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Peroxisomal protein PEX13 functions in selective autophagy

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1st Editorial Decision 02 May 2016

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all three referees acknowledge the potential interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript or to strengthen the data and the conclusions drawn. As the reports are below, I will not detail them here, but in particular the quality of the IF images needs to be improved. Also point 1 of referee #1 and point 3 of referee #2 (in particular the use of fibroblasts derived from patients with PEX13 mutants) are very important.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns (as detailed in their reports) must be fully addressed 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

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REFEREE REPORTS

Referee #1:

In this manuscript, Lee et al. reported that the peroxisomal membrane protein PEX13 is involved in selective autophagy of Sindbis virus (virophagy) and damaged mitochondria (mitophagy). Interestingly, the authors showed that mutations in PEX13 associated with Zellweger syndrome spectrum (ZSS) disorders, including PEX13(I326T) and PEX13(W313G), showed defective mitophagy. The authors further showed that PEX3 knockdown causes a defect in mitophagy, while PEX14 and PEX19 are required for general autophagy. This study provides novel insights into the pathogenesis of ZSS, which was previously thought to be a peroxisome biogenesis disorder, but may in fact be due to dysfunction of PEX13-mediated mitophagy.

1. Confocal images should be used to examine the colocalization of mCherry-Capsid and GFP-LC3. ATG7 is essential for LC3 lipidation and thus for formation of LC3 puncta. In Fig. 1b, GFP-LC3 still forms a large number of puncta in ATG7 KD cells, indicating that ATG7 was not sufficiently depleted. Knocking down other autophagy genes should be used as a control. The authors used GFP-LC3 for measuring autophagy activity throughout the study. GFP-LC3 is sometimes incorporated into aggregates. Endogenous LC3, detected by anti-LC3, should be examined.

2. Baf A1 treatment blocks autophagic flux. Levels of p62 shown in Fig. 1f (lane 4 and 8) were still reduced in Baf A1-treated cells upon starvation. Levels of LC3-II were not induced (lane 2) upon autophagy induction. These experiments apparently were not properly performed.

3. Fig. 2A, Enlarged images should be included to show mitochondrial morphology (Fig. 2A) and fragmented mitochondria (Fig. 2e).

4. Molecular weights should be labeled in immunoblotting images.

Referee #2:

Remarks to the Authors:
The authors report that PEX13, one of the peroxins involved in peroxisomal matrix protein import, is required for selective autophagy of Sindbis virus (virophagy) and of damaged mitochondria (mitophagy), and that disease-associated PEX13 mutants I326T and W313G are defective in mitophagy. The selective mitophagy function of PEX13 is shared with another peroxin family member PEX3, but not with two other peroxins, PEX14 and PEX19, which are required for general autophagy. Based on these findings, the authors suggested that dysregulation of PEX13-mediated mitophagy may contribute to ZSS pathogenesis. However, molecular mechanism underlying how PEX13 mediated mitophagy is totally uncovered. There are quite a few issues that need to be addressed as listed below.

Suggestions to authors:
Major comments:

1. The authors clearly showed that peroxisome membrane protein PEX13 is a crucial factor of mitophagy (and virophagy). However, this finding is based only on the observation for mitopahgy in PEX13-deficient cells and there is no sufficient data to address how PEX13 functions in mitophagy in normal cells. Molecular mechanism of PEX13-mediated mitophagy is totally unclear.

2. Fig. 2: e, f, and g: Mitochondrial activities such as oxygen consumption and membrane potential should be assessed.

3. Fig. 3: As described in abstract, the authors claimed that disease-associated PEX13 mutants I326T and W313G are defective in mitophagy. But this conclusion is weak because rescue experiments of mitophagy in Fig. 3, the basis of the authors claim, is performed under the condition that PEX13 with disease-associated mutations are much highly expressed as compared to endogenous PEX13.

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This might artifactually cause alteration of mitochondrial morphology under the basal condition (Supplementary Fig. 3) as similar in <I>Pex13-/-</I> MEF under the same condition (Supplementary Fig. 2), results in protective phenotype to mitophagy upon CCCP treatment. Therefore, the results of Fig. 3 should be interpreted very carefully. To identify more directly the relationship between disease-associated PEX13 mutants and mitophagy defect, mitophagy should be addressed in fibroblasts derived from patients with PEX13 mutants I326T and W313G and the experiments shown in Fig. 3 should be performed under the condition at a lower level of PEX13 expression. Quantitative data need to be shown.

4. In regard to the role of PEX13 in mitophagy in normal cells, the authors represented only morphological data that PEX13 localized to peroxisomes even upon mitophagy induction (Fig. 4). Does PEX13 show any change in its protein level and any modifications such as ubiquitination and phosphorylation? Western blotting data on PEX13 in mitophagy-induced normal cells should be shown.

5. To address the function of PEX13 in selective autophagy, other selective autophagic pathways such as pexophagy, aggrephagy, ER-phagy, lipophagy should be verified.

Minor comments:
1. Fig. 3c: The authors indicated that difference of cell number with less than 10 mitochondria is not significant (NS) between the cells transfected with control siRNA and empty vector (solid bar) and those with <I>PEX13#2</I> siRNA and <I>PEX13WT</I> plasmid (gray bar). But it seems to be significantly different between these two.

Referee #3:

This report follows a genome-wide screen from the Levine lab identifying Pex13 as one of ~140 proteins required for mitophagy and virophagy (Nature 2011). Here they have collaborated with the Crane team, who have long-standing expertise in the function of Pex13, using mouse models and patient fibroblasts with Zellweger syndrome. The authors confirm the requirement for Pex13 in selective autophagy, showing an additional requirement for Pex3, but not Pex19 or Pex14 in mitophagy. These effects are mediated from the peroxisomal surface, as there is no obvious mitochondrial recruitment of these proteins. Loss of Pex13 leads to cells with abnormal mitochondrial cristae, as has been reported within mouse models lacking Pex5, and in patients. Indeed, the disruption of peroxisomes has long been known to have consequences on mitochondrial homeostasis - although the real mechanism underlying this has not been proven. Overall the results are very interesting, and the idea that peroxisomal machinery is critical in mitochondrial quality control pathways will hopefully drive a great deal of research into an understudied organelle (the peroxisome). The authors provide no mechanistic explanation for the apparent indirect effect of these peroxins on mitochondrial turnover (or virophagy), however I'm not so concerned with this point. The functional relationship between peroxisomes and mitochondria is well established, yet the field has only a rudimentary understanding of any mechanisms that may link them. For this reason I am supportive of this finding. However, some experiments require additional controls, and I'm dissatisfied with the quality of some of their immunofluorescence imaging. Therefore I have some suggestions to clarify some of the more confusing points and to focus the discussion.

- The authors show the peroxisome localization of Pex13 during CCCP, and in Figure 4 I see the clumping of mitochondria while peroxisomes remain peripherally localized. What happens to the peroxisomal ghosts (label with PMP70, for example) in Pex13-/- cells upon CCCP treatment? Do they remain associated with mitochondria? The study focuses on mitochondria or virus particles but peroxisomes (or ghosts) are not imaged in most of the figures. It would be important to see how they are behaving in each condition as well since the study is about peroxisomal proteins.

- In cells lacking peroxisomes many membrane proteins can target mitochondria, which could potentially interfere with PINK1 arrest? While this is observed in Pex3, 16 or 19 null cells (without ghosts), perhaps some of this is occurring in the Pex13 null cells as well. In the Parkin overexpressed, CCCP treated HeLa model, does PINK1 still accumulate in the absence of Pex13 or Pex3? This would help us understand where the block is occurring. I cannot see from the images

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supplied whether Parkin is still recruited to the blurry clump of Tom20 stained mitochondria.

- An obvious missing peroxin to look at is Pex16, which targets the ER for de novo peroxisomal biogenesis. Where is Pex16 in the Pex13 null cells, and does loss of Pex16 alter mitophagy? To make the dataset complete, this would be an important control as it is the binding partner of Pex3.

- In addition, the siRNA experiments in Figure 5 must show the level of peroxisomal loss in these cells. It can take some days to deplete peroxisomes in mammalian cells upon silencing the core import machinery, and even then they stubbornly remain. Are the peroxisomes gone at this point? Are they ghosts? Are they associated with mitochondria with CCCP? These controls should be included for each of the siRNA targets in Figure 5. (As mentioned above, I also cannot see whether Parkin is still recruited or not in siPex3 cells within Supp figure 4)

- The dsDNA puncta quantifications are extremely noisy and not very convincing. If the concern is proteosomal degradation of Tom20 mediated by Parkin, then why not use a matrix marker for IF instead? Also, "dsDNA" should be labeled mtDNA to be more explicit.

- All figures require much higher resolution to examine the morphology of mitochondria, recruitment of LC3 and Parkin, etc. As stated above, is Parkin recruited to mitochondria in Fig 2A? I can't resolve this from the single image shown. In 3C the TOM20 stain in siRNA 2 rescued with wt Pex13 looks very strange. What is this? How is it possible to quantify number of Tom20 punct in a cell like this? The figures are of very low magnification in general, making it difficult to visualize the details critical to support the conclusions.

- While I appreciate that the CCCP or Anti/OA models of induced mitophagy are standard in the field, it remains controversial the extent of mitophagy that may occur in vivo, whether Parkin dependent or not. This is particularly obvious when thinking of the extremely broad half lives of mitochondrial proteins characterized in multiple tissues. Perhaps more importantly, it is not known what the actual contribution of mitophagy for mitochondrial turnover may be for quality control - as this accompanies internal proteases, proteasomal turnover and MDVs. Indeed, the mitochondria are highly "sculpted", making it difficult to know how much of their content is cleared through mitophagy in vivo. Therefore the conclusion that the altered cristae structure relates specifically to a loss in mitophagy is an assumption. The authors state "Since autophagy is the only pathway for degrading large cellular components such as organelles and protein aggregates, these observations are consistent with an important homeostatic role of Pex13-mediated mitophagy in regulating mitochondria quality in vivo." The functional links between mitochondria and peroxisome are extensive, and this caveat should be included.

- That Pex14 and Pex19 did not alter mitophagy implies that the Pex3 or Pex13 effects were not due to overall peroxisomal biogenesis, rather a separate function is at play (again, I'd love to see Pex16). Was Pex3 also required for virophagy? The virophagy is also very intriguing, but there is very little speculation of this. Is there any previous mitochondrial involvement in virophagy? Are there any parallels beyond the requirement for the Atg proteins?

- As the authors mention in the discussion, Pex3 overexpression can trigger pexophagy, so did the authors observe any increase in pexophagy upon overexpression of Pex13? There are no robust or well-established triggers for pexophagy so far, but perhaps this could be tested by comparing Pex13 and Pex3 overexpression.

- My last point is a comment, leading to a suggestion that I leave to the authors to include if they think it makes sense. I think the coupling of peroxisomal biogenesis with pexophagy through Pex3 is very interesting, as it suggests the accumulation of Pex3 ultimately signals their degradation, likely through an import competition model. Importantly, this may mirror the mechanisms at the mitochondria where mitochondrial protein import failure locks PINK1 in (or near) the import channel, launching the phosphorylation of ubiquitin and Parkin for mitophagy. While the data here focus on the requirement for these peroxins in CCCP induced mitophagy, does this infer that peroxisomal import failure could signal through Pex13 and Pex3 to drive mitophagy? The sensors of peroxisomal "import failure" are specifically Pex3 and 13, as loss of 14 or 19 did not inhibit mitophagy, but would presumably lead to import failure (which I need to see in this study). But in the absence of Pex14 and 19, Pex3 and 13 would still be able to signal if CCCP is added. This hints

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that peroxisomal import is functionally coupled to mitochondrial health, which is a major take-away message of the study. Is there any evidence that mitochondrial dysfunction leads to peroxisomal import failure? Does addition of CCCP (blocking mitochondrial import) lead to an accumulation of their GFP-SKL markers, or catalase?

1st Revision - authors' response 01 September 2016

Below we provide a detailed response to each referee's comments. First, we summarize the changes/new data in the revised manuscript.

1. New, better quality images in Figure 1B obtained by deconvolution microscopy.
2. New virophagy experiments in Figure 1A-C, using new control siRNAs that target previously established selective virophagy factors, SMURF1 and FANCC.
3. New virophagy experiments in Figure EV1C-F, assessing colocalization of endogenous LC3 with Sindbis virus mCherry-capsid protein.
4. New autophagic flux experiments assessing endogenous LC3 puncta +/- lysosomal inhibitor in cells with knockdown of different *PEX* genes (Figure EV5D).
5. We have replaced the images in Figure 2A and 2E with better quality deconvolution microscopy images from new experiments to more clearly show mitochondrial morphology (Figure 2A) and the fragmented mitochondria (Figure 2E). We have also added additional images with insets related to Figure 2E in Figure EV2C to more clearly show the fragmented mitochondria that accumulate in Pex13-deficient MEFs after CCCP treatment.
6. New data measuring mitochondrial oxygen consumption and extracellular acidification rate in Pex13 wild-type and Pex13 knockout cells (Figure EV 2E, F).
7. New data with PEX13 W313G patient fibroblasts showing a defect in oligomycin/antimycin A-induced mitophagy (Figure 3E, F) and abnormal baseline mitochondria (Figure EV 3C).
8. New western blot data showing levels of PEX13 expression at serial points after mitophagy induction (Figure 4D).
9. New data in Figure EV 5C showing peroxisomal ghost labeling in *Pex13*$^{-/-}$ MEFs.
10. Better quality images in Figure 2A.
11. New experiment showing Parkin colocalization with mitochondria (TOMM20) in CCCP-treated *PEX13* siRNA cells (Figure EV 2B) and *PEX3* siRNA cells (Figure EV 4B).

Referee #1:

In this manuscript, Lee et al. reported that the peroxisomal membrane protein PEX13 is involved in selective autophagy of Sindbis virus (virophagy) and damaged mitochondria (mitophagy). Interestingly, the authors showed that mutations in PEX13 associated with Zellweger syndrome spectrum (ZSS) disorders, including PEX13(I326T) and PEX13(W313G), showed defective mitophagy. The authors further showed that PEX3 knockdown causes a defect in mitophagy, while PEX14 and PEX19 are required for general autophagy. This study provides novel insights into the pathogenesis of ZSS, which was previously thought to be a peroxisome biogenesis disorder, but may in fact be due to dysfunction of PEX13-mediated mitophagy.

1. Confocal images should be used to examine the colocalization of mCherry-Capsid and GFP-LC3. ATG7 is essential for LC3 lipidation and thus for formation of LC3 puncta. In Fig. 1b, GFP-LC3 still forms a large number of puncta in ATG7 KD cells, indicating that ATG7 was not sufficiently depleted. Knocking down other autophagy genes should be used as a control.

Authors' Response: We replaced the images in Figure 1B with better quality immunofluorescence images obtained by widefield deconvolution epifluorescence microscopy (which provides better quality images for these types of samples than confocal images). We agree that ATG7 knockdown in the subtype of HeLa cells used, HeLa/VS cells (which have been specifically adapted for viral growth) does not meaningfully decrease the formation of LC3 puncta. However, the level of knockdown achieved does block viral targeting to autophagosomes in these cells in the current study, as well as in the original RNAi screen published by our group in 2011 (Orvedahl et al. Nature 2011) that identified PEX3 and PEX13 as candidate selective autophagy factors. In fact, the same siRNA targeting ATG7 was used as the on-plate positive control in the original screen for virophagy

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factors in the same cell type, HeLa/VS cells used in this figure. If the knockdown decreased GFP-LC3 puncta, then it could not be used as a positive control for factors that decrease viral targeting to autophagosomes without decreasing GFP-LC3 puncta numbers. Although we do not know why in this particular cell type, the knockdown of ATG7 does not decrease GFP-LC3 puncta (it does in regular HeLa cells using the same experimental conditions in our hands), we believe that it represents a suitable control and that the knockdown of a core autophagy gene which decreases GFP-LC3 puncta would not represent a suitable control – as it would not be possible to assess whether targeting red viral nucleocapsids to green autophagosomes was decreased if the numbers of green autophagosomes was also decreased. Therefore, to address what we believe to be the underlying bases of the referee's concern (i.e. puzzling phenotype of ATG7 knockdown, need for more control knockdowns), we have performed new experiments knocking down two selective virophagy factors which our laboratory has previously shown to be required for Sindbis virus virophagy but not general autophagy, including SMURF1 (Orvedahl et al. Nature 2011) and FANCC (Sumpter et al. Cell 2016). Our new results shown in Figure 1A-C indicate that PEX13 knockdown blocks SIN virophagy as effectively as knockdown of these previously established virophagy factors.

The authors used GFP-LC3 for measuring autophagy activity throughout the study. GFP-LC3 is sometimes incorporated into aggregates. Endogenous LC3, detected by anti-LC3, should be examined.

Authors' Response: We performed new experiments to quantify the colocalization of SIN mCherry-capsid colocalization with endogenous LC3 in HeLa/VS cells treated with either control or PEX13 siRNA. The results are shown in the new Figure EV1C-F, and indicate that PEX13 also blocks the colocalization of SIN mCherry-capsid with endogenous LC3. For analysis of autophagic activity in Pex13 knockout MEFs, we included western blot analyses of p62 and LC3 in the presence and absence of bafilomycin A1; thus, these studies did not rely exclusively on using GFP-LC3. For analysis of the effects of knockdown of different PEX genes on autophagic flux in Figure 5 using GFP-LC3 puncta +/- lysosomal inhibitor (Baf A1), we have performed new experiments using similar knockdown conditions quantitating endogenous LC3 puncta. These new data are shown in Figure EV5D and show that knockdown of PEX14 and PEX19 but not PEX3 and PEX13 block autophagic flux.

2. Baf A1 treatment blocks autophagic flux. Levels of p62 shown in Fig. 1f (lane 4 and 8) were still reduced in Baf A1-treated cells upon starvation. Levels of LC3-II were not induced (lane 2) upon autophagy induction. These experiments apparently were not properly performed.

Authors' Response: We respectfully submit that the experiments shown in Figure 1F were properly performed and provide convincing evidence that autophagic flux is normal in baseline and starvation conditions in Pex13 knockout MEFs. In both $Pex13^{+/+}$ and $Pex13^{-/-}$ MEFs, there is an increase in p62, LC3-II, and total LC3 in baseline conditions with bafilomycin A1 conditions (lanes 3 vs. 1 and lanes 7 vs. 5). Moreover, in both $Pex13^{+/+}$ and $Pex13^{-/-}$ MEFs, starvation results in p62 degradation and LC3-II conversion (compare lanes 2 vs. 1 and 6 vs. 5). This represents increased autophagic flux as bafilomycin A1 partially blocks p62 degradation and increases levels of LC3-II accumulation in both genotypes (compare lanes 4 vs. 2 and lanes 8 vs. 6. Depending on the timing of measurement of LC3 protein measurement after starvation (in relation to increased translation, increased autophagolysosomal degradation), one can see different patterns in terms of the amounts of total LC3, and we find the ratio of LC3-II to LC3-I (normalized to a protein loading control such as actin) to be a much more reliable measurement of starvation-induced autophagy than absolute levels of LC3-II (since following the acute transcriptional burst when total LC3 and LC3-II increases, there is increased turnover of LC3 by the autophagolysosomal pathway). While the block in p62 degradation with bafilomycin A1 is not complete, it is our experience that even the use of 100 nM bafilomycin A1 (which is generally the highest dose tolerated by cells) does not completely block p62 degradation. Our results, a partial decrease in starvation-induced p62 degradation with bafilomycin A1, are quite typical of what is commonly seen in the literature with these types of autophagic flux assays. It is unknown whether the partial effects are due to some role of lysosomal-independent pathways in p62 degradation during starvation, incomplete block in lysosomal function, or some other factor.

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3. Fig. 2A, Enlarged images should be included to show mitochondrial morphology (Fig. 2A) and fragmented mitochondria (Fig. 2e).

Authors' Response: We have replaced the original images in Figure 2A with better quality images obtained by deconvolution microscopy that better show the mitochondrial morphology. We also replaced the images in Figure 2E with better quality images obtained by deconvolution microscopy that more clearly show the diffuse cellular accumulation of fragmented mitochondria that occurs upon CCCP treatment in Pex13$^{-/-}$ MEFs (versus the perinuclear compaction of mitochondria in wild-type MEFs). We have indicated the cell boundaries with white lines so that this phenotype can be more readily visualized by readers. We have also included a new supplemental figure showing insets of both genotypes of TOMM20-stained MEF cells in the presence and absence of CCCP treatment to allow better visualization of mitochondrial morphology (Figure EV 2C).

4. Molecular weights should be labeled in immunoblotting images.

Authors' Response: We have added molecular weight markers to all immunoblots throughout the manuscript (Figure 1A, D, F; Figure 3A; Figure 4D; Figure EV2A).

Referee #2:

Remarks to the Authors:
The authors report that PEX13, one of the peroxins involved in peroxisomal matrix protein import, is required for selective autophagy of Sindbis virus (virophagy) and of damaged mitochondria (mitophagy), and that disease-associated PEX13 mutants I326T and W313G are defective in mitophagy. The selective mitophagy function of PEX13 is shared with another peroxin family member PEX3, but not with two other peroxins, PEX14 and PEX19, which are required for general autophagy. Based on these findings, the authors suggested that dysregulation of PEX13-mediated mitophagy may contribute to ZSS pathogenesis. However, molecular mechanism underlying how PEX13 mediated mitophagy is totally uncovered. There are quite a few issues that need to be addressed as listed below.

Suggestions to authors:
Major comments:

1. The authors clearly showed that peroxisome membrane protein PEX13 is a crucial factor of mitophagy (and virophagy). However, this finding is based only on the observation for mitopahgy in PEX13-deficient cells and there is no sufficient data to address how PEX13 functions in mitophagy in normal cells. Molecular mechanism of PEX13-mediated mitophagy is totally unclear.

Authors' Response: We agree that the question of how PEX13 functions in mitophagy is an important open question that remains to be understood, and acknowledge this limitation in the discussion of our manuscript. However, it may take many years to define the mechanism (in part because of the point made by reviewer #3 that understanding of the mechanisms that link peroxisomes and mitochondria are so rudimentary) and we believe that such studies are beyond the scope of the present manuscript. Despite the absence of definitive mechanistic insights, we believe that our findings that certain peroxins function in selective autophagy (mitophagy and virophagy) are novel and open up a new and important area of cell biology. Moreover, they have relevance for understanding the potential pathogenesis of a human genetic disorder, Zellweger Syndrome.

2. Fig. 2: e, f, and g: Mitochondrial activities such as oxygen consumption and membrane potential should be assessed.

Authors' Response: In response to the reviewer's suggestion, we collaborated with a colleague, Prashant Mishra, who is an expert on mitochondrial metabolism, and measured mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in Pex13$^{+/+}$ and Pex13$^{-/-}$ primary MEFs after sequential treatment with oligomycin, CCCP, and antimycin A. We observed no difference in these two mitochondrial functional readouts between MEFs of the two genotypes during basal state and during maximal mitochondrial respiration (after CCCP treatment). (Our collaborator has unpublished data on immortalized MEFs lacking core autophagy genes which are

deficient in both autophagy and mitophagy which show a similar lack of difference in these mitochondrial activities in mitophagy-competent versus mitophagy-incompetent cells.) Our new findings suggest that there is no major defect in mitochondrial biogenesis in Pex13 KO cells, and are consistent with our hypothesis that mitophagy defect contributes to the accumulation of abnormal mitochondria in the cells. These new data have been added as Figure EV 2D-E.

3. Fig. 3: As described in abstract, the authors claimed that disease-associated PEX13 mutants I326T and W313G are defective in mitophagy. But this conclusion is weak because rescue experiments of mitophagy in Fig. 3, the basis of the authors claim, is performed under the condition that PEX13 with disease-associated mutations are much highly expressed as compared to endogenous PEX13. This might artifactually cause alteration of mitochondrial morphology under the basal condition (Supplementary Fig. 3) as similar in *Pex13-/-* MEF under the same condition (Supplementary Fig. 2), results in protective phenotype to mitophagy upon CCCP treatment. Therefore, the results of Fig. 3 should be interpreted very carefully. To identify more directly the relationship between disease-associated PEX13 mutants and mitophagy defect, mitophagy should be addressed in fibroblasts derived from patients with PEX13 mutants I326T and W313G and the experiments shown in Fig. 3 should be performed under the condition at a lower level of PEX13 expression. Quantitative data need to be shown.

Authors' Response: We appreciate the referee's concern about the need for caution in interpreting phenotypes observed in the setting of overexpression. However, we point out that wild-type PEX13, when overexpressed to the same levels as PEX13 mutants I326T and W313G, does not result in mitochondrial morphology abnormalities in basal conditions or enhanced suppression of mitophagy in the setting of PEX13 knockdown. This suggests that the phenotypes of PEX13 mutants I326T and W313G are not due to overexpression per se. We also controlled for the total amount of plasmid DNA transfected into each sample (with the addition of empty vector in the WT PEX13 sample). Thus, the phenotype observed in the PEX13 mutants is not due to increased plasmid transfection. However, we acknowledge that it is possible that these phenotypes would not be observed with endogenous levels of PEX13 mutants I326T and W313G and appreciate the referee's excellent suggestion to assess mitophagy in mutant patient fibroblasts. We have contacted several groups in different countries around the world to collaborate with us on these studies and provide such patient fibroblasts. We were able to find a collaborator, Dr. Heindrik Rosewich, at the University Medical Center Göttingen at Georg August University, who was able to share with us PEX13 W313G fibroblasts (we were not able to find a source for PEX13 I326T fibroblasts). We have added new data in the revised manuscript showing that PEX13 W313G mutant patient fibroblasts have both a defect in mitophagy (Figure 3E, F) as assessed using the same assay used for primary MEFs (% of cells diffuse accumulation of fragmented mitochondria after treatment with a mitochondrial uncoupling agent) as well as abnormal mitochondria during basal conditions (Figure 3 EVC) as assess by super-resolution microscopy of mitochondria stained with Mitotracker Red. With respect to the referee's request to perform the rescue experiment shown in Figure 3 under conditions of a lower level of PEX13 expression, this is not technically possible because we need to determine which knockdown cells have a rescue of PEX13 expression by IF staining (for mitophagy quantification) and the PEX13 antibody does not detect lower levels of PEX13 expression. Nonetheless, we believe that our new data showing a defect in mitochondria and mitophagy in primary fibroblasts from a patient with the PEX13 homozygous W313G rules out the possibility that the phenotypes observed in the rescue experiments in HeLa cells are artifacts due to overexpression, and significanty strengthen the revised manuscript.

4. In regard to the role of PEX13 in mitophagy in normal cells, the authors represented only morphological data that PEX13 localized to peroxisomes even upon mitophagy induction (Fig. 4). Does PEX13 show any change in its protein level and any modifications such as ubiquitination and phosphorylation? Western blotting data on PEX13 in mitophagy-induced normal cells should be shown.

Authors' Response: In response to the referee's request, we have performed western blot analysis of PEX13 protein levels at serial time points after CCCP treatment and induction of mitophagy. The new data, added as Figure 4D, do not show any differences in PEX13 protein levels during mitophagy induction. In addition, we do not see any band shifts or new higher molecular weight bands that might be suggestive of post-translational modifications such as ubiquitination and

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phosphorylation; however, a complete assessment of whether PEX13 undergoes post-translational modifications is beyond the scope of the present study.

5. To address the function of PEX13 in selective autophagy, other selective autophagic pathways such as pexophagy, aggrephagy, ER-phagy, lipophagy should be verified.

Authors' Response: As discussed in the text, previous studies have shown that PEX3 is required for pexophagy in yeast and mammalian cells. We attempted to evaluate whether PEX13 is required for pexophagy, but were unable to induce pexophagy in our HeLa PEX13 knockdown cells or in the Pex13 knockout MEFs (data not shown). We believe that the identification of a role for PEX13 in two forms of selective autophagy, mitophagy and virophagy, in which the peroxisome is not a target, represents a novel and important finding. While it would be interesting to know whether PEX13 is also required for other forms of selective autophagy, we believe that such studies are beyond the scope of the present manuscript and that the mere identification of additional forms of selective autophagy in which PEX13 plays a role would not directly address the function of PEX13 in selective autophagy.

Minor comments:

Fig. 3c: The authors indicated that difference of cell number with less than 10 mitochondria is not significant (NS) between the cells transfected with control siRNA and empty vector (solid bar) and those with *PEX13#2* siRNA and *PEX13WT* plasmid (gray bar). But it seems to be significantly different between these two.

Authors' Response: We apologize that the labeling of the statistics on the original figure was confusing. The relevant comparisons are empty vector + PEX13 siRNA vs. each different siRNA-resistant PEX13 plasmid. These comparisons (adjusted for multiple comparisons) reveal that wild-type PEX13 partially rescues mitophagy in PEX13 knockdown HeLa/Parkin cells, whereas PEX13 mutants (I326T and W313G) actually further suppress mitophagy in PEX13 knockdown HeLa/Parkin cells.

Referee #3:

This report follows a genome-wide screen from the Levine lab identifying Pex13 as one of ~140 proteins required for mitophagy and virophagy (Nature 2011). Here they have collaborated with the Crane team, who have long-standing expertise in the function of Pex13, using mouse models and patient fibroblasts with Zellweger syndrome. The authors confirm the requirement for Pex13 in selective autophagy, showing an additional requirement for Pex3, but not Pex19 or Pex14 in mitophagy. These effects are mediated from the peroxisomal surface, as there is no obvious mitochondrial recruitment of these proteins. Loss of Pex13 leads to cells with abnormal mitochondrial cristae, as has been reported within mouse models lacking Pex5, and in patients. Indeed, the disruption of peroxisomes has long been known to have consequences on mitochondrial homeostasis - although the real mechanism underlying this has not been proven. Overall the results are very interesting, and the idea that peroxisomal machinery is critical in mitochondrial quality control pathways will hopefully drive a great deal of research into an understudied organelle (the peroxisome). The authors provide no mechanistic explanation for the apparent indirect effect of these peroxins on mitochondrial turnover (or virophagy), however I'm not so concerned with this point. The functional relationship between peroxisomes and mitochondria is well established, yet the field has only a rudimentary understanding of any mechanisms that may link them. For this reason I am supportive of this finding. However, some experiments require additional controls, and I'm dissatisfied with the quality of some of their immunofluorescence imaging. Therefore I have some suggestions to clarify some of the more confusing points and to focus the discussion.

1.) The authors show the peroxisome localization of Pex13 during CCCP, and in Figure 4 I see the clumping of mitochondria while peroxisomes remain peripherally localized. What happens to the peroxisomal ghosts (label with PMP70, for example) in Pex13-/- cells upon CCCP treatment? Do they remain associated with mitochondria? The study focuses on mitochondria or virus particles but peroxisomes (or ghosts) are not imaged in most of the figures. It would be important to see how they are behaving in each condition as well since the study is about peroxisomal proteins.

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Authors' Response: Figure EV5C shows perosixomal ghosts (labeled with PMP70) are present in PEX13 KO MEFs +/- 24 h CCCP treatment. In both basal and mitophagy conditions, peroxisomal ghosts largely do not associate with mitochondria (labeled with mitochondrial matrix protein ATP5B). Our observations in PEX13-deficient MEFs are consistent with those of PEX13-deficient HeLa cells (Figure 4B).

2.) In cells lacking peroxisomes many membrane proteins can target mitochondria, which could potentially interfere with PINK1 arrest? While this is observed in Pex3, 16 or 19 null cells (without ghosts), perhaps some of this is occurring in the Pex13 null cells as well. In the Parkin overexpressed, CCCP treated HeLa model, does PINK1 still accumulate in the absence of Pex13 or Pex3? This would help us understand where the block is occurring. I cannot see from the images supplied whether Parkin is still recruited to the blurry clump of Tom20 stained mitochondria.

Authors' Response: We have added improved images in Figure 2A. These images are intended only to show which cells are expressing Parkin (and therefore, would be used for quantitating mitochondrial clearance) and not for examining the colocalization of Parkin and mitochondria during mitophagy as we are looking at a time point (16 h) when mitochondrial clearance has occurred in Parkin-expressing cells. To address the question of whether Parkin mitochondrial colocalization is altered by PEX13 and PEX3 knockdown, we have performed new experiments (Figure EV 2B and Figure EV 4B) at an earlier time point (4 h) in CCCP-treated cells, prior to mitochondrial clearance. Our results indicate that Parkin has a diffuse cytoplasmic localization in the absence of CCCP in NC-treated, PEX13 siRNA-treated and PEX3 siRNA-treated cells. After short-term CCCP-treatment (4 h), the pattern of Parkin localization to mitochondria is identical between cells treated with siRNAs targeting NC, PEX13, and PEX3. Thus, our new data clearly demonstrate that the PINK1-Parkin pathway is not affected by PEX13 or PEX3 knockdown.

3.) An obvious missing peroxin to look at is Pex16, which targets the ER for de novo peroxisomal biogenesis. Where is Pex16 in the Pex13 null cells, and does loss of Pex16 alter mitophagy? To make the dataset complete, this would be an important control as it is the binding partner of Pex3.

Authors' Response: In this manuscript, we studied PEX19 as a peroxin binding partner of PEX3 and PEX14 as a peroxin binding partner of PEX13. Both PEX19 and PEX14 are known to be essential for peroxisomal biogenesis; however, we found that they are not required for mitophagy. These findings convincingly dissociate the role of PEX3 and PEX13 in mitophagy from a canonical role in peroxisomal biogenesis. A complete investigation of all peroxins in selective autophagy is beyond the scope of the present study.

4.) In addition, the siRNA experiments in Figure 5 must show the level of peroxisomal loss in these cells. It can take some days to deplete peroxisomes in mammalian cells upon silencing the core import machinery, and even then they stubbornly remain. Are the peroxisomes gone at this point? Are they ghosts? Are they associated with mitochondria with CCCP? These controls should be included for each of the siRNA targets in Figure 5. (As mentioned above, I also cannot see whether Parkin is still recruited or not in siPex3 cells within Supp figure 4)

Authors' Response: In response to the referee's request, we have stained for PMP70 (a peroxisomal marker) in the siRNA conditions used to assess mitophagy in Figure 5. As the referee notes, it takes some days to deplete peroxisomes in mammalian cells upon silencing the core import machinery and accordingly, we do not see a depletion of peroxisomes in the cells treated with any of the siRNAs (control, PEX3, PEX13, PEX14, and PEX19) in the presence or absence of CCCP (Figure EV5A and B). In addition, as noted above, to visualize Parkin recruitment to mitochondria during mitophagy, we performed experiments to co-stain mitochondria and Parkin in baseline conditions and at an early time point after CCCP treatment, prior to mitochondrial degradation in control siRNA-treated cells. The new data, shown in Figure EV2B and Figure EV4B of the revised manuscript, indicate that Parkin is recruited normally to depolarized mitochondria in the setting of PEX13 or PEX3 knockdown.

5.) The dsDNA puncta quantifications are extremely noisy and not very convincing. If the concern is proteosomal degradation of Tom20 mediated by Parkin, then why not use a matrix marker for IF instead? Also, "dsDNA" should be labeled mtDNA to be more explicit.

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Authors' Response: The quantification of mtDNA puncta is a standard approach used in the mitophagy field to quantitate mitophagy (Lazarou et al. Nature 2015). We graph the mtDNA puncta quantification as box-and-whisker plots instead of bar graphs because (1) it is the more rigorous approach to display non-normally distributed data sets such as the mtDNA puncta quantification; and (2) most journals are now encouraging graphs that show the full range of actual data points rather than just means and error bars. With mitophagy, there is a wide range of puncta per cell in any condition and it is necessary to count a large number of cells per condition (~300 cells per experiment, 3 independent experiments) as we do in this study. If one takes the same dataset and simply shows a bar graph of the mean and error bars, the data will look extremely "tight" and "non-noisy" but this is not the correct statistical approach for non-normally distributed data. The boxes in the box-and-whisker plot mark the first, second, and third quartile of the dataset, and the outliers (highest and lowest 5%) are plotted as individual points. The differences between PEX13 siRNA, PEX3 siRNA, and ATG7 siRNA vs. NC control are highly significant ($p<0.0001$). We also modified our statistical analysis from the Mann-Whitney U-test to the Kruskal-Wallis H-test, which is an extension of the Mann-Whitney U-test with correction for multiple comparisons. This test was applied for the data shown in Figure 2D, Figure 5C, and Figure EV4E. As per the referee's suggestion, we have changed the label "dsDNA" to "mtDNA".

6.) All figures require much higher resolution to examine the morphology of mitochondria, recruitment of LC3 and Parkin, etc. As stated above, is Parkin recruited to mitochondria in Fig 2A? I can't resolve this from the single image shown. In 3C the TOM20 stain in siRNA 2 rescued with wt Pex13 looks very strange. What is this? How is it possible to quantify number of Tom20 puncta in a cell like this? The figures are of very low magnification in general, making it difficult to visualize the details critical to support the conclusions.

Authors' Response: In response to this referee's comment and those of the other referees, we have both improved the quality of existing immunofluorescence images and added new data to address additional questions posed by the referees. Specifically, the changes we made include:

1. *Images in Figures 2A and EV4B were replaced with higher quality widefield deconvolution epifluorescence images for Parkin-mediated mitophagy in HeLa cells with siPEX13 or siPEX3 treatment.*
2. *Images in Figures 2E and EV2C were replaced with higher quality widefield deconvolution epifluorescence images for MEF mitophagy with inset showing mitochondria morphology.*
3. *We included representative images of TOMM20 and Parkin colocalization at an early time point during mitophagy (4 h after CCCP treatment) in Figures EV2B and EV4D.*
4. *We replaced the representative image for siPEX13 + WT PEX13 rescue in Figure 3B. The previous image showed a large TOMM20 aggregate compacted to the perinuclear region. These aggregates may appear in some overexposed images as we increase the signal to display peripheral mitochondria morphology more clearly. During our quantification, we counted cells with more than 10 TOMM20 puncta or the presence of large perinuclear aggregates as cells with defective mitophagy.*
5. *We have performed super-resolution imaging of mitochondria in primary human fibroblasts derived from a patient with wild-type PEX13 and from a patient with a homozygous PEX313G mutation (Figure EV 3C). We note that the capacity for super-resolution microscopy first became available to us at our institution of July 2016. Hence, we were able to use super-resolution microscopy for the new experiments on primary human patient fibroblasts performed within the time frame of the revision. However, it would be impractical to repeat all of the experiments in the manuscript using this technique within the scope of a revision.*

7.) While I appreciate that the CCCP or OA models of induced mitophagy are standard in the field, it remains controversial the extent of mitophagy that may occur in vivo, whether Parkin dependent or not. This is particularly obvious when thinking of the extremely broad half lives of mitochondrial proteins characterized in multiple tissues. Perhaps more importantly, it is not known what the actual contribution of mitophagy for mitochondrial turnover may be for quality control - as this accompanies internal proteases, proteasomal turnover and MDVs. Indeed, the mitochondria are highly "sculpted", making it difficult to know how much of their content is cleared through mitophagy in vivo. Therefore the conclusion that the altered cristae structure relates specifically to a loss in mitophagy is an assumption. The authors state "Since autophagy is the only pathway for

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degrading large cellular components such as organelles and protein aggregates, these observations are consistent with an important homeostatic role of Pex13-mediated mitophagy in regulating mitochondria quality in vivo." The functional links between mitochondria and peroxisome are extensive, and this caveat should be included.

Authors' Response: We fully agree with the referee that the functional links between mitochondria and peroxisomes are extensive, that the precise role of mitophagy in mitochondrial turnover in vivo remains incompletely understood, and that the accumulation of abnormal mitochondria in Pex13 knockout mouse tissues could have multiple different causes. However, we note that knockout of essential core autophagy genes in tissues such as liver, heart, brain, and skeletal muscle invariably results in the accumulation of damaged mitochondria; this extensive literature has convincingly established that the core autophagy machinery is essential for mitochondrial quality control in vivo in post-mitotic tissues. (We have added this statement and a reference in the revised manuscript.) The mitochondrial phenotype that we observe in Pex13 knockout tissues is consistent with that observed in core autophagy gene knockout tissues, and therefore, it is reasonable to speculate that – in addition to peroxisomal dysfunction, defects in mitophagy (which require PEX3 and PEX13) may contribute to the pathogenesis of a subset of ZSS disorders. We have revised the text in the paragraph describing the abnormal mitochondria in Pex13 knockout animals to soften the conclusion and more clearly highlight the caveats noted by the referee.

8.) That Pex14 and Pex19 did not alter mitophagy implies that the Pex3 or Pex13 effects were not due to overall peroxisomal biogenesis, rather a separate function is at play (again, I'd love to see Pex16). Was Pex3 also required for virophagy? The virophagy is also very intriguing, but there is very little speculation of this. Is there any previous mitochondrial involvement in virophagy? Are there any parallels beyond the requirement for the Atg proteins?

Authors' Response: PEX3 scored as a hit for both virophagy and mitophagy in our previous genome-wide siRNA screen (Orvedahl et al. Nature 2011). However, for reasons that are unclear, we were unable to consistently reproduce the virophagy defective phenotype in PEX3 siRNA knockdown experiments (i.e. sometimes they were observed; sometimes they were not observed). We chose to focus on evaluating PEX13 in more detail because we have Pex13 knockout MEFs and mice and antibodies that detect PEX13/Pex13 protein in human and mouse cells. We also focused in more detail on mitophagy instead of virophagy because of technical reasons rescuing siRNA knockdown phenotypes in viral infection (described in the paper). Yes, indeed there are many parallels between virophagy and mitophagy beyond Atg proteins. In our previous genome-wide siRNA screen to identify virophagy factors (Orvedahl et al. Nature 2011), 96 of 141 virophagy factors also scored positive in our secondary screen for factors involved in Parkin-mediated mitophagy. In the Orvedahl et al. paper, we focused on one of these factors, SMURF1, and in a recently published paper (Sumpter et al. Cell 2016), we focused on another factor, FANCC – both of which bind to Sindbis virus nucleocapsids and also are required for mitophagy. The precise mechanisms that function in common in virophagy and mitophagy is a fascinating question about which little is currently known.

9.) As the authors mention in the discussion, Pex3 overexpression can trigger pexophagy, so did the authors observe any increase in pexophagy upon overexpression of Pex13? There are no robust or well-established triggers for pexophagy so far, but perhaps this could be tested by comparing Pex13 and Pex3 overexpression.

Authors' Response: As the referee notes, there are no robust or well-established triggers for pexophagy in mammalian cells or good assays for detecting pexophagy. Thus, we were not able to measure whether knockdown or overexpression of Pex13 affected pexophagy. With Pex13 overexpression, we did not observe a qualitative difference in PMP70 puncta numbers, but we did not perform detailed quantitative imaging.

10.) My last point is a comment, leading to a suggestion that I leave to the authors to include if they think it makes sense. I think the coupling of peroxisomal biogenesis with pexophagy through Pex3 is very interesting, as it suggests the accumulation of Pex3 ultimately signals their degradation, likely through an import competition model. Importantly, this may mirror the mechanisms at the mitochondria where mitochondrial protein import failure locks PINK1 in (or near) the import channel, launching the phosphorylation of ubiquitin and Parkin for mitophagy. While the data here

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focus on the requirement for these peroxins in CCCP induced mitophagy, does this infer that peroxisomal import failure could signal through Pex13 and Pex3 to drive mitophagy? The sensors of peroxisomal "import failure" are specifically Pex3 and 13, as loss of 14 or 19 did not inhibit mitophagy, but would presumably lead to import failure (which I need to see in this study). But in the absence of Pex14 and 19, Pex3 and 13 would still be able to signal if CCCP is added. This hints that peroxisomal import is functionally coupled to mitochondrial health, which is a major take-away message of the study. Is there any evidence that mitochondrial dysfunction leads to peroxisomal import failure? Does addition of CCCP (blocking mitochondrial import) lead to an accumulation of their GFP-SKL markers, or catalase?

Authors' Response: We agree that the question of whether and if so, how, peroxisomal import failure is coupled to mitochondrial health is an interesting and important question that arises from our study. Since, as the reviewer points out, the loss of PEX14 and PEX19 did not affect mitophagy (as did loss of PEX3 and PEX19) but would presumably result in a general impairment in peroxisomal import, we hypothesize that PEX3 PEX13 have unique functions at the peroxisome that couple them to mitochondrial health that would not be revealed by general assays of peroxisomal import. It is possible that PEX13 and PEX3 function in the specific import of certain proteins (for which PEX14 and PEX19 are not required) which somehow link to the mitophagy pathway and/or that PEX3 or PEX13 have additional functions beyond peroxisomal import and biogenesis that function in mitophagy. We believe that unbiased proteomic experiments to detect proteins that uniquely interact with PEX3 and PEX13, but not with PEX14 and PEX19, during mitophagy conditions would be the best starting point to dissect the potential unique functions of these specific peroxins in mitophagy. Such experiments will be an important focus of future investigations.

2nd Editorial Decision 22 September 2016

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the three referee reports that you will find enclosed below.

As you will see, referees #1 and #3 support publication of your manuscript in EMBO reports and find that the revised manuscript has significantly improved and that their concerns have been adequately addressed. However, referee #2 has still concerns and thinks that the manuscript needs further revision. After discussing with the other referees, we feel that comment 1 of referee #2 needs not to be addressed experimentally and is beyond scope of the present study (of course we invite you to add any additional data you might have addressing this point). Nevertheless, we feel that the other two comments of referee #2 should be addressed in a final revised version.

It is our policy at EMBO reports have that submitted manuscripts need to be accepted within 6 months of the initial decision, which in your case would be the 2nd of November 2016. Otherwise novelty would need to be re-assessed. Presently novelty seems not to be impacted, but depending on when you submit the very final version, I might need to re-assess this again.

REFEREE REPORTS

Referee #1:

The data quality in this revised manuscript has been significantly improved. All my concerns have been addressed. It is acceptable.

Referee #2:

Most of the issues pointed out by the reviewer #2 are adequately addressed in the revised manuscript. Experiments using fibroblasts from the patient with PEX13-W131G mutation particularly strengthened the essential role of PEX13 in mitophagy. However, the reviewer #2 still has several concerns as follows.

Reply to comment 1- There is no practical response to clarifying molecular mechanism of PEX13-

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mediated mitophagy. This reviewer think that it is essential to show the evidence for which process is affected by the defect of peroxisomal PEX13 during mitophagy.

Reply to comment 3 - The authors showed abnormal mitochondrial morphology, lower membrane potential, and defects in mitochondrial clearance in fibroblasts from a patient defective with PEX13 mutants W313G in the revised manuscript. However, cell morphology seems to be intrinsically and quite different in fibroblasts from between healthy and W313G patient. For instance, wild-type fibroblasts treated with OA shows narrow shape, whereas spread shaped-W313G cells was selected as a typical cell, in which several narrow shaped-cells were observed in the same field (Fig. 3E). In addition, mitochondrial morphology in Fig. 3E requires much higher resolution and/or enlarged images to discriminate the abnormal structure of mitochondria in W313G fibroblasts.

Reply to comment 5 - Did the authors induce pexophagy by overexpression of PEX3 and observe no decrease of peroxisomes in PEX13-depleted HeLa cells or in PEX13-KO MEFs? If so, the authors should be better to describe the results.

Referee #3:

The authors have responded to each of my concerns, and (although I would have really liked to see Pex16...) I am satisfied with the revision. The images are much clearer, and the controls I requested were performed. This study will certainly trigger a renewed interest in carefully examining the peroxisome/mitochondrial relationship within mammalian systems, which has high relevance to multiple disease paradigms I'm sure.

2nd Revision - authors' response 04 October 2016

Referee #2

Reply to comment 1 - There is no practical response to clarifying molecular mechanism of PEX13-mediated mitophagy. This reviewer think that it is essential to show the evidence for which process is affected by the defect of peroxisomal PEX13 during mitophagy.

Authors' Response: We appreciate the importance of clarifying the molecular mechanism of PEX13-mediated mitophagy and hope that publication of our work will stimulate further research into this area. From a practical point of view, such studies may take a long time and are beyond the scope of the present manuscript.

Reply to comment 3 - The authors showed abnormal mitochondrial morphology, lower membrane potential, and defects in mitochondrial clearance in fibroblasts from a patient defective with PEX13 mutants W313G in the revised manuscript. However, cell morphology seems to be intrinsically and quite different in fibroblasts from between healthy and W313G patient. For instance, wild-type fibroblasts treated with OA shows narrow shape, whereas spread shaped-W313G cells was selected as a typical cell, in which several narrow shaped-cells were observed in the same field (Fig. 3E). In addition, mitochondrial morphology in Fig. 3E requires much higher resolution and/or enlarged images to discriminate the abnormal structure of mitochondria in W313G fibroblasts.

Authors' Response: The reviewer is correct that (1) the cell morphology is variable among PEX13 mutant cells; and (2) cell morphology may be intrinsically different between the control and PEX13 W313G mutant cells. It is precisely for this reason that we show fields in Fig. 3E which accurately reflect this variability in PEX13 W313 mutant cells (in both the DMSO- and OA-treated conditions), and that we performed structured illumination microscopy imaging to more closely examine the mitochondrial structure in PEX13 W313G fibroblasts in baseline conditions. The abnormal mitochondrial structure in PEX13 W313G fibroblasts is clearly shown by the super-resolution microscopy images shown in Fig EV3C. Despite this abnormality in mitochondria during baseline conditions observed at the level of super-resolution microscopy, we did not observe cells which had the same diffuse accumulation of fragmented mitochondria detectable at the level of standard deconvolution immunofluorescence microscopy (i.e. the conditions used for imaging the cells in the experiment in Fig. 3E-F) that would be scored as positive in the quantitative assessment (Fig. 3F).

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Therefore, the differences in baseline mitochondrial structure observed at the level of super-resolution microscopy did not affect our ability to detect an increased accumulation of cells with diffuse fragmented mitochondria after OA treatment in Fig. 3F in PEX13 W313G versus wild-type human fibroblasts. We note that the variability among cells within each genotype is controlled for by counting a large number of cells per genotype by an observer blinded to experimental group. Details of this quantification are provided in the legend for Fig. 3F. Importantly, we significantly revised the paragraph describing the characterization of the PEX13 W313G fibroblasts to more clearly explain the points made in this response to the referee's comment and we also revised the figure legend for Fig. 3E to more clearly explain what information the outlined W313G cells is intended to convey.

Reply to comment 5 - Did the authors induce pexophagy by overexpression of PEX3 and observe no decrease of peroxisomes in PEX13-depleted HeLa cells or in PEX13-KO MEFs? If so, the authors should be better to describe the results.

Authors' Response: As we mentioned previously in comment 5, we were unable to induce pexophagy in our control HeLa or HeLa PEX13-depleted cells or in the wild-type or Pex13 knockout MEFs. We did not perform any pexophagy experiments with overexpression of PEX3.

3rd Editorial Decision 07 October 2016

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.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Beth Levine, M.D.
Journal Submitted to: EMBO
Manuscript Number: EMBOR-2016-42443V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 justified
- 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 $\chi 2$ 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	For the mitophagy, virophagy, and starvation-induced general autopahgy immunofluorescent imaging experiments, we counted 100 cells per sample in triplicates, and repeated each experiment independently at least 3 times. Based on our previous studies with these experiments (Orvedahl et al, 2011, Sumpter et al, 2016), the selected sample size is sufficient to detect effect size in positive controls .
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The only animal studies performed in this study involved electron microscopic analyses of tissues from embryonic wlld-type and mutant mice. These analyses are qualitative in nature and no quantitative assessments were made. Tissues from three mice of each genotype were evaluated to ensure that qualitative differences observed by electron microscopy were due to genotype-specific differences.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Immunofluorescent imaging experiments were quantified by a blinded observer to minimize bias.

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4.b. For animal studies, include a statement about blinding even if no blinding was done	Electron microscopic analyses of tissues from Pex13 wild-type and Pex13 knockout mice were performed by an observer blinded to mouse genotype.
5. For every figure, are statistical tests justified as appropriate?	Yes. Student's t-test was used to compare the means of two groups. ANOVA with adjustment for multiple comparisons was used to compare the means of multiple groups to a control. Mann-Whitney U-test and Kruskal-Wallis H-test were used to comparemultiple non-normally distributed datasets.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Student's t-test and ANOVA with adjustment for multiple comparisons were used to analyze data that were normally distributed. Mann-Whitney U-test and Kruskal-Wallis H-test were used to analyze data that were non-normally distributed. We asked a biostatistician at UT Southwestern Medical Center to assess our datasets for normality using standard statistical programs prior to performing the appropriate parametric or non-parametric analyses.
Is there an estimate of variation within each group of data?	Standard error was calculated for each sample and presented in the bar graphs as an estimate of variation within each group.
Is the variance similar between the groups that are being statistically compared?	The variation are mostly simimlar between the groups being compared. However, to be more stringent on our statistical analysis, we did not assume that the variance is the same between groups for this study.

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).	All antibodies used in this study are cited by catalog number and clone number in the Materials and Methods section of the manuscript.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The sources of all cell lines used in this experiment are included in the Materials and Methods section of the manuscript. All cell lines were tested for mycoplasma contamination and HeLa cells were recently authenticated by STR profiling.

* 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.	Pex13+- mice (reference 21 of manuscript) were provided by our co-author Dennis Crane (reference 21 of manuscript) and GFP-LC3 transgenic mice (reference 36 of manuscript) were provided by Noboru Mizushima of the University of Tokyo Medical School.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were in compliance with AALUC regulations and approved by the UT Southwestern Institutional Animal Care and Use Committee.
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.	The relevant information is reported for our animal experiments.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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 accession codes for deposited data. See author guidelines, under '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	NA
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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	All referenced data are cited in the References section of the manuscript.
22. 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

23. 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.