Assurance of mitochondrial integrity and mammalian longevity by the p62-Keap1-Nrf2-Nqo1 cascade

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Transaction Report:

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

1st Editorial Decision 05 July 2011

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of reports from the referees. As the three referees feel that the manuscript is interesting, I would like to invite you to revise it according to their comments.

Referee #1 has three major concerns mostly related to the comparison of your results to other findings previously reported. First, s/he points out that the role of p62 in mitophagy could contribute to the aging effect observed in the mutant and this point is further expanded under “minor points”. This relates to Referee #3 concern regarding the contribution of other pathways to the accelerated aging phenotype. Second, s/he thinks that previous phenotypes reported for p62 KO mice, particularly neurodegeneration and obesity, should be discussed. Also, s/he mentions previous studies showing that increased p62 levels are related to increased oxidative stress. S/he has some minor points that should be addressed as well. Referee #2 is very positive towards the manuscript. S/he has two minor comments. S/he believes that sex differences in lifespan should be discussed. Referee #1 agrees on this point. S/he is also concerned, together with Referee #3 with the data regarding H2O2 generation. Finally, Referee #3 has a single major concern. S/he thinks that additional data supporting the main claim of the study - that the p62/Keap1/Nrf2/Nqo1 signaling cassette is directly involved in aging control - needs to be provided, and suggests three possible
approaches to that end.

Given the reviewers constructive comments and the potential interest of your study, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely,

Editor

EMBO reports

REFEREE REPORTS:

Referee #1:

The current manuscript from the group of Jaekyoon Shin reports data showing that p62 KO mice age prematurely and have a reduced life span, particularly the male mice. This very interesting, novel and dramatic finding is explained by a decline of mitochondrial function by age leading to an increasingly pro-oxidant cellular environment. The authors attribute this to the role of p62 in enhancing the basal Nrf2 activity to yield a higher steady state expression of the NAD(P)H: quinone oxidoreductase 1 (Nqo1) and thereby maintaining the mitochondrial membrane potential and limit generation of reactive oxygen species (ROS). The authors convincingly show that ROS levels and mitochondrial integrity, membrane potential and function are clearly compromised in the p62 KO mice. Hence, considering a substantial literature on a correlation between mitochondrial function and longevity, it is not farfetched to conclude that there is a connection between loss of p62 function and reduced life-span caused by an age-dependent decline in mitochondrial function. The authors, however, explain this only with a role of p62 in enhancing the basal level of Nrf2 which in turn enhances the steady state level of Nqo1 resulting in healthy mitochondria. Although, the evidence they present supports this hypothesis fairly well, these studies are largely done in cell culture models and the aspect of time involved is lost compared to the old mice they otherwise study. Another aspect is that the authors do not discuss previously described phenotypes reported for p62 KO mice or other processes or pathways, such as defects in selective autophagy of damaged mitochondria (mitophagy) caused by loss of p62 that likely is an important contributing factor to the reduced life-span observed for the p62 KO mice.

Major points:
1. The authors should discuss the relevant literature on the role of p62 in selective autophagy and particularly in mitophagy and if this is not a likely contributing factor in addition to the p62-Nrf2-Nqo1 pathway they present as the sole explanation for the accelerated aging observed. Surprisingly, the manuscript does not contain any references to the potential role that p62 may play in selective autophagy and clearance of damaged mitochondria.
2. It would be very interesting to learn if there is any sign of a neurodegenerative phenotype in the old p62 KO mice. A previous study of 6 month old p62 KO mice concluded that neurodegeneration occurred and the mice displayed increased anxiety, depression, loss of working memory, and reduced serum brain-derived neurotrophic factor levels as well as aggregated K63-ubiquitinated tau protein (Ramesh Babu et al., J. Neurochem. (2008) 106, 107-120.)

3. p62 KO mice have been made independently earlier by the groups of Jorge Moscat and Komatsu/Ishii/Tanaka and a number of papers have been published involving p62 KO mice. These studies have reported that p62 KO mice get overtly obese by 6 months of age (Duran) and have neurodegenerative disease features (see point 2 above). Several papers by Komatsu and others document that autophagy deficiency leads to accumulation of p62 causing liver dysfunction. Some comments to these other studies should be made in the Results and Discussion and perhaps also in the Introduction section of the manuscript.
4. Autophagy inhibition has been shown to increase p62 levels and high levels of p62 have been correlated with increased oxidative stress and tumor promotion. A discussion of the importance of the level of p62 for the outcome on oxidative stress status seems pertinent.

Minor points:
5. In Figure 3D the authors use semiquantitative RT-PCR to determine transcript levels of a small set of Nrf2 target genes. They conclude from that analysis that only the Nqo1 mRNA levels are reduced in p62 KO skeletal muscle. However, this type of analysis is not sensitive enough. They also use only a small collection of Nrf2 target genes which should be increased by at least 3 more Nrf2 target genes and they need to perform a quantitative RT-PCR assay in order to conclude if only Nqo1 mRNA levels are affected in the p62 KO muscles.
6. On top of page 11 it is stated that ectopic expression of p62 in Nrf2-knockdown HCT116 cells did not have any effect on oxidant concentration (Fig. 4J) or Nrf2 activity (Fig. 4O). However, although small there seems to be an effect in these two cases. Is this statistically insignificant or caused by residual Nrf2 that become activated by p62, or do we see a glimpse of a Nrf2-independent effect of p62 that perhaps would be much more prevalent in more long term experiments? I am here thinking on an effect of p62 exerted via selective autophagy of damaged mitochondria that will contribute to keep the mitochondria healthy and lower ROS levels. Autophagy has been shown to play a role in determining longevity so is there a link between p62 and its role in clearing damaged organelles and protein aggregates and increased longevity? See point 1 above.
7. Would the authors like to speculate about the sex difference in longevity of the p62 KO mice? I think this difference needs some comments.
8. The alignment in Supplementary Figure 1 lacks an indication on the location of the crucial N-terminal PB1 domain of p62. Also the use of the term conserved domains I to VI is discouraged since it may cause confusion relative to the established domains (PB1, ZZ, UBA and the LIR and KIR) of p62. It is better to define these regions more stringently based on % identity scores and use the term conserved regions instead of conserved domains.
9. A few typographical errors: "kepa1", second line of page 10 should be "Keap1"; first line page 11, "knockdowned" should be "knockdown"; page 11, line 3 from bottom, "effector" to "effector";

Referee #2:

The authors have generated a sqstm1/p62 knockout mouse to study the physiological relevance of the protein. Sqstm1/p62 is known to interact with Keap1, an adaptor of a ubiquitin ligase complex, via its Keap1 binding region (KIR). This interaction interferes with Keap1’s role in degradation of Nrf1. Stabilization of Nrf1 has been shown to promote longevity in worms and fruit-flies.

The authors show that the sqstm1/p62 knockout mouse age faster than the wild-type, that the mitochondrial function is compromised, and that Nrf2 activity is inhibited. They suggest that the p62-Nrf2-Nqo1 cascade assures mammalian longevity by stabilizing mitochondrial integrity.

Minor comments:
1. The authors observe that p62 knockout mice have a shorter lifespan compared to wild-type mice. This is seen both for male and female knockout mice, but the phenotype is more pronounced for male mice. May the authors propose an explanation for this?
2. On page 8 of the manuscript, the authors report age-dependent (20/90 weeks) differences in H2O2 generation in wild-type and knockout mice. These are not shown and should be indicated as such or included in Fig 2D.
3. On page 10 of the manuscript, "Keap1" has been spelled "kepa1".

Referee #3:

This manuscript reports data from the characterization of a knock-out mouse in which p62, a protein being part of the p62-Keap1-Nrf2-Nqo1 regulatory cascade is characterized. The key finding of this characterization is the expression of accelerated aging phenotype and a decrease in lifespan of
knock-out mice. This phenotype is linked to mitochondrial dysfunction. While the work is well performed and technically sound a major critique concerns the conclusions about the relevance of this study in respect to the aging process. It is undisputed that there is a strong effect on lifespan as the result of the ablation of one component of the studied regulatory pathway (p62). However, it is questionable at this time whether the identified pathway has any relevance for natural aging. A lifespan decrease may indeed be the result of affecting many molecular pathways which do not play a general role in aging. In order to provide more robust data supporting the KEY CONCEPT of the submitted study, the impact of p62 on mouse aging, which will be of interest for a larger readership, the authors are asked to provide convincing data to support such an impact. Among other kinds of investigations which can generate such data there are:

1. An analyses of wild-type mice of different age to show whether under natural conditions components of the studied regulatory pathway are declining in abundance, become modified (e.g., damaged) or whether there is an effect on the expression of the corresponding genes.
2. Demonstration of a lifespan extending effect via manipulation of components of the studied pathway (e.g., stimulation of the expression of components, or over-expression of the p62 gene in mice).
3. Identification of long-lived mice strain and/or cell culture types in which stimulation of the regulatory pathway has a kind of a positive effect related to aging and lifespan (e.g., lifespan at the organismal level (preferable) or on replicative lifespan of cells).

Apart from this major point there are few issues the authors are asked to correct/clarify/consider:

1. Fig. 2 C. The quality of this Figure is poor (too small). Please increase to a size mitochondria can clearly be identified.
2. Since mitochondria are dynamic organelles constantly undergoing fission and fusion it would be interesting to see the light microscopic structure (filamentous network punctuate).
3. In Fig 2 D % mitochondrial H2O2 production is indicated as being measured. This appears not to be correct. It is probably the 'amount' which is depending on the generation and the removal of H2O2 (e.g. via scavenging enzymes like catalase, peroxiredoxin). Correct?
4. In Figure 2 E deletions of the mitochondrial DNA are analyzed. The conceptual point to this analysis is not explained in the paper.
5. The authors report electron dense material in mitochondria of the deletion strain. Is it clear what this material is? Such material has been previously reported in cell cultures in which Lon protease as a protease removing damaged proteins from mitochondria is affected. Is there an effect on Lon expression in the ko mice?

Specific Responses to Referee #1’s comments:

Major points:
1. The authors should discuss the relevant literature on the role of p62 in selective autophagy and particularly in mitophagy and if this is not a likely contributing factor in addition to the p62-Nrf2-Nqo1 pathway they present as the sole explanation for the accelerated aging observed. Surprisingly, the manuscript does not contain any references to the potential role that p62 may play in selective autophagy and clearance of damaged mitochondria.

Response: We have discussed with relevant references on the potential role of p62 for selective autophagy and particularly for mitophagy, in relation to the rapid mitochondrial and organismal aging phenotypes that arise in p62−/− mice. (pp. 13, line 15 to pp. 14, line 5).

2. It would be very interesting to learn if there is any sign of a neurodegenerative phenotype in the old p62 KO mice. A previous study of 6 month old p62 KO mice concluded that neurodegeneration occurred and the mice displayed increased anxiety, depression, loss of working memory, and reduced serum brain-derived neurotrophic factor levels as well as aggregated K63-ubiquitinated tau protein (Ramesh Babu et al., J. Neurochem. (2008) 106, 107-120.)
Response: Due to time constraints, preparation of a cohort of old p62KO mice and measuring neurodegenerative phenotypes during the revision period was not possible. Instead, we examined the levels of aggregated K63-ubiquitinated tau protein in the available frozen brain samples of 20 month-old p62KO mice (n = 4) relative to those of age-matched wild-type controls. However, we failed to see any significant changes, probably due to the small number of samples that may amplify individual variation or due to differences in sample preparation (fresh vs. frozen). On the other hand, consistent with the previous results (Ramesh Babu et al., J. Neurochem. 106, 107-120), we could also observe the increased anxiety and depression in 2 month-old p62KO mice. Nevertheless, in order to avoid unnecessary dispute with our incomplete data, we discussed the reported neurodegenerative phenotypes in p62 KO mice in relation to rapid aging observed in our current study (pp. 15, line 14 to pp. 16, line 8).

3. p62 KO mice have been made independently earlier by the groups of Jorge Moscat and Komatsu/Ishii/Tanaka and a number of papers have been published involving p62 KO mice. These studies have reported that p62 KO mice get overly obese by 6 months of age (Duran) and have neurodegenerative disease features (see point 2 above). Several papers by Komatsu and others document that autophagy deficiency leads to accumulation of p62 causing liver dysfunction. Some comments to these other studies should be made in the Results and Discussion and perhaps also in the Introduction section of the manuscript.

Response: We have discussed previously reported obesity and neurodegeneration phenotypes in the Results and Discussion section in conjunction with point 2 above in pp. 15, line 14 to pp. 16, line 8. In addition, we also briefly mentioned those previous reports in the Introduction section (pp. 5, lines 13 to 15). The adverse effect of p62 accumulation in the autophagy-deficient liver has been mentioned in the original manuscript (pp. 5, lines 5 and 10) and further discussed in the revised manuscript (pp. 14, second paragraph) in conjunction with point 4 below.

4. Autophagy inhibition has been shown to increase p62 levels and high levels of p62 have been correlated with increased oxidative stress and tumor promotion. A discussion of the importance of the level of p62 for the outcome on oxidative stress status seems pertinent.

Response: We have discussed this in conjunction with point 3 above (pp. 14, second paragraph). For this, we included new data showing age-dependent decrease in the expression of both p62 and autophagy components, Atg5 and Atg7, in wild-type mice livers (Fig. 5), which indicates the difference between pathologic environments of autophagy-deficient mice and normally aged wild-type mice.

Minor points:
5. In Figure 3D the authors use semiquantitative RT-PCR to determine transcript levels of a small set of Nrf2 target genes. They conclude from that analysis that only the Ngo1 mRNA levels are reduced in p62 KO skeletal muscle. However, this type of analysis is not sensitive enough. They also use only a small collection of Nrf2 target genes which should be increased by at least 3 more Nrf2 target genes and they need to perform a quantitative RT-PCR assay in order to conclude if only Ngo1 mRNA levels are affected in the p62 KO muscles.

Response: Following the reviewer’s comments, we performed quantitative RT-PCR of previously tested Nrf2 target genes and five additional genes including Gstp1, Mt2, Prdx1, Txnrd1 and Ugt1a6a. This new result was presented in the revised Fig. 3D.

6. On top of page 11 it is stated that ectopic expression of p62 in Nrf2-knockdown HCT116 cells did not have any effect on oxidant concentration (Fig. 4J) or Nrf2 activity (Fig. 4O). However, although small there seems to be an effect in these two cases. Is this statistically insignificant or caused by residual Nrf2 that become activated by p62, or do we see a glimpse of a Nrf2-independent effect of p62 that perhaps would be much more prevalent in more long term experiments? I am here thinking on an effect of p62 exerted via selective autophagy of damaged mitochondria that will contribute to keep the mitochondria healthy and lower ROS levels. Autophagy has been shown to play a role in determining longevity so is there a link between p62 and its role in clearing damaged organelles and protein aggregates and increased longevity? See point 1 above.
Response: The effect of ectopic expression of p62 in Nrf2-knockdown HCT116 cells was statistically insignificant, and thus we described this in Fig. 4O. In addition, we do not think that this small effect was due to the loss of p62 function for mitophagy, because (i) the wild-type and UBA mutant of p62 showed similar effects on Nrf2 activity (Fig. 3C) and on ROS levels (data not shown), and (ii) a recent report (Narendra D. et al.) also demonstrated that p62 and its LC3 binding region were dispensable for mitophagy.

7. Would the authors like to speculate about the sex difference in longevity of the p62 KO mice? I think this difference needs some comments.

Response: We have discussed the sexually dimorphic effect of p62 knockout on the mice longevity in pp. 14, line 16 to pp. 15, line 12.

8. The alignment in Supplementary Figure 1 lacks an indication on the location of the crucial N-terminal PB1 domain of p62. Also the use of the term conserved domains I to VI is discouraged since it may cause confusion relative to the established domains (PB1, ZZ, UBA and the LIR and KIR) of p62. It is better to define these regions more stringently based on % identity scores and use the term conserved regions instead of conserved domains.

Response: Following the reviewer’s comment, we have used the established domains (PB1, ZZ, UBA and the LIR and KIR) of p62 throughout the text with related references. In addition, to cope with the EMBOR policy limiting the number of supplementary figures to five, we deleted the previously Supplementary Fig. S1.

9. A few typographical errors: “kepa1”, second line of page 10 should be ”Keap1”; first line page 11, “knockdowned” should be “knockdown”; page 11, line 3 from bottom, “effecter” to “effector”;

Response: We have corrected typographical errors.

Specific Responses to Referee #2’s comments:

Minor comments:
1. The authors observe that p62 knockout mice have a shorter lifespan compared to wild-type mice. This is seen both for male and female knockout mice, but the phenotype is more pronounced for male mice. May the authors propose an explanation for this?

Response: We have discussed on this in point 7 of referee #1.

2. On page 8 of the manuscript, the authors report age-dependent (20/90 weeks) differences in H2O2 generation in wild-type and knockout mice. These are data not shown and should be indicated as such or included in Fig 2D.

Response: There was a mistake in labeling of Fig 2D of original manuscript. We have corrected to 20, 60 and 90-weeks of age in the revised Fig. 2D as such indicated in the text.

3. On page 10 of the manuscript, "Keap1" has been spelled "kepal1".

Response: We have corrected the typographical error.

Specific Responses to Referee #3’s comments:

Major comment:
It is questionable at this time whether the identified pathway has any relevance for natural aging. A lifespan decrease may indeed be the result of affecting many molecular pathways which do not play a general role in aging. In order to provide more robust data supporting the KEY CONCEPT of the submitted study, the impact of p62 on mouse aging, which will be of interest for a larger readership, the authors are asked to provide convincing data to support such an impact. Among other kinds of investigations which can generate such data there are:
1. Analyses of wild-type mice of different age to show whether under natural conditions components of the studied regulatory pathway are declining in abundance, become modified (e.g., damaged) or whether there is an effect on the expression of the corresponding genes.

2. Demonstration of a lifespan extending effect via manipulation of components of the studied pathway (e.g., stimulation of the expression of components, or over-expression of the p62 gene in mice).

3. Identification of long-lived mice strain and/or cell culture types in which stimulation of the regulatory pathway has a kind of a positive effect related to aging and lifespan (e.g., lifespan at the organismal level (preferable) or on replicative lifespan of cells).

Response: Due to limitations in time and resources, we analyzed the expression levels of p62 and Nqo1 in wild-type mice of different ages (12-, 67- and 132-week-old) following the reviewer’s suggested #1 investigation. Data showing the declining expression of p62 and Nqo1 with age in wild-type mice were presented in the Fig. 5. In addition, we have discussed this and recent reports describing the enhanced Nrf2 activity in the tissues and cells derived from long-lived mice strains in the text (pp. 13, first paragraph) of revised manuscript.

In relation to reviewer’s suggested #2 investigation, it should be mentioned that we already tried to establish a transgenic mouse strain overexpressing p62 without success, probably due to deleterious effects of p62 accumulation.

Minor comments:

1. Fig. 2 C. The quality of this Figure is poor (too small). Please increase to a size mitochondria can clearly be identified.

Response: The size of EM image has been enlarged and presented in the revised Fig. 2C.

2. Since mitochondria are dynamic organelles constantly undergoing fission and fusion it would be interesting to see the light microscopic structure (filamentous-network-punctuate).

Response: We have performed immunocytochemistry to examine the changes in dynamic mitochondrial structure in control and p62 knockdown HeLa cells. As mitochondrial morphology varies according to the stages of cell cycle progression, it was difficult to measure the effect of p62 knockdown in asynchronized cells. However, under the G1 arrested condition, we clearly saw the increase in the number of cells containing fragmented mitochondria with p62 knockdown, compared to control cells showing mostly tubular mitochondrial structure. This result was presented in Supplementary Fig. S3 and described in pp. 9, lines 1 to 3 of the revised manuscript.

3. In Fig 2D % mitochondrial H2O2 production’ is indicated as being measured. This appears not to be correct. It is probably the ‘amount’ which is depending on the generation and the removal of H2O2 (e.g. via scavenging enzymes like catalase, peroxiredoxin). Correct?

Response: Following the reviewer’s comment, we have changed the legend to ‘Relative rates of H2O2 generation’ in Fig. 2D of the revised manuscript.

4. In Figure 2E deletions of the mitochondrial DNA are analyzed. The conceptual point to this analysis is not explained in the paper.

Response: Due to the page limitations, we have used a reference rather than explaining the conceptual point in the original manuscript. In addition, we addressed this in the figure legend. However, in order to clarify this, we added a sentence ‘Deletions in this region provide a robust measure of age-dependent mitochondrial mutation’ right next to the reference cited in the text (pp. 9, lines 15 and 16).

5. The authors report electron dense material in mitochondria of the deletion strain. Is it clear what this material is? Such material has been previously reported in cell cultures in which Lon protease as a protease removing damaged proteins from mitochondria is affected. Is there an effect on Lon expression in the ko mice?

Response: Currently, we do not know the nature of electron dense material present in the mitochondria of p62KO mice. In order to examine the expression of Lon protease in p62KO mice,
we performed quantitative RT-PCR and Western blot as suggested by the reviewer. We observed that both message and protein levels of Lon protease were not different between tissues of wild-type and p62KO mice. This was presented in Supplementary Fig. S3 and text (pp. 8, last sentence) of the revised manuscript.

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

Referee #1 particularly suggests several minor changes that I will not repeat here. I would like however to draw your attention to one of them, point #4, regarding the length of certain figure legends. As it is, you manuscript is already about 2000 characters above our limit. Although we understand the need for explanatory figure legends, I would like to ask you to stay below the 30000 character count including references.

After these remaining corrections have been introduced, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the publication process to continue.

Thank you for your contribution to EMBO reports.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In the revised version the authors have addressed and answered the questions/criticisms I had after reading the original manuscript in an acceptable manner. The authors have also added new results including data showing an age-dependent decrease in the mRNA levels of p62, Nqo1, Atg5 and Atg7 in wild type mice as well as qPCR on five additional Nrf2 target genes.

I accept that more detailed and clarifying answers regarding a possible neurodegenerative phenotype in old p62 KO mice and more on the possible contribution of selective autophagy, particularly mitophagy, are impossible to give as part of the current paper.

Some minor points:
1. I could not find any description of the UBA mutant used in this study. This must be included in the manuscript text and relevant figure legend. From Figure 3C it is clear that it cannot be a deletion mutant. What point mutant is it? And is it one shown to be deficient in ubiquitin -binding?
2. Page 9, line 12 and Fig. 2E. The authors say that "two major fragments corresponding to age-associated deletions are seen from 20-week old p62/− samples"... However, There are no bands in the relevant samples from 20-week old p62/− mice as shown in Fig. 2E. They appear clearly first at 60 weeks.
3. Page 11, line 10: I guess "conferring a higher steady state expression" is meant, not only "conferring a higher steady expression"?
4. Several of the figure legends are too short. Some important info is lacking that would help the reader understand the data more quickly. Also some important labeling is lacking in some figures.
Fig. 2E it should be indicated that the upper panel is from WT mice, the next from p62 KO mice and the third WT (?) and the forth p62 KO mice.

5. In Fig. 3A the blots are not labeled with p62, Keap1 and GAPDH (or is it actin?)

6. In Fig. 5 p62, the panels A-D could be labeled with p62, Nqo1, Atg5 and Atg7, respectively and "Relative expression" should be changed to "Relative mRNA levels". It should also be explained in the figure legend how the data are normalized (to GAPDH or other housekeeping gene mRNA levels?).

Referee #3:

This paper reports very interesting data from the characterization of a p62 knock-out mouse. The key finding is the expression of an accelerated aging phenotype and a decrease in lifespan. This phenotype is related to mitochondrial dysfunction. Most importantly, a link to a role of the analyzed p62-Keap1-Nrf2-Nqo1 regulatory pathway to normal aging (i.e., aging of wild-type mice) is described by demonstrating a reduction expression p62 and Nqo1 in liver samples from wild-type mice.

In the revised version the authors have sufficiently addressed my initial concerns. In particular providing additional data on the expression on genes coding for p62 and Nqo1 in wild-type strains of different age strengthens a link of the corresponding molecular pathways with NORMAL aging. Overall, this is an important piece of work with interesting perspectives for follow-up studies to uncover the detailed role of quality control pathways (e.g., autophagy-mitophagy, mitochondrial dynamics) in aging and disease.

2nd Revision - authors’ response 23 November 2011

Specific Responses to Referee #1’s comments:

Minor points:

1. I could not find any description of the UBA mutant used in this study. This must be included in the manuscript text and relevant figure legend. From Figure 3C it is clear that it cannot be a deletion mutant. What point mutant is it? And is it one shown to be deficient in ubiquitin -binding?

Response: A mutant p62, in which Leu416 and Leu417 were replaced with alanines, was used as the UBA mutant. We confirmed the loss of ubiquitin binding activity by this mutation. This information was included in the text (pp. 11, lines 1 and 2) and also in the figure legend for Fig. 3.

2. Page 9, line 12 and Fig. 2E. The authors say that "two major fragments corresponding to age-associated deletions are seen from 20-week old p62-/- samples"... However, There are no bands in the relevant samples from 20-week old p62-/- mice as shown in Fig. 2E. They appear clearly first at 60 weeks.

Response: Although those two bands appeared indeed from 20-week old p62−/- samples, their signal intensities were so low to clearly demonstrate in the figure. Thus, we modified the sentence in a way that "---revealed the largely increased appearance of two major fragments corresponding to age-associated deletions in p62−/- samples from 60- and 90-week-old mice" (pp. 9, lines 10 to 13).

3. Page 11, line 10; I guess "conferring a higher steady state expression" is meant, not only "conferring a higher steady expression"?

Response: In order to describe more properly, we corrected the wording from "conferring a higher steady state expression" to "conferring a higher steady state expression" (pp. 11, line 8)
4. Several of the figure legends are too short. Some important info is lacking that would help the reader understand the data more quickly. Also some important labeling is lacking in some figures. In Fig. 2E it should be indicated that the upper panel is from WT mice, the next from p62 KO mice and the third WT (?) and the forth p62 KO mice.

Response: In conjunction with points #1 and #6, we added more information to figure legends for Fig. 3 and 5. We also added the figure labeling properly.

5. In Fig. 3A the blots are not labeled with p62, Keap1 and GAPDH (or is it actin?)

Response: We added the figure labeling properly.

6. In Fig. 5 p62, the panels A-D could be labeled with p62, Nqo1, Atg5 and Atg7, respectively and "Relative expression" should be changed to "Relative mRNA levels". It should also be explained in the figure legend how the data are normalized (to GAPDH or other housekeeping gene mRNA levels?).

Response: We labeled p62, Nqo1, Atg5 and Atg7 to corresponding figures as suggested, and also changed their labels from "Relative expression" to "Relative mRNA levels". In addition, we explained the way of data normalization (to 18S rRNA) in the Fig. 5 legend.

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports