JCB

Peroxisome retention involves Inp1-dependent peroxisome-plasma membrane contact sites in yeast

Arjen M. Krikken, Huala Wu, Rinse de Boer, Damien Devos, Tim Levine, and Ida J van der Klei

Corresponding Author(s): Ida J van der Klei, University of Groningen

Review Timeline:

Submission Date:	2019-06-04
Editorial Decision:	2019-08-07
Revision Received:	2020-03-15
Editorial Decision:	2020-04-20
Revision Received:	2020-06-23
Accepted:	2020-06-23

Monitoring Editor: Jodi Nunnari

Scientific Editor: Marie Anne O'Donnell

Transaction Report:

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

DOI: https://doi.org/10.1083/jcb.201906023

July 29, 2019

Re: JCB manuscript #201906023

Prof. Ida J van der Klei
University of Groningen
Molecular Cell Biology
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Netherlands

Dear Prof. van der Klei,

Thank you for submitting your manuscript entitled "Peroxisome retention involves Inp1 dependent peroxisome-plasma membrane contact sites in yeast". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

The reviewers note that the identification of Pex3-Inp1 as the first tether of peroxisomes to the plasma membrane is novel and interesting but they are quite critical of the overall advance as Pex3-Inp1 has already been proposed to control peroxisomal inheritance via their tethering to the ER. To be suitable for JCB, we feel that some expansion to the scope would be necessary, such as providing more insight into the relationship between ER and plasma membrane tethering by Inp1 in this process, how tethering to the plasma membrane specifically alters peroxisomal retention, or identifying other peroxisome processes that may require tethering to the plasma membrane, or the role of Pex3 versus Pex19 binding for tethering.

In addition, the reviewers note that further evidence is necessary to bolster the main claims that Inp1 binds the plasma membrane and clarify if this alone, or in conjunction with ER tethering, is necessary for Inp1 to mediate peroxisomal inheritance. For example, Rev#1 and #3 recommend characterizing in more molecular detail the domains of Inp1 necessary for interaction with the plasma membrane and confirmation that this binding is necessary for peroxisome retention.

Although your manuscript is intriguing, I feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic, I would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the

journal office with any questions, cellbio@rockefeller.edu.

As an alternative to expanding the scope for JCB, we have discussed your manuscript with the editors of Life Science Alliance (http://www.life-science-alliance.org/) and they would like to invite a revision that provides: a pbp response and accordingly text changes and inclusion of further references, as well as addressing the request for additional images (rev#1), and addressing the specific comments of rev#3. Rev#3's point on the decrease of Pex3-GFP patches from 80% to 15% does not need to get addressed. Further insight into PO-PM tethering as requested by rev#3 would significantly strengthen the paper, the requested full mechanistic understanding is, however, not expected for publication in LSA. LSA is our academic editor-led, open access journal launched as a collaboration between RUP, EMBO Press and Cold Spring Harbor Press. You can use the link below to initiate an immediate transfer of your manuscript files and reviewer comments to LSA.

Link Not Available

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Wu et al identify Inp1 as a peroxisome-plasma membrane tether in H. polymorpha. In S. cerevisiae and H. polymorpha, Inp1 has been previously shown to be required for retention of peroxisomes in mother cells, and S. cerevisiae Inp1 is thought to tether peroxisomes to the cortical ER. Here the authors use CLEM to show that Inp1 localizes to sites of peroxisome-PM contact in H. polymorpha and that these sites are devoid of ER. Overexpression of Inp1 increases peroxisome-PM contact. In the absence of Inp1, the close apposition of peroxisomes to the PM is decreased, while peroxisomal-ER contacts remain unchanged. Using Inp1 truncation constructs, the authors identify that a region in the N-terminus of Inp1 associates with the plasma membrane and that the C-terminal half associates with peroxisomes. The data demonstrating that Inp1 functions as a peroxisome-PM tether are solid and challenge the idea that Inp1 is a peroxisome-ER tether. That being said, the study would benefit from the addition of experiments that 1) further narrow down the peroxisome and PM interacting domains and demonstrate that these domains are functionally required for peroxisome tethering and retention and 2) provide more insight into the mechanism/molecular basis for the interaction with the PM and peroxisomes.

Additional comments:
1. In Fig. 3E, it would be very beneficial to the reader if images in which peroxisomes are visualized using DsRED-SKL are shown for WT cells as they are for the Inp1 overexpressing cells since Pex3 localization looks so different.

2. The N-terminus of Inp1 is found to be important for the interaction with the plasma membrane. Constructs that lack the N-terminus and associate with peroxisomes still appear to be cortical in Fig. 5. Is that due to the fact that WT Inp1 is still around?

3. Inp1 driven from the TEF1 promoter is said to be "slightly" overproduced, however, based on the western blot shown in Fig. 4B, it appears more than slightly overproduced. The authors should modify their wording.

4. The authors state that because their Inp1 truncation constructs are all driven by the TEF promoter the differences in protein levels observed are due to post-translational processes. While this is likely to be true the authors cannot rule out differences in mRNA stability and should modify their wording.

5. In the discussion, the authors state that all peroxisomes are transported to the bud in inp1 mutants but no references are given. References need to be included.

6. For the cell images shown, it should be stated if they are maximum intensity projects or single focal planes.

7. When previous studies are referred to, it is not always clear if the authors are referring to work done in S. cerevisiae or H. polymorpha. This needs to be better clarified throughout the manuscript.

Reviewer #2 (Comments to the Authors (Required)):

In S. cerevisiae, the proteins Inp1 and Pex3 form a tether that links peroxisomes to the ER and plays a critical role in peroxisome inheritance (Knoblach et al, EMBO 2013). This study convincingly shows the same proteins tether peroxisomes to the plasma membrane in H. polymorpha. It suggests that the Inp1-Pex3 tether plays a role in inheritance but does not provide evidence. However, even if it did, the study is only a modest conceptual advance on Knoblack et al. It would be substantially stronger if it provided insight into how tether formation is regulated.

Reviewer #3 (Comments to the Authors (Required)):

Summary:
Wu et al. have identified Inp1 as a crucial factor for formation of peroxisome (PO)-plasma membrane (PM) contacts in the budding yeast H. polymorpha. Overexpression of Inp1 increases, whereas INP1 deletion reduces PO-PM contacts. The authors propose that PO-PM contacts are mediated by the PM-Inp1-Pex3-PO interaction, since the two colocalise on the peripheral edge of POs, INP1 deletion results in Pex3 redistribution on POs, and Inp1 localises to the cell periphery in the absence of Pex3. Importantly, Inp1 is not required for PO-ER contacts as they are maintained in strains lacking INP1. They identify the N-terminal region of Inp1 as being necessary for its peripheral localisation. They also observe a mistargeting of PO matrix proteins in strains in which Inp1 is localised to PO.

General comments:
The characterisation of the proteins regulating PO-PM contacts is novel, interesting and timely, and it is convincingly shown that Inp1 plays a direct role in PO-PM contacts through sophisticated CLEM experiments and deletion/overexpression strains. It is interesting that Inp1 seems to be dispensable

for the formation of PO-ER contacts in H. polymorpha, and it should be discussed further. However, direct interaction of Inp1 and Pex3 is not shown here, and has already been established in S. cerevisiae. In addition, some of the conclusions are rather speculative from the data presented, particularly concerning the function of such PO-PM contacts - for example, the reference to PO retention in the title is erroneous, as no data is presented to support this. I would suggest significant further experiments to more extensively characterise this putative 'tether' on the molecular level and to support a physiological role for PO-PM contacts (e.g. is PO retention/inheritance disrupted in strains with reduced/enhanced PO-PM contacts?).

Specific comments:
Introduction:
• A brief introduction to POs might be helpful.
• The description of ER-PO contacts in S. cerevisiae is a little confusing - one paragraph says they are mediated by Inp1-Pex3, another mentions Pex30 and Pex31? This should be clarified.
• The authors may want to add a clearly explained hypothesis (e.g. providing more of a rationale).

Results and discussion:
• The authors make the statement '...in budding cells generally POs contain a second, relatively small spot of Pex3-GFP fluorescence...'. Can this be characterised more robustly? For example, is this only seen in actively budding cells, and in what proportion of cells? It might also be necessary to define what is considered to be a 'patch' of Pex3, since Pex3 is detected around the entire PO and not just in distinct puncta.
• The authors state that '...these findings are consistent with the view that the peripheral Pex3 spot is involved in Inp1-dependent PO retention in mother cells...', based on Figure 1. While it is true that these findings might be consistent, this is far too speculative at this stage and should be removed. The later interpretation of the data from Figures 1 and 2 suggesting '...POs form contacts with the PM, to which Inp1 and Pex3 localise...' is more appropriate, and actually very convincing from the data presented.
• The authors observe that, in inp1 deletion cells, the percentage of cells containing a peripheral Pex3-GFP patch dropped from 80% to 15%. Can the authors determine what the remaining 15% of patches correspond to? Are they PO-ER contacts, or with another organelle? This could be checked by colocalisation with organelle markers or CLEM.
• The result that INP1 deletion does not affect PO-ER contacts is interesting, because this seems to be different from the observations in S. cerevisiae - the authors should discuss this disparity. Does this mean that Inp1-Pex3 does not regulate PO-ER contacts in H. polymorpha, or that a currently unknown component can compensate following Inp1 deletion?
• The authors observe that 'In Inp1+++ cells Inp1-GFP and Pex3-mKate2 co-localized to an elongated patch at the cell periphery (Fig. 3E). At the same time, the intensity of the Pex3-GFP patch at VAPCONS decreased, suggesting that bulk of the peroxisomal Pex3 protein was recruited to the peroxisome-PM contacts'. Only one example is shown - is this typical? Is total Pex3 expression the same in Inp1+++ cells and WT cells? This would be important for the conclusion that Pex3 is only redistributed upon Inp1 overexpression. Furthermore, DsRed-SKL staining appears to be at the PO membrane - should this not label the Po lumen?
• The authors should show that Inp1 deletion/overexpression alter PO retention or inheritance during budding. This would be important to support some of the later conclusions about the function of PO-PM contacts.
• Figure 3 - the WT example of Inp1 and Pex3 localisation in Figure 3E seems to show considerably less colocalisation than previously seen - is this a consequence of the growth in glycerol/methanol media? If so, the rationale for using this media should be more clearly explained. If not, a better example should be found.

• Similarly to before, whilst the statement 'Our data are consistent with the view that Pex3-bound Inp1 connects peroxisomes to the PM' is technically true, this seems a bit misleading as a direct Pex3-Inp1 interaction at PO-PM contacts is not shown here. Perhaps 'a Pex3-Inp1 complex connects PO to the PM' might be more reasonable?
• The authors observe that Inp1-GFP localises to the cell periphery in the absence of Pex3 - the authors should confirm that this represents the PM and not, for example, cortical ER (e.g. colocalisation with a PM marker, ER control, and/or immuno-EM).
• In line with this, N-terminal portions of Inp1 appear to localise to different locations, potentially unspecific due to positively charged residues. The manuscript would benefit from a more thorough molecular analysis of the N-terminal and C-terminal regions mediating (specific) PM affinity and Pex3 binding. For example, can a membrane-bound fusion protein containing part of the Inp1 N-terminus associate PO (or other organelles) with the PM; are the positively charged residues required for this?; is the N-terminus of Inp1 interacting directly with lipids of the PM? What is the molecular mechanism?
• The authors suggest that '...The presence of cytosolic Inp1-GFP [in Pex3 deletion cells] may be related to Inp1-GFP overproduction...'. Could another possibility be that a subpopulation of Inp1 normally bridges Pex3 at the ER and POs, but becomes cytosolic in the absence of Pex3? The authors may wish to discuss this. Similarly, I disagree with the conclusion that 'Our data do not support the view that Inp1 functions as a molecular hinge by binding to ER- and peroxisome localized Pex3, because according to this model Inp1 would become fully cytosolic in the absence of Pex3' - my interpretation is that this data only excludes the possibility that the sole function of Inp1 is bridging ER- and PO-localised Pex3.
• Figure 5B: What happens to Pex3 localisation when Inp1 truncations are expressed? Do you lose the peripheral Pex3 patch if Inp1 is not localised to the cell periphery?

Concluding remarks:
• The concluding remarks seem particularly speculative. These should be tempered considerably as 'discussion' and, if based on the literature, should be better referenced.
• References to 'VAPCONS' should be 'EPCONS'?
• 'The VAPCON (EPCON?) is apparently not sufficiently strong to retain peroxisomes in the mother cell of INP1 deletion mutants, because in such mutants all peroxisomes are transported to the newly formed buds.' Is this based on the literature? Certainly no data is presented to show this. If so, it would be important to mention this more extensively in the introduction. References should also be provided.
• The final model suggested: 'Upon Dnm1 dependent asymmetric fission, the original peroxisome remains associated to the PM (and possibly the ER), whereas the newly formed organelle is transported to the newly formed bud, a process that requires Inp2, Myo2 and the actin cytoskeleton. Finally, upon reaching the new bud, the peroxisome detaches from Myo2 and becomes anchored to the PM of the bud via Inp1' seems plausible, but should be presented more clearly as just a hypothesis, since the data presented do not address this. References (if available) would make the case more convincing. The authors could consider adding a schematic of this proposed model to make it clearer.

• Statistical analysis is not presented in all cases and should be added if appropriate. All data seems to be collected from two independent experiments?

REBUTTAL

Reviewer #1:
The data demonstrating that Inp1 functions as a peroxisome-PM tether are solid and challenge the idea that Inp1 is a peroxisome-ER tether. That being said, the study would benefit from the addition of experiments that 1) further narrow down the peroxisome and PM interacting domains and demonstrate that these domains are functionally required for peroxisome tethering and retention and 2) provide more insight into the mechanism/molecular basis for the interaction with the PM and peroxisomes.

Reply: We are very grateful for the constructive criticism of the reviewer, which helped us to improve our manuscript.

Reply:
1) We have performed a detailed analysis of the different domains in Inp1 (new Fig. 3) and analysed the localization and function of several truncated species. Also, we studied the role of the conserved positively charged residues in the extreme N-terminus (Fig. 3). These studies indicated that the extreme N-terminus as well as the charged residues are not essential of Inp1 function, but play a role in the regulation of Inp1 levels. We show that the central, conserved domain is a Pleckstrin Homology-like (PH-like) domain, which is essential for association to the plasma membrane.
2) We show that the PH-like domain is required for plasma membrane binding. Moreover, in the absence of Pex3 Inp1 accumulates in patches near the bud neck and in the bud cortex, which are lost upon incubation of cells with latrunculin A, suggesting that Inp1 binds an actin skeleton associated protein (new Fig. 4B).

Additional comments:
1. In Fig. 3E, it would be very beneficial to the reader if images in which peroxisomes are visualized using DsRED-SKL are shown for WT cells as they are for the Inp1 overexpressing cells since Pex3 localization looks so different.

Reply: We have replaced the original image by a better one (new Fig. 2E). The cells are grown for 16 h on glycerol/methanol, resulting in cells with multiple peroxisomes. This has been clarified in the legend.

2. The N-terminus of Inp1 is found to be important for the interaction with the plasma membrane. Constructs that lack the N-terminus and associate with peroxisomes still appear to be cortical in Fig. 5. Is that due to the fact that WT Inp1 is still around?

Reply: Inp1 was indeed still around. We have removed these experiments and instead analysed all truncated species in an *inp1* background (new Fig. 3). Also, we used the Inp1 promoter for the production of the truncated proteins.

3. Inp1 driven from the TEF1 promoter is said to be "slightly" overproduced, however, based on the western blot shown in Fig. 4B, it appears more than slightly overproduced. The authors should modify their wording.

Reply: We agree. We modified the wording.

4. The authors state that because their Inp1 truncation constructs are all driven by the TEF promoter the differences in protein levels observed are due to post-translational processes. While this is likely to be true the authors cannot rule out differences in mRNA stability and should modify their wording.

Reply: These experiments have been removed. We now produce all trunctations under control of the endogenous *INP1* promoter in an *inp1* strain. .

5. In the discussion, the authors state that all peroxisomes are transported to the bud in inp1 mutants but no references are given. References need to be included.
Reply: We removed this part.

6. For the cell images shown, it should be stated if they are maximum intensity projects or single focal planes.
Reply: We have included this information in the legends.

7. When previous studies are referred to, it is not always clear if the authors are referring to work done in S. cerevisiae or H. polymorpha. This needs to be better clarified throughout the manuscript.

Reply: We agree and apologize. We have better clarified this.

Reviewer #2 (Comments to the Authors (Required)):

In S. cerevisiae, the proteins Inp1 and Pex3 form a tether that links peroxisomes to the ER and plays a critical role in peroxisome inheritance (Knoblach et al, EMBO 2013). This study convincingly shows the same proteins tether peroxisomes to the plasma membrane in H. polymorpha. It suggests that the Inp1-Pex3 tether plays a role in inheritance but does not provide evidence. However, even if it did, the study is only a modest conceptual advance on Knoblack et al. It would be substantially stronger if it provided insight into how tether formation is regulated.

Reply: We would like to thank the reviewer for the comments. We have significantly improved our manuscript, added several new experiments and included important new findings.
We have added more evidence that the Inp1 is essential for retention of peroxisomes in mother cells (new Fig. 3E). We show that in *H. polymorpha* Inp1 does not tether peroxisomes to the ER. Also, we show that in the absence of Inp1, the peroxisome-ER contact is not sufficient for retention of peroxisomes in the mother cells (new Fig. 5). Importantly, we show that Inp1 has a PH-like domain, which is essential for binding to the plasma membrane. Moreover, we show that disruption of the actin cytoskeleton affects PM localisation of Inp1.

Reviewer #3 (Comments to the Authors (Required)):

General comments:
I would suggest significant further experiments to more extensively characterise this putative 'tether' on the molecular level and to support a physiological role for PO-PM contacts (e.g. is PO retention/inheritance disrupted in strains with reduced/enhanced PO-PM contacts?).

Reply: We thank the reviewer for the very constructive report and the suggestions to improve our manuscript. We have substantially revised the manuscript and characterized the tether on the molecular level. Moreover, we included data on the function of Inp1 in peroxisome inheritance (new Fig. 3).

Specific comments:
Introduction:
• A brief introduction to POs might be helpful.
• The description of ER-PO contacts in S. cerevisiae is a little confusing - one paragraph says they are mediated by Inp1-Pex3, another mentions Pex30 and Pex31? This should be clarified.

• The authors may want to add a clearly explained hypothesis (e.g. providing more of a rationale).

Reply: We have improved the introduction of the manuscript as suggested.

Results and discussion:
• The authors make the statement '...in budding cells generally POs contain a second, relatively small spot of Pex3-GFP fluorescence...'. Can this be characterised more robustly? For example, is this only seen in actively budding cells, and in what proportion of cells? It might also be necessary to define what is considered to be a 'patch' of Pex3, since Pex3 is detected around the entire PO and not just in distinct puncta.

Reply: This information was presented in our previous publication on peroxisome-vacuole contact sites (Wu et al., BBA-MCR 2019). We have clarified this in the introduction section. In the published paper we show that approximately 30 % of the peroxisomes contain a second Pex3-GFP patch at the experimental conditions used. A patch was defined as a region where the signal was 50% higher compared to the lowest signal measured on the same peroxisome.

• The authors state that '...these findings are consistent with the view that the peripheral Pex3 spot is involved in Inp1-dependent PO retention in mother cells...', based on Figure 1. While it is true that these findings might be consistent, this is far too speculative at this stage and should be removed. The later interpretation of the data from Figures 1 and 2 suggesting '...POs form contacts with the PM, to which Inp1 and Pex3 localise...' is more appropriate, and actually very convincing from the data presented.
Reply: We agree and removed the sentence.

• The authors observe that, in inp1 deletion cells, the percentage of cells containing a peripheral Pex3-GFP patch dropped from 80% to 15%. Can the authors determine what the remaining 15% of patches correspond to? Are they PO-ER contacts, or with another organelle? This could be checked by colocalisation with organelle markers or CLEM.

Reply: We were unable to determine what the remaining patches correspond to. The level of fluorescence is too low for CLEM.

• The result that INP1 deletion does not affect PO-ER contacts is interesting, because this seems to be different from the observations in S. cerevisiae - the authors should discuss this disparity. Does this mean that Inp1-Pex3 does not regulate PO-ER contacts in H. polymorpha, or that a currently unknown component can compensate following Inp1 deletion?

Reply: We have added the new figure 5, in which we compare peroxisome-ER and peroxisome-plasma membrane contacts. This shows that the Inp1-dependent contact with the plasma membrane is crucial for peroxisome retention in mother cells.

• The authors observe that 'In Inp1+++ cells Inp1-GFP and Pex3-mKate2 co-localized to an elongated patch at the cell periphery (Fig. 3E). At the same time, the intensity of the Pex3-GFP patch at VAPCONS decreased, suggesting that bulk of the peroxisomal Pex3 protein was recruited to the peroxisome-PM contacts'. Only one example is shown - is this typical? Is total Pex3 expression the same in Inp1+++ cells and WT cells? This would be important for the conclusion that Pex3 is only redistributed upon Inp1 overexpression. Furthermore, DsRed-SKL staining appears to be at the PO membrane - should this not label the Po lumen?

Reply: We have quantified the peripheral Pex3-GFP patch in Inp1 overproduction cells and were unable to identify this in any of the analysed cells (2 x 20 cells from two independent cultures were analysed). This is included in the manuscript. We also included a Pex3 blot (new Fig. 2G), which shows a slight increase in Pex3 levels. DsRed-SKL is not in the peroxisome

lumen because in methanol-grown *H. polymorpha* the peroxisomal lumen contains an alcohol oxidase crystalloid. We have added this information in the legend.

• The authors should show that Inp1 deletion/overexpression alter PO retention or inheritance during budding. This would be important to support some of the later conclusions about the function of PO-PM contacts.

Reply: We fully agree and included this information (New Fig. 3 E).

• Figure 3 - the WT example of Inp1 and Pex3 localisation in Figure 3E seems to show considerably less colocalisation than previously seen - is this a consequence of the growth in glycerol/methanol media? If so, the rationale for using this media should be more clearly explained. If not, a better example should be found.

Reply: Inp1 overproduction affects peroxisome biogenesis. Therefore, cells are unable to grow on methanol as sole carbon source. We therefore add glycerol as a second carbon source (which does not require functional peroxisomes). We added this information in the legend.

• Similarly to before, whilst the statement 'Our data are consistent with the view that Pex3-bound Inp1 connects peroxisomes to the PM' is technically true, this seems a bit misleading as a direct Pex3-Inp1 interaction at PO-PM contacts is not shown here. Perhaps 'a Pex3-Inp1 complex connects PO to the PM' might be more reasonable?

Reply: We agree and removed this statement.

• The authors observe that Inp1-GFP localises to the cell periphery in the absence of Pex3 - the authors should confirm that this represents the PM and not, for example, cortical ER (e.g. colocalisation with a PM marker, ER control, and/or immuno-EM).

Reply: To our opinion the fluorescence pattern is not consistent with ER localization. Co-localization studies with fluorescence microscopy do not allow discriminating between plasma membrane and cortical ER because of the limited resolution of light microscopy. We now added the new experiment shown in Fig. 4B, which shows that the peripheral localisation is lost upon treatment with LatA. This is not consistent with ER localization.

• In line with this, N-terminal portions of Inp1 appear to localise to different locations, potentially unspecific due to positively charged residues. The manuscript would benefit from a more thorough molecular analysis of the N-terminal and C-terminal regions mediating (specific) PM affinity and Pex3 binding. For example, can a membrane-bound fusion protein containing part of the Inp1 N-terminus associate PO (or other organelles) with the PM; are the positively charged residues required for this?; is the N-terminus of Inp1 interacting directly with lipids of the PM? What is the molecular mechanism?

Reply: We have included detailed analysis of truncated and mutated proteins (New Fig. 3 D,E). This shows that the middle domain is responsible for associating peroxisomes to the plasma membrane. Also, the conserved positive charges are not essential for Inp1 function. Most likely they contribute to the regulation of Inp1 levels.

• The authors suggest that '...The presence of cytosolic Inp1-GFP [in Pex3 deletion cells] may be related to Inp1-GFP overproduction...'. Could another possibility be that a subpopulation of Inp1 normally bridges Pex3 at the ER and POs, but becomes cytosolic in the absence of Pex3? The authors may wish to discuss this. Similarly, I disagree with the conclusion that 'Our data do not support the view that Inp1 functions as a molecular hinge by binding to ER- and peroxisome localized Pex3, because according to this model Inp1 would become fully cytosolic in the

absence of Pex3' - my interpretation is that this data only excludes the possibility that the sole function of Inp1 is bridging ER- and PO-localised Pex3.

Reply: We agree that our statements were too strong and have removed them from the text.

• Figure 5B: What happens to Pex3 localisation when Inp1 truncations are expressed? Do you lose the peripheral Pex3 patch if Inp1 is not localised to the cell periphery?

Reply: We agree that this is an interesting point. However, given the large amount of extra data already presented in this Short Report, we feel that this is outside the scope of the current study.

Concluding remarks:
• The concluding remarks seem particularly speculative. These should be tempered considerably as 'discussion' and, if based on the literature, should be better referenced.
• References to 'VAPCONS' should be 'EPCONS'?
• 'The VAPCON (EPCON?) is apparently not sufficiently strong to retain peroxisomes in the mother cell of INP1 deletion mutants, because in such mutants all peroxisomes are transported to the newly formed buds.' Is this based on the literature? Certainly no data is presented to show this. If so, it would be important to mention this more extensively in the introduction. References should also be provided.
• The final model suggested: 'Upon Dnm1 dependent asymmetric fission, the original peroxisome remains associated to the PM (and possibly the ER), whereas the newly formed organelle is transported to the newly formed bud, a process that requires Inp2, Myo2 and the actin cytoskeleton. Finally, upon reaching the new bud, the peroxisome detaches from Myo2 and becomes anchored to the PM of the bud via Inp1' seems plausible, but should be presented more clearly as just a hypothesis, since the data presented do not address this. References (if available) would make the case more convincing. The authors could consider adding a schematic of this proposed model to make it clearer.

Reply: We fully agree with the reviewer and have significantly shortened our concluding remarks. We have added a model (new Fig. 5C) as requested.

• Statistical analysis is not presented in all cases and should be added if appropriate. All data seems to be collected from two independent experiments?

Reply: We have added statistical analyses if appropriate. Indeed, all data are collected from two independent experiments.

May 20, 2020

Re: JCB manuscript #201906023R-A

Prof. Ida J van der Klei
University of Groningen
Molecular Cell Biology
Groningen Biomolecular Sciences and Biotechnology Institute
P.O. Box 11103
Groningen 9700 CC
Netherlands

Dear Prof. van der Klei,

Thank you for submitting your revised manuscript entitled "Peroxisome retention involves Inp1 dependent peroxisome-plasma membrane contact sites in yeast" and and for your patience as the editorial process was delayed by staff shortage and the current pandemic. The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

All of the points raised points of Reviewer #1 and #3 are valid and overlapping. Specifically, the need to bolster the evidence that the modified PH domain is responsible for PM interaction. As pointed out by Reviewer #3, data in Figure S2 do not fully support this model. We feel that this point could be addressed by better or more quantification of existing data. In addition, the text should be amended to more accurately reflect the conclusions that can be drawn, where pointed out by the reviewers, and to improve the clarity as suggested by Reviewer #1.

Please also attend to the following formatting requests to expedite production:

- Provide the main and supplementary texts as separate, editable .doc or .docx files
- Provide main and supplementary figures as separate, editable files according to the instructions for authors on JCB's website *paying particular attention to the guidelines for preparing images and blots at sufficient resolution for screening and production*
- Format references for JCB
- Display data for individual samples where appropriate and clearly state the sample size / replicates (see Rev#1, point #2c)
- Provide tables as excel files
- Check font size in Fig S2 meets our formatting guidelines
- Add paragraph after the Materials and Methods section briefly summarizing all "Online Supplementary Materials" - i.e. use figure titles, current version is too brief.
- Move supplementary references to main text and cite where appropriate

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision within one month, along with a cover letter that includes a point by point response to the remaining reviewer comments. Please let us know if additional time may be required as a result of lab closure due to the pandemic.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have made significant changes to the manuscript and in doing so have strengthened it. The finding that Inp1 functions to tether peroxisomes to the plasma membrane is interesting and challenges the current idea that Inp1 is a peroxisome-ER tether. That being said, there are still some points that need to be addressed to make the manuscript suitable for publication.

Comments:

1. The authors have done additional work to further narrow down the peroxisome and PM interacting domains. However, there are still some points that need to be addressed.

a. From the Inp1 deletion experiments, it is clear that amino acids 217-405 are sufficient for association of Inp1 with peroxisomes. However, data that demonstrate that the MHD (PH-like domain) is responsible for the association with the PM are not shown. The authors show that the MHD is required for peroxisome retention but not for the association of peroxisomes with the plasma membrane. The authors should quantify the fraction of peroxisomes that are cortical in cells expressing the Inp1 deletion constructs to show that the two activities are correlated.

b. The conclusions made from the Inp1mut data are overstated. While the steady-stead level of Inp1mut is increased compared to WT, the authors try to suggest that the mutated lysine residues may be ubiquitinated in the wild type protein. While this is one possibility, many other possibilities exist that could explain the elevated levels of the protein.

c. Also, the entire section could benefit from being reworked for better clarity.

2. Robust quantification is lacking for many of the figures in the manuscript.

a. Quantification of the colocalization of Inp1 and Pex3 patches for the data shown in Fig. 1A

should be included.

b. Figure 4 lacks quantification.

c. For many experiments, the quantification shown including error represent data from two independent experiments. Typically, three independent experiments are quantified. I leave it to the journal to decide if two independent experiments are sufficient. If so, I would recommend showing the individual data points for each independent experiment in addition to the mean.

3. The authors perform additional experiments to better understand the molecular basis of the Inp1-plasma membrane interaction. However, the conclusions made are overstated. For example, the statement in the abstract that "the pleckstrin homology-like domain in Inp1 binds a yet unknown plasma membrane protein that is a component of the actin cytoskeleton" is overstated based on the data shown. The conclusion that can be made from the LatA results is that Inp1 localization is dependent on the presence of an intact actin cytoskeleton.

4. The figures would benefit from better labeling, especially Figure 3. Fig. 3B needs better labeling within the figure itself. Perhaps add a legend to the figure panel so it is more obvious what the green, magenta, etc. refer to. Labels for the N- and C-termini or the amino acid numbers need to be added to the structure shown in Fig. 3C.

5. For consistency, the authors should refer to the cortical accumulations of Pex3-GFP as patches. They go back and forth between patches and spots and patches is a more accurate description. In contrast to Inp1, which is found in discrete spots, Pex3 is found all along the peroxisomal membrane as well as in accumulations at peroxisome-vacuole contacts and peroxisome-PM contacts.

6. The statement "These contacts disappeared in the absence of Pex32, whereas associations with the PM were unaffected in these cells (Fig. 2 CD)." near the top of page 5 is not accurate. While the difference between the peroxisome-PM contacts in the pex32 mutant and wild type cells is not statistically significant, the mean in the pex32 mutant is clearly lower.

7. There are arrows in Fig 1A but the legend does not describe what these arrows are pointing to.

Reviewer #2 (Comments to the Authors (Required)):

This study has been been improved. My only suggestion is that the legend for Fig 4A should explain what is shown in each panel, as in the text.

Reviewer #3 (Comments to the Authors (Required)):

The authors have improved their manuscript. They have substantially revised the manuscript and performed additional experiments to characterize the tether and its physiological function. They have also included data on the function of Inp1 in peroxisome inheritance. Overall, the data convincingly demonstrate that Inp1 functions as a peroxisome-plasma membrane (PM) tether which is important for peroxisome retention and inheritance though the molecular details remain unclear. The study describes peroxisome-plasma membrane contact sites in yeast for the first time and challenges the existing model that peroxisome-ER contacts are essential for peroxisome inheritance.

A few concerns need to be addressed:

The authors claim that "An internal domain in Inp1 is responsible for association to the PM". However, this is only based on data showing the localisation of Inp1 mutants to peroxisomes (restricted to those cases where fluorescence was strong enough to be detected), not the PM, and using peroxisome retention as a readout. As a result, their claim that "the central, conserved domain is a Pleckstrin Homology-like (PH-like) domain, which is essential for association to the plasma membrane" is not necessarily valid from the data shown.

Fig. 3 - Since overexpression/deletion of WT Inp1 affects peroxisome retention, the results for peroxisome inheritance may be confounded by the different expression levels of the different mutants.

The authors state: "The protein levels of Inp1MUT are strongly increased compared to the WT control (Fig. 3F; note that 100 x less protein is loaded for Inp1++). This suggests that the positive charges are important for the stability of the protein." This is true, but they must also have some impact on function that is not addressed, otherwise this mutant would be expected to have the same phenotype as overexpression of the WT (which is not the case, and it also only partially rescues Inp1 deletion). The claim that the conserved positive charges are not essential for Inp1 function is therefore misleading.

Figure S2: When overexpressed, the MHD domain (containing PH-like) alone appears to be cytosolic, and does not localise to the plasma membrane. However, overexpression of the 1-99 mutant (lacking the supposed PH-like domain) does appear to localise to the PM. This does not necessarily fit into the model proposed for Inp1-plasma membrane interaction.

The interaction of Inp1 (FL and mutants) with Pex3 (peroxisomes) was not investigated, e.g. by pulldown experiments.

Fig. 4B. The latrunculin A experiment needs more controls - does the disruption of the actin cytoskeleton prevent any other plasma membrane-associated proteins from localising to the plasma membrane? It would be useful to know if this is specific to Inp1 or if the actin skeleton is just required for plasma membrane function in general (e.g. PI lipid content). If so, is this via the PH-like domain? Does a mutant lacking this domain resemble the latrunculin-treated condition in pex3Δ strains? Phenotypes are not especially clear in the images and should be quantified.

Dear Dr. Nunnari,

Thank you very much for the positive and constructive comments to our paper. We are very happy that you invited us to submit a revised version. Unfortunately we have very little access to the laboratory because of the Corona crisis. However, we were able to perform a few small experiments and in addition could include additional data that was already available. We feel that the changes made improve the quality of the paper.

Below you find our point-to-point response to the comments of the reviewers.

We sincerely hope that our manuscript is acceptable for publication.

With best regards,

Ida van der Klei

Rebuttal:

Reviewer #1
Comments:

1. The authors have done additional work to further narrow down the peroxisome and PM interacting domains. However, there are still some points that need to be addressed.

a. From the Inp1 deletion experiments, it is clear that amino acids 217-405 are sufficient for association of Inp1 with peroxisomes. However, data that demonstrate that the MHD (PH-like domain) is responsible for the association with the PM are not shown. The authors show that the MHD is required for peroxisome retention but not for the association of peroxisomes with the plasma membrane. The authors should quantify the fraction of peroxisomes that are cortical in cells expressing the Inp1 deletion constructs to show that the two activities are correlated.
Reply: We agree with the reviewer that we did not proof that the MHD is required for association to the PM. However, the suggested experiment is not feasible because the cortical localization is not lost in the absence of Inp1, because of Pex32 dependent association of peroxisomes to the peripheral ER (Wu, F. et al., 2020 https://doi.org/10.1101/2020.03.05.977884). Instead of the requested experiment, we quantified the distance between the peroxisomal membrane and the PM in strains producing $Inp1_{217-405}$ *and* $Inp1_{100-405}$ *by EM (new Fig. 4 AB). This shows that the tight association between peroxisome and PM is lost upon removing the MHD (residues100-216), supporting the conclusion that the MHD is important for PM binding.*

b. The conclusions made from the Inp1mut data are overstated. While the steady-

stead level of Inp1mut is increased compared to WT, the authors try to suggest that the mutated lysine residues may be ubiquitinated in the wild type protein. While this is one possibility, many other possibilities exist that could explain the elevated levels of the protein.
Reply: the reviewer is correct. We have removed this statement.

c. Also, the entire section could benefit from being reworked for better clarity.
Reply: We have rewritten this section and hope it is more clear now.

2. Robust quantification is lacking for many of the figures in the manuscript.
a. Quantification of the colocalization of Inp1 and Pex3 patches for the data shown in Fig. 1A should be included.
Reply: We have added the quantification in the results section.

b. Figure 4 lacks quantification.
Reply: We quantified the data of Fig. 4F and added this in the results section.

c. For many experiments, the quantification shown including error represent data from two independent experiments. Typically, three independent experiments are quantified. I leave it to the journal to decide if two independent experiments are sufficient. If so, I would recommend showing the individual data points for each independent experiment in addition to the mean.
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 current limited access to the laboratory. We thank the reviewer for pointing this out and will make 3 biological replicates in future studies.
We have changed Figs 2 D,I and 4 B, which now include all individual data points.

3. The authors perform additional experiments to better understand the molecular basis of the Inp1-plasma membrane interaction. However, the conclusions made are overstated. For example, the statement in the abstract that "the pleckstrin homology-like domain in Inp1 binds a yet unknown plasma membrane protein that is a component of the actin cytoskeleton" is overstated based on the data shown. The conclusion that can be made from the LatA results is that Inp1 localization is dependent on the presence of an intact actin cytoskeleton.
Reply: We agree with the reviewer and have adapted the statement as suggested.

4. The figures would benefit from better labeling, especially Figure 3. Fig. 3B needs better labeling within the figure itself. Perhaps add a legend to the figure panel so it is more obvious what the green, magenta, etc. refer to. Labels for the N- and C-termini or the amino acid numbers need to be added to the structure shown in Fig. 3C.
Reply: We thank the reviewer for these suggestions and adapted the figure accordingly.

5. For consistency, the authors should refer to the cortical accumulations of Pex3-GFP as patches. They go back and forth between patches and spots and patches is a more accurate description. In contrast to Inp1, which is found in discrete spots, Pex3 is found all along the peroxisomal membrane as well as in accumulations at peroxisome-vacuole contacts and peroxisome-PM contacts.

Reply: We have made the suggested changes throughout the text.

6. The statement "These contacts disappeared in the absence of Pex32, whereas associations with the PM were unaffected in these cells (Fig. 2 CD)." near the top of page 5 is not accurate. While the difference between the peroxisome-PM contacts in the pex32 mutant and wild type cells is not statistically significant, the mean in the pex32 mutant is clearly lower.
Reply: We agree that in the presented figure, the mean was lower. We now show all individual data points (new figure 2D). From this it is clear that in WT and pex32 cells the average distance between peroxisomes and PM is similar (approx. 28 nm), whereas this is almost 100 nm in inp1 cells.

7. There are arrows in Fig 1A but the legend does not describe what these arrows are pointing to.
Reply: *We have added this information in the legend.*

Reviewer #2 (Comments to the Authors (Required)):

This study has been improved. My only suggestion is that the legend for Fig 4A should explain what is shown in each panel, as in the text.
Reply: We have added the explanation in the legend as requested.

Reviewer #3:

1. The authors claim that "An internal domain in Inp1 is responsible for association to the PM". However, this is only based on data showing the localisation of Inp1 mutants to peroxisomes (restricted to those cases where fluorescence was strong enough to be detected), not the PM, and using peroxisome retention as a readout. As a result, their claim that "the central, conserved domain is a Pleckstrin Homology-like (PH-like) domain, which is essential for association to the plasma membrane" is not necessarily valid from the data shown.
Reply: *We agree with the reviewer that we did not proof that the MHD is required for association to the PM. We quantified the distance between the peroxisomal membrane and the PM in strains producing* $Inp1_{217\text{-}405}$ *and* $Inp1_{100\text{-}405}$ *by EM (new Fig. 4 AB). This shows that the tight association between peroxisome and PM is lost upon removing the MHD (residues100-216), supporting the conclusion that the MHD is important for PM binding.*

Fig. 3 - Since overexpression/deletion of WT Inp1 affects peroxisome retention, the results for peroxisome inheritance may be confounded by the different expression levels of the different mutants.
Reply: This is a good point. For the truncated proteins we studied it is unlikely that the changes in protein levels are responsible for the phenotypes. For instance, the protein level of protein 217-405 is strongly enhanced, but its function in peroxisome retention is lost. Conversely, the protein level of truncation 100-405 is not detectable by Western blotting (like the WT control), still it is largely functional. We have added this information in the results section.

The authors state: "The protein levels of Inp1MUT are strongly increased compared to the WT control (Fig. 3F; note that 100 x less protein is loaded for Inp1++). This suggests that the positive charges are important for the stability of the protein." This is true, but they must also have some impact on function that is not addressed, otherwise this mutant would be expected to have the same phenotype as overexpression of the WT (which is not the case, and it also only partially rescues Inp1 deletion). The claim that the conserved positive charges are not essential for Inp1 function is therefore misleading.
Reply: We agree that it is better to compare the phenotype of this mutant with the overproduction strain. Indeed, this indicates that the mutations do have an effect on the function of the protein. We have added this information in the text.

Figure S2: When overexpressed, the MHD domain (containing PH-like) alone appears to be cytosolic, and does not localise to the plasma membrane. However, overexpression of the 1-99 mutant (lacking the supposed PH-like domain) does appear to localise to the PM. This does not necessarily fit into the model proposed for Inp1-plasma membrane interaction.

Reply: Indeed, our data indicate that both the MHD and the positive charged residues in 1-99 contribute to PM binding. We have adapted this throughout the manuscript.

The interaction of Inp1 (FL and mutants) with Pex3 (peroxisomes) was not investigated, e.g. by pulldown experiments.
Reply: Indeed we did not do that. However, we added the new figure 4 CD, which strongly suggests that Inp1 binds to Pex3, like established for S. cerevisiae.

Fig. 4B. The latrunculin A experiment needs more controls - does the disruption of the actin cytoskeleton prevent any other plasma membrane-associated proteins from localising to the plasma membrane? It would be useful to know if this is specific to Inp1 or if the actin skeleton is just required for plasma membrane function in general (e.g. PI lipid content). If so, is this via the PH-like domain? Does a mutant lacking this domain resemble the latrunculin-treated condition in pex3Δ strains? Phenotypes are not especially clear in the images and should be quantified.
Reply: In the literature we could not find an example of a yeast protein, whose PM localization depends on the actin cytoskeleton. We agree with the reviewer that several questions remain. Given the very limited access to the laboratory because of the Corona crisis, we are unable to address these questions experimentally. We have added available data on the localization of construct 216-405 in inp1 pex3 cells (in figure S3). Also, we have quantified the data of the latrunculin A experiment and added this information in the text.