THE EMBO JOURNAL

Manuscript EMBO-2013-84987

An ER-peroxisome tether exerts peroxisome population control in yeast

Barbara Knoblach, Xuejun Sun, Nicolas Coquelle, Andrei Fagarasanu, Richard L. Poirier and Richard A. Rachubinski

Corresponding author: Richard Rachubinski, University of Alberta

Review timeline:

Submission date:	06 March 2013
Editorial Decision:	03 April 2013
Revision received:	28 June 2013
Editorial Decision:	02 July 2013
Accepted:	02 July 2013

Transaction Report:

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

1st Editorial Decision 03 April 2013

Thank you for submitting your manuscript entitled 'An ER-peroxisome tether exerts peroxisome population control in yeast' to us. Again, I am sorry for the delay in getting back to you, but I have now finally received the third review on your work.

As you will see, all three referees find your study very interesting, well performed and suitable for publication at The EMBO Journal. They suggest a few amendments that would improve the paper. Notably, referee #2 suggests to test Pex3 mutants associated with human disorders for a possible problem with Pex3-Inp1 interaction. I am in agreement with the referee on this point and find that such an analysis (of course depending on the result) would nicely extend the broader significance of your work. I am happy to discuss this issue further if needed.

Given the comments provided, I would like to invite you to submit a revised version of the manuscript, addressing the concerns of the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

Peroxisomes are single membrane bound organelles with high number of diverse functions adapted to cellular need and environmental changes. The number of these organelles per cell is regulated by either de novo synthesis or division of existing peroxisomes. In yeast, however, the main source of peroxisomes is the division of present ones, while the de novo synthesis seems to displays a rescue system. Within the cell division process, it has to be ensured that the mother cells and the bud both contain peroxisomes when the division is completed. Therefore, a fraction of the peroxisomes is transportes to daughter cells via the actin cables, mediated by peroxisome specific adaptor Inp2p and driven by the myosin motor protein Myo2p. In addition, some peroxisomes are retained in the mother cell by the action of a Pex3p/Inp1p complex. The detail of how the retention of peroxisomes in mother cell takes place remains still unclear and is addressed by the manuscript of Knoblach et al. The authors analyzed the Pex3p/Inp1p-interaction and based on their data they draw a model of Inp1p bridging the ER and peroxisomes. Due to an asymmetric division of this organelle, an Inp1p deficient part segregates to the bud, whereas the other part containing Inp1p remains in the mother cell.
The data provided by the authors are without exception of high quality and the topic is of interest not only for specialists in the field. The manuscript is well written and the data presentation is excellent. A few comments need to be addressed.

Major comments
Figure 2A: The pattern of Inp1/Pex3p in wild-type and pex19Δ is similar and thus gives the impression of an identical localization of both proteins. However, it is more likely that the visible foci are peroxisomes (wild-type) or specialized regions of the ER (pex19Δ). To make this more clearly, it would be necessary to show co-localization with marker of both organelles. Moreover, co-localization studies are also important as it is assumed that formation of the complex takes part at the ER (page 7).
To this reviewer, the order of events is not suffieciently clear. Please clarify. Inp1p is localized to vesicles/ER even when Pex3p is missing. The pattern changes upon deletion of Pex19p, which indicates that Pex3p recruits Inp1p. The question is what anchors Inp1p to the membrane? Moreover, when Inp1p binds Pex3p in the ER and in addition retains peroxisomes via peroxisomal Pex3p, Inp1p has to form either a dimer or displays at least two different interactions-sites for Pex3p. Please comment.

Minor comments
Figure 2A/legend: It is mentioned that the cells displayed all express Pex3p-mCherry. In this case, one would expect a labeling also in pex3Δ cells. Please comment.
Figure 2B: The signals for Inp1p and Pex3p in the PNS are lower than in the 20kgP fractions. Shouldn't the sum of the single fractions ad to the amount of the PNS? Please clarify. Why is the soluble marker protein present in the 200kgP fractions?
Figure 2C: Detection of G6PDH in pex3Δ and pex3-V81E would be of interest as it also indicated the load fractions of the gradient.
Figure 3A: Please add a molecular weight marker.
Figure 3B: As this reviewer understands, the Inp1p-HA and the Tom70p-Inp1p-HA fusions were separately expressed in yeast. Thus, the gradients shown seem to belong to two different preparations. What about the immunoblots for thiolase and Tom70p, which preparation has been used? Please label more precisely. Moreover, Pex3p detection would support the statement headings of the figure legend.
Figure 3C: Arrows are hard to see.
Figure 4: The lower band in the anti-GST blot most likely represents a degradation product of the fusion proteins or GST alone. Why is the so much GST in these eluate fractions? It could not be explained by dimerization as the ration between GST and GST-fusion is not 1. Please comment in the text. Moreover, binding capacity should be quantified.
It is known that interactions not seen by two-hybrid assays are still present and could be visualized in an in vitro pull-down. However, it is still surprising that the direct interaction is not affected when

Pex3pV81E is used. At this point, the yeast phenotype is hard to explain. When interaction is possible, why is there no retention of peroxisomes visible?
Figure 7B: The GFP labeling is hard to see.

Referee #2

The manuscript of Knoblach et al. presents new interesting data on the mechanisms underlying peroxisome population control in yeast. In particular, the authors identified the molecular nature of peroxisomes-ER tethers in Saccharomices cerevisiae: Pex3p and Inp1p, the latter acting as a bridge that is able, through its N and C terminus, to bind Pex3p molecules located in different subcellular compartments. Their conclusions are supported by the following evidence:
- The overexpression of six pex3 mutant alleles in the pex3D strain, previously reported to be defective in peroxisome biogenesis: only pex3-V81E did not rescue the phenotype of the pex3D strain. This was due to lack of interaction between Pex3p and Inp1p, as assessed by yeast two-hybrid experiments.
- The colocalization of Inp1p with structures at the cell periphery containing Pex3p (but not with the bud-localized punctae where pex3-V81E was detected) further suggests that the two interact.
- The analysis of subcellular fractions showed that in pex3-V81E cells Inp1p and Pex3p segregate into different membrane compartments.
- The interaction of Inp1p and Pex3p in trans was confirmed by targeting Inp1p to the mitochondrial surface: in cells expressing mito-Inp1p, peroxisomes preferentially attach to mitochondria rather than to the cell cortex.
- Both N and C terminus of Inp1p bind Pex3p independently, suggesting that Inp1p could bind Pex3p molecules residing in different membranes. They successfully verified this idea by fluorescence complementation experiments in cells expressing the Pex3p: Pex3p-V81E and Pex3p-W128L mutants.
- Finally, photoactivation experiments highlighted that division of peroxisomes prevails on their de novo formation, peroxisomes transport thus likely being a key component of their transfer to the bud.

The manuscript is well written and the conclusions are supported by strong experimental evidence.

I have only minor suggestions that could further improve the study.
1) In the first paragraph of the results, the authors claim that, compared to the Pex3p-V81E mutant, "other pex3 mutant alleles... never lost their entire peroxisome population to the bud". Could the authors provide a more quantitative analysis, e.g. calculate the percentage of peroxisomes that are retained in the mother cell in presence of the different mutants?
2) Several mutations in Pex3p associated to human disorders have already been described, including the D347Y mutation (Matsui et al, 2012) or mutations leading to C-terminal truncation (Muntau et al 2000). The relevance of this story for human health would be higher if the authors tested how mutants in homologous residues of S. cerevisiae Pex3p (as the authors described for the residue W128) impair Pex3p function. One wonders if the phenotype associated to these mutations can be due for example to a defect in the interaction with Inp1p.

Referee #3

In this manuscript, the authors present data indicating that Inp1 functions as a tether that anchors peroxisomes via the cortical ER in the mother cell and acts in an antagonistic manner with Inp2, which facilitates the transport of peroxisomes into daughter cells. In general, the experiments are high quality and represent a significant advance in the field. The weakest part of the manuscript is support for the authors' molecular model of the Inp1 tether, which they suggest acts as a linker to facilitate a trans Pex3-Inp1-Pex3 interaction between the ER and peroxisomes.

Comments:

What membrane compartments do Inp1 and localize to in the absence of Pex3 and with Pex3-V81E expression. Markers would be appropriate in both biochemical and cell based experiments. Does Inp1 localize to subdomains of the ER, for example ER exit sites?

The use of Gal1/10 driven peroxisomal and mitochondrial reporters is not ideal. Why have the authors chosen these instead of appropriate constitutive promoters? Does changing carbon source affect the outcome of the experiment?

In cells expressing Inp1, there is a portion of peroxisomes that in in persistent proximity to mitochondria. Can the authors comment on the potential significance of this result?

Data supporting the model that Inp1 bridges ER bound Pex3 with peroxisomal Pex3 is indirect. Do the authors detect an Inp1/Pex3 complex with a stoichiometry consistent with this model? Also, examining the localization of the ER relative to Inp1 foci /peroxisome would be helpful, especially in a background where the surface area of cortical ER is reduced (reticulon deletions for example). In this situation, does Inp1 remain co-localized with the ER?

Is the ER associated with Inp1/Inp2foci in the large peroxisomes observed in the vps1/dnm1 mutant?

What is the peroxisomal distribution in an inp1/inp2/vps1/dnm1 mutant?

Minor comments:

The organization of the manuscript could be better. For example, Figure 7 could be presented early to form the functional rationale for the study.

In many of the cell image panels, it would help the reader to have a zoomed in version of a relevant portion of the cell in the figure.

Additional correspondence (author) 8 April 2013

Thank you for providing us with the reviews. We will be happy to address the reviewers' comments on a point-by-point basis in the revised manuscript.

Today we are writing to you regarding the suggestion of Reviewer 2 to analyze previously published mutations in human Pex3p for defects in interaction with Inp1p. Obviously, we could generate mutations in the homologous residues of yeast Pex3p and perform protein-protein interaction studies with Inp1p; however, we believe that these experiments would not enhance the novel findings presented in our manuscript.

First, active retention of organelles in the mother cell and transport of organelles to the daughter cell is observed only in cell types that divide asymmetrically, such as budding yeasts. Inp1p homologues are restricted to these organisms.

Second, human cells, in contrast, divide by median fission, and the organelles are allocated stochastically between the two resulting daughter cells at cytokinesis. As humans do not contain an Inp1p homologue, we have every reason to expect that any mutation in human Pex3p that leads to a disease phenotype would likely affect only peroxisome biogenesis (such as the ability of Pex3p to interact with Pex19p) and not peroxisome segregation.

Please let us know how you would like us to proceed in light of this information.

Additional correspondence (editor) 9 April 2013

Thank you very much for your note and for the information about Inp1p. In light of this it is of course not necessary to test the proposed mutants, but to simply respond to the referee's point in your letter giving the same information as you have given to me.

1st Revision - authors' response 28 June 2013

Answers to Reviewers' comments, EMBO Journal
Reviewer 1

Figure 2A: The pattern of Inp1/Pex3p in wild-type and pex19Δ is similar and thus gives the impression of an identical localization of both proteins. However, it is more likely that the visible foci are peroxisomes (wild-type) or specialized regions of the ER (pex19Δ). To make this more clearly, it would be necessary to show co-localization with marker of both organelles. Moreover, co-localization studies are also important as it is assumed that formation of the complex takes part at the ER (page 7).

To differentiate between wild-type and *pex19D* cells, we now co-localize Inp1p-GFP with peroxisomal and ER-markers in cells expressing wild-type Pex3p (revised Figure 2A). In wild-type cells, the peroxisomal matrix protein mCherry-PTS1 co-localizes with Inp1p-GFP, thus demonstrating that Inp1p associates with peroxisomes. In the *pex19D* mutant, Inp1p-GFP forms foci, but these foci do not correspond to functional peroxisomes, as is evident by the cytosolic localization of mCherry-PTS1. The Inp1p-GFP foci are in close proximity to the cortical ER marker Rtn1p-mCherry, but are non-overlapping with the ER-exit site marker Sec13p-mCherry (related question, Reviewer 3). These data support our findings that Inp1p enriches at the ER-peroxisome interface in wild-type cells and in pre-peroxisomal subdomains of the ER in *pex19D* cells.
To this reviewer, the order of events is not suffieciently clear. Please clarify. Inp1p is localized to vesicles/ER even when Pex3p is missing. The pattern changes upon deletion of Pex19p, which indicates that Pex3p recruits Inp1p. The question is what anchors Inp1p to the membrane?

Figure 2A shows that Inp1p is recruited to structures containing wild-type Pex3p both in the presence and absence of Pex19p, i.e. regardless of whether cells contain functional peroxisomes or not. In *pex3D* or *pex3-V81E* mutants, Inp1p takes on a diffuse localization throughout the cell, which partially overlaps with Pex30p (revised Figure 2B). Our findings thus point to the following order of events:

1. Inp1p targets to a vesicular compartment in a Pex3p-independent manner. Work from our and other labs has previously shown that many peroxisomal membrane proteins traffic through the ER *en route* to peroxisomes or exhibit a dual ER/peroxisome localization. It is not known which factor(s) target Inp1p to the membrane.
2. Wild-type Pex3p recruits Inp1p to specific pre-peroxisomal subdomains of the ER (termed "foci") even in peroxisome-deficient cells.
3. In cells containing peroxisomes, these "foci" are the sites of attachment of peroxisomes with the ER (see also Figure 5C), as peroxisomes remain mobile when cortical foci do not assemble.

Moreover, when Inp1p binds Pex3p in the ER and in addition retains peroxisomes via peroxisomal Pex3p, Inp1p has to form either a dimer or displays at least two different interactions-sites for Pex3p. Please comment.

Inp1p does not appear to self-interact (Figure 1C, yeast 2-hybrid analysis). But Inp1p binds Pex3p both via its N-terminal and C-terminal domains (see revised Figure 4). Having two "arms" that can independently bind to Pex3p enables Inp1p to bridge two Pex3p molecules across membranes.

Figure 2A/legend: It is mentioned that the cells displayed all express Pex3p-mCherry. In this case, one would expect a labeling also in pex3Δ cells. Please comment.

Our mistake, we mislabeled this figure. In this cell the PEX3 gene is deleted and mCherry is not integrated at the PEX3 locus. We have corrected the figure in the revised manuscript.

Figure 2B: The signals for Inp1p and Pex3p in the PNS are lower than in the 20kgP fractions. Shouldn't the sum of the single fractions ad to the amount of the PNS? Please clarify. Why is the soluble marker protein present in the 200kgP fractions?

Total protein was adjusted to comparable concentrations for all fractions after centrifugation. The 20kgP and 200kgP fractions (which contain less protein) were re-suspended in a smaller volume than the PNS or supernatant fractions. A protein assay was performed, and the same amount of protein was loaded in each lane (please see legend to revised Figure 2C). Pex3p and Inp1p are thus enriched in the 20kgP fraction relative to the PNS per unit of protein. The presence of G6PDH in the 200kgP fraction could be caused by its association with larger structures such as vesicles and partial sedimentation at 200,000 x g. Note that G6PDH – unlike Inp1p in samples from pex3D and pex3-V81E cells – does not enrich in the 200kgP fraction.

Figure 2C: Detection of G6PDH in pex3Δ and pex3-V81E would be of interest as it also indicated the load fractions of the gradient.

Blots for G6PDH and Nyv1p have now been included for pex3D and pex3-V81E samples in revised Figure 2D.

Figure 3A: Please add a molecular weight marker.

Done.

Figure 3B: As this reviewer understands, the Inp1p-HA and the Tom70p-Inp1p-HA fusions were separately expressed in yeast. Thus, the gradients shown seem to belong to two different preparations. What about the immunoblots for thiolase and Tom70p, which preparation has been used? Please label more precisely. Moreover, Pex3p detection would support the statement headings of the figure legend.

Yes, the Inp1p-HA and Tom70p-Inp1p-HA blots belong to two different fractionations. We now include blots for thiolase and Tom70p for each of the gradients, as well as blots for Pex3p.

Figure 3C: Arrows are hard to see.

We have made them bigger.

Figure 4: The lower band in the anti-GST blot most likely represents a degradation product of the fusion proteins or GST alone. Why is the so much GST in these eluate fractions? It could not be explained by dimerization as the ration between GST and GST-fusion is not 1. Please comment in the text. Moreover, binding capacity should be quantified.

Lower bands in the anti-GST and anti-MBP blots are degradation products of GST-Pex3p and MBP-Inp1p, respectively. They have been labeled by arrows in the revised manuscript (see also legend to Figure 4A). Please note that the degradation products in the GST blot run at a higher molecular mass than GST alone, i.e. they appear to contain a portion of Pex3p. Both Pex3p and Inp1p are membrane proteins and prone to degradation. Inp1p contains a PEST motif in its C-terminal domain. Both full-length Inp1p and Inp1p-C are thus rapidly degraded, whereas Inp1p-N is stable (see revised Figure 4A). Binding of Inp1p and its domains to Pex3p is now quantified in new Figures 4B and 4C.

It is known that interactions not seen by two-hybrid assays are still present and could be visualized in an in vitro pull-down. However, it is still surprising that the direct interaction is not affected when Pex3pV81E is used. At this point, the yeast phenotype is hard to explain. When interaction is possible, why is there no retention of peroxisomes visible?

Our data point to a regulatory event that modulates the initial recruitment of Inp1p to Pex3p *in vivo*. This recruitment could be due to a conformational change in Pex3p leading to the exposure of the Inp1p-binding site. Alternatively, an accessory factor bound to the V81-region of Pex3p could be

required for the initial recruitment of Inp1p in cells. Our *in vitro* data clearly show that the V81-region on Pex3p does not act as a site for direct binding of Inp1p.

Figure 7B: The GFP labeling is hard to see.

We have increased the intensity of the GFP signal in the revised figure.

Reviewer 2

1) In the first paragraph of the results, the authors claim that, compared to the Pex3p-V81E mutant, "other pex3 mutant alleles... never lost their entire peroxisome population to the bud". Could the authors provide a more quantitative analysis, e.g. calculate the percentage of peroxisomes that are retained in the mother cell in presence of the different mutants?

For the peroxisome inheritance assay presented in Figure 1B of the original manuscript, we used a stringent all-or-none criterion to determine whether cells expressing a *pex3* mutant allele do or do not contain peroxisomes. More subtle differences in phenotypes emerge when total peroxisome counts in mother cells are evaluated (see modified Figure 1B, lower panel). Fewer peroxisomes are present in mother cells of the *inp1D* and *pex3-V81E* mutants, *i.e.* these cells exhibit a peroxisome retention defect.

2) Several mutations in Pex3p associated to human disorders have already been described, including the D347Y mutation (Matsui et al, 2012) or mutations leading to C-terminal truncation (Muntau et al 2000). The relevance of this story for human health would be higher if the authors tested how mutants in homologous residues of S. cerevisiae Pex3p (as the authors described for the residue W128) impair Pex3p function. One wonders if the phenotype associated to these mutations can be due for example to a defect in the interaction with Inp1p.

We could make mutations in the homologous residues of yeast Pex3p and perform protein-protein interaction studies with Inp1p; however, we believe that these experiments would not enhance the novel findings presented in our manuscript.

First, active retention of organelles in the mother cell and transport of organelles to the daughter cell are observed only in cell types that divide asymmetrically, such as budding yeasts. Inp1p homologues are restricted to these organisms.

Second, human cells, in contrast, divide by median fission, and the organelles are apparently allocated stochastically between the two resulting daughter cells at cytokinesis. As humans do not contain an Inp1p homologue, we have every reason to expect that any mutation in human Pex3p that leads to a disease phenotype would likely affect peroxisome biogenesis (such as the ability of Pex3p to interact with Pex19p) but not peroxisome segregation.

Reviewer 3

What membrane compartments do Inp1 and localize to in the absence of Pex3 and with Pex3-V81E expression. Markers would be appropriate in both biochemical and cell based experiments.

In *pex3-V81E* cells, Inp1p partially overlaps with the integral membrane protein Pex30p (Figure 2B), which has previously been shown to compartmentalize both to the ER and to peroxisomes (Yan M, Rachubinski DA, Joshi S, Rachubinski RA, Subramani S (2008) Dysferlin domain-containing proteins, Pex30p and Pex31p, localized to two compartments, control the number and size of oleate-induced peroxisomes in *Pichia pastoris*. *Mol Biol Cell* **19:** 885–898).

Does Inp1 localize to subdomains of the ER, for example ER exit sites?

We have analyzed the distribution of Inp1p-GFP with respect to other ER markers (see revised Figures 2A, 2B).

In cells expressing wild-type Pex3p, Inp1p and Pex3p localize to cortical foci that are in close proximity to the cortical ER-marker Rtn1p (revised Figure 2A). These foci are pre-peroxisomal subdomains of the ER as they assemble in the peroxisome-deficient mutant *pex19D*, in which all of Pex3p is trapped in the ER. The foci do not overlap with ER-exit sites (no colocalization with the COP II marker Sec13p).

The cortical localization of Inp1p is lost in cells lacking Pex3p or expressing Pex3p-V81E. In these strains Inp1p shows some degree of colocalization with Pex30p. It does not colocalize with other *bona fide* ER-markers (see revised Figure 2B).

The use of Gal1/10 driven peroxisomal and mitochondrial reporters is not ideal. Why have the authors chosen these instead of appropriate constitutive promoters? Does changing carbon source affect the outcome of the experiment?

We use both inducible and constitutive promoters for the mitochondrial redirection assay. A galactose-inducible system is shown in Figures 3B-D, as well as in Movies 2 and 4. A constitutive expression system using the endogenous *INP1* promoter is shown in Movie 3. In the first scenario, peroxisome-mitochondria tethering is dependent on the carbon source (*i.e.* galactose-inducible, glucose-repressible). In the second scenario, peroxisomes are permanently attached to mitochondria and a change in carbon source has no effect.

In cells expressing Inp1, there is a portion of peroxisomes that in in persistent proximity to mitochondria. Can the authors comment on the potential significance of this result?

Several studies have described a close association of peroxisomes with mitochondria. The organelles are known to exchange metabolites, share common proteins, and exchange materials through a vesicular trafficking pathway (for reviews see: Schrader & Yoon, 2007: Bioessays 29:1105-14; Thoms et al, 2009: Trends Mol. Med. 15:293-302). We therefore expect both organelles to be in proximity to each other in cells expressing wild-type Inp1p, but not to be permanently tethered to each other like in cells expressing Tom70p-Inp1p or Inp1p-Tom22p.

Data supporting the model that Inp1 bridges ER bound Pex3 with peroxisomal Pex3 is indirect. Do the authors detect an Inp1/Pex3 complex with a stoichiometry consistent with this model?

Please re-evaluate Figure 5; it demonstrates directly that Inp1p forms a molecular bridge between ER-bound and peroxisomal Pex3p *in vivo*. In Figures 5A and 5B we first show that Inp1p is recruited to foci by a mutant form of Pex3p that cannot egress the ER. In the cell mating assay in Figure 5C, we go on to demonstrate that peroxisomes tether to these foci in the diploid cell. Finally, we demonstrate in the split-GFP/cell mating assay in Figures 5D and 5E that a molecular interaction is formed between Inp1p that is present in foci at the ER and Pex3p that is bound to peroxisomes.

We have also characterized the binding of Pex3p to full-length Inp1p and its N- and C-terminal domains *in vitro* (see revised Figures 4B, 4C). The N- and C-terminal domains of Inp1p can bind Pex3p independently of each other. Full-length Inp1p binds more Pex3p than either the N- or C-terminal domain alone. These biochemical data support our findings that Inp1p acts as a molecular hinge connecting Pex3p molecules *in vivo*.

Also, examining the localization of the ER relative to Inp1 foci /peroxisome would be helpful, especially in a background where the surface area of cortical ER is reduced (reticulon deletions for example). In this situation, does Inp1 remain co-localized with the ER?

We have analyzed reticulon deletion mutants (*rtn1D*, *rtn1D*/*rtn2D*, *rtn1D*/*rtn2D*/*yop1D*) and find that in all cases Inp1p localized to cortical foci similar to the ones observed in wild-type cells and that peroxisome tethering to the cell cortex remained unaffected. As these data are negative, we did not include them in the revised manuscript.

Is the ER associated with Inp1/Inp2foci in the large peroxisomes observed in the vps1/dnm1 mutant?

In the *vps1D*/*dnm1D* double mutant, the cortical ER is in close association with the part of the peroxisome that contains Inp1p and which is retained in the mother cell. The tip of the peroxisome tubule that is enriched for Inp2p, on the other hand, is not directly associated with the ER (see revised Figure 8B). These findings are consistent with our previous observation that Inp2p connects peroxisomes to Myo2p.

What is the peroxisomal distribution in an inp1/inp2/vps1/dnm1 mutant?

Peroxisomes in this mutant are neither actively retained in the mother cell nor actively transported to the daughter cell. In a growing cell population of this mutant, we do observe occasional peroxisome insertion into the bud. This is in line with the anticipated random distribution of peroxisomes between mother and daughter cells in the *inp1Δ/inp2Δ/vps1Δ/dnm1Δ* mutant. Peroxisomes also seem to be ruptured and broken apart into smaller entities in this mutant (see revised Figure S2).

The organization of the manuscript could be better. For example, Figure 7 could be presented early to form the functional rationale for the study.

We have considered a change in the order in which the data are presented but hold that the original form of presentation is most suitable for this story. In the first part of the manuscript we identify the molecular components of the ER-peroxisome tether, while in the second part we identify the mechanism by which peroxisomes are shared between mother and daughter cells. The other two reviewers also liked the data presentation of our manuscript.
In many of the cell image panels, it would help the reader to have a zoomed in version of a relevant portion of the cell in the figure.

We now include inserts at higher magnification in Figures 5B and 5D (to show the assembly of the ER-peroxisome tether) and in Figure 8B (to show the formation of Inp1p-foci in peroxisome-deficient cells of the *vps1D*/*dnm1D*/*inp2D* mutant).

2nd Editorial Decision 02 July 2013

Thank you for submitting your revised manuscript 'An ER-peroxisome tether exerts peroxisome population control in yeast' to the EMBO Journal. I appreciate the introduced changes and I am pleased to accept the manuscript for publication here.

I would be grateful if you were to provide original source data, particularly uncropped/-processed electrophoretic blots for the main figures of your manuscript (Figs 2, 3, 4). This is in accord with our policy to make original results better accessible for the community and thus increase reliability of published data. We would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files. You can send them via email to me.
Furthermore, we would require statements about author contributions and possible conflicts of interest.
Finally, may I suggest altering Figure 9 a bit? I think it might be helpful to either remove the V81E molecules all together to display the wild-type situation or to include a Pex3pV81E-rich ER site that fails to recruit Inp1/peroxisomes.

Please see below for important information on how to proceed. Make sure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.
Thank you for contributing to the EMBO Journal. Please allow me to congratulate you to this study at this point!

Additional correspondence (author) 04 July 2013

Thank you for this wonderful news.

Please find attached three pdf files for source data for Figures 2, 3 and 4. I am also attaching a revised Figure 9 showing only the wild-type condition (no changes to the manuscript text were needed to accommodate this change).

Conflict of interest: All authors declare that they have no conflict of interest.

Author contributions: BN, AF and RAR provided a conceptual framework for the study, interpreted data and wrote the manuscript. BN also performed the experiments. NC provided the structural analysis of yeast Pex3p. XS provided confocal microscopy support and expertise. RLP constructed recombinant plasmids and yeast strains.

We have already provided the completed Page Charge Authorization and Licence to Publish forms with our revised manuscript submission.

Again, thank you for handling our manuscript so expeditiously.

