EMBO reports

MOAP-1-mediated dissociation of p62/SQSTM1 bodies releases Keap1 and suppresses Nrf2 signaling

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DOI: 10.15252/embr.202050854

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Review Timeline:

Submission Date:	11th May 20
Editorial Decision:	4th Jun 20
Revision Received:	28th Sep 20
Editorial Decision:	27th Oct 20
Revision Received:	2nd Nov 20
Accepted:	3rd Nov 20

Editor: Martina Rembold

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 4th Jun 2020

Dear Dr. Yu

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, referees 1 and 2 also point out several technical concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.

Given the constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We invite you to submit your manuscript within three months of a request for revision. This would be September 4th in your case. Yet, given the current COVID-19 related lockdowns of laboratories, we have extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact me if you wish to discuss the time needed and the revisions further (martina.rembold@embo.org).

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

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Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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6) Please note that a maximum of 5 EV Figures can be typeset (you currently have 8). Please move part of the EV figures into an Appendix. The Appendix is a single PDF file called *Appendix*, which should start with a short Table of Content including page numbers. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc.
EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures. Appendix figure legends are part of the Appendix pdf.

See detailed instructions regarding expanded view here:

Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>).
Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (state if the replicate is

biological or technical), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Editor
EMBO reports

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Referee #1:

In this manuscript, Tan CT et al demonstrate MOAP-1 as a negative regulator for the formation of p62-bodies. They found that MOAP-1 directly interacts with p62, which interferes with the oligomerization and the phase-separation of p62. Thus, p62 bodies increased in size and number due to loss of MOAP-1, and then Keap1, which is an adaptor protein for Cullin 3-based ubiquitin ligase responsible for the degradation of a transcription factor Nrf2, was translocated onto the p62-bodies. As a result, Nrf2 was activated by ablation of MOAP-1, which promoted the DEN-induced liver carcinogenesis. This manuscript contains interesting findings warrant to publish in EMBO Reports, but some issues that should be addressed remain.

Major comments
1. In stress conditions including the impairment of proteostasis, the phase-separation of p62 is induced upon the interaction with ubiquitinated proteins, resulting in the increased number of p62-bodies (Sun D et al., Cell Res 2018). And, the p62-liquid droplets cause the activation of Nrf2 through the sequestration of Keap1 (Sanchez-Martin P et al., EMBO Rep 2020). In accordance to

the author's model, MOAP-1 inhibits the condensation of p62 and inactivates Nrf2. However, since the impairment of proteostasis (e.g., proteasomal inhibition) increases the level of MOAP-1 protein (Fu NY et al., PNAS 2007), a futile cycle (simultaneous p62-phase separation and the dissociation, and concomitant activation and inactivation of Nrf2) is easily postulated. The authors should show a role of endogenous MOAP-1 in the p62-body formation. For instance, the treatment of cells with arsenite induces the formation of p62-positive structures, and the removal of the arsenite decreases the number of the p62-structures (Eino A et al., J Cell Sci 2015). In such conditions, endogenous MOAP-1 may increase and dissociate p62-bodies.
2. The authors showed that MOAP-1 does not have any effect on total level of p62 protein. The PB1 mutant of p62 is indispensable for the degradation of p62 (Ichimura Y et al., J Biol Chem 2008). If the MOAP-1 blocks the self-oligomerization of p62 through the PB1-domain, the degradation of p62 should be inhibited, resulting in the accumulation of p62. The author should explain this discrepancy.
3. In the case of selective autophagy for Ape1 complex, P-body, and stress granule, liquidity or interacting protein(s) have been shown to be crucial factors for the structures to be surrounded by autophagosomes (Yamasaki A et al., Mol Cell 2020, Zhang G et al., Cell 2018). The authors showed that RFP-p62 bodies become gel-like structures in MOAP-1 knockout cells. The author should examine the autophagic degradation of p62-bodies with and without MOAP-1.

Minor comments
1. Quantitative analyses are needed.
Figure 3A and B: quantification of the number of p62-puncta.
Figure 6B and C: quantification of signal intensity for FLAG and oligomerized p62, respectively.
Figure 7B: quantification of signal intensity for immunoprecipitated Keap1.
Figure 7D: quantification of cells with nuclear Nrf2.
Fig. EV2: quantification of number of the number of p62-puncta.
Fig. EV3: quantification of number of the number of p62-puncta.
2. The authors generated several knockout cell lines by CAS9-CRISPR technology. They should present the data showing loss of those proteins.
Fig. EV1 C: p62
Fig. EV2B: MOAP-1.

Referee #2:

In the current work, the authors identify a new role of Modulator of apoptosis 1 (MOAP-1) as a negative regulator in the p62-Keap1-NRF2 signaling. The authors claim that under cellular stress, MOAP-1 is recruited to the p62 bodies where negatively regulates p62 oligomerization. Loss of MOAP-1 leads to the upregulation of p62 bodies that sequestrates Keap1 resulting in the hyperactivation of NRF2. This study concludes that MOAP-1 has an inhibitory role in liver carcinogenesis induced by DEN. Although the proposed model is of potential biological relevance and the results support their conclusions, several concerns should be addressed to definitively demonstrate the proposed model.

Specific Comment

1- Since MOAP-1 is a protein that is constantly degraded via the ubiquitin-proteasome system, the experiments should be performed under proteasome blockade conditions after transfection (Figures 2A-F). , Also, because MOAP-1 expression and distribution in the cell changes in a time-

dependent manner after transfection, experiments should be done in cells stably expressing MOAP-1 instead of in transient expression (Figures 2A, 2B, and 2E). Otherwise, it is quite difficult to do a rigorous analysis of the role of MOAP-1 in the dissociation of the p62 bodies. Since p62 acts as a cargo adaptor for both proteasomal- and autophagy-mediated degradation, the authors should clarify whether the loss of the colocalization of MOAP-1 and p62 is due to a disassembling of the p62 bodies or an increment in the degradation of the p62-MOAP-1 complex by the proteasome.

2- The authors claim that MOAP-1 expression does not affect the levels of p62 protein (Figures 2G and 2J). However, the method used to analyze the expression levels of p62 is not suitable for the analysis of the p62 ubiquitin aggregates that are generally found in the insoluble protein fraction. To demonstrate that MOAP-1 only regulates the disassembling of the p62 bodies and not the p62 levels, the authors should generate detergent-soluble and insoluble protein fractions (ubiquitin aggregates) to demonstrate that MOAP-1 overexpression or MOAP-1 depletion only affects the p62 distribution in the p62 bodies and not its expression.

3- After transfection, the distribution of MOAP-1 in normal and cancer cells is different. While, in normal cells, MOAP-1 has a diffuse distribution in the cytosol, in cancer cells, MOAP-1 colocalize directly with p62. The authors should explain the criteria they have followed to use normal hepatocytes (LO2 cells) or cancer cells (HepG2, HCT16…) in the different experiments of this work since the distribution of this protein is critical for its effect in the p62 bodies (Figure 3A and 3B). Is the MOAP-1 protein turnover rate different in LO2 cells compared with HepG2 cells?

4- The authors should analyze whether MOAP-1 WT or MOAP-1 M13 overexpression affects the exchange of p62 in the RFP-p62 bodies after photobleaching (FRAP) assay (Figure 4F).

5- In Figures 4I and 5E, overexpression of MOAP-1 (24h after transfection) presents a diffuse pattern in the cytosol, while in Figure 2E (24h after transfection), MOAP-1, colocalizes with the p62 bodies. The authors should explain this inconsistency.

6- To understand the mechanism by which MOAP-1 promotes dissociation of the p62 bodies, the authors should analyze whether MOAP-1 overexpression affects the interaction of p62 with K48/K63-linked polyubiquitin or with proteins that mediate the formation of p62 bodies such as NBR1, DAXX or TAK1.

7- The authors should reconstitute MOAP-1 KO cells with MOAP-1 WT or MOAP-1 M13 to analyze the effect of the MOAP-1/p62 complex in p62-Keap interaction (Figure 7A-B), Keap1-NRF2 interaction (Figure 7C), NRF2 nucleus translocation (Figure 7D) and the expression of NRF2 targets (Figure 7E).

8- In Figure EV8-A, the authors failed to demonstrate that there is an induction of the p62/Keap1 interaction in MOAP-1 KO livers because the increment of the interaction correlates with the increment of the Keap1 levels. Therefore, the interaction is just secondary to the amount of Keap1.

9- Figures 5D and 5E are redundant.

10- The labeling of Figure EV4D should be corrected.

11- p62 bodies should be quantified in Figures 3A and 3B as in Figures 2D, 2F, and 2I.

Referee #3:

This paper conveys a novel and well-demonstrated central finding that is well summarized in its title. The role of MOAP1 in the dissociation of p62 bodies and the regulation of Keap1/Nrf2 signaling is demonstrated using multiple approaches, including extensive cell-based characterization as well as in vivo validation and phenotypic relevance. I have a couple general comments (primarily for the editors) that do not require a response, and only a few minor comments for the authors to consider:

General comments:

1. The manuscript has probably been submitted elsewhere before, as a result of which it has become very thorough and solid. There is really not much to criticize or improve about it. I expect it will receive several citations.

2. With 8 main figures and 8 supplemental ones, the manuscript does not really fit the short-format original concept of EMBO Reports. Nevertheless, I expect that the editors are well aware of that, and since they are sending it out for review, I take that to mean that they are fully happy to publish manuscripts of this length. Besides, other manuscript I have reviewed for EMBO Reports were also quite long and exhaustive. Therefore, I don't really take this factor into account in my assessment and recommendation.

Minor comments:

1. On page 6, last sentence of the second paragraph: From the data shown, it is difficult to draw conclusions about the physical properties of the p62 bodies ("were more viscous or gel-like"). I suggest to rephrase using a more hypothetical formulation.

2. On page 6, last 3 sentences at the bottom of the page: From the data shown, it appears difficult to draw firm conclusions about the shape of the p62 bodies. Perhaps it is just a certain phase of the fusion-fission process?

3. The Y axis in Fig. 4G is too busy and can be simplified.

Faculty of Science
Department of Pharmacy

National University of Singapore

September 28th, 2020

Martina Rembold, PhD
Editor
EMBO Reports

Re: Submission of Revised Manuscript (EMBOR-2020-50854V1).

Dear Dr. Rembold,

We are pleased to learn that our manuscript entitled, "MOAP-1-mediated dissociation of the p62/SQSTM1 bodies suppresses the p62-Keap1-Nrf2 signaling axis" by Tan et al., was judged to be of interest and is potentially suitable to be published as an Article in *EMBO Reports*.

The referees raised a number of concerns, but also offered many valuable suggestions on potential experiments that would help strengthening the manuscript. We are pleased to inform that we have essentially completed all the additional experiments and revisions required for addressing the concerns and comments raised.

Specifically, we would like to elaborate in details of our responses to the concerns and comments raised, point-by-point, as the following:

Referee #1

"In this manuscript, Tan CT et al demonstrate MOAP-1 as a negative regulator for the formation of p62-bodies. They found that MOAP-1 directly interacts with p62, which interferes with the oligomerization and the phase-separation of p62. Thus, p62 bodies increased in size and number due to loss of MOAP-1, and then Keap1, which is an adaptor protein for Cullin 3-based ubiquitin ligase responsible for the degradation of a transcription factor Nrf2, was translocated onto the p62-bodies. As a result, Nrf2 was activated by ablation of MOAP-1, which promoted the DEN-induced liver carcinogenesis. This manuscript contains interesting findings warrant to publish in EMBO Reports, but some issues that should be addressed remain."

1. (i) *"In stress conditions including the impairment of proteostasis, the phase-separation of p62 is induced upon the interaction with ubiquitinated proteins, resulting in the increased number of p62-bodies (Sun D et al., Cell Res 2018). And, the p62-liquid droplets cause the activation of Nrf2 through the sequestration of Keap1 (Sanchez-Martin P et al., EMBO Rep 2020). In accordance to the author's model, MOAP-1 inhibits the condensation of p62 and inactivates Nrf2. However, since the impairment of proteostasis (e.g., proteasomal inhibition) increases the level of MOAP-1 protein (Fu NY et al., PNAS 2007), a futile cycle (simultaneous p62-phase separation and the dissociation, and concomitant activation and inactivation of Nrf2) is easily postulated. The authors should show a role of endogenous MOAP-1 in the p62-body formation. For instance, the treatment of cells with arsenite induces the formation of p62-positive structures, and the removal of the arsenite decreases the number of the p62-structures (Eino A et al., J Cell Sci 2015). In such conditions, endogenous MOAP-1 may increase and dissociate p62-bodies."*

   We thank you for the valuable comment and suggestion. Using the arsenite washout experiment *(Eino A et al., J Cell Sci 2015)* as suggested, reduction in levels of p62 bodies during the washout period of post-arsenite treatment was found to be significantly lower in the MOAP-1 KO cells. The new data are now shown in Fig EV1E and F. Together with the observation that loss of MOAP-1 promotes a more robust elevation of p62 bodies triggered by arsenite (Fig 2G and H), our data therefore support a role

of endogenous MOAP-1 in regulating levels of p62 bodies. Regarding to the question whether arsenite would promote upregulation of MOAP-1 protein, our data showed that arsenite upregulated MOAP-1 as well as enhanced MOAP-1/p62 interaction. The new data are now shown in Figs 2J and EV3D. It is therefore reasonable to postulate that higher levels of MOAP-1 induced by arsenite could be the mechanism for reducing levels of the p62 bodies observed. In the DEN treatment condition, however, endogenous MOAP-1 was not upregulated in the LO2 hepatocytes nor in the mouse livers *in vivo* (Fig EV3B and C), yet MOAP-1/p62 interaction was enhanced significantly (Fig EV3B and C). Hence, the enhanced binding of MOAP-1 to p62 mediated by stress signals is likely to play an important role in activating the function of MOAP-1 to facilitate disassembly of the p62 bodies. It would certainly be of interest in future study to investigate the mechanism underlying the enhanced MOAP-1/p62 interaction mediated by the stress signals.

2. *"The authors showed that MOAP-1 does not have any effect on total level of p62 protein. The PB1 mutant of p62 is indispensable for the degradation of p62 (Ichimura Y et al., J Biol Chem 2008). If the MOAP-1 blocks the self-oligomerization of p62 through the PB1- domain, the degradation of p62 should be inhibited, resulting in the accumulation of p62. The author should explain this discrepancy."*

   Thank you for sharing the interesting insight. While we don't yet have conclusive data to explain the apparent discrepancy, we would like to share our ideas which we hope would be helpful to shed light on explaining the discrepancy. We have also incorporated these ideas into the discussion section (p.13 in the revised manuscript) as the following:

   "Self-oligomerization of p62, mediated by its PB1 domain, allows p62 to aggregate and sequester ubiquitylated substrate for facilitating their degradation through the autophagy-lysosome pathway (Bjorkoy et al., 2005; Komatsu et al., 2007; Lamark et al., 2003; Wilson et al., 2003). The K7A and D69A mutations of the two critical residues of the PB1 domain of p62 render it unable to self-oligomerize and form aggregates, resulting in impaired autophagic degradation of p62 (Ichimura et al, 2008). Our finding that MOAP-1 blocks the PB1-mediated self-oligomerization of p62 should theoretically also result in inhibition of autophagic degradation of p62, leading to its accumulation, especially under the condition of MOAP-1 overexpression. However, as stress-induced formation of p62 bodies remains robust in the presence of endogenous and exogenous MOAP-1 (Figs 1C, 2A and G, and EV1A), it is conceivable that MOAP-1 may only exert an effect on disrupting the oligomeric states of p62. This may potentially be achieved through enhancement of the MOAP-1/p62 interaction in the p62 bodies which may play a part for disrupting the integrity of the p62 bodies. Thus, a hypothetical model can be considered that upon stress stimulation, p62 bodies are formed to sequester Keap1 to result in the activation of Nrf2 signaling, MOAP-1 is then recruited to the p62 bodies to promote their dissociation, thereby releasing Keap1 from p62. This can serve as a negative feedback mechanism to modulate the activity of the p62-Keap1-Nrf2 signaling, as well as allowing p62 to re-distribute to non-aggregated state, so that it can standby and re-initiate another round of signaling to activate Nrf2 upon stress."

3. *"In the case of selective autophagy for Ape1 complex, P-body, and stress granule, liquidity or interacting protein(s) have been shown to be crucial factors for the structures to be surrounded by autophagosomes (Yamasaki A et al., Mol Cell 2020, Zhang G et al., Cell 2018). The authors showed that RFP-p62 bodies become gel-like structures in MOAP-1 knockout cells. The author should examine the autophagic degradation of p62-bodies with and without MOAP-1."*

   Thank you for the interesting comment and suggestion. p62 can be degraded via autophagy through direct binding to LC3 *(Komatsu M et al., Cell, 2007; Pankiv S et al., J Biol Chem 2007).* As pointed out, in the case of selective autophagy for Ape1 complex, P-body, and stress granule in the *C elegans* and yeast systems, liquidity or interacting protein(s) have been shown to be crucial factors for defining these structures that exist as condensates to be surrounded and engulfed by autophagosomes, which are marked by ATG8 homologs *(Yamasaki A et al., Mol Cell 2020, Zhang G et al., Cell 2018)*. Our analysis of the liquidity of RFP-p62 bodies in the MOAP-1 deficient cells suggests that they are less dynamic

than those in the WT cells (Fig 4F and G). In the case of PGL granules in *C. elegans*, size of the granules is also one of the crucial determinants for influencing their susceptibility to autophagic degradation (*Zhang G et al., Cell 2018*). Thus, reduced liquidity and increased in the size of the p62 bodies in the MOAP-1 deficient cells could also potentially influence their ability to be degraded via the autophagy pathway. To evaluate this possibility, we performed co-staining of p62 and LC3 using immunofluorescence analysis. GFP-LC3 co-localized well with p62 bodies in both the WT and MOAP-1 deficient LO2 cells (new data included as Appendix Fig S3A). We also stained for endogenous LC3 using a well-described commercial LC3 antibody (Cell Signaling Technology, #2275), but unlike GFP-LC3, endogenous LC3 appeared to be poorly co-localized with the p62 bodies in both the WT and *MOAP-1* KO LO2 cells (new data included as Appendix Fig S3B). We believe this conundrum could be due to sensitivity of the antibodies (anti-GFP vs anti-LC3) or a consequence from overexpression of LC3. Nevertheless, these observations support the argument that an increase in size and levels of p62 bodies in the MOAP-1 deficient cells are not likely caused by alteration in the recruitment of LC3 to the p62 bodies. While localization of LC3 has been generally perceived as an indication for autophagic targeting *(Klionsky D et al., Autophagy, 2016)*, it has also been reported by Rubinsztein and colleagues that in cells with defective autophagy, LC3-I could still be sequestered by the p62 aggregates *(Runwal G et al., Sci Rep, 2019, 9:10147)*, raising the question on the reliability of using LC3 as a definitive marker for autophagic degradation of p62 in all circumstances. Moreover, LAMP1, which is a lysosome-resident protein frequently used as a marker for lysosome (*Klionsky D et al., Autophagy, 2016*), did not appear to localize in close proximity with p62 (new data included as Appendix Fig S3C). Thus, while p62 bodies serve to sequester Keap1 for activation of the Nrf2 signaling, it remains unclear whether they are targeted for autophagic degradation.

4. *"Minor comments*
   Quantitative analyses are needed.
   Figure 3A and B: quantification of the number of p62-puncta.
   Figure 6B and C: quantification of signal intensity for FLAG and oligomerized p62, respectively.
   Figure 7B: quantification of signal intensity for immunoprecipitated Keap1. Figure 7D: quantification of cells with nuclear Nrf2.
   Fig. EV2: quantification of number of the number of p62-puncta. Fig. EV3: quantification of number of the number of p62-puncta.
   The authors generated several knockout cell lines by CAS9-CRISPR technology. They should present the data showing loss of those proteins.
   Fig. EV1 C: p62
   Fig. EV2B: MOAP-1."

   We apologize for the lack of quantification in some of the data and missing Western data documenting the loss of p62 and MOAP-1 in the knockout cell lines. In the revised manuscript, we have added the quantifications to these data as the followings: for Fig 3A and B (the quantification of data are now shown in Fig 3B and E), Fig 6B and C (the quantification of the data are now shown in Fig 6C and E), Fig 7B and D (the quantification of the data are now shown in Fig 7C and G), Fig EV2A (the quantification of the data are now shown in Fig 2H) and Fig EV3 (the quantification of the data are now shown in Fig EV2C). The Western blots for validating the loss of p62 or MOAP-1 protein in the KO cell-lines are now shown in Fig 1F and Fig 2J.

Referee #2

"In the current work, the authors identify a new role of Modulator of apoptosis 1 (MOAP-1) as a negative regulator in the p62-Keap1-NRF2 signaling. The authors claim that under cellular stress, MOAP-1 is recruited to the p62 bodies where negatively regulates p62 oligomerization. Loss of MOAP-1 leads to the upregulation of p62 bodies that sequestrates Keap1 resulting in the hyperactivation of NRF2. This study concludes that MOAP-1 has an inhibitory role in liver carcinogenesis induced by DEN. Although the proposed model is of potential biological relevance and the results support their conclusions, several concerns should be addressed to definitively demonstrate the proposed model."

1. *"Since MOAP-1 is a protein that is constantly degraded via the ubiquitin-proteasome system, the experiments should be performed under proteasome blockade conditions after transfection (Figures 2A- F). Also, because MOAP-1 expression and distribution in the cell changes in a time-dependent manner after transfection, experiments should be done in cells stably expressing MOAP-1 instead of in transient expression (Figures 2A, 2B, and 2E). Otherwise, it is quite difficult to do a rigorous analysis of the role of MOAP-1 in the dissociation of the p62 bodies."*

   Thank you for the valuable insights and suggestions. We have now generated cells stably expressing MOAP-1 to facilitate further analysis of the role of MOAP-1 in regulating the p62 bodies. Overexpression of MOAP-1 via transient transfection was able to downregulate p62 bodies induced by DEN (Fig 2A) and similar observation was made in LO2 cells stably overexpressing MOAP-1 (new data included as Appendix Fig S2A). Using transient transfection to overexpress MOAP-1 in HepG2 cells, downregulation of the levels of the p62 bodies was also observed (Fig 2B). We have now repeated the experiment using HepG2 cells stably overexpressing GFP-MOAP-1 and similar observation was made (new data included as Fig EV1A). We also observed that treatment of these HepG2 cells with MG132 resulted in upregulation of GFP-MOAP-1 as well as levels of the p62 bodies. However, despite the initial elevation of p62 bodies at 12 and 16 hours post-MG132 treatment, levels of the p62 bodies were found to be gradually declined at later time points (at 20 and 24 hours post-MG132 treatment) in cells overexpressing the GFP-MOAP-1 (new data shown in Fig EV1A). Moreover, we have now also performed rescue experiment using the *MOAP-1* KO LO2 cell-line stably expressing MOAP-1 and similar result was obtained as the rescue experiment performed using transient transfection (Fig 2E). The new data are now shown in Appendix Fig S2B. With regarding to the suggestion to perform the experiments in Figures 2A-F under proteasome blockade conditions, we noted that proteasome inhibition *per se* can also lead to enhancement of levels of p62 bodies which has prevented us from analyzing the effect on p62 bodies brought upon by DEN. Furthermore, LO2 cells treated with MG132 for more than 16 hours would start showing signs of cellular toxicity which will also affect our ability to conduct analysis using DEN and MG132 at longer time points. For these reasons, we are unable to include data on the analysis using proteasome inhibitor for Fig 2A. For Fig 2C and D where knockout of MOAP-1 was shown to lead to a spontaneous increase in the levels of p62 bodies, additional experiment with proteasome inhibition have now been conducted. In both the WT and *MOAP-1* KO LO2 cells treated with MG132 for 8 hours, p62 bodies were found to be elevated, but significant increase in the sizes and numbers of p62 bodies in the *MOAP-1* KO LO2 cells were also be detected. The new data are now shown in Appendix Fig S2C and D. For Fig 2E and F, as overexpression of MOAP-1 alone was already sufficient to achieve potent downregulation of p62 bodies, we therefore did not repeat the experiment with proteasome inhibitor.

   "Since p62 acts as a cargo adaptor for both proteasomal- and autophagy-mediated degradation, the authors should clarify whether the loss of the colocalization of MOAP-1 and p62 (presented in Figure 2A and B) is due to a disassembling of the p62 bodies or an increment in the degradation of the p62-MOAP-1 complex by the proteasome"

   Thanks for the comment. Our data suggest that loss of the co-localization of the MOAP-1 and p62 is not due to the degradation of the p62-MOAP-1 complex by the proteasome, as inhibition of the

proteasome using MG132 did not result in downregulation of p62 bodies by MOAP-1. The new data are now shown in Fig EV1A.

2. "*The authors claim that MOAP-1 expression does not affect the levels of p62 protein (Figures 2G and 2J). However, the method used to analyze the expression levels of p62 is not suitable for the analysis of the p62 ubiquitin aggregates that are generally found in the insoluble protein fraction. To demonstrate that MOAP-1 only regulates the disassembling of the p62 bodies and not the p62 levels, the authors should generate detergent-soluble and insoluble protein fractions (ubiquitin aggregates) to demonstrate that MOAP-1 overexpression or MOAP-1 depletion only affects the p62 distribution in the p62 bodies and not its expression."*

   Thank you for raising the concern that our analysis may only account for the p62 protein in the insoluble fraction which may be insufficient to lead us to the conclusion that MOAP-1 only affects p62 distribution in the p62 bodies but not its expression. In our work, we used RIPA lysis buffer containing 1% SDS to prepare the total protein lysates for Western blotting of p62, as described *(Pan et al. Mol. Cell, 2016, 61:720-733)*. This lysis buffer should be adequate for extracting all the soluble and insoluble cellular p62 protein. Using another lysis buffer with stronger detergent content (i.e. 1X Laemmli sample buffer containing 2% SDS, 60 mM Tris pH 6.8 and supplemented with 150 mM NaCl, 1 mM EDTA, 5% glycerol and 7 mM beta-mercaptoethanol), similar observations were made that loss of MOAP-1 did not alter the total protein level of p62 in both the LO2 and HeLa cell-lines (Fig 2I and EV1D in the revised manuscript). We have now also examined the detergent-soluble and insoluble protein fractions to demonstrate further that MOAP-1 deficiency resulted in an enhancement of distribution of p62 in the insoluble fraction of the cell-lines prepared using the lysis buffer containing 1% Triton-X. The new data are now shown in Fig EV1H. In addition, we have also conducted the Halo-pulse chase assay, which is an established method for determining protein stability *(Yamaguchi et al., 2009, 577:121-132)*, and noted that no significant difference in the turn-over of Halo-tagged p62 protein between the WT and *MOAP-1* KO LO2 cells (Fig 2K and L).

3- *"After transfection, the distribution of MOAP-1 in normal and cancer cells is different. While, in normal cells, MOAP-1 has a diffuse distribution in the cytosol, in cancer cells, MOAP-1 colocalize directly with p62. The authors should explain the criteria they have followed to use normal hepatocytes (LO2 cells) or cancer cells (HepG2, HCT16...) in the different experiments of this work since the distribution of this protein is critical for its effect in the p62 bodies (Figure 3A and 3B).*

   Thank you for pointing out there has been inadequate explanation on the rationale for the choice of cell-lines in various experiments in our work.

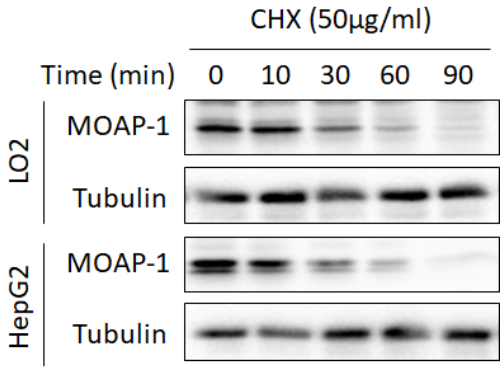
   The study began with our observation that MOAP-1 is recruited to the p62-enriched protein aggregates in HCT116 cells and mouse embryonic fibroblasts (MEFs) treated with MG132. We subsequently utilized liver-derived cell-lines including LO2 human normal hepatocytes and HepG2 and Huh1 human liver cancer cell-lines due to the well-established physiopathological role of the p62 bodies in the liver context, though how this signaling is regulated in the liver, at the level of homeostatic control of the p62 bodies, remain poorly understood. We have revised the writing in the manuscript (p.4, second paragraph of the results section) to help improving the explanation on the rationale of using the liver-derived and other cell-lines to conduct most of the analysis of the role of MOAP-1 in regulating p62 bodies as the following:

   "Formation of p62-enriched protein aggregates, which are insoluble cytoplasmic inclusion bodies hereon referred to as the p62 bodies, is normally induced upon exposure to various types of cellular stresses in many cell types including hepatocytes (Pan et al., 2016; Sanchez-Martin & Komatsu, 2018). In these cells, p62 normally exists in a soluble and non-aggregate state under basal condition. In some HCC cell-lines, however, high abundance of p62 bodies can be readily detected at the resting state, even without any stress signals (Inami et al., 2011; Zatloukal et al., 2002). Due to the well-established

physiopathological role of the p62-Keap-1-Nrf2 signaling in the liver and availability of many established hepatic cell lines with known status of p62 bodies at the basal and stress states, we decided to initially focus our investigation on defining the relationship between MOAP-1 and p62 bodies using hepatic cell-lines as our cellular models. LO2 human normal hepatocytes, which retain hepatocellular properties (Hu et al, 2013), are known to exhibit low level of insoluble p62 at the resting state (Xu et al., 2019). In these cells, treatment with the proteasome inhibitor MG132 or the oxidative stressor, arsenic trioxide (As_2O_3), potently triggered formation of p62 bodies (Fig 1C). The localization patterns of MOAP-1 were then examined in the LO2 hepatocytes under basal and stress conditions."

Is the MOAP-1 protein turnover rate different in LO2 cells compared with HepG2 cells?"

Thanks for raising the question. Half-life ($T_{1/2}$) of MOAP-1 in HepG2 cells had been reported in our previous work *(Supplemental Figure 3, Fu et al., PNAS, 2007, 104:10051-6)* to be about 30 minutes, which is similar to the half-life detected in the HCT116 cells. Based on the cycloheximide (CHX)-chase experiment conducted, the protein turn-over rate of endogenous MOAP-1 in the LO2 hepatocytes is comparable to that in the HepG2 cells. Please refer to the new data are shown below for the purpose of review only. Thus, the different distribution patterns of MOAP-1 in the LO2 and HepG2 cells at resting states is unlikely due to difference in the turn-over rate of MOAP-1 protein. Collectively, our data presented in Fig 1 suggest that the abundance of p62 bodies plays an important role to influence the distribution of MOAP-1 at the p62 bodies.



4- *"The authors should analyze whether MOAP-1 WT or MOAP-1 M13 overexpression affects the exchange of p62 in the RFP-p62 bodies after photobleaching (FRAP) assay (Figure 4F)."*

Thanks for the suggestion. We have now performed the analysis to compare the effect of overexpression of MOAP-1 or its M13 mutant on the exchange of p62 in the RFP-bodies after photobleaching assay. We observed that MOAP-1, but not the M13 mutant, promoted an increase rate of exchange of RFP-p62 in p62 bodies. The new data are now shown in Fig 5E and F.

5- *"In Figures 4I and 5E, overexpression of MOAP-1 (24h after transfection) presents a diffuse pattern in the cytosol, while in Figure 2E (24h after transfection), MOAP-1, colocalizes with the p62 bodies. The authors should explain this inconsistency."*

Thanks for pointing out the inconsistency in the data. These experiments involved transient transfection with constant amount of plasmid encoding MOAP-1 for overnight incubation after preforming the transfection. At 24 hours post transfection, the transfected cells were processed for analysis. Appearance of MOAP-1 expression patterns, however, can vary from aggregated (Fig 2E), semi-aggregated (Fig 4J) to even diffuse patterns (Fig 5H). It is very likely that the varying patterns of MOAP-1 signals detected were due to the transitionary nature of MOAP-1 in facilitating the dissociation of p62

bodies as we noted that the state of aggregation of MOAP-1 correlates well with the residual levels of p62 bodies observed in these cells.

6- *"To understand the mechanism by which MOAP-1 promotes dissociation of the p62 bodies, the authors should analyze whether MOAP-1 overexpression affects the interaction of p62 with K48/K63-linked polyubiquitin or with proteins that mediate the formation of p62 bodies such as NBR1, DAXX or TAK1."*

Thank you for the suggestion. We have now performed the analysis as suggested to evaluate the effect of overexpression of MOAP-1 on the interaction of p62 with K48/K63-linked polyubiquitin or with the proteins that mediate the formation of p62 bodies such as NBR1, DAXX or TAK1. These proteins, as expected, associated with p62 in HepG2 cells, but no notable difference in the binding of p62 to these known regulators of p62 bodies was observed in the HepG2 cells stably overexpressing MOAP-1 in comparison to the control HepG2 cells stably expressing just the empty vector. The new data are now shown in Appendix Fig S4B. As levels of p62 bodies were unaffected by overexpressing MOAP-1 in HepG2 cells treated with MG132 (new data in Fig EV1A), it may not be surprising that overexpression of MOAP-1 does not have a notable effect on affecting the interaction of p62 with these proteins.

7- *"The authors should reconstitute MOAP-1 KO cells with MOAP-1 WT or MOAP-1 M13 to analyze the effect of the MOAP-1/p62 complex in p62-Keap interaction (Figure 7A-B), Keap1-NRF2 interaction (Figure 7C), NRF2 nucleus translocation (Figure 7D) and the expression of NRF2 targets (Figure 7E)."*

Thank you for the suggestions. We have now performed the reconstitution experiments as suggested in the *MOAP-1* KO LO2 cells with MOAP-1 or its M13 mutant through stable expression and analyzed their effects on the p62-Keap interaction, Keap1-NRF2 interaction, nucleus translocation of NRF2 as well as expression of target genes of NRF2. MOAP-1 and its M3 mutant were expressed at comparable levels in the MOAP-1 KO LO2 cells (new data in Fig EV5K). Consistent with the inability of the M3 mutant to bind p62, it also failed to restore p62/Keap1 interaction (Fig 7J) and suppress Nrf2 activity (Fig 7K). MOAP-1, but not its M3 mutant, was able to reduce recruitment of Keap1 to the p62 bodies (new data in Fig EV5E), restoring Keap1/Nrf2 interaction (new data in Fig EV5F and G), suppressing Nrf2 nuclear localization (new data in Fig EV 5H and I) and the expression of Nrf2 target genes (new data in Figure EV5J).

8- *"In Figure EV8-A, the authors failed to demonstrate that there is an induction of the p62/Keap1 interaction in MOAP-1 KO livers because the increment of the interaction correlates with the increment of the Keap1 levels. Therefore, the interaction is just secondary to the amount of Keap1."*

Thank you for pointing out the weakness of the data presented in the MOAP-1 KO livers in EV8-A. We have now repeated the experiments and managed to obtain better quality data. The new data clearly showed an enhancement of the p62/Keap1 interaction in the livers of the *MOAP*-1 KO mice administered with the DEN (revised data shown as Appendix Figure S6A).

9- "*Figures 5D and 5E are redundant."*

Thank you for raising this point. Although Fig 5D and E seemingly appear to be redundant due to their similar experimental set-up, the data in Fig 5D aimed at demonstrating that upon re-expression of MOAP-1 and its M3 mutant in the *MOAP-1* KO cells background, MOAP-1, but not its M3 mutant, was found to co-localize to the p62 bodies at early time point post-transfection. For Fig 5E, which is now shown as Fig 5H in the revised manuscript, the rescue experiment was mainly designed to evaluate the effect of MOAP-1 and the M3 mutant to downregulate p62 bodies at late time points (i.e. 24h post-transfection). Together, the data in these two figures serve to provide evidence to suggest that localization of MOAP-1 to the p62 bodies is required to exert an effect on dissociating p62 bodies. To clearly indicate the co-localization of MOAP-1 and p62 at the early time points, we have now revised the data presentation in the Fig 5D to use arrowheads to highlight the co-localization of MOAP-1 with

p62. We have also amended the figure legends for these two figures to improve the clarity of the main points of the data in these two figures.

10- "*The labeling of Figure EV4D should be corrected."*

Thank you for alerting us of the error. The error has now been correct by removing the word "liver" (Fig EV3C in the revised version). We have also checked and ensured no other mislabeling in this panel.

11- "*p62 bodies should be quantified in Figures 3A and 3B as in Figures 2D, 2F, and 2I."*

Thank you for the suggestion. We have now quantified the p62 bodies in Fig 3A and 3B. The data on the quantitation of p62 bodies are now shown as Fig 3B and 3E in the revised manuscript.

……

Referee #3:

"This paper conveys a novel and well-demonstrated central finding that is well summarized in its title. The role of MOAP1 in the dissociation of p62 bodies and the regulation of Keap1/Nrf2 signaling is demonstrated using multiple approaches, including extensive cell- based characterization as well as in vivo validation and phenotypic relevance. I have a couple general comments (primarily for the editors) that do not require a response, and only a few minor comments for the authors to consider:"

General comments:

1. *"The manuscript has probably been submitted elsewhere before, as a result of which it has become very thorough and solid. There is really not much to criticize or improve about it. I expect it will receive several citations."*

2. *"With 8 main figures and 8 supplemental ones, the manuscript does not really fit the short-format original concept of EMBO Reports. Nevertheless, I expect that the editors are well aware of that, and since they are sending it out for review, I take that to mean that they are fully happy to publish manuscripts of this length. Besides, other manuscript I have reviewed for EMBO Reports were also quite long and exhaustive. Therefore, I don't really take this factor into account in my assessment and recommendation."*

   Thank you very much for the supportive comments.

Minor comments:

1. *"On page 6, last sentence of the second paragraph: From the data shown, it is difficult to draw conclusions about the physical properties of the p62 bodies ("were more viscous or gel-like"). I suggest to rephrase using a more hypothetical formulation."*

   Thank you for the suggestion. We have now revised the description in the text (p.7) to suggest that MOAP-1 deficiency may alter the rate of the exchange of RFP-p62 in the p62 bodies in the photobleaching experiment.

2. *"On page 6, last 3 sentences at the bottom of the page: From the data shown, it appears difficult to draw firm conclusions about the shape of the p62 bodies. Perhaps it is just a certain phase of the fusion-fission process?"*

Thank you for the comment and suggestion. We apologize for our over-interpretation and we acknowledge that based on the data shown in Fig 4, it would be difficult to draw firm conclusion about the shape of the p62 bodies. We have revised the text (p.7 and 8) to suggest that it is likely that upon overexpression of MOAP-1, disintegration of p62 bodies proceeds from spherical, phase-separated to smaller, asymmetrical states, before transforming into diffuse patterns in the cytosol.

3. "*The Y axis in Fig. 4G is too busy and can be simplified."*

Thank you for the suggestion. We have now simplified the Y-axis of Fig 4G, and corrected the error that the numbers are in percentile instead of the fraction of 1 as we incorrectly annotated in the earlier version.

……

In summary, we believe that we have addressed the comments and concerns raised by all the three referees. We thank you and the referees for the all the constructive criticisms and comments, which are undoubtedly useful for supporting development of a clearer and more vigorous manuscript.

Lastly, we would also like to take this opportunity to inform that we had addressed all the requests from the editorial office such as reducing to five Expanded View Figures and moved some of these figures to the Appendix (Please refer to Appendix Fig S1-S6 in the revised manuscript). The data availability section, source data, justification for the use of statistical test, have also been included in the revised manuscript, along with the author checklist.

Yours sincerely,

Victor C. Yu, Ph.D.

Associate Professor, National University of Singapore

Email: phayuv@nus.edu.sg Tel: +65 6516 8216

1st Revision - Editorial Decision 27th Oct 2020

Dear Dr. Yu,

Thank you for the submission of your revised manuscript to EMBO reports. It was sent back to former referee #1 and #2 and we have now received their reports (copied below).

As you will see, both referees are very positive about the study and support its publication without further changes.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

1) Appendix: Please list each figure and its corresponding page number in the table of content.

2) Please provide a separate header for the "Expanded View Figure legends" and move all figure legends to the end of the article file.

3) Materials and Methods: please specify the meaning of 'DEN" in the paragraph "Acute DEN experiment..."

4) Source data: I have only spot checked the source data provided in the form of xls files but noted the following:

a) Source data for Figure 2D: the average number of p62 bodies shown in the .xls file (8,3 and 22,49) does not match the numbers shown in the graph in Figure 2D. Please double-check. It might have been mixed up with Fig. EV1C. Here, the .xls file states 4,117 and 11,372 as average number but the graph in the figure shows approx. 8 and 22. Please double check the source data and the figure panels.

b) Source data for Figure 3 (blots): please correct the label for panel "K" to "F"

c) Source data for Figure EV1E/F: the .xls file is labeled as EV1E, but the numbers correspond to panel EV1F. Please correct.

d) Please note that we need one file per figure, i.e., please ZIP the pdf file with the Western blots together with the respective .xls file for this figure.

5) As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find the edited versions below my signature and let me know if you do NOT agree with any of the changes.

6) Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Title and Abstract

MOAP-1-mediated dissociation of p62/SQSTM1 bodies releases Keap1 and suppresses Nrf2 signaling

Nrf2 signaling is vital for protecting cells against oxidative stress. However, its hyperactivation is frequently found in liver cancer through excessive build-up of p62/SQSTM1 bodies that sequester Keap1, an adaptor of the E3-ubiquitin ligase complex for Nrf2. Here we report that the Bax-binding protein MOAP-1 regulates p62-Keap1-Nrf2 signaling through disruption of p62 bodies. Upon induction of cellular stresses that stimulate formation of p62 bodies, MOAP-1 is recruited to p62 bodies and reduces their levels independent of the autophagy pathway. MOAP-1 interacts with the PB1-ZZ domains of p62 and interferes with its self-oligomerization and liquid-liquid phase separation, thereby disassembling the p62 bodies. Loss of MOAP-1 can lead to marked upregulation of p62 bodies, enhanced sequestration of Keap1 by p62 and hyperactivation of Nrf2 antioxidant target genes. MOAP-1 deficient mice exhibit an elevated tumor burden with excessive levels of p62 bodies and Nrf2 signaling in a diethylnitrosamine (DEN)-induced hepatocarcinogenesis model. Together, our data define MOAP-1 as a negative regulator of Nrf2 signaling via dissociation of p62 bodies.

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Referee #1:

I am satisfied with the responses of the authors to my criticism. The revised manuscript is suitable for publication in EMBO Rep.

Referee #2:

The authors have addressed reasonably well all my comments.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 3rd Nov 2020

Dr. Victor Yu
National University of Singapore
Department of Pharmacy
18 Science Drive 4
Singapore, Singapore 117543
Singapore

Dear Dr. Yu,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

Thank you again 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,

Martina Rembold, PhD
Senior Editor
EMBO reports

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

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Corresponding Author Name: Victor C. Yu
Journal Submitted to: EMBO REPORTS
Manuscript Number: EMBOR-2020-50854V1

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was applied to pre-determine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	A minimum of three mice were used in each experiment, and at least three independent experiments were conducted. For tumorigenesis analysis, 13-14 mice were used for the 8.5 month stage, whereas 18-23 mice were used for the 4.5 month stage.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from the analyses
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization procedure was included in the execution of experiment and data analyses.
For animal studies, include a statement about randomization even if no randomization was used.	No randomizaton was used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done for animal studies
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, all data points were analyzed using GraphPad PRISM software to assess the distribution of the data.
Is there an estimate of variation within each group of data?	No

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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The sources and clone or catalogue numbers were provided in the materials section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines used were not recently authenticated, but they were sequenced to ensure the gene editing of MOAP-1 and p62, where applicable. Mycoplasma contamination was tested routinely during the study by PCR test.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Yes, the info is included in the materials and methods section as follows: MOAP-1 KO mice were first generated in the 129 background and backcrossed with the C57/BL6 strain for at least ten generations as previously described (Tan et al., 2016). All the wild type and MOAP-1 KO mice used for experiments were from the same heterozygous founders in C57/BL6 genetic background and were in-bred for not more than six generations. Mice were weaned at 21 day old and maintained on the Chow diet and 12 h light/12 h dark cycle at 23ºC. Only male mice were used for all experiments, which were approved by and conducted in accordance to the regulations and guidelines of the Institutional Animal Care and Use Committee of National University of Singapore.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were approved by and conducted in accordance to the regulations and guidelines of the Institutional Animal Care and Use Committee of National University of Singapore.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA, no human subject was included in this study.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Yes
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No