Pex24 and Pex32 are required to tether peroxisomes to the ER for organelle biogenesis, positioning and segregation in yeast

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Original submission

First decision letter

MS ID#: JOCES/2020/246983

MS TITLE: Pex24 and Pex32 tether peroxisomes to the ER for organelle biogenesis, positioning and segregation

AUTHORS: Fei Wu, Rinse de Boer, Arjen M. Krikken, Arman Aksit, Nicola Bordin, Damien P. Devos, and Ida J. J Van der Klei
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

You will see that they both raise concerns that relate to the way in which the data support your conclusions. In particular, whether Pex24 and PEx32 are indeed tethers that bridge peroxisomes to the ER. I find myself in agreement with them that this would require further experimental work that meetsthe criteria set out by Eisenberg-Bord et al. (2016). If that is not possible then you might consider reframing your conclusions around the role of Pex23 family proteins in mediating contact between ER and peroxisomes and that these contacts are required for biogenesis and segregation. That would leave open for further experiments the proof that these proteins are indeed a bona fide tether.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This manuscript entitled “Pex24 and Pex32 tether peroxisomes to the ER for organelle biogenesis, positioning and segregation” by Wu et al. describes the discovery and functional characterization of four Pex23 family proteins in H. polymorpha. The main conclusions of this manuscript are that all of these proteins (Pex24, Pex32, Pex23 and Pex29) localize to the ER and that two of them, Pex24 and Pex32, specifically localize to ER-peroxisome contact sites. Loss of these peroxins results in the loss of ER-peroxisome contacts, defects in peroxisomal matrix import, membrane growth, as well as peroxisome positioning in the cell and peroxisome inheritance. Interestingly, expression of an artificial tether molecule that reestablishes ER-peroxisome contact sites in cells devoid of Pex24 and Pex32 results in a rescue of most of the observed phenotypes. Furthermore, the authors identify PEX11 as a potential counterpart for contact site formation between the ER and peroxisomes.

This is a very well-written manuscript and the observations described here are of potential high interest to a broad readership in the fields of membrane trafficking, organelle biogenesis and inter-organelle communication including physical contact site formation. While there is data about PEX23 family members in other species available in the literature, this manuscript systematically analyzes the localization and potential function of these proteins in H. polymorpha, which nicely complements existing data and contributes to the establishment of new functional models in the field.

Comments for the author

Most of the conclusions that the authors state in this manuscript are well supported by the experimental data, especially their main conclusion that Pex24 and Pex32 tether peroxisomes to the ER for organelle biogenesis, positioning and segregation, as the title states. Overall, the quality of the provided data is high. However a few points should be addressed before accepting the manuscript for publication:

1. My major concern is about the statement that Pex23 proteins are not crucial for the formation of PPVs, which is in contradiction to what has been published for Pex23 proteins in S. cerevisiae. The data provided in this manuscript are not strong enough for such a big statement. In Fig. 7 the authors quantify PEX14-GFP signals in atg1pex3 and atg1pex3pex32 cells and cannot detect any differences. PEX14 is a peroxisomal membrane protein and not an exclusive marker for PPVs. The authors refer to a published paper, which apparently shows that PEX14 localizes to PPVs in this genetic background but it would be important to provide a better characterization about what is actually detected with this fluorescent spots also in this manuscript. While the rest of Fig. 7 addresses this question by CLEM and shows that PEX14-positive signals indeed correlate with vesicular structures, there is no comparison with the respective control cells. The basis for their conclusion that PPV formation is not affected by Pex32 is solely the quantification of GFP spots. It may, however, be possible that the number, size or morphology of individual PPVs is in fact altered

upon pex32 deletion. The authors should either add CLEM data for the respective control and provide a detailed comparison based on CLEM or alternatively tone down their conclusion.

2. No molecular mechanism has been directly addressed in this manuscript. While I believe that the manuscript in its current form is interesting, the impact and mechanistic insight could be improved by for example mapping interaction sites between PEX32 and PEX11. Do these proteins directly interact with each other in these cells? Which domains are required for this?

Minor points:
1. Fig. 5 shows that expression of ER-PER restored growth defects caused by the deletion of pex24 and pex32, respectively. The authors should include the data for the WT and WT-ER-PER expressing cells into the graphs for comparison. These data are available in the supplement but incorporating them here in the main figure would help the reader to directly appreciate the effects.

2. On page 20 the authors state “In these cells Pex32-GFP accumulation in a spot was lost. Instead, multiple fainter Pex32-GFP spots were observed that showed a typical ER pattern (Fig. 6B).” I cannot see that ER pattern. The signals are too faint and a colocalization experiment would be required for such a statement.

3. The order of some figure panels is somewhat confusing. On page 11, the text first refers to Fig 3D and then later to Fig. 3C. I recommend rearranging the figure panels or alternatively the text. Also the arrangement of panels in Fig. 5 could be improved, so that E is not in between panels B and D.

4. On page 18, the text refers to Fig. 6 G,H but I believe the correct reference would be Fig. 5 G,H instead.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Wu et al., describes the systematic analysis of the Pex23 protein family in the yeast Hansenula polymorpha.
All 4 proteins tagged at their C-terminus with a fluorescent protein (GFP), localise to the ER upon overexpression. Upon expression driven by their own promoter, the proteins localise to subdomains of the ER.
Of particular interest for this manuscript is the localisation of Pex24 and Pex32 that localise to areas where peroxisomes and ER are in very close proximity.
The authors report that deletion of PEX24 or PEX32 results in loss of peroxisome-ER contacts, a decrease in peroxisomal membrane area per cell as a consequence of membrane growth and peroxisome positioning and inheritance. PEX32 deletion also strongly affects peroxisomal protein import, whereas minor import defects were observed in pex23- and pex24-deficient cells. Introduction of an artificial peroxisome-ER tether partially restores the peroxisomal phenotypes. The authors conclude that Pex24 and Pex32 function as contact site tethers.
Pex11, a peroxisomal membrane protein is shown to be required for peroxisome-ER contacts and for localisation of Pex32 to these sites. In addition, they show that ER-peroxisome contacts are required for peroxisome retention in mother cells and that these contacts are made independent of Inp1.

Comments for the author

The study is well performed and contains novel information that extends our understanding of the Pex23 family of proteins specific to Hansenula polymorpha. This protein family in S. cerevisiae has been reported to share features with the reticulon family of proteins and may therefore affect ER subdomain structure. The S. cerevisiae homologues Pex30 and Pex31 have been shown to be required for peroxisome-ER contacts and that they affect de novo peroxisome formation.
The observations that Hansenula polymorpha Pex24 and Pex32 are required for the formation of peroxisome-ER contact sites in conjunction with Pex11 and that this contact contributes to biogenesis and peroxisome retention in mother cells, are important and worth publishing as such.

However, the conclusion that Pex24 and Pex32 form a tether is premature (see for instance guidelines set out in Eisenberg-Bord et al., 2016). Showing that it is forming an actual tether needs more experiments. Furthermore, whether Pex24 and Pex32 work together or whether they are partially redundant is unclear. The role of Pex11 in tether formation is also unclear.
The limited analysis of the role of only one member of the Pex23 protein family in preperoxisomal vesicle (ppv) formation is not sufficient for a study that aims to be a systematic analysis of the Pex23 family.

Minor comments
Line 106 The authors mention that some of the Pex23 family proteins contain potential di-lysine motifs that are important for retention in the ER. These motifs require to be at the carboxy-terminus of the proteins (Nilsson et al., 1989), exposed to the cytosol so that coatomer can bind. Are the c-termini predicted to be exposed to the cytosol or is there any experimental evidence for this?
C-terminal tagging would interfere with the function of the di-lysine motif Are the GFP tagged fusion proteins fully functional?

Line 178 Pex24 and Pex32 are important for peroxisome biogenesis and proliferation.
I agree that peroxisome number is reduced in the mutants but the number still increases under conditions of peroxisome proliferation, so the authors should consider changing this conclusion.

Line 271 Fig. 4F.
The strongest segregation defects were observed in cells with the least peroxisomes except for Inp1 cells. For instance, based on data in Figure 3, most of the cells with a pex24 or 32 mutation that contain peroxisomes, contain only 1 peroxisome. Is peroxisome segregation also defective in those cells with multiple peroxisomes in these mutants?

Figure 3,4: Only 2 biological replicates were counted throughout these figures.

Figure 5D Are peroxisomes really labelled with GFP-SKL, it looks more like a membrane marker. Does the artificial tether restore import into peroxisomes in pex32-deficient cells?
Figure 5E is missing.

Data presented in Figure 7 only focusses on Pex32. As this paper is a systematic study on the Pex23 family it is surprising that only pex32 was analysed here and not the other members of the family.

First revision

Author response to reviewers' comments

Reviewer #1.
Major concerns:
1. My major concern is about the statement that Pex23 proteins are not crucial for the formation of PPVs, which is in contradiction to what has been published for Pex23 proteins in S. cerevisiae. The data provided in this manuscript are not strong enough for such a big statement. In Fig. 7 the authors quantify PEX14-GFP signals in atg1pex3 and atg1pex3pex32 cells and cannot detect any differences. PEX14 is a peroxisomal membrane protein and not an exclusive marker for PPVs. The authors refer to a published paper, which apparently shows that PEX14 localizes to PPVs in this genetic background but it would be important to provide a better characterization about what is actually detected with this fluorescent spots also in this manuscript. While the rest of Fig. 7 addresses this question by CLEM and shows that PEX14-positive signals indeed correlate with vesicular structures, there is no comparison with the respective control cells. The basis for their conclusion that PPV formation is not affected by Pex32 is solely the quantification of GFP spots. It may, however, be possible that the number, size or morphology of individual PPVs is in fact altered

upon pex32 deletion. The authors should either add CLEM data for the respective control and provide a detailed comparison based on CLEM or alternatively tone down their conclusion.
REPLY: We agree that our conclusions were too strong. All studies in S. cerevisiae indicate that Pex30 and Pex31 are required for the regulation of PPV formation, but are not essential for their formation. To clarify this point, we have described the current knowledge on the role of Pex23 proteins in PPV formation in more detail. We are currently unable to perform the requested CLEM experiment for the control strain, because of the Corona crisis. Instead, we have added available electron microscopy images of chemically fixed pex3 atg1 pex32 and pex3 atg1 control cells (new figure 7D). This data shows that in terms of size and morphology the membrane structures are similar in both strains. We have toned down our conclusions and wrote that deletion of PEX32 in pex3 atg1 does not affect the number of Pex14-marked spots or the morphology of the vesicles.

2. No molecular mechanism has been directly addressed in this manuscript. While I believe that the manuscript in its current form is interesting, the impact and mechanistic insight could be improved by for example mapping interaction sites between PEX32 and PEX11. Do these proteins directly interact with each other in these cells? Which domains are required for this?
REPLY: We do not know yet whether Pex32 and Pex11 interact directly. Given the limited access to the laboratory, we are unable to perform additional work to address this issue. Also, we feel that the requested structure-function studies are outside the scope of our current manuscript. We have rephrased the title and text indicating that Pex24, Pex32 and Pex11 contribute to tethering instead of writing that these proteins are bona fide tethers.

Minor points:
1. Fig. 5 shows that expression of ER-PER restored growth defects caused by the deletion of pex24 and pex32, respectively. The authors should include the data for the WT and WT-ER-PER expressing cells into the graphs for comparison. These data are available in the supplement but incorporating them here in the main figure would help the reader to directly appreciate the effects.
REPLY: We thank the reviewer for this suggestion and added the data on WT and WT ER-PER in Figure 5F, as requested.

2. On page 20 the authors state “In these cells Pex32-GFP accumulation in a spot was lost. Instead, multiple fainter Pex32-GFP spots were observed that showed a typical ER pattern (Fig. 6B).” I cannot see that ER pattern. The signals are too faint and a colocalization experiment would be required for such a statement.
REPLY: We agree that GFP signals are too faint and do not support our conclusion. We have adjusted the text accordingly.

3. The order of some figure panels is somewhat confusing. On page 11, the text first refers to Fig 3D and then later to Fig. 3C. I recommend rearranging the figure panels or alternatively the text. Also the arrangement of panels in Fig. 5 could be improved, so that E is not in between panels B and D.
REPLY: We agree that the figures should be placed in the proper order. We rearranged the text and figures as suggested.

4. On page 18, the text refers to Fig. 6 G,H but I believe the correct reference would be Fig. 5 G,H instead.
REPLY: We apologize for the mistake and corrected this.

Reviewer #2.
Major remark:
The conclusion that Pex24 and Pex32 form a tether is premature (see for instance guidelines set out in Eisenberg-Bord et al., 2016). Showing that it is forming an actual tether needs more experiments.
REPLY: Our data suggest that Pex24 and Pex32 are components of tether complexes that bridge peroxisomes to the ER. Indeed, we do not meet all three criteria suggested by Eisenberg-Bord et al. (2016). These criteria include: “(1) Defined location: A tether must reside exclusively in, or be enriched in, the contact site. This can be determined by methods such as fluorescence or electron microscopy. (2) Structural capacity: A tether/tether complex must mediate binding to the membranes of the opposing organelles forming the contact site. Structure-function analyses are

currently the best way to prove this point. (3) Functional activity: A tether must exert a tethering force. This can either be measured directly in vivo or in reconstituted systems or indirectly by the effect of the tether on the extent of the contact site or the rescue of tether loss by artificial tethers." We show that H. polymorpha Pex24 and Pex32 have a defined location (criterion 1) and that an artificial tether rescues the phenotypes caused by the loss of these proteins (criterion 3). We included this information in the discussion of the revised manuscript. Also, we rephrased the title and text of our manuscript indicating that Pex24 and Pex32 may not be bona fide tethers, but are proteins that contribute to peroxisome-ER association.

Furthermore, whether Pex24 and Pex32 work together or whether they are partially redundant is unclear. The role of Pex11 in tether formation is also unclear.
We have added new data (Figure 6A, B), which show that overproduction of Pex23, Pex24 or Pex29 in pex32 cells does not restore peroxisome abundance in glucose-grown cells or the methanol growth defect of pex32 cells. These data indicate that these proteins are not functionally redundant with Pex32. Further studies are required to understand the composition and function of the tether complex and the role of Pex11. So far we cannot exclude that Pex11 plays an indirect role in association of peroxisomes to the ER. We added this information to the discussion section.

The limited analysis of the role of only one member of the Pex23 protein family in preperoxisomal vesicle (ppv) formation is not sufficient for a study that aims to be a systematic analysis of the Pex23 family.
REPLY: We agree with the reviewer and have rephrased in the discussion that we only know for Pex32 that it has no major role in the regulation of PPV formation. In the first part of our manuscript we analyzed all four proteins (Figs. 1-5), but in the last part (Figs. 6-7) we focused on Pex32, the protein that has the most important role in peroxisome biogenesis. We have removed the word "systematic", where appropriate.

Minor comments:
The authors mention that some of the Pex23 family proteins contain potential di-lysine motifs that are important for retention in the ER. These motifs require to be at the carboxy-terminus of the proteins (Nilsson et al., 1989), exposed to the cytosol so that coatomer can bind. Are the c-termini predicted to be exposed to the cytosol or is there any experimental evidence for this?
REPLY: We have not studied the orientation of the four proteins. It is hard to say whether the C-termini are exposed to the cytosol, also given the differences in predicted membrane spans (see Figure 1).

C-terminal tagging would interfere with the function of the di-lysine motif. Are the GFP tagged fusion proteins fully functional?
REPLY: Pex23-GFP, Pex24-GFP and Pex32-GFP are fully functional. pex23, pex24 and pex32 mutants all show retarded growth on methanol. However, growth analysis of the strains producing the C-terminally tagged proteins showed that these strains grow like wild-type controls. We have added this new information in supplementary table S1.

Line 178 Pex24 and Pex32 are important for peroxisome biogenesis and proliferation. I agree that peroxisome number is reduced in the mutants but the number still increases under conditions of peroxisome proliferation, so the authors should consider changing this conclusion.
REPLY: We thank the reviewer for pointing this out. We have adapted the title of the paragraph.

Line 271 Fig. 4F. The strongest segregation defects were observed in cells with the least peroxisomes, except for Inp1 cells. For instance, based on data in Figure 3, most of the cells with a pex24 or 32 mutation that contain peroxisomes, contain only 1 peroxisome. Is peroxisome segregation also defective in those cells with multiple peroxisomes in these mutants?
REPLY: It is very hard to find budding pex24 or pex32 cells that contain more than one peroxisome. In these rare cases, a peroxisome was generally observed in both the mother cell and bud. Because the number of budding cells with > 2 peroxisomes was very low, we were unable to quantify these data properly.

Figure 3,4: Only 2 biological replicates were counted throughout these figures.
REPLY: We routinely analyze two biological replicates in our research. Indeed, we recognize that several journals nowadays ask for 3 biological replicates. We would need to repeat almost all

experiments to obtain triplicates, which is very hard because of the limited access to the laboratory. We thank the reviewer for pointing this out and will make 3 biological replicates in future studies.

Figure 5D Are peroxisomes really labelled with GFP-SKL, it looks more like a membrane marker.
REPLY: Yes, these cells produce GFP-SKL. Because peroxisomes of methanol-grown H. polymorpha cells contain an alcohol oxidase crystalloid, GFP is not evenly distributed over the peroxisomal matrix, but present between the crystalloid and peroxisomal membrane. We have clarified this in the legend.

Does the artificial tether restore import into peroxisomes in pex32-deficient cells?
REPLY: As shown in Fig. 5D, the matrix marker GFP-SKL localizes to peroxisomes. This is in line with the observed restoration of methanol-growth. We have clarified this in the text.

Figure 5E is missing.
REPLY: Fig. 5E was not missing, but difficult to find due to the confusing arrangement of the figure. We have rearranged the figure to avoid this confusion.

Data presented in Figure 7 only focusses on Pex32. As this paper is a systematic study on the Pex23 family it is surprising that only pex32 was analyzed here and not the other members of the family.
REPLY: Indeed we only studied all proteins for the overall characterizations (localization, phenotype, contact sites, etc.; Figs. 1-5), which revealed that Pex32 was most important for peroxisome biology. Therefore the later studies, presented in Figs 6-7, were only performed for Pex32. We have explained this better in the revised version of the text.

Second decision letter

MS ID#: JOCES/2020/246983

MS TITLE: Pex24 and Pex32 are required to tether peroxisomes to the ER for organelle biogenesis, positioning and segregation

AUTHORS: Fei Wu, Rinse de Boer, Arjen M. Krikken, Arman Aksit, Nicola Bordin, Damien P. Devos, and Ida J. J Van der Klei
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

Thank you for the careful revisions to your paper. In order to prevent unnecessary work for reviewers and delays in publication, I have reviewed the revised version myself. I find that you have dealt with the comments raised well. The only further amendment that I would require is that you remove the statistical testing of the data in Figure 4 and else where in the text as this is not valid from a duplicate (n=2) sampling only. Since you clearly state n=2 I am satisfied that this is acceptable.I hope that you will be able to carry this out, because I would like to be able to accept your paper.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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Third decision letter

MS ID#: JOCES/2020/246983

MS TITLE: Pex24 and Pex32 are required to tether peroxisomes to the ER for organelle biogenesis, positioning and segregation

AUTHORS: Fei Wu, Rinse de Boer, Arjen M. Krikken, Arman Aksit, Nicola Bordin, Damien P. Devos, and Ida J. Van der Klei
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.