delay in communicating our decision to you. Your study has now been seen by four referees whose comments are shown below. As you will see, while the referees all express interest in the work and topic in principle, they all do not offer strong support for publication in The EMBO Journal - at least at this stage of analysis.

I will not repeat all their individual points of criticism here, but it becomes clear that the referees raise a number of technical concerns - especially with regard to the statistical analysis conducted - that question the validity of the conclusions drawn in the study. In addition, referee #4 expresses concern about the sensitivity and localization of the GFP-reporters used. In our view, these are serious concerns of an unclear outcome that could potentially undermine the entire study, and we therefore have little choice but to conclude that we cannot invite a revised manuscript at the current stage.

However, if you were to fully address the technical concerns raised by all four referees - and if the conclusion of the study remains intact - we would be happy to consider a new submission of this work at a later stage. One thing I need to mention, though, is that both refs #2 and #3 - in addition to the technical concerns - are concerned about the descriptive nature of the work and specifically mention that further-reaching mechanistic insight would be required for this manuscript to be a strong candidate for publication in The EMBO Journal.

Given these additional concerns, I have therefore taken the liberty to discuss both your manuscript and the referee reports with my colleagues at EMBO reports and they would be happy to consider a new version of the manuscript addressing the technical issues and control experiments only. For the revised study to be a candidate for publication at The EMBO Journal we would need it to provide additional mechanistic and functional insight, as exemplified by the request from ref#2 to expand the analysis to a number of well-established worm models for aging, ER stress and proteostasis.

In summary, I am afraid that we are unable to make a direct commitment to publication at the current stage, but if you were to significantly extend the work along the lines described above we would be happy to consider a new version for either EMBO Reports or The EMBO Journal.

I am sorry that I cannot be more positive at this stage, but I hope nevertheless that you will find our referees' comments helpful in deciding the future strategy for this work. Thank you for giving us the opportunity to consider this manuscript and please feel free to contact me with any questions.

REFEREE COMMENTS

Referee #1:

In this manuscript, the authors describe the interesting phenomenon that challenges in proteostasis induce cross-compartimental changes in redox homeostasis. The authors come to these conclusions by making use of two different in vivo redox sensors, which are either expressed in the cytosol or the ER of C. elegans or HeLa cells. For both model organisms, the authors show that accumulation of aggregation prone proteins change the redox homeostasis in the ER and cytosol. Interestingly, while the cytosol becomes generally more oxidizing during proteotoxic stress (induced in either the cytosol or the ER), the ER becomes more reducing when aggregation prone proteins are expressed in the cytosol. Even more astounding was the finding that expression of aggregation prone proteins in the muscle cells affect the redox status of neurons (and vice versa), providing further support for trans-tissue signaling.

There are some important aspects that the authors need to consider and address however:

1. The authors need to provide more detail about their image analysis. The authors state in the material and methods part that about 20 worms were imaged per data point - however, how many cells were imaged per worm, and how was the data analysis conducted? Moreover, what did the authors use as internal standard particularly to account for day-to-day variations in the confocal microscope?
2. More statistical analysis is required. Ideally, the authors should show the Hyper/roFGP ratios for each individual worm that they analyzed, conduct a student t-test and list the p-values rather than showing the mean value and including the standard error of the mean. This analysis would be much more accurate and would also reveal whether the expression of Q77-RFP does indeed significantly affect the redox status of the ER of HeLa cells as stated by the authors. This significance does not become obvious the way the data are currently presented.
3. The argument that aggregated proteins change the redox homeostasis of the cell would be more convincing if the authors performed kinetic analyses, in which they correlated the changes in the redox status directly with the appearance of protein aggregates.

Minor comments:
4. The authors might consider removing most of the images - while pretty to look at, they are really not very informative. This is best illustrated in Fig. 5 where it is not clear what we learn from the merged images shown.
5. The authors should consider changing the labeling of their graph in Fig. 5 to illustrate that day 1-day 3 represents different larval stages, while day 4 is in fact day 0/1 of adulthood. This would make the presentation more consistent with other publications in the field, and avoids unnecessary confusion. Also, day 9 of adulthood should not be considered "late stage of aging", given that the median lifespan of worms at 20{degree sign}C is around 10-12 days.

6. The authors might want to cite Back et al, who previously showed already that peroxide levels increase in aging worms using HyPer.

Referee #2:

While it is pretty well established that both protein homeostasis and the oxidation/reduction (redox) environment of the cell play critical roles in disease and age-associated physiological decline, the relationship between the two remains mysterious. Cytosolic proteins become oxidized over time, resulting in dysfunction and cellular damage; the Ubiquitin Proteasome System (UPS) helps keep oxidation in check by removing such oxidized proteins. By contrast, secreted and ER resident proteins require some level of oxidation for disulfide bond formation and thus proper folding; a failure of oxidation in the ER triggers the Unfolded Protein Response (UPR) and its host of chaperones. We don't know if or how the redox state between the ER and the cytosol is coordinated, or how that coordination changes over time or in disease states.

Here, Kirstein et al. have taken advantage of the simple genetics and transparency of C. elegans to monitor the oxidation state of the ER and cytosol using previously published fluorescent reporters (roGFP and HyPer). They show that roGFP and HyPer can be used to measure oxidative state and H2O2 levels in both the ER and cytosol in two different tissues (muscle and neurons) in the worm. They also conduct parallel studies with these reagents in HeLa cells. They suggest that proteotoxic stress in the cytosol (e.g., blocking the proteasome or expressing a known aggegration-prone protein) results in the loss of the ER's reducing environment and an increase in the cytosol's oxidative environment. They suggest that proteotoxic stress in the ER (e.g., by tunicamycin treatment) results in the cytosol becoming more oxidative. They find that these compartments lose their respective redox states in older animals, suggesting that collapse of compartmental redox identify is a hallmark of aging. Finally, they suggest that proteotoxic stress in the cytosol of one tissue (e.g., muscle) can trigger changes in the redox state of another tissue (e.g., neurons), implying the existence of an intercellular signaling mechanism.

The manuscript is tackling an interesting and important set of questions. The authors do a nice job of showing that their reporters are truly reporting redox state, and they establish the dynamics range for each reporter, compartment, and tissue. Some of the experiments are quite convincing. However, there are technical problems that limit the interpretation of their findings and thus the manuscript's overall potential impact on the field. In addition, the paper is rather descriptive: the reader is left with a tantalizing model in which there is coordinated signaling for proteotoxicity and redox state between the ER and the cytosol, but there is no mechanistic insight about how this coordination happens. While the overall topic is a great fit for EMBO, the technical problems and lack of mechanistic insight fall short of what I would expect for an EMBO publication.

My main technical concern is the statistics used to validate the authors' findings. The authors use a Student t-test and consider $p<0.05$ as significant. First, $p<0.05$ is a liberal cut-off for the notoriously forgiving Student t-test; that alone likely causes a rejection of the null hypothesis in several of the presented sets of data. Second, and more worrisome, is the use of the Student t-test to evaluate more than two groups of data. The Student t-test was designed to evaluate two groups of data, but is totally inappropriate for more than two groups of data. More than two groups of data require an adjustment for the multiple pairwise comparisons that can be drawn once the number of groups surpasses two. Indeed, this is the case for the data presented in this manuscript. A more appropriate analysis would require ANOVA followed by a suitable post-hoc test between different groups (e.g., with Bonferoni's correction).

This is not an esoteric issue; it's Stats 101. From my simple examination of the error bars on the graphs throughout the manuscript, I strongly suspect that several data set comparisons would fail an ANOVA combined with a reasonable post-hoc test: Figure 2B (the Q77 column compared to the Q0, Q19, and Q40 columns), Figure 3A (the Q40 column compared to the Q0 column), Figure 4B (the epoxomicin column compared to the control column), Figure 4D (the epoxomicin column compared to the control column), Figure 4E (the epoxomicin column compared to the control column for both tissues), Figure 6A (the Q40 column compared to the Q0 column). Figure 6B (the Q40 column compared to the "muscle roGFP" column), and Figure E4 (the TG column compared to

the control column). Even if some of these comparisons turn out to be statistically significant after an ANOVA analysis, the magnitude of the difference for many of them is extremely subtle (e.g., Figure 2B, there is barely a difference between Q77 and the other Q's in the graph, something exaggerated further by the fact that the authors start numbering the Y axis at "1" rather than "0"). This is not a trivial issue as these comparisons provide the critical support for the authors' model.

Additional concerns:

The authors use false color to show roGFP and HyPer in their two potential states, but they don't ever formally state what color represents each state (the reader is left to guess this on their own). Also, the chosen colors (e.g., I'm guessing green for excitation at 488 nm and blue for excitation at 405 nm for roGFP) make it very difficult to interpret the images in the figures. I would recommend either that the authors present separate side-by-side gray scale images for 405 and 488 excitation wavelengths, or they should use a false color scale that represents the 405/488 image ratio for each picture.

In Figure 1, quantification of the 405/488 and 488/405 ratios for roGFP and HyPer, respectively, are listed, but there are no sem values. The authors should graph these and include sem values.

In Figure 2A, the RFP control gives a 405/488 ratio of about 1.1 for roGFP in the ER, whereas Figure 1A gives a control 405/488 ratio of 1.81 for roGFP in the ER. From these experiments, it seems that we can conclude that expression of cytosolic RFP is triggering a more oxidative state in the ER. How do the authors reconcile this with their model?

Figure 2B, the Qn-mCherry panel for Q19 looks blank (the cells here are not as bright as in other panels).

Figure 3C,D, 405/488 ratios are present for C. elegans expressing HyPer. It's unclear if this is in muscle or neuron.

The authors obtain different results using tunicamycin and thapsigargin to induce the UPR. They propose that thapsigargin might be the giving its result because of its effect on internal calcium homeostasis. It's unclear what they are concluding about ER stress and redox state in the cytosol. Also, I was surprised that they did not look at xbp-1 or ire-1 mutants, which have an impaired UPR and collect unfolded proteins in the ER. Do these mutants have altered redox states in the cytosol?

The examination of redox states over developmental time and aging is a nice touch. However, I was surprised that the authors did not look at a few classic aging mutants to see if this developmental redox profile changes. For example, one model for how insulin signaling regulates lifespan is through its regulation of redox gene expression, among other things. What does the developmental time course of the redox state look like in daf-2 and daf-16 mutants? How about skn-1 mutants, for which there is a clear role in regulating redox states.

The second paragraph of the Discussion section is overly long. The first half is clear: the authors list reasonable explanations for the phenomena they are observing. However, the second half of the paragraph, starting with "Alternatively, it is possible that the redox surveillance system fails..." is nearly inscrutable. I've read that passage over a dozen times, and I'm still not sure what the authors' point is or how that point relates to their findings and their central hypothesis. The authors need to break this paragraph in two and clarify the later section.

Referee #3:

In the manuscript "Loss of redox homeostasis by trans-compartmental stress and ageing" Kirstein J. et al. studied changes of ER and cytosolic redox state under different proteotoxic stress conditions and during ageing as well as the cross-talk between compartments and tissues in terms of redox homeostasis. First, Kirstein and colleagues established redox sensors that monitor changes in general redox state and H2O2 content either in the cytosol or ER of C. elegans muscle cells through

a shift in their excitation spectra. A similar response towards redox homeostasis in the ER und cytosol was shown in HeLa cells. Next, the authors demonstrated that misfolded proteins in the cytosol as well as inhibition of the proteasome lead to a more reduced environment in the ER while the cytosol shift to more oxidative conditions. Furthermore, changes in ER homeostasis by a block of N-linked protein glycosylation results in a more oxidative environment of the cytosol. The authors identified that the redox state in the ER and the cytosol changes in an opposing manner during ageing. While the ER is more oxidative on day 1 with a second peak on day 4 and 5 and decline to more reduced condition during ageing, the cytosolic milieu is more reduced on day 1 with a second peak on day 4 and 5 and decline to more oxidative conditions with time. Next, they proposed that redox homeostasis could change across different tissues. Proteotoxic stress in muscle cells resulted in a shift of the redox state towards oxidative condition in the cytosol of neuronal cells, and the expression of misfolded proteins in neurons shifts the redox homeostasis to more oxidative conditions in the cytosol of muscles cells.

The manuscript describes two new redox sensitive model-substrates in a model organism. Using these substrates identified a so far undiscovered crosstalk between the ER and cytosolic redox homeostasis affected by different proteotoxic conditions and during ageing, which is very interesting for a broad readership. However, the data are mainly descriptive and do not provide mechanistic insight how redox homeostasis might be regulated and coordinated with known proteostasis pathways.

Major points
1) The authors focused on the redox homeostasis in the ER and the cytosol using redox sensors in both compartments. It is important to show that the sensors for ER redox homeostasis (ER-roGFP and ER-HyPerGFP) are correctly localized to the ER, for example by co-localization studies with already known ER residual proteins. For non elegans experts it is not clear what is shown in Fig. 1A and Fig. 1F.

2) In most cases changes in the redox states are shown statistically. In the individual experiments it remains however unclear if these changes are significant. It is necessary to provide these informations in the figures. In general, the redox changes are quite small. Surprisingly, expression of the Q0 form that serves as a control for expression of aggregation prone polyQ proteins causes already a strong change in several experiments (for example Fig. 2B right panel). Compared with Q0, Q77 has a very mild effect, which questions the physiological relevance of this result. Please also explain what is meant with "fully oxidized" and "fully reduced" controls; are this DPS and DTT treated samples?

3) The opposing effects of proteotoxic conditions on redox homeostasis of the cytosol and the ER are mechanistically interesting and suggest a regulated crosstalk between both compartments. It would be important to test if protein aggregation in the ER affects the cytosolic redox state and if proteotoxicity in the cytosol interferes with ERAD.

4) While interesting, the cell-non-autonomous effects of redox homeostasis remained unclear and need further control experiments. Are these effects unc-13 (regulation of neurotransmitter release) or unc-31 (dense core vesicle fusion) dependent?

Minor Points
1) Fig. 2C and Fig. 4E demonstrate the changes in redox state of neuronal cells towards misfolded proteins and proteasome inhibition. However, for a better overview on the properties of this redox-sensors, the authors should demonstrate once their ratio metric characteristics under known oxidative or reduced conditions for example upon treatment with DTT, paraquat or DPS.
2) Fig. 2B right panel should be Fig. 2C
2) Fig. 3D: The block of N-linked protein glycosylation by tunicamycin treatment results in a shift towards oxidative conditions in the cytosol. The authors conclude from this a general cross compartmental change of the redox state in the cytosol in response to proteotoxic stress in the ER. Since the ER-redox sensors do not respond towards tunicamycin, the authors should at least demonstrate their findings using another ER proteostasis drug or by expressing an aggregation prone protein in the ER.
3) Fig3E/D: the authors should label both graphs with C.elegans HyPer-GFP like it was done before.
4) Figure legend of Fig4C and f: the authors should mention the meaning of NT and nt.

5) In Fig. 6B the authors should include a sample that expresses muscle roGFP and neuronal Q0 in an analogues manner to Fig 6A, since the expression of Q0 in neuronal cells on its own already influenced the redox homeostasis in muscle cells. If this is indeed the case the authors should add it to both graph and figure legend.

Referee #4:

Review of EMBO paper-Loss of redox homeostasis by trans compartmental stress and aging.

This manuscript deals with the important question regarding the redox balance in the ER and how this is affected by proteostasis and aging.
The authors use genetically encoded in vivo roGFP sensors in the model organism Caenorhabditis elegans and also compare this to similar sensors expressed in mammalian HeLa cells. The sensors were used to measure changes in the redox status of the ER and cytosol with aging and also provide evidence that redox homeostasis can be regulated across compartments and tissues in C. elegans. While these findings are important there are some major issues concerning the experimental approach, the choice of and validation of the GFP sensors. The validation of the sensor requires major clarification since the results that follow are absolutely dependent on this.

MAJOR CONCERNS
1. It is not entirely clear which version of roGFP was used for the C. elegans analysis and indeed whether this was the most suitable version to examine REDOX changes in the ER. If as stated this was based on roGFP1, namely the version described in the quoted reference (Hanson et al 2004), then this is adapted for the mitochondria and does not have the correct changes required to efficiently report on changes in ER environment. The most suitable roGFP sensor for the ER environment are described in the reference Lohman and Remington (2008) Development of a family of redox-sensitive green fluorescent protein indicators for use in relatively oxidizing subcellular environments. Biochemistry, 47, 8678-8688, PMID:18652491. This key reference has not been quoted, however one of the derived sensor is used in the human cell studies but not in the C. elegans studies.
Indeed, the authors results using ER roGFP1 in mammalian cells FigE2c (the version of the sensor used in their C. elegans studies) support the point that ER roGFP1 is an inappropriate sensor for the ER.

2. Related to this point is the observation that the images in Fig 1A show blobs of fluorescence and it is impossible to establish if they are indeed expressed in the ER. There is a need for higher magnification images, and better still a clear demonstration of ER expression through the co-localization with known ER markers. This point is pertinent since the authors point out that for a different protein, namely Aβ1-42.. "Notably, although Aβ1-42 is fused to a signal sequence for targeting to the ER, it resides mainly in the cytosol (C. Link, personal communication)."
This ER localization confirmation is essential. Also it is not made clear why the muscle cell was selected, since as afar as I can discern muscle is not one of the major secretory organs in C. elegans.

3. Figure 1 A shows the changes in sensor emission ratios following DTT and Paraquat and then compares this data to a Western blot to detect the oxidized and reduced forms of roGFP following DTT and DPS treatment. These studies must be done with the same oxidant- either use paraquat or DPS There is also the distinct possibility that paraquat, being highly toxic, may induce a more general stress response. It would also be pertinent to show the response of UPR sensor hsp-3 to paraquat in addition to DTT as shown in Fig E1A. In addition, it would be informative to see the Western blot image covering the whole gel rather than a low molecular weight slice since aggregation of GFP has previously been reported for roGFP sensors (Birk et al, 2013, J. Cell Sci 126, 1604-17, PMID: 23424194 and Van Lith et al, 2011, J. Cell Sci, 124, 2349-2356, PMID: 21693587).

4. The authors then attempt to validate their results by exposing sensors expressed in HeLa cells to DTT and DPS and measuring ratio changes over time. Why was this type of experiment not carried out in the C. elegans system?

The mammalian cell system is then used extensively throughout the following experiments, however this is not reflected in the manuscript abstract, which focuses exclusively on C. elegans.

5. A large proportion of the experiments in this manuscript are carried out in mammalian cells and therefore it is unusual that the authors failed to quote (Van Lith et al, 2011, J. Cell Sci, 124, 2349-2356,PMID: 21693587) as it covers the robust validation and detailed characterization of roGFP sensors in the ER of mammalian cells.

6. Another technical point relating to the sensor in live C.elegans relates the fact that the authors have used levamisole to immobilize worms prior to viewing muscle cell expressed sensors. This drug specifically affects muscle cell function, therefore controls using non-muscle immobilizing agents should be used to rule out a direct effect of levamisole on the muscle cell expressed roGFP.

Until these major issues are resolved (initial characterization and validation of the roGFP sensors and the use of different oxidants in the assay) it is difficult to assess the potentially interesting conclusions drawn from the experiments that follow.

Minor points
Scale bars in Figure 1 A and C need careful checking.

Resubmission 05 April 2015

Point-by-point response:

Referee #1:

In this manuscript, the authors describe the interesting phenomenon that challenges in proteostasis induce cross-compartimental changes in redox homeostasis. The authors come to these conclusions by making use of two different in vivo redox sensors, which are either expressed in the cytosol or the ER of C. elegans or HeLa cells. For both model organisms, the authors show that accumulation of aggregation prone proteins change the redox homeostasis in the ER and cytosol. Interestingly, while the cytosol becomes generally more oxidizing during proteotoxic stress (induced in either the cytosol or the ER), the ER becomes more reducing when aggregation prone proteins are expressed in the cytosol. Even more astounding was the finding that expression of aggregation prone proteins in the muscle cells affect the redox status of neurons (and vice versa), providing further support for trans-tissue signaling.

There are some important aspects that the authors need to consider and address however:

1. The authors need to provide more detail about their image analysis. The authors state in the material and methods part that about 20 worms were imaged per data point - however, how many cells were imaged per worm, and how was the data analysis conducted? Moreover, what did the authors use as internal standard particularly to account for day-to-day variations in the confocal microscope?

The reviewer asks for more detail on image analysis. In response, we have completely revised the description of the imaging analysis in the Materials & Methods section and have added more information on data processing. Our analysis of the redox state was performed at a single cellular level using a confocal microscope to minimize background and variation of multiple cell layers and tissues. Notably, we did not detect any variation between the same cell type e.g. muscle cells or neuronal cells within the same animal and consequently decided to analyze a minimum of 20 animals. Regarding the internal standard to account for any variations stemming from the confocal microscope, we always included a suitable control set e.g. unstressed, day 4 old nematodes as reference when we performed our experiments. Any variations are reflected in the error bars and we present the raw data of the ratios for each sample including statistical information of the mean, standard deviation and p-value in Table 1.

2. More statistical analysis is required. Ideally, the authors should show the Hyper/roFGP ratios for each individual worm that they analyzed, conduct a student t-test and list the p-values rather than showing the mean value and including the standard error of the mean. This analysis would be much more accurate and would also reveal whether the expression of Q77-RFP does indeed significantly affect the redox status of the ER of HeLa cells as stated by the authors. This significance does not become obvious the way the data are currently presented.

The reviewer asks for more statistical analysis and a presentation of the raw data of the ratios for both HyPer and roGFP. As noted before, in the revised manuscript we are presenting the ratios for each sample in Table 1. We have also expanded our statistical evaluation by performing either pair-wise ANOVA (analysis of variance) or students t-test analyses (depending on the number of samples and experimental set-up). The statistical analyses were followed by a post-hoc Turkey HSD (Honest Significant Difference) test that represents the minimum distance between two groups that must exist to consider the difference between these groups significant. The values of the post-hoc Turkey HSD tests are displayed together with the ANOVA results in Table 1.
In addition, we have added two additional polyQ proteins (HttQ22 and HttQ134) for the analyses conducted in HeLa cells that are shown in Figs. 2B and 3B. We can now clearly demonstrate that the expression of aggregation-prone polyQ proteins e.g. HttQ134 correlates with a change of the redox state in the ER and the cytosol.

3. The argument that aggregated proteins change the redox homeostasis of the cell would be more convincing if the authors performed kinetic analyses, in which they correlated the changes in the redox status directly with the appearance of protein aggregates.

The reviewer asks for a kinetic analysis of the correlation between protein aggregates and the redox state. We have addressed this by analyzing the effect of polyQ and HttpolyQ proteins with an increasing polyQ stretch. The pathogenic threshold length of polyQ is 40 in muscle and neuronal cells of C. elegans and more than 40 in HeLa cells (see our revised Figures 2B and 3B). Through the addition of the longer and more aggregation-prone polyQ stretch of 134 glutamine residues, we can now convincingly demonstrate the tight correlation of protein aggregation and changes in the redox states also in HeLa cells.

Minor comments:

4. The authors might consider removing most of the images - while pretty to look at, they are really not very informative. This is best illustrated in Fig. 5 where it is not clear what we learn from the merged images shown.

We would prefer to retain these images as they aid in conveying the message of substantial fluctuations of the redox state in response to proteotoxic stress and during ageing. We have however improved the data presentation by accompanying also the raw data on the ratios for each sample, time point and conditions, etc. All imaging data are also presented as graph as part of the same figure. In addition, we indicated more clearly the developmental stages and progression of ageing of the respective time points in Fig 5.

5. The authors should consider changing the labeling of their graph in Fig. 5 to illustrate that day 1-day 3 represents different larval stages, while day 4 is in fact day 0/1 of adulthood. This would make the presentation more consistent with other publications in the field, and avoids unnecessary confusion. Also, day 9 of adulthood should not be considered "late

stage of aging", given that the median lifespan of worms at 20{degree sign}C is around 10-12 days.

The reviewer suggests to change the labeling of the graph in Fig 5. We have revised the labeling to indicate the developmental stages (larval stages, young adults and aging nematodes). However, we kept the labeling of day 1 representing day 1 old nematodes, as we somehow need to refer to this age as well. Day 4 / 5 old animals are young adults and are labeled as such. Our data cover the period of the median lifespan (cytosol: day 1- day 12 and for the ER: day 1- day 13).

6. The authors might want to cite Back et al, who previously showed already that peroxide levels increase in aging worms using HyPer.

We thank the reviewer for pointing out this missing reference. We have previously only acknowledged Knoefler et al (2012). We have added the reference of Back et al. (2012) in the revised manuscript.

Referee #2:

Review Kirstein and Morimoto

While it is pretty well established that both protein homeostasis and the oxidation/reduction (redox) environment of the cell play critical roles in disease and age-associated physiological decline, the relationship between the two remains mysterious. Cytosolic proteins become oxidized over time, resulting in dysfunction and cellular damage; the Ubiquitin Proteasome System (UPS) helps keep oxidation in check by removing such oxidized proteins. By contrast, secreted and ER resident proteins require some level of oxidation for disulfide bond formation and thus proper folding; a failure of oxidation in the ER triggers the Unfolded Protein Response (UPR) and its host of chaperones. We don't know if or how the redox state between the ER and the cytosol is coordinated, or how that coordination changes over time or in disease states.

Here, Kirstein et al. have taken advantage of the simple genetics and transparency of C. elegans to monitor the oxidation state of the ER and cytosol using previously published fluorescent reporters (roGFP and HyPer). They show that roGFP and HyPer can be used to measure oxidative state and H2O2 levels in both the ER and cytosol in two different tissues (muscle and neurons) in the worm. They also conduct parallel studies with these reagents in HeLa cells. They suggest that proteotoxic stress in the cytosol (e.g., blocking the proteasome or expressing a known aggegration-prone protein) results in the loss of the ER's reducing environment and an increase in the cytosol's oxidative environment. They suggest that proteotoxic stress in the ER (e.g., by tunicamycin treatment) results in the cytosol becoming more oxidative. They find that these compartments lose their respective redox states in older animals, suggesting that collapse of compartmental redox identify is a hallmark of
aging. Finally, they suggest that proteotoxic stress in the cytosol of one tissue (e.g., muscle) can trigger changes in the redox state of another tissue (e.g., neurons), implying the existence of an intercellular signaling mechanism.

The manuscript is tackling an interesting and important set of questions. The authors do a nice job of showing that their reporters are truly reporting redox state, and they establish the dynamics range for each reporter, compartment, and tissue.

We thank the reviewer for the positive feedback especially on the validation of our reporters.

Some of the experiments are quite convincing. However, there are technical problems that limit the interpretation of their findings and thus the manuscript's overall potential impact on the field. In addition, the paper is rather descriptive: the reader is left with a tantalizing model in which there is

coordinated signaling for proteotoxicity and redox state between the ER and the cytosol, but there is no mechanistic insight about how this coordination happens. While the overall topic is a great fit for EMBO, the technical problems and lack of mechanistic insight fall short of what I would expect for an EMBO publication.

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This is not an esoteric issue; it's Stats 101. From my simple examination of the error bars on the graphs throughout the manuscript, I strongly suspect that several data set comparisons would fail an ANOVA combined with a reasonable post-hoc test: Figure 2B (the Q77 column compared to the Q0, Q19, and Q40 columns), Figure 3A (the Q40 column compared to the Q0 column), Figure 4B (the epoxomicin column compared to the control column), Figure 4D (the epoxomicin column compared to the control column), Figure 4E (the epoxomicin column compared to the control column for both tissues), Figure 6A (the Q40 column compared to the Q0 column). Figure 6B (the Q40 column compared to the "muscle roGFP" column), and Figure E4 (the TG column compared to the control column). Even if some of these comparisons turn out to be statistically significant after an ANOVA analysis, the magnitude of the difference for many of them is extremely subtle (e.g., Figure 2B, there is barely a difference between Q77
and the other Q's in the graph, something exaggerated further by the fact that the authors start numbering the Y axis at "1" rather than "0"). This is not a trivial issue as these comparisons provide the critical support for the authors' model.

We appreciate the feedback from this reviewer and have completely revised the statistical analysis of our data and have expanded our data sets and feel confident that we have thereby strengthened our findings. We are presenting now all raw data of the ratios for each cell, sample and time point in Table 1 and have performed pairwise ANOVA analysis as well as students t-test (when applicable) as statistical evaluation of the data. The statistical analyses were followed by a post-hoc Turkey HSD test. A p-value of below 0.01 was considered significant. We want to point out that the statistical analyses for all data were evaluated with $p< 0.01$ and most of our data were actually evaluated with $p< 0.001$ (Table 1).
In addition, we have revised the data presentation and the y-axes of the graphs displaying the ratios of HeLa cell samples also start now at 0.

Additional concerns:

The authors use false color to show roGFP and HyPer in their two potential states, but they don't ever formally state what color represents each state (the reader is left to guess this on their own). Also, the chosen colors (e.g., I'm guessing green for excitation at 488 nm and blue for excitation at 405 nm for roGFP) make it very difficult to interpret the images in the figures.

We have added this information in the figure legends and in the Materials and Methods section: "The images resulting upon excitation at 405 nm are false-coloured in blue and those upon excitation at 488 nm in green.".

I would recommend either that the authors present separate side-by-side gray scale images for 405 and 488 excitation wavelengths, or they should use a false color scale that represents the 405/488 image ratio for each picture.

As stated above, in this revised manuscript we present the raw ratio-metric data for each cell, time point and condition in an accompanying table for a better evaluation of the data distribution.

In Figure 1, quantification of the 405/488 and 488/405 ratios for roGFP and HyPer, respectively, are listed, but there are no sem values. The authors should graph these and include sem values.

The reviewer wishes to add a graphical presentation of the data in Figure 1A, C, F and G. We have added graphs for each of them (Figs. E1A-D) and the mean +/-standard deviation values are presented underneath the images (Figs 1 A, C, F and G). In addition the data can be found in Table 1 listing all the raw data of the ratio-metric analyses.

In Figure 2A, the RFP control gives a 405/488 ratio of about 1.1 for roGFP in the ER, whereas Figure 1A gives a control 405/488 ratio of 1.81 for roGFP in the ER. From these experiments, it seems that we can conclude that expression of cytosolic RFP is triggering a more oxidative state in the ER. How do the authors reconcile this with their model?

We thank the reviewer for pointing out this discrepancy and decided to repeat this experiment. The mean value for the redox state in the ER is 1.8 irrespective of a cytosolic expression of RFP (Fig. 1A + 2A).

Figure 2B, the Qn-mCherry panel for Q19 looks blank (the cells here are not as bright as in other panels).

We have repeated the imaging analysis and the new figure is of much better quality and the expression of all polyQ proteins (Q19, Q77, HttQ22 and HttQ134) is clearly visible (Fig. 2B).

Figure 3C,D, 405/488 ratios are present for C. elegans expressing HyPer. It's unclear if this is in muscle or neuron.

We thank the reviewer for pointing out the missing information and have added the information that these images were taken from muscle cells in the corresponding figure legends.

The authors obtain different results using tunicamycin and thapsigargin to induce the UPR. They propose that thapsigargin might be the giving its result because of its effect on internal calcium homeostasis. It's unclear what they are concluding about ER stress and redox state in the cytosol.

The reviewer refers to the connection between ER stress and the redox state in the cytosol. It is established that both reagents induce the UPR^{ER}. We used both, tunicamycin and thapsigargin, to test their effect on the cytosolic as well as ER redox state. We observed that the cytosolic redox state is indeed affected by the drug-induced UPR^{ER}. However, as the reviewer pointed out, we obtained different results using tunicamycin and thapsigargin on the ER redox state. While we did observe a change of the ER redox state towards reducing conditions upon treatment with thapsigargin, we failed to detect a response upon addition of tunicamycin.
To solve this conundrum and along with this reviewer's suggestion (see next paragraph), we examined the effect of ire-1 knockdown on the ER redox state. Depletion of ire-1 is known to induce the UPR^{ER} and we could verify the UPR^{ER} induction using hsp-3::yfp (ER Hsp70) as

reporter (Fig E3). Upon depletion of ire-1, we could indeed observe that a change of the ER redox state towards reducing conditions (Fig. 2D). This observation demonstrates that an imbalance of ER proteostasis leads to perturbation of ER redox. We thank the reviewer for the suggestion to use ire-1 knockdown as a genetic modulation to induce UPR^{ER}.

Also, I was surprised that they did not look at xbp-1 or ire-1 mutants, which have an impaired UPR and collect unfolded proteins in the ER. Do these mutants have altered redox states in the cytosol?

The reviewer suggests employing genetic modifiers to perturb protein-folding conditions in the ER such as ire-1. We thank the reviewer for this suggestion and analyzed the redox state of the cytosol upon knockdown of ire-1. We observed that RNAi-mediated knockdown of ire-1 was indeed associated with a change of the ER redox state towards reducing conditions (Fig. 2D). We also demonstrated that the knockdown of ire-1 indeed causes the induction of UPR^{ER} by analyzing the expression of hsp-3 (ER Hsp70), which is an established marker for the UPR^{ER} in C. elegans (Fig. E3).

The examination of redox states over developmental time and aging is a nice touch.

We thank the editor for the positive feedback.

However, I was surprised that the authors did not look at a few classic aging mutants to see if this developmental redox profile changes. For example, one model for how insulin signaling regulates lifespan is through its regulation of redox gene expression, among other things. What does the developmental time course of the redox state look like in daf-2 and daf-16 mutants? How about skn-1 mutants, for which there is a clear role in regulating redox states.

We thank the reviewer for this suggestion and have examined the redox state of the ER at specific time points in mutants of daf-2 and daf-16 (Fig 5C). The effect of the insulin signaling and lifespan on the redox state in the cytosol has been analyzed before (Knoefler et al., 2012). It was however not known yet, if mutants that either extend or shorten the lifespan affect the redox state of the ER. We have analyzed the ER redox state of day 4, day 6 and day 10 old daf-2 and daf-16 mutants and in control nematodes. We observed that the daf-2 mutants that extend lifespan exhibit a delayed decline in the oxidizing conditions compared to the control animals. Contrary to that, daf-16 mutant animals exhibit a more rapid decline in the oxidizing conditions during ageing (Fig. 5C).

To gain more mechanistic insight into the redox regulation the reviewer suggested analyzing the redox state upon depletion of skn-1. Interestingly, a knockdown of skn-1 displayed a very strong effect on the ER redox state (Fig. 2E). Depletion of skn-1 resulted in a pronounced change towards more reducing conditions in the ER and also correlated with an induction of the UPR^{ER} as demonstrated by the induced expression of the hsp-3 (ER Hsp70) reporter (Fig. E3). This finding further supports our conclusion of a strong correlation between proteostasis imbalances and redox state.
The cytosolic redox state shifted to slightly more oxidizing conditions in response to a knockdown of skn-1 (Fig. 3).

The second paragraph of the Discussion section is overly long. The first half is clear: the authors list reasonable explanations for the phenomena they are observing. However, the second half of the paragraph, starting with "Alternatively, it is possible that the redox surveillance system fails..." is nearly inscrutable. I've read that passage over a dozen times, and I'm still not sure what the authors' point is or how that point relates to their findings and their central hypothesis. The authors need to break this paragraph in two and clarify the later section.

We appreciate the constructive criticism by this reviewer that this section of our discussion can be improved. We have thoroughly revised the discussion in particular to include all new data and we think this made the logical flow clearer.

Referee #3:

In the manuscript "Loss of redox homeostasis by trans-compartmental stress and ageing" Kirstein J. et al. studied changes of ER and cytosolic redox state under different proteotoxic stress conditions and during ageing as well as the cross-talk between compartments and tissues in terms of redox homeostasis. First, Kirstein and colleagues established redox sensors that monitor changes in general redox state and H2O2 content either in the cytosol or ER of C. elegans muscle cells through a shift in their excitation spectra. A similar response towards redox homeostasis in the ER und cytosol was shown in HeLa cells. Next, the authors demonstrated that misfolded proteins in the cytosol as well as inhibition of the proteasome lead to a more reduced environment in the ER while the cytosol shift to more oxidative conditions. Furthermore, changes in ER homeostasis by a block of N-linked protein glycosylation results in a more oxidative environment of the cytosol. The authors identified that
the redox state in the ER and the cytosol changes in an opposing manner during ageing. While the ER is more oxidative on day 1 with a second peak on day 4 and 5 and decline to more reduced condition during ageing, the cytosolic milieu is more reduced on day 1 with a second peak on day 4 and 5 and decline to more oxidative conditions with time. Next, they proposed that redox homeostasis could change across different tissues. Proteotoxic stress in muscle cells resulted in a shift of the redox state towards oxidative condition in the cytosol of neuronal cells, and the expression of misfolded proteins in neurons shifts the redox homeostasis to more oxidative conditions in the cytosol of muscles cells.

The manuscript describes two new redox sensitive model-substrates in a model organism. Using these substrates identified a so far undiscovered crosstalk between the ER and cytosolic redox homeostasis affected by different proteotoxic conditions and during ageing, which is very interesting for a broad readership. However, the data are mainly descriptive and do not provide mechanistic insight how redox homeostasis might be regulated and coordinated with known proteostasis pathways.

Major points

1) The authors focused on the redox homeostasis in the ER and the cytosol using redox sensors in both compartments. It is important to show that the sensors for ER redox homeostasis (ER-roGFP and ER-HyPerGFP) are correctly localized to the ER, for example by co-localization studies with already known ER residual proteins. For non elegans experts it is not clear what is shown in Fig. 1A and Fig. 1F.

We have added an experiment addressing the correct localization of C. elegans ER-roGFP by performing a co-localization analysis of ER-roGFP and an immunofluorescence analysis using antibodies directed against KDEL (Fig. E1E). To demonstrate the correct ER targeting of HeLa ER-roGFPiE we performed a co-localization analysis of ER-roGFPiE and ER-targeted DsRed (Fig E1E). The images depicted in Figure E1E convincingly demonstrate the correct targeting to the ER for C. elegans ER-roGFP and HeLa ER-roGFPiE.

2) In most cases changes in the redox states are shown statistically. In the individual experiments it remains however unclear if these changes are significant. It is necessary to provide these informations in the figures. In general, the redox changes are quite small. Surprisingly, expression of the Q0 form that serves as a control for expression of aggregation prone polyQ proteins causes already a strong change in several experiments (for example Fig. 2B right panel). Compared with Q0, Q77 has a very mild effect, which questions the physiological relevance of this result. Please also explain what is meant with "fully oxidized" and "fully reduced" controls; are this DPS and DTT treated samples?

The reviewer notes that the expression of mCherry alone (Q0) already leads to a strong change in the redox state. This however is not correct. The reference "fully reduced" and fully oxidized" are samples of cells treated with DTT and DPS, respectively and only serve to indicate the dynamic range of the sensor. The Q0 sample serves as control for the longer polyQ stretches. We have however completely revised this experiment and removed the fully oxidized and fully reduced columns (the data can still be found in Table 1) and also used a longer polyQ stretch (HttQ134) that shows more robust aggregation and consequently has a stronger effect on the redox state (revised Fig. 2B). The control for the aggregating HttQ134-mCherry is the non-aggregating HttQ22-mCherry and the soluble Q19-mCherry serves as control for the aggregating Q77-mCherry, respectively.

3) The opposing effects of proteotoxic conditions on redox homeostasis of the cytosol and the ER are mechanistically interesting and suggest a regulated crosstalk between both compartments. It would be important to test if protein aggregation in the ER affects the cytosolic redox state and if proteotoxicity in the cytosol interferes with ERAD.

We thank the reviewer for this suggestion and have addressed the question of whether ER stress leads to changes in the cytosolic redox state by depleting ire-1 and analyzing the redox state in the cytosol (Fig. 3D). Indeed, this cross-compartmental analysis revealed that perturbation of protein folding conditions in the ER affect the redox state of the cytosol (shift towards more oxidizing conditions; Fig. 3D).

4) While interesting, the cell-non-autonomous effects of redox homeostasis remained unclear and need further control experiments. Are these effects unc-13 (regulation of neurotransmitter release) or unc-31 (dense core vesicle fusion) dependent?

The reviewer raises the question whether the signaling of the redox state upon perturbation of proteostasis in a distant tissue is regulated via a neurotransmitter release or dense core vesicle fusion.
The question of the underlying mechanism for the trans-tissue signaling of the redox response is challenging. There have recently been described a number of different cell non-autonomous signal transduction pathways to adjust proteostasis in C. elegans (Prahlad et al., 2008; Durieux et al., 2011; van Oosten-Hawle et al., 2013, Nussbaum-Krammer et al., 2013; Taylor & Dillin, 2013; and reviewed in Taylor et al., 2014).
E.g. besides a neuronal signaling as suggested by this reviewer, the adjustment of the redox state in response to proteotoxic stress in a distal tissue can also be mediated by e.g. transmission of misfolded and aggregated proteins to a neighboring cell or tissue (Nussbaum-Krammer et al., 2013). The proteotoxicity is thus transmitted and the redox homeostasis responds to the intracellular imbalance of proteostasis as shown in Fig. 3A+B. Moreover, it has recently been shown that chaperone levels are adjusted in a trans-tissue manner (van Oosten-Hawle et al., 2013) and thus could also contribute to the observed redox signaling events. Importantly, we have observed a signaling in both directions, not only from neurons to muscle, but also from muscle to neurons. The latter would exclude a signaling via neurotransmitters.
We are currently investigating the underlying mechanism of the trans-tissue signaling of the redox state, that is beyond the scope of this study.

Minor Points

1) Fig. 2C and Fig. 4E demonstrate the changes in redox state of neuronal cells towards misfolded proteins and proteasome inhibition. However, for a better overview on the properties of this redox-sensors, the authors should demonstrate once their ratio metric

characteristics under known oxidative or reduced conditions for example upon treatment with DTT, paraquat or DPS.

The reviewer asks to demonstrate the sensitivity of the neuronal redox sensor as we have shown for the muscle lines. We have added the experiments for neuronal HyPer targeted to the cytosol and the ER (Fig. E1H).

2) Fig. 2B right panel should be Fig. 2C

The depicted images and the graph belong to one sub figure (Fig. 2B). However, to display the figure in the same order as the previous and subsequent figures (Figs. 2A and 3A+B), we have changed the arrangement of the graph and the images and present the graph on the left and the corresponding images on the right of Fig 2B.

3) Fig. 3D: The block of N-linked protein glycosylation by tunicamycin treatment results in a shift towards oxidative conditions in the cytosol. The authors conclude from this a general cross-compartmental change of the redox state in the cytosol in response to proteotoxic stress in the ER. Since the ER-redox sensors do not respond towards tunicamycin, the authors should at least demonstrate their findings using another ER proteostasis drug or by expressing an aggregation prone protein in the ER.

The reviewer asks for a clarification of the effect of perturbations of protein-folding conditions in the ER on the redox state of the ER. As an alternative way to cause proteotoxic stress specific to the ER we have analyzed the redox state upon knockdown of ire-1. Interestingly, knockdown of ire-1 affects the redox state of the ER (Fig. 2E). This finding strengthens our conclusion of a correlation between the ER proteostasis and redox homeostasis of the ER.

4) Fig3E/D: the authors should label both graphs with C.elegans HyPer-GFP like it was done before.

We have modified the figure accordingly.

5) Figure legend of Fig4C and f: the authors should mention the meaning of NT and nt.

We have added this information. NT refers to non–treated (control).

5) In Fig. 6B the authors should include a sample that expresses muscle roGFP and neuronal Q0 in an analogues manner to Fig 6A, since the expression of Q0 in neuronal cells on its own already influenced the redox homeostasis in muscle cells. If this is indeed the case the authors should add it to both graph and figure legend.

The value for Q0 in Fig. 6B serves as control and thus sets the baseline for this experiment. The columns labeled with "fully reduced" and "fully oxidized" only indicate the dynamic range of the sensor. To avoid any confusion we have decided to remove the fully reduced / fully oxidized columns in the revised manuscript while still listing the data in Table 1.

Referee #4:

Review of EMBO paper-Loss of redox homeostasis by trans compartmental stress and aging.

This manuscript deals with the important question regarding the redox balance in the ER and how this is affected by proteostasis and aging.
The authors use genetically encoded in vivo roGFP sensors in the model organism Caenorhabditis elegans and also compare this to similar sensors expressed in mammalian HeLa cells. The sensors were used to measure changes in the redox status of the ER and cytosol with aging and also provide evidence that redox homeostasis can be regulated across compartments and tissues in C. elegans.
While these findings are important there are some major issues concerning the experimental approach, the choice of and validation of the GFP sensors. The validation of the sensor requires major clarification since the results that follow are absolutely dependent on this.

MAJOR CONCERNS
1. It is not entirely clear which version of roGFP was used for the C. elegans analysis and indeed whether this was the most suitable version to examine REDOX changes in the ER. If as stated this was based on roGFP1, namely the version described in the quoted reference (Hanson et al 2004), then this is adapted for the mitochondria and does not have the correct changes required to efficiently report on changes in ER environment. The most suitable roGFP sensor for the ER environment are described in the reference Lohman and Remington (2008) Development of a family of redox-sensitive green fluorescent protein indicators for use in relatively oxidizing subcellular environments. Biochemistry, 47, 8678-8688, PMID:18652491. This key reference has not been quoted, however one of the derived sensor is used in the human cell studies but not in the C. elegans studies.
Indeed, the authors results using ER roGFP1 in mammalian cells FigE2c (the version of the sensor used in their C. elegans studies) support the point that ER roGFP1 is an inappropriate sensor for the ER.

The reviewer expresses his/her concern about the choice and validity of the ER redox sensor. We have carefully tested and analyzed all sensors. The other reviewers have also acknowledged this point. In C. elegans we have used roGFP1 and correctly cited the appropriate reference (Hanson et al., 2004). We agree with the reviewer and have also demonstrated here that the very same version (roGFP1) is not suitable for the ER of mammalian cells. However, ERroGFP1 works fine to access the redox state in C. elegans as demonstrated in Figs. 1A+B, 2A, 4A, 5A, E1A and E1F. We have addressed this issue on page 7 of our manuscript and it reads as follows:

"For validation of the sensitivity of the cytosolic roGFP sensor, ratio metric analysis was performed following DTT or DPS treatment analogous to the ER sensor (Figs. 1C + E1B). The cytosolic roGFP responded to reducing and oxidizing conditions from which we conclude that roGFP can also be used as a tool to report on redox changes towards reducing and oxidizing conditions in the ER and the cytosol of C. elegans.
The roGFP sensors expressed in the cytosol (roGFP1) and the ER (ER-roGFPiE) in HeLa cells were validated by exposing the sensor expressing cells first to DTT and subsequently to DPS, after which the ratios of 405/488 nm were monitored (Figs. 1D+E). The sensors responded to reducing and oxidizing changes, in both compartments (Figs. 1D+E). The correct targeting of ER-roGFPiE to the ER was verified by co-localization with an ER-targeted DsRed (Fig. E1E). In contrast to ER-roGFPiE, ER-roGFP1 expressed in HeLa cells did not respond to treatment with 1 mM DPS, indicating that this roGFP variant is completely oxidized in the ER of mammalian cells as previously described ((Schwarzer et al, 2007; van Lith et al, 2011); Fig. E2). These differences in the sensitivity of ER-roGFP1 between C. elegans and mammalian cells may be due to the ER redox equilibrium associated with the robustness of ER redox catalysts. For example, in addition to ero1, mammalian cells express peroxiredoxin 4 and glutathione peroxidase 7 and 8 as ER thiol-oxidases (Araki & Nagata, 2012). Additionally, while knockdown of ero-1 in C. elegans has been reported to up-regulate UPR^{ER} broadly in

different tissues (Harding et al, 2003), Ero1α/β knockout mice only display defects in insulin biogenesis and glucose tolerance (Zito et al, 2010).“

2. Related to this point is the observation that the images in Fig 1A show blobs of fluorescence and it is impossible to establish if they are indeed expressed in the ER. There is a need for higher magnification images, and better still a clear demonstration of ER expression through the co-localization with known ER markers.

To validate the correct subcellular localization and targeting of the C. elegans ER redox sensor to the ER we performed immunofluorescence analysis using an antibody directed against KDEL and analyzed the co-localization of the immunostaining and the GFP signal stemming from the ER sensors (Fig. E1E). We have extended the analysis to the ER sensor of HeLa cells where we used an ER-targeted DsRed probe for co-localization analysis with the redox sensor.

This point is pertinent since the authors point out that for a different protein, namely Aβ1-42.. "Notably, although Aβ1-42 is fused to a signal sequence for targeting to the ER, it resides mainly in the cytosol (C. Link, personal communication)."

We are very careful with our analysis and are aware of mis-targeting issues and thus contacted Chris Link who generated the Ab1-42 C. elegans model to clarify the subcellular localization. We take the same measure for our strains and therefore performed a co-localization analysis with ER markers as described above for both, C. elegans as well as HeLa cells (Fig. E1E).

This ER localization confirmation is essential. Also it is not made clear why the muscle cell was selected, since as afar as I can discern muscle is not one of the major secretory organs in C. elegans.

The reviewer is correct in that there are other tissues with higher secretory load than muscles such as the intestine. However, the intestine accumulates gut granules (lysosome like organelles) during ageing and stress that have auto fluorescent properties and are especially excitable in the wavelengths of 405 and 488 nm (Coburn and Gems., 2013). Those are the wavelengths we use for the ratio-metric analysis and thus infer with the function of the redox sensors. Therefore, we switched to the other major tissues of an animal: muscle and neurons, whose assessment is not hampered by auto fluorescent granules.

3. Figure 1 A shows the changes in sensor emission ratios following DTT and Paraquat and then compares this data to a Western blot to detect the oxidized and reduced forms of roGFP following DTT and DPS treatment. These studies must be done with the same oxidant- either use paraquat or DPS There is also the distinct possibility that paraquat, being highly toxic, may induce a more general stress response.

The reviewer is correct and we have performed the imaging analysis in Figs. 1A+C now with DPS as oxidizing agent and replaced the images and data sets accordingly.

It would also be pertinent to show the response of UPR sensor hsp-3 to paraquat in addition to DTT as shown in Fig E1A.

We have added this experiment, but used DPS as oxidizing agent instead of paraquat to stay conform with the experiments shown in Figs. 1A+C (Fig. E1G).

In addition, it would be informative to see the Western blot image covering the whole gel rather than a low molecular weight slice since aggregation of GFP has previously been reported for roGFP sensors (Birk et al, 2013, J. Cell Sci 126, 1604-17, PMID: 23424194 and Van Lith et al, 2011, J. Cell Sci, 124, 2349-2356, PMID: 21693587).

The reviewer asks to see the complete western blot of the data depicted in figure 1b. We have included this as Fig. E1J.

4. The authors then attempt to validate their results by exposing sensors expressed in HeLa cells to DTT and DPS and measuring ratio changes over time. Why was this type of experiment not carried out in the C. elegans system?

In order to perform confocal imaging analysis of living C. elegans, the nematodes need to be anaesthetized and mounted prior to imaging. This procedure takes up to 120 min and thus makes it impossible to perform a ratio-metric analysis over time as carried out for the HeLa cells.

The mammalian cell system is then used extensively throughout the following experiments, however this is not reflected in the manuscript abstract, which focuses exclusively on C. elegans.

The abstract size is limited to 150 words and we thus focused on the major findings of our manuscript. The model system C. elegans enabled us to monitor changes of the redox state throughout the lifespan of an animal as well as the redox signaling between different tissues. Thus we feel it is appropriate to focus on the animal model in the word-limited abstract.

5. A large proportion of the experiments in this manuscript are carried out in mammalian cells and therefore it is unusual that the authors failed to quote (Van Lith et al, 2011, J. Cell Sci, 124, 2349-2356,PMID: 21693587) as it covers the robust validation and detailed characterization of roGFP sensors in the ER of mammalian cells.

We thank the reviewer for pointing out the missing reference and have added it in the revised manuscript.

6. Another technical point relating to the sensor in live C. elegans relates the fact that the authors have used levamisole to immobilize worms prior to viewing muscle cell expressed sensors. This drug specifically affects muscle cell function, therefore controls using non-muscle immobilizing agents should be used to rule out a direct effect of levamisole on the muscle cell expressed roGFP.

The reviewer expresses concern about the effect of the anesthetic Levamisole and its potential effect on muscle function. Levamisole is an established reagent for confocal imaging of C.

elegans and is routinely used. However, to exclude any interference with the assessment of the redox sensors we have performed an imaging analysis with nematodes mounted in high percentage (10%) agarose pads that restrict movement and enable confocal imaging. We did not observe any changes in the ratio-metric analysis for muscle or neuronal cells compared to nematodes that were anaesthetized using levamisole. We thank the reviewer to point out this potential technical problem, but are now confident that the anesthetic reagent does not affect the data sets.

Until these major issues are resolved (initial characterization and validation of the roGFP sensors and the use of different oxidants in the assay) it is difficult to assess the potentially interesting conclusions drawn from the experiments that follow.

Minor points
Scale bars in Figure 1 A and C need careful checking.

We have checked and adjusted the scale bars for Figs. 1A + C.

2nd Editorial Decision 11 May 2015

Thank you for submitting a new version of your manuscript for consideration by the EMBO Journal. It has now been seen by three of the original four referees and their comments are shown below.

As you will see from the reports, the referees express interest in the findings reported in your manuscript and ref#1 and ref#2 find that the main concerns of the original study have been largely addressed, although ref#2 still points out the need for further statistical validation of the data to ensure the conclusiveness of the study. In contrast, ref #4 (original numbering is maintained) remains rather critical of the revised manuscript, raising concerns about ER localization data, choice of tissue inspected and the possible aggregation of the redox reporter.

Given the rather strong discrepancy in the reports we conducted a second round of cross-referee commenting and received extensive feedback from ref #2 in support of your manuscript. More specifically this ref pointed out the established use of KDEL as an ER marker for C elegans, the functional relevance for skeletal muscle when looking at ageing, and that only a minor amount of the reporter is seen in the slower migrating band in the Western blot (making this unlikely to grossly alter the experimental outcome).

In light of the positive recommendations from ref#1 and ref #2, I would thus invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

I would in particular like to emphasise that the remaining issues from ref#2 will need to be fully addressed for this revised version. Concerning the points raised by ref #4 we will not require you to address all these experimentally, but we would ask you to comment on and discuss the issues raised. If you should have further data at hand to help clarify this we would also encourage you to include it.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the 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://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your 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 your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

The authors adequately addressed most of my comments. The authors need to check their graphs since it appears that at least in some graphs (e.g., fig. 4D) they depicted the standard deviation in their graphs but wrote in the figure legends that they show the standard error of the mean. This needs to be corrected. Also Fig. 2B is missing the control, which is important since it serves as necessary reference for this data set.

Referee #2:

Many of my technical concerns from the initial review have been addressed. However, one of those concerns was that the original manuscript did not employ suitable statistical tests for multiple comparisons presented in their graphed data (i.e., they used a Student t test to compare more than two sample means, which is a big no-no in statistics). The authors have responded by either stripping down data from the graphs to yield pairwise data (suitable to their original method for statistical analysis) or applying an ANOVA. P values for the ANOVA are presented in the figure legends; however, there is no indication in the paper of what post-hoc test was used to perform individual comparisons between different bars in the graphs. In addition, there is no indication on the graphs to tell the reader which pairwise comparisons passed the post-hoc tests and which failed, leaving me to wonder if they even did a pair-wise test. It's not enough to just do the ANOVA and present a P value for the whole data set. The P value is only telling you that one or more data points in the graph are significant, but it is not telling you which ones, or comparing them to each other - you need a post-hoc test. Without this, the data in Figures 1A, 1C, 1E, 2C, 2D, 3E, 4B, 4C, 4E, E1A, E1B, E1I, E4A, and E4B is pretty meaningless. EMBO would be publishing a very technically flawed paper in the absence of this.

Referee #4:

I believe that the majority of the points raised in the initial review have not been dealt with adequately by the authors and have listed my comments below in italics.

Review of EMBO paper-Loss of redox homeostasis by trans compartmental stress and aging.

This manuscript deals with the important question regarding the redox balance in the ER and how this is affected by proteostasis and aging.
The authors use genetically encoded in vivo roGFP sensors in the model organism Caenorhabditis elegans and also compare this to similar sensors expressed in mammalian HeLa cells. The sensors were used to measure changes in the redox status of the ER and cytosol with aging and also provide evidence that redox homeostasis can be regulated across compartments and tissues in C. elegans. While these findings are important there are some major issues concerning the experimental approach, the choice of and validation of the GFP sensors. The validation of the sensor requires major clarification since the results that follow are absolutely dependent on this.

MAJOR CONCERNS

1. It is not entirely clear which version of roGFP was used for the C. elegans analysis and indeed whether this was the most suitable version to examine REDOX changes in the ER. If as stated this was based on roGFP1, namely the version described in the quoted reference (Hanson et al 2004), then this is adapted for the mitochondria and does not have the correct changes required to efficiently report on changes in ER environment. The most suitable roGFP sensor for the ER environment are described in the reference Lohman and Remington (2008) Development of a family of redox-sensitive green fluorescent protein indicators for use in relatively oxidizing subcellular environments. Biochemistry, 47, 8678-8688, PMID:18652491. This key reference has not been quoted, however one of the derived sensor is used in the human cell studies but not in the C. elegans studies.
Indeed, the authors results using ER roGFP1 in mammalian cells FigE2c (the version of the sensor used in their C. elegans studies) support the point that ER roGFP1 is an inappropriate sensor for the ER.

The reviewer expresses his/her concern about the choice and validity of the ER redox sensor. We have carefully tested and analyzed all sensors. The other reviewers have also acknowledged this point. In C. elegans we have used roGFP1 and correctly cited the appropriate reference (Hanson et al., 2004). We agree with the reviewer and have also demonstrated here that the very same version (roGFP1) is not suitable for the ER of mammalian cells. However, ERroGFP1 works fine to access the redox state in C. elegans as demonstrated in Figs. 1A+B, 2A, 4A, 5A, E1A and E1F. We have addressed this issue on page 7 of our manuscript and it reads as follows:

"For validation of the sensitivity of the cytosolic roGFP sensor, ratio metric analysis was performed following DTT or DPS treatment analogous to the ER sensor (Figs. 1C + E1B). The cytosolic roGFP responded to reducing and oxidizing conditions from which we conclude that roGFP can also be used as a tool to report on redox changes towards reducing and oxidizing conditions in the ER and the cytosol of C. elegans.
The roGFP sensors expressed in the cytosol (roGFP1) and the ER (ER-roGFPiE) in HeLa cells were validated by exposing the sensor expressing cells first to DTT and subsequently to DPS, after which the ratios of 405/488 nm were monitored (Figs. 1D+E). The sensors responded to reducing and oxidizing changes, in both compartments (Figs. 1D+E). The correct targeting of ER-roGFPiE to the ER was verified by co-localization with an ER-targeted DsRed (Fig. E1E). In contrast to ER-roGFPiE, ER-roGFP1 expressed in HeLa cells did not respond to treatment with 1 mM DPS, indicating that this roGFP variant is completely oxidized in the ER of mammalian cells as previously described ((Schwarzer et al, 2007; van Lith et al, 2011); Fig. E2). These differences in the sensitivity of ER-roGFP1 between C. elegans and mammalian cells may be due to the ER redox equilibrium associated with the robustness of ER redox catalysts. For example, in addition to ero1, mammalian cells express peroxiredoxin 4 and glutathione peroxidase 7 and 8 as ER thiol-oxidases (Araki & Nagata, 2012). Additionally, while knockdown of ero-1 in C. elegans has been reported to up-regulate UPRER broadly in different tissues (Harding et al, 2003), Ero1α/β knockout mice only display defects in insulin biogenesis and glucose tolerance (Zito et al, 2010).

2. Related to this point is the observation that the images in Fig 1A show blobs of fluorescence and it is impossible to establish if they are indeed expressed in the ER. There is a need for higher magnification images, and better still a clear demonstration of ER expression through the co-localization with known ER markers.

To validate the correct subcellular localization and targeting of the C. elegans ER redox sensor to the ER we performed immunofluorescence analysis using an antibody directed against KDEL and analyzed the co-localization of the immunostaining and the GFP signal stemming from the ER sensors (Fig. E1E). We have extended the analysis to the ER sensor of HeLa cells where we used an ER-targeted DsRed probe for co-localization analysis with the redox sensor.

The images still resemble blobs, there is no bright field image to indicate what we are looking at and no labeling to indicate what tissues, organs or cells are being depicted.
Shouldn't the merge image in Figure E1-E for C. elegans be orange?

Why use an anti KDEL antibody and not an antibody direct to an endogenous ER resident protein?

This point is pertinent since the authors point out that for a different protein, namely Aβ1-42.. "Notably, although Aβ1-42 is fused to a signal sequence for targeting to the ER, it resides mainly in the cytosol (C. Link, personal communication)."

We are very careful with our analysis and are aware of mis-targeting issues and thus contacted Chris Link who generated the Aβ1-42 C. elegans model to clarify the subcellular localization. We take the same measure for our strains and therefore performed a co-localization analysis with ER markers as described above for both, C. elegans as well as HeLa cells (Fig. E1E).

This ER localization confirmation is essential. Also it is not made clear why the muscle cell was selected, since as afar as I can discern muscle is not one of the major secretory organs in C. elegans.

The reviewer is correct in that there are other tissues with higher secretory load than muscles such as the intestine. However, the intestine accumulates gut granules (lysosome like organelles) during ageing and stress that have auto fluorescent properties and are especially excitable in the wavelengths of 405 and 488 nm (Coburn and Gems., 2013). Those are the wavelengths we use for the ratio-metric analysis and thus infer with the function of the redox sensors. Therefore, we switched to the other major tissues of an animal: muscle and neurons, whose assessment is not hampered by auto fluorescent granules.

The authors have not addressed this question. The muscle is not a tissue with high secretory load. The gut is not the only alternative to the muscle and various tissues including the gonad, the hypodermis and the gland tissues all have a much higher secretory load than muscle. Indeed a recent paper on tissue specific roGFP ratio measurements did not report any problems regarding auto fluorescence in the gut (http://www.ncbi.nlm.nih.gov/pubmed/25262602)

3. Figure 1 A shows the changes in sensor emission ratios following DTT and Paraquat and then compares this data to a Western blot to detect the oxidized and reduced forms of roGFP following DTT and DPS treatment. These studies must be done with the same oxidant- either use paraquat or DPS There is also the distinct possibility that paraquat, being highly toxic, may induce a more general stress response.

The reviewer is correct and we have performed the imaging analysis in Figs. 1A+C now with DPS as oxidizing agent and replaced the images and data sets accordingly.

It would also be pertinent to show the response of UPR sensor hsp-3 to paraquat in addition to DTT as shown in Fig E1A.

We have added this experiment, but used DPS as oxidizing agent instead of paraquat to stay conform with the experiments shown in Figs. 1A+C (Fig. E1G).

In addition, it would be informative to see the Western blot image covering the whole gel rather than a low molecular weight slice since aggregation of GFP has previously been reported for roGFP sensors (Birk et al, 2013, J. Cell Sci 126, 1604-17, PMID: 23424194 and Van Lith et al, 2011, J. Cell Sci, 124, 2349-2356, PMID: 21693587).

The reviewer asks to see the complete western blot of the data depicted in figure 1b. We have included this as Fig. E1J.

The Western blot confirms that indeed in the non-reduced sample there is clear evidence of disulfide-linked aggregation, indicating that a less than ideal sensor has been used.

4. The authors then attempt to validate their results by exposing sensors expressed in HeLa cells to DTT and DPS and measuring ratio changes over time. Why was this type of experiment not carried out in the C. elegans system?

In order to perform confocal imaging analysis of living C. elegans, the nematodes need to be anaesthetized and mounted prior to imaging. This procedure takes up to 120 min and thus makes it impossible to perform a ratio-metric analysis over time as carried out for the HeLa cells.

This response does not address the question and the author's inability to immobilize nematodes is not a valid reason for switching to Hela cells. No such problems were reported in the following manuscript http://www.ncbi.nlm.nih.gov/pubmed/25262602
In addition, there are numerous well document methods of immobilizing worms prior to confocal microscopy, for example http://www.wormbook.org/wbg/articles/volume-18-number-1/agarose-immobilization-of-c-elegans/ and http://www.ncbi.nlm.nih.gov/pubmed/23301069

Indeed the authors also go on to describe an alternative method that they have used (see point 1 below regarding 10% agarose pads).

The mammalian cell system is then used extensively throughout the following experiments, however this is not reflected in the manuscript abstract, which focuses exclusively on C. elegans.

The abstract size is limited to 150 words and we thus focused on the major findings of our manuscript. The model system C. elegans enabled us to monitor changes of the redox state throughout the lifespan of an animal as well as the redox signaling between different tissues. Thus we feel it is appropriate to focus on the animal model in the word-limited abstract.

I agree that the abstract should focus on the C. elegans finding but the main experiments should therefore be carried out in C. elegans and not HeLa cells (see immobilization point above).

5. A large proportion of the experiments in this manuscript are carried out in mammalian cells and therefore it is unusual that the authors failed to quote (Van Lith et al, 2011, J. Cell Sci, 124, 2349-2356,PMID: 21693587) as it covers the robust validation and detailed characterization of roGFP sensors in the ER of mammalian cells.

We thank the reviewer for pointing out the missing reference and have added it in the revised manuscript.

1. Another technical point relating to the sensor in live C. elegans relates the fact that the authors have used levamisole to immobilize worms prior to viewing muscle cell expressed sensors. This drug specifically affects muscle cell function, therefore controls using non-muscle immobilizing agents should be used to rule out a direct effect of levamisole on the muscle cell expressed roGFP.

The reviewer expresses concern about the effect of the anesthetic Levamisole and its potential effect on muscle function. Levamisole is an established reagent for confocal imaging of C. elegans and is routinely used. However, to exclude any interference with the assessment of the redox sensors we have performed an imaging analysis with nematodes mounted in high percentage (10%) agarose pads that restrict movement and enable confocal imaging. We did not observe any changes in the ratio-metric analysis for muscle or neuronal cells compared to nematodes that were anaesthetized using levamisole. We thank the reviewer to point out this potential technical problem, but are now confident that the anesthetic reagent does not affect the data sets.

Until these major issues are resolved (initial characterization and validation of the roGFP sensors and the use of different oxidants in the assay) it is difficult to assess the potentially interesting conclusions drawn from the experiments that follow.

Minor points
Scale bars in Figure 1 A and C need careful checking.

We have checked and adjusted the scale bars for Figs. 1A + C.

Revision - authors' response 05 June 2015

Detailed point-to-point response to EMBOJ-2015-91711

Our responses to the reviewers' comments are shown in red. Our previous responses that were kept in the text by reviewer 4 are italicized.

Referee #1:

The authors adequately addressed most of my comments. The authors need to check their graphs since it appears that at least in some graphs (e.g., fig. 4D) they depicted the standard deviation in their graphs but wrote in the figure legends that they show the standard error of the mean. This needs to be corrected. Also Fig. 2B is missing the control, which is important since it serves as necessary reference for this data set.

We thank the reviewer for the positive evaluation of our revised manuscript and for pointing out the incorrect assignment of the error evaluation in Figure 4D. We have changed the figure legend accordingly and indicate standard deviation (STDEV) rather than standard error. In Figure 2B, the data set of the soluble protein HttQ22 serves as control for the aggregation-pone Htt134, and likewise the soluble Q19 serves as control for the aggregation-prone Q77.

Referee #2:

Many of my technical concerns from the initial review have been addressed.

We thank the reviewer for the positive feedback and again for the constructive criticism of the previous version of the manuscript that have improved the revision.

However, one of those concerns was that the original manuscript did not employ suitable statistical tests for multiple comparisons presented in their graphed data (i.e., they used a Student t test to compare more than two sample means, which is a big no-no in statistics). The authors have responded by either stripping down data from the graphs to yield pairwise data (suitable to their original method for statistical analysis) or applying an ANOVA. P values for the ANOVA are presented in the figure legends; however, there is no indication in the paper of what post-hoc test was used to perform individual comparisons between different bars in the graphs. In addition, there is no indication on the graphs to tell the reader which pairwise comparisons passed the post-hoc tests and which failed, leaving me to wonder if they even did a pair-wise test. It's not enough to just do the ANOVA and present a P value for the whole data set. The P value is only telling you that one or more data points in the graph are significant, but it is not telling you which ones, or comparing them to each other - you need a post-hoc test. Without this, the data in Figures 1A, 1C, 1E, 2C, 2D, 3E, 4B, 4C, 4E, E1A, E1B, E1I, E4A, and E4B is pretty meaningless. EMBO would be publishing a very technically flawed paper in the absence of this.

We have now clarified these concerns. Every ANOVA evaluated data set was followed up by a post-hoc analysis using the Tukey HSD (honest significance difference) test. The results were listed together with all raw data and the p values of the ANOVA test in Table S1. The reviewer is correct that we did not mention the post-hoc analysis in the main text or in the figure legend. We have now added this information to each figure legend and have also added a description of the post-hoc Tukey HSD test into the statistical section of the Materials & Methods part. We have also indicated when the statistical evaluation passed the post-hoc test in each figure legend as well as in Table S1.

Referee #4:

I believe that the majority of the points raised in the initial review have not been dealt with adequately by the authors and have listed my comments below in bold.

Review of EMBO paper-Loss of redox homeostasis by trans compartmental stress and aging.

This manuscript deals with the important question regarding the redox balance in the ER and how

this is affected by proteostasis and aging.
The authors use genetically encoded in vivo roGFP sensors in the model organism Caenorhabditis elegans and also compare this to similar sensors expressed in mammalian HeLa cells. The sensors were used to measure changes in the redox status of the ER and cytosol with aging and also provide evidence that redox homeostasis can be regulated across compartments and tissues in C. elegans. While these findings are important there are some major issues concerning the experimental approach, the choice of and validation of the GFP sensors. The validation of the sensor requires major clarification since the results that follow are absolutely dependent on this.

MAJOR CONCERNS
1. It is not entirely clear which version of roGFP was used for the C. elegans analysis and indeed whether this was the most suitable version to examine REDOX changes in the ER. If as stated this was based on roGFP1, namely the version described in the quoted reference (Hanson et al 2004), then this is adapted for the mitochondria and does not have the correct changes required to efficiently report on changes in ER environment. The most suitable roGFP sensor for the ER environment are described in the reference Lohman and Remington (2008) Development of a family of redox-sensitive green fluorescent protein indicators for use in relatively oxidizing subcellular environments. Biochemistry, 47, 8678-8688, PMID:18652491. This key reference has not been quoted, however one of the derived sensor is used in the human cell studies but not in the C. elegans studies.
Indeed, the authors results using ER roGFP1 in mammalian cells FigE2c (the version of the sensor used in their C. elegans studies) support the point that ER roGFP1 is an inappropriate sensor for the ER.

The reviewer expresses his/her concern about the choice and validity of the ER redox sensor. We have carefully tested and analyzed all sensors. The other reviewers have also acknowledged this point. In C. elegans, we have used roGFP1 and correctly cited the appropriate reference (Hanson et al., 2004). We agree with the reviewer and have also demonstrated here that the very same version (roGFP1) is not suitable for the ER of mammalian cells. However, ERroGFP1 works fine to access the redox state in C. elegans as demonstrated in Figs. 1A+B, 2A, 4A, 5A, E1A and E1F. We have addressed this issue on page 7 of our manuscript and it reads as follows:

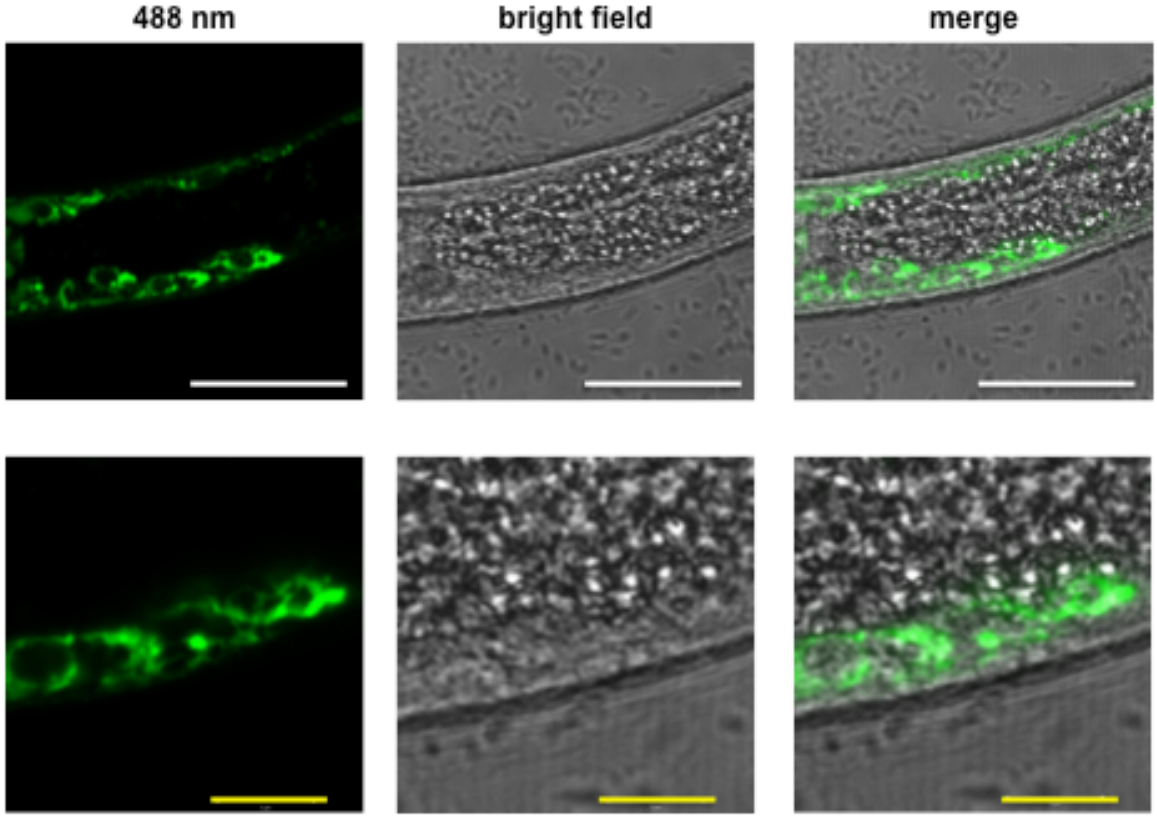
"For validation of the sensitivity of the cytosolic roGFP sensor, ratio metric analysis was performed following DTT or DPS treatment analogous to the ER sensor (Figs. 1C + E1B). The cytosolic roGFP responded to reducing and oxidizing conditions from which we conclude that roGFP can also be used as a tool to report on redox changes towards reducing and oxidizing conditions in the ER and the cytosol of C. elegans.
The roGFP sensors expressed in the cytosol (roGFP1) and the ER (ER-roGFPiE) in HeLa cells were validated by exposing the sensor expressing cells first to DTT and subsequently to DPS, after which the ratios of 405/488 nm were monitored (Figs. 1D+E). The sensors responded to reducing and oxidizing changes, in both compartments (Figs. 1D+E). The correct targeting of ER-roGFPiE to the ER was verified by co-localization with an ER-targeted DsRed (Fig. E1E). In contrast to ER-roGFPiE, ER-roGFP1 expressed in HeLa cells did not respond to treatment with 1 mM DPS, indicating that this roGFP variant is completely oxidized in the ER of mammalian cells as previously described ((Schwarzer et al, 2007; van Lith et al, 2011); Fig. E2). These differences in the sensitivity of ER-roGFP1 between C. elegans and mammalian cells may be due to the ER redox equilibrium associated with the robustness of ER redox catalysts. For example, in addition to ero1, mammalian cells express peroxiredoxin 4 and
glutathione peroxidase 7 and 8 as ER thiol-oxidases (Araki & Nagata, 2012). Additionally, while knockdown of ero-1 in C. elegans has been reported to up-regulate UPRER broadly in different tissues (Harding et al, 2003), Ero1α/β knockout mice only display defects in insulin biogenesis and glucose tolerance (Zito et al, 2010)."

2. Related to this point is the observation that the images in Fig 1A show blobs of fluorescence and it is impossible to establish if they are indeed expressed in the ER. There is a need for higher magnification images, and better still a clear demonstration of ER expression through the co-localization with known ER markers.

To validate the correct subcellular localization and targeting of the C. elegans ER redox sensor to the ER we performed immunofluorescence analysis using an antibody directed against KDEL and analyzed the co-localization of the immunostaining and the GFP signal stemming from the ER sensors (Fig. E1E). We have extended the analysis to the ER sensor of HeLa cells where we used an ER-targeted DsRed probe for co-localization analysis with the redox sensor.

The images still resemble blobs, there is no bright field image to indicate what we are looking at and no labeling to indicate what tissues, organs or cells are being depicted.

The figure legend states that the images are *C. elegans* muscle cells. To address the concern of the reviewer we add in this rebuttal letter images of our muscle ER-roGFP sensor.



On the left you can see the GFP channel (488 nm), in the middle the bright field image and on the right the merge of both of them. We show two image sets of different magnification. The roGFP sensor is expressed in the body wall muscle driven by the *unc-54* promoter as can be accessed in these images. The scale bars of the top panel are 20 µm (depicted in white) and the scale bars of the bottom panel are 5 µm (depicted in yellow). Additional images that were also taken at a higher magnification (100x) can be found in figure 5 that show single cell analyses of roGFP either expressed in the cytosol or ER of a single cell each.

Shouldn't the merge image in Figure E1-E for C. elegans be orange?

The intensities of the antibody staining and the endogenous fluorescent protein are not equivalent in intensity and stronger for the endogenous ER-roGFP.

Why use an anti KDEL antibody and not an antibody direct to an endogenous ER resident protein.

The KDEL antibody is a well-established maker to analyze the ER localization of proteins within the *C. elegans* field as well as for all other model system (e.g. for *C. elegans*: Basham & Rose et al., 2001; Marciniak et al., 2004).

This point is pertinent since the authors point out that for a different protein, namely Aβ1-42.. "Notably, although Aβ1-42 is fused to a signal sequence for targeting to the ER, it resides mainly in the cytosol (C. Link, personal communication)."

We are very careful with our analysis and are aware of mis-targeting issues and thus contacted Chris Link who generated the Aβ1-42 C. elegans model to clarify the subcellular localization. We take the same measure for our strains and therefore performed a co-localization analysis with ER markers as described above for both, C. elegans as well as HeLa cells (Fig. E1E).

This ER localization confirmation is essential. Also it is not made clear why the muscle cell was selected, since as afar as I can discern muscle is not one of the major secretory organs in C. elegans.

The reviewer is correct in that there are other tissues with higher secretory load than muscles such as the intestine. However, the intestine accumulates gut granules (lysosome like organelles) during ageing and stress that have auto fluorescent properties and are especially excitable in the wavelengths of 405 and 488 nm (Coburn and Gems, 2013). Those are the wavelengths we use for the ratio-metric analysis and thus infer with the function of the redox sensors. Therefore, we switched to the other major tissues of an animal: muscle and neurons, whose assessment is not hampered by auto fluorescent granules.

The authors have not addressed this question. The muscle is not a tissue with high secretory load. The gut is not the only alternative to the muscle and various tissues including the gonad, the hypodermis and the gland tissues all have a much higher secretory load than muscle. Indeed a recent paper on tissue specific roGFP ratio measurements did not report any problems regarding auto fluorescence in the gut (http://www.ncbi.nlm.nih.gov/pubmed/25262602)

The reference the reviewer is listing (Romero-Aristizabal et al., 2014) did not analyze the redox state on a single cell level, but rather analyzed sections of the animal. We would like to point out that the listed reference only used a 10X objective that excludes the analysis of single cells. We have performed all confocal imaging analyses using a 100x objective. In addition, the authors only analyzed animals that reached day 1 or day 2 of adulthood (represents day 4 and 5 of life). Gut granules are known to accumulate during ageing and in response to stress or starvation that were not encountered under the conditions tested in this reference.

3. Figure 1 A shows the changes in sensor emission ratios following DTT and Paraquat and then compares this data to a Western blot to detect the oxidized and reduced forms of roGFP following DTT and DPS treatment. These studies must be done with the same oxidant- either use paraquat or DPS There is also the distinct possibility that paraquat, being highly toxic, may induce a more general stress response.

The reviewer is correct and we have performed the imaging analysis in Figs. 1A+C now with DPS as oxidizing agent and replaced the images and data sets accordingly.

It would also be pertinent to show the response of UPR sensor hsp-3 to paraquat in addition to DTT as shown in Fig E1A.

We have added this experiment, but used DPS as oxidizing agent instead of paraquat to stay conform with the experiments shown in Figs. 1A+C (Fig. E1G).

In addition, it would be informative to see the Western blot image covering the whole gel rather than a low molecular weight slice since aggregation of GFP has previously been reported for roGFP sensors (Birk et al, 2013, J. Cell Sci 126, 1604-17, PMID: 23424194 and Van Lith et al, 2011, J. Cell Sci, 124, 2349-2356, PMID: 21693587).

The reviewer asks to see the complete western blot of the data depicted in figure 1b. We have included this as Fig. E1J.

The Western blot confirms that indeed in the non-reduced sample there is clear evidence of disulfide-linked aggregation, indicating that a less than ideal sensor has been used.

We have repeated this analysis (revised Fig. E1J). In order to address the concern of this reviewer we have fractionated protein samples of nematodes that express ER-roGFP in muscle into the soluble and insoluble fraction using detergents (1% NP40) and a sonication step (5 min) followed by centrifugation for 5 min at 12.000 rpm. (A detailed description can be found in the Materials & Method section). Samples of the soluble and insoluble fraction were subjected to SDS-PAGE under non-reducing condition and after the electrophoresis step further analyzed by Western blot using a GFP antibody. The left lane shows the soluble fraction with signals for the reduced (upper and slower migrating band) and oxidized (lower and faster migrating band) roGFP. We show the entire western blot and there are no signals in the higher molecular weight fraction that might indicate SDS-resistant (often due to inter-molecular disulphide formation) aggregation. In addition the absence of any GFP signal in the insoluble fraction (right lane) further strengthens our conclusion that roGFP expressed at this level is not prone to aggregation.

4. The authors then attempt to validate their results by exposing sensors expressed in HeLa cells to DTT and DPS and measuring ratio changes over time. Why was this type of experiment not carried out in the C. elegans system?

In order to perform confocal imaging analysis of living C. elegans, the nematodes need to be anaesthetized and mounted prior to imaging. This procedure takes up to 120 min and thus makes it impossible to perform a ratio-metric analysis over time as carried out for the HeLa cells.

This response does not address the question and the author's inability to immobilize nematodes is not a valid reason for switching to Hela cells. No such problems were reported in the following manuscript http://www.ncbi.nlm.nih.gov/pubmed/25262602
In addition, there are numerous well document methods of immobilizing worms prior to confocal microscopy, for example http://www.wormbook.org/wbg/articles/volume-18-number-1/agarose-immobilization-of-c-elegans/ and http://www.ncbi.nlm.nih.gov/pubmed/23301069

Indeed the authors also go on to describe an alternative method that they have used (see point 1 below regarding 10% agarose pads).

We did not switch to HeLa cells in order to confirm the sensors. We just argued that an analysis of an immediate response of the redox sensors towards reducing and oxidizing agents can be easily

performed in Hela cells and thus we did a time-resolved analysis of the Hela cells and only analyzed end point data for *C. elegans*. An analysis of single cells of *C. elegans* (that were not done in the listed references) would require an immobilization. Even an immobilization in 10% agarose as we have done as well or using nano particles (Kim et al., 2013) takes several minutes to mount the nematodes prior to confocal imaging using a 100X objective.

The mammalian cell system is then used extensively throughout the following experiments, however this is not reflected in the manuscript abstract, which focuses exclusively on C. elegans.

The abstract size is limited to 150 words and we thus focused on the major findings of our manuscript. The model system C. elegans enabled us to monitor changes of the redox state throughout the lifespan of an animal as well as the redox signaling between different tissues. Thus we feel it is appropriate to focus on the animal model in the word-limited abstract.

I agree that the abstract should focus on the C. elegans finding but the main experiments should therefore be carried out in C. elegans and not HeLa cells (see immobilization point above).

We disagree with this point, as the main findings of this manuscript were obtained using *C. elegans*. In fact, the analysis of the redox state throughout the lifespan of an intact animal and the comparison of the response of different tissues and especially across tissues were solely analyzed in *C. elegans*. In addition, we employed this model system further to analyze the oxidative stress and UPR stress response by organism-wide knockdown using RNAi against *skn-1* and *ire-1* or treatment with tunicamycin. Analyses upon inhibition of the proteasome or expression of aggregation prone proteins such as polyQ were performed equally using both systems. Thus the majority of experiments were performed in *C. elegans* and we therefore think it is justified to focus on the main findings obtained using *C. elegans* in the limited space of the abstract.

We have added however a statement that we obtained the same findings in HeLa cells arguing for the conservation of the adjustment of the redox state to proteotoxic challenges.

Our abstract reads now as follows:

The cellular proteostasis network integrates the protein folding and clearance machineries in multiple subcellular compartments of the eukaryotic cell. The endoplasmic reticulum (ER) is the site of synthesis and folding of membrane and secretory proteins. A distinctive feature of the ER is its tightly controlled redox homeostasis necessary for the formation of inter- and intra-molecular disulphide bonds. Employing genetically encoded *in vivo* sensors reporting on the redox state in an organelle-specific manner, we show in the nematode *C. elegans* that the redox state of the ER is subject to profound changes during its lifetime. In young animals, the ER of is oxidizing and shifts towards reducing conditions during ageing, whereas in the cytosol the redox state becomes more oxidizing. Likewise, the redox state in the cytosol and the ER change in an opposing manner in response to proteotoxic challenges in *C. elegans* and in HeLa cells revealing conservation of redox homeostasis. Moreover, we show that organellar redox homeostasis is regulated across tissues within *C. elegans* providing a new measure for organismal fitness.

5. A large proportion of the experiments in this manuscript are carried out in mammalian cells and therefore it is unusual that the authors failed to quote (Van Lith et al, 2011, J. Cell Sci, 124, 2349-2356,PMID: 21693587) as it covers the robust validation and detailed characterization of roGFP sensors in the ER of mammalian cells.

We thank the reviewer for pointing out the missing reference and have added it in the revised manuscript.

1. Another technical point relating to the sensor in live C. elegans relates the fact that the authors have used levamisole to immobilize worms prior to viewing muscle cell expressed sensors. This drug specifically affects muscle cell function, therefore controls using non-muscle immobilizing agents should be used to rule out a direct effect of levamisole on the muscle cell expressed roGFP.

The reviewer expresses concern about the effect of the anesthetic Levamisole and its potential effect on muscle function. Levamisole is an established reagent for confocal imaging of C. elegans and is routinely used. However, to exclude any interference with the assessment of the redox sensors we have performed an imaging analysis with nematodes mounted in high percentage (10%) agarose pads that restrict movement and enable confocal imaging. We did not observe any changes in the ratio-metric analysis for muscle or neuronal cells compared to nematodes that were anaesthetized using levamisole. We thank the reviewer to point out this potential technical problem, but are now confident that the anesthetic reagent does not affect the data sets.

Until these major issues are resolved (initial characterization and validation of the roGFP sensors and the use of different oxidants in the assay) it is difficult to assess the potentially interesting conclusions drawn from the experiments that follow.

Minor points
Scale bars in Figure 1 A and C need careful checking.

We have checked and adjusted the scale bars for Figs. 1A + C.

3rd Editorial Decision 24 June 2015

Thank you again for submitting your revised manuscript for The EMBO Journal. Your study has now been seen by one of the original referees (comments included below, including specific comments on the concerns raised by the other referees) and as you will see he/she finds that all criticisms have been addressed. I am therefore happy to inform you that your manuscript is in-principle ready to be accepted for publication with us.

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

REFEREE COMMENTS

Referee #2:

I have summarized the findings in my previous review so I will not repeat that here.

My previous concerns regarding the statistical analysis (i.e., the requirement that the authors provide post-hoc tests for their ANOVA data) has been addressed. I am satisfied with the paper.

Mu understanding is that EMBO invites reviewers to make cross-reviewer evaluations and comments. Reviewer 4 appeared to have several concerns that he or she felt were not addressed in the initial revision. I have analyzed the new version of the manuscript along with the authors rebuttal to reviewer 4. In my opinion, the authors have satisfied the concerns raised by reviewer 4. Below I elaborate on my own assessment of reviewer 4's concerns (numbered according to reviewer 4's original numbering scheme):

Point 2. The reviewer's original concern was that the fluorescence images of the ER-localized reporter are not actually showing ER localization. The authors addressed this point by doing a double-label antibody staining using an anti-KDEL antibody. The authors also ported these markers in HeLa cells, where the larger size of these cells make it easier to do this kind of confirmation. The reviewer rebutted by suggesting that KDEL is not a good ER marker, and that the merged image in Figure E1-E should be orange.

I disagree with this reviewer's rebuttal. KDEL is a well established ER marker. The images presented in Figure 1 and Figure E1 are consistent within the discipline and commonly observed in C. elegans cells, which are notoriously small and compact, making resolution difficult. Unlike in mammalian cells, there are not a huge number of commercially available antibodies against C. elegans proteins that one can employ for colocalization studies. To my knowledge, there is no available antibody against an endogenous C. elegans ER resident protein. Also, the results of Figure E1-E are clear (both single channels are presented). Perhaps the gain on the red channel could be increased so that image in E1-E would have more of a yellow tint to it. Overall, these concerns were cosmetic, not substantive.

In this same point, the reviewer originally raised the concern that muscle might not be the best tissue to examine for ER function because muscles do not have a large ER load (they don't secrete much). The authors responded by indicating that they chose muscle instead of intestine (presumed to have a higher ER load) because intestine has autofluorescent organelles, which hamper analysis of the reporter. The reviewer's rebuttal was that there are other tissues besides the intestine that have a high secretory load (e.g., the gonad, the hypodermis), and that at least one paper has used these reporters in the gut without problem.

On principle, I agree with the reviewer's rebuttal. There are other tissues that the authors could have examined. The autofluorescence problem in the intestine can be dealt with using spectral analysis (commonly done in the worm cell biology field). However, I would not allow this point to preclude accepting the paper. First, one could make the counterargument that the results would be skewed if the authors had looked at a tissue with high-secretory load instead (i.e., their findings would only apply to high-secretory tissues). Second, and more important, is that the muscle is one of the key tissues to undergo age-associated decline during aging. This is true in humans and in C. elegans. Researchers in the aging and stress fields are going to want to know what is happening in muscle.

Point 3. The reviewer's original concern was that the Western blot used to detect the oxidized and reduced forms of roGFP cropped out the large molecular weight portion of the gel, and that the reader has no way of knowing whether aggregates of roGFP are accumulating. The authors responded by providing the complete Western blot in Figure E1J. The reviewer's rebuttal indicated that he or she felt that there was clear evidence of disulfide-linked aggregation in the non-reduced sample, suggesting that the sensor is not ideal.

I disagree with this reviewer's rebuttal. First, the higher molecular weight bands observed in previous Figure E1J correspond in size to roGFP dimers, not to aggregates. Second, the presence of dimers and/or aggregates is only a concern when they are the predominant form of roGFP being detected. For example, in the two papers listed by the reviewer, dimers and aggregates together make up 50% or more of the total roGFP signal on the blot when examining inferior roGFP reagents. For the paper under review here, dimers and aggregates appear to make up less than 10% of the total signal for roGFP on the blot. It is unlikely that this small fraction of roGFP would dramatically alter interpretation of the fluorescence from the reporters and data shown in Figure 1. The revised figure for E1J repeats this experiment, and extends it by examining fractionation of detergent-soluble and insoluble fractions; this approach further supports that there is no major aggregation of the roGFP reporter.

Point 4. The reviewer's original concern was that the authors did not repeat their time lapse analysis from HeLa cells in worms. The authors responded by indicating that it would not be technically feasible to perform such an experiment in intact animals (there is no easy way to expose the animals to the reagents and then rapidly mount them, let alone wash them out quickly). The reviewer's rebuttal suggested that other papers have found ways to immobilize worms for such experiments.

I think that this reviewer's rebuttal was unfair. Yes, other researchers have monitored roGFP in worms in real time; however, they did this at low magnification, examining whole tissues (not subcellular organelles), with worms on growth plates (not worms immobilized on microscope slides). The authors are focussing on subcellular structures like ER, which require high-resolution optics and hence coverslips and immobilization. There is no way to do the experiments that the reviewer requested. Moreover, the experiments, even if they could be performed, would not add anything new or substantive to the paper.

The reviewer also had an original concern that the abstract did not reflect the heavy use of HeLa cells in the paper. The authors responded by stating that the abstract word limit is short and that the ability to examining aging and this phenomena in C. elegans made it important to emphasize the worm studies in the abstract. The reviewer rebutted that the main experiments should therefore be done in C. elegans and not HeLa cells.

I do not agree with the reviewer that this is a major concern. The combination of C. elegans and HeLa cell studies is a strength, not a weakness. It shows that what is being observed is not just a "worm-only" phenomena. That said, it seems like an easy fix would just be to require that the authors add the words "and HeLa cells" after the word "C. elegant," which is what they have done.

Overall, I feel that reviewer 4 is overly critical. His or her concerns are not really substantive.

