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Insights into ubiquitin-conjugating enzyme/co-activator interactions from the structure of the Pex4p:Pex22p complex

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(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 07 June 2011

Thank you again for submitting your manuscript to our editorial office. Enclosed below are the reports of the three expert referees that have evaluated it. As indicated in my previous email, the referees acknowledge the structural insights into the Pex4-Pex22 complex as well as their potential mechanistic implications, but at the same time raise a number of caveats that in our view preclude publication of the study in The EMBO Journal, at least in the current form. While I am not going to repeat all the individual points of criticism in detail here, I think it is fair to summarize the main concerns under two main issues:
- that is remains unclear on the biochemical level how Pex22 stimulates or 'coactivates' Pex4, or whether it may really act as noncanonical E3 enzyme instead
- that it is not clear how the observed in vitro effects of Pex22 on Pex4 would bear out for in vivo ubiquitination of Pex5, especially in light of the likely additional involvement of Pex5 ubiquitin ligases

Given the number of (often overlapping) concerns raised in this respect by all three referees, we feel that decisively addressing them will likely require substantial further time and effort (of unclear outcome), which appears to be beyond the scope of the single round of revision we usually ask for. As we only publish a small percentage of the many manuscripts submitted to The EMBO Journal, we can really only invite revision for those few submissions that receive elevated enthusiasm from the referees already upon initial review, and that appear to be sufficiently close to becoming acceptable during a limited revision period. Since this is unfortunately not the case here, I see little choice but to return the paper to you with the message that we cannot consider a revision in this case, and that you may at this point be best advised to seek rapid publication without major changes in a more structural journal. Should you however be prepared to develop the study further in line of

the comments and suggestions of our referees, I would in light of the potential interest of this study not exclude the possibility of discussing a single resubmission on this topic at some point in the future; this would however have to be treated as a new submission and only go back to our referees if we thought that the main issues had been largely answered and the key conclusions decisively substantiated, and if the conceptual novelty should still be warranted by then.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,
Editor
The EMBO Journal

Referee reports

Referee #1 (Remarks to the Author):

This manuscript describes the structure of a complex of Pex4p and the soluble domain of Pex22p. Pex22p enhances the activity of Pex4p in vitro. The structure of the complex shows a novel interaction surface between the Pex4p and Pex22p. Mutation in a critical residue on the E2, Y172A, abrogates interaction with Pex22p and reduces the activity of Pex4p in the presence of Pex22p.

The ability of E2 binding protein to stimulate E2 activity has been reported for Cue1p/Ubc7p. The cytosolic domain of Cue1p promotes the formation of polyubiquitin chain on Ubc7p in the absence of E3 in vitro although the structural basis for Cue1p/Ubc7p interaction is not yet known. This study is interesting and could provide insights into how an E2 binding partner could stimulate E2 activity in the absence of E3. However, this study can benefit from more analysis of the mechanism by which Pex22p stimulates Pex4p activity.

However, it is also not clear whether the structure of Pex4p changes in the presence of Pex22p. This may be difficult in the absence of a structure of the E2 alone. The authors propose that binding of Pex22p could make the E2 more "rigid." Are the authors proposing that rigidity of the E2 is associated with more stable structure or that rigidity is associated with ore favorable conformation for ubiqutin transfer?

One major question is whether Pex22p stimulate the rate of ubiquitin conjugation or the elongation of ubiquitin chain by Pex4p. In Fig 1, the effect of Pex22p appears to be enhancing the formation of polyubiquitin chain. On the other hand, Fig 5 suggests that the rate of reaction may be slower for Y172A mutant. Since Y172A mutation completely abrogates interaction with Pex22p, one would expect Y172A to function similarly in the presence or absence of Pex22p. This is not the case however.
Experiments that distinguish between enhanced rate of reaction and polyubiquitin chain elongation will be needed to better answer this question.

Another question is whether Pex22p affects the expression of Pex4p in vivo. It would be important to show the levels of Pex4 and mutants in Fig 7. The ubiquitin status of Pex5p is confusing and would require elaboration. The authors show also discuss the difference between the effects of Ubc4p and Pex22p ubiquitination on Pex5p.

Finally the effect of Pex22p on E2:E3 interaction is not investigated. Although it is interesting that Pex22p could stimulate polyubiquitination of the E2 in the absence of E3, it is not clear whether that is the major function of Pex22p in vivo.

Referee #2 (Remarks to the Author):

In this manuscript, Williams and coworkers determined the crystal structure of the Pex415-

183:Pex22S complex from the yeast Saccharomyces cerevisiae. They also demonstrated that Pex22S stimulates Pex4(15-183)'s E2 ubiquitin-conjugating activity in vitro, which helps the formation of additional Ub moieties to make lysine 48 linked Ub chains on Pex415-183. Using in vivo experiments involving Pex4 mutations that fail to interact with Pex22, they conclude that Pex4:Pex22 assembly, but not Pex4 alone, is required for Pex5 ubiquitination in vivo.

The conclusions from the crystal structure and in vitro experiments are clear and justified and represent, in principle, an important advance for the peroxisome biogenesis field. However, while the paper does a fine job on the structure, it could do more on the functional implications of the structure and the conclusions made for the in vivo significance. The authors should be encouraged to revise the MS, as explained below.

It is interesting that in vitro Pex22 appears to stimulate the attachment of multiple Ub (linked via K48) to Pex4. However, in vivo the authors have not focused on whether Pex22 binding allows Pex4 to attach two Ub moieties to the cycling receptor Pex5 as has been shown in vivo by Kragt et al. (2005). Fig 7 only shows mono-ubiquitinated Pex5.

What the authors show nicely is that the Pex4:Pex22 interaction is needed in vitro for charging the E2, Pex4, efficiently, but this is then extrapolated to state that "Pex22 binding is essential for Pex4 to ubiquitinate its target, the Pex5 import receptor in vivo". How can the authors distinguish whether the inefficiency in ubiquitinating Pex5 in vivo is due to one or more of the following - (a) inefficient charging of Pex4 with Ub, (b) inefficient interaction of Pex4 with the appropriate E3 ligase, which they state is still unknown, (c) instability of Pex4, as suggested by the ITC experiments, or (d) inefficient ubiquitination of the Pex5 target alone? In other words, what step/s is Pex22 co-activating exactly?

The authors appear to have the tools to identify the E3 ligase needed for Pex4-dependent ubiquitination of Pex5, and should do this work.

The conclusions from the in vivo experiments could be more convincing. The major problem is that they did not provide strong data to demonstrate that Pex22 is an E2/co-activator required for Pex5 ubiquitination in vivo. For example, the authors made a mutation of Pex4 (eg. Pex4-Y172A) to detect the effect of the binding site of Pex4 on Pex4's E2 ubiquitin-conjugating activity in vivo, but they did not show any data to demonstrate that Pex22 serves as an E2/co-activator in vivo. To demonstrate that Pex22 is essential for Pex4's E2 ubiquitin-conjugating activity and functions as an E2/co-activator required for Pex5 ubiquitination in vivo, the authors need to add more experimental data, such as -

(1) What is the effect of mutations in the Pex4 binding site of Pex22 on Pex22-Pex4 interaction in vivo? To prove this, they need to show co-immunoprecipitation of Pex22 (and its binding site mutations) with Pex4, in addition to the effect on Pex5 ubiquitination in vivo.
(3) How does Pex22 affect the Pex5 ubiquitination as an E2/co-activator in vivo? To prove this, they need to do some in vivo ubiquitination assays to show how Pex22 (and its binding sites mutations) affects Pex5 ubiquitination in vivo.

Other Concerns:
1. On page 3, line 25.
I suggest adding Zolman's paper "Identification and functional characterization of Arabidopsis PEROXIN4 and the interacting protein PEROXIN22. Zolman BK, Monroe-Augustus M, Silva ID, Bartel B. Plant Cell. 2005 Dec;17(12):3422-35." as another reference for Pex22.

2. On page 7, line 11. What are the roles of the conserved Asp151, Ala165, Gly168 and Ile169 in the function of Pex4 and its interaction with Pex22?

3. In Fig.5, we can still see some Ub2-Pex4 at T120. It looks like the ubiquitination of Pex4Y172A+Pex22S may just be delayed compared to wild type Pex4+Pex22S. Have longer incubations been attempted?

4. In Fig. 7A, how do the authors know that ubiquitination of Pex5 is Ubc4 dependent as stated?

5. Please explain residue average B factors (page 6) for general readers.

Referee #3 (Remarks to the Author):

This manuscript describes the structure of a complex between Pex4, a ubiquitin E2 enzyme and Pex22, a socalled coactivator and attempts to establish that the complex is the active form of the E2 enzyme. The paper makes the argument that the role of Pex22 is therefore not solely localization to the peroxisome (which was shown before), but also functions to activate the E2.

The paper suffers from a lack of controls as detailed below, but this important point, the dual recruitment and activation function will hopefullly hold up with the proper controls as detailed below and it is a very interesting finding. However, the manuscript does not address the fundamental question whether this should be called a coactivator or an E3 ligase. Clearly Pex22 stimulates intrinsic activity of the E2 and in the usual nomenclature this would be called an E3 enzyme. In this case there are additional RING E3s present in the cellular environment, and I guess that is why the designation 'coactivator' was chosen by the authors.

Ideally it would therefore be very interesting to figure out if these are really working simultaneously, since one could also envisage two step models, where Pex22 is required first and then the ring E3s come in. Although that may be too much for this manuscript, it is important to discuss this possibility. In the light of the role as an alternative type of E3 enzyme, the paper should probably discuss the other E3 variants such as RanBP2 and the viral ligases and their mode of action.

In addition there are a number of important technical issues in this manuscript that need to be addressed
-The biochemical analysis should show full gels (not cut off at 43 kD), show the free ubiquitin and include controls in the absence of E1 and E2 respectively.
-The assay descriptions are missing: e.g. buffer conditions, method of stopping reactions, time points in several different assays.
-ITC data should include the titration curve and the fitting to be acceptable for publication. Also ITC can never be used to prove lack of binding, since interaction could be purely entropic and then not be visible either.
-The CD data don't add anything to the story. It would be better to remove them or at least move them to the supplemental data.
-Please mention what methods were used to compare the fold to the PDB and give the cutoff that was used to define lack of significance (usually dali/ssm will find some homologs, so z-score cut-off would be appreciated)
-Please include the Ubc9/importin complex in the comparisons, since it seems to interact in this region also

1st Revision - authors' response 28 September 2011

Comments to Referee #1

This study is interesting and could provide insights into how an E2 binding partner could stimulate E2 activity in the absence of E3. However, this study can benefit from more analysis of the mechanism by which Pex22p stimulates Pex4p activity.
However, it is also not clear whether the structure of Pex4p changes in the presence of Pex22p. This may be difficult in the absence of a structure of the E2 alone. The authors propose that binding of Pex22p could make the E2 more "rigid." Are the authors proposing that rigidity of the E2 is associated with more stable structure or that rigidity is associated with more favorable conformation for ubiqutin transfer?

We have tried to obtain structural data on Pex4p alone, unfortunately without success. Our CD data suggest that no significant change in the overall secondary structure of Pex4p

occurs upon Pex22p binding although as we state in the manuscript, we cannot rule out subtle changes in Pex4p's conformation associated with Pex22p binding. Such effects, induced by the binding of ligands or other interacting partners, have been reported in the literature for this class of enzyme. Consequently, we can only speculate on whether this is indeed the case and have expanded our comments on this in the Discussion. Related to this issue, describing the effect Pex22p binding has on Pex4p as "rigidification" is based on our CD results. Pex4p undergoes gradual transition from a folded to unfolded state when shifted from lower to higher temperatures, suggesting a loosely associated structure capable of exploring several conformational states (Supplementary Figure S5B). The complex, on the other hand retains much of its secondary structure at higher temperatures, indicating a more rigid arrangement (Supplementary Figure S5F). Although the rigidification of Pex4p could very well result in enhanced stability *in vivo*, such an effect is likely to be supplemental to the more important role played by Pex22p binding; i.e. locking Pex4p in a conformation which is favourable for ubiquitin to substrate transfer.

One major question is whether Pex22p stimulate the rate of ubiquitin conjugation or the elongation of ubiquitin chain by Pex4p. In Fig 1, the effect of Pex22p appears to be enhancing the formation of polyubiquitin chain. On the other hand, Fig 5 suggests that the rate of reaction may be slower for Y172A mutant. Since Y172A mutation completely abrogates interaction with Pex22p, one would expect Y172A to function similarly in the presence or absence of Pex22p. This is not the case however. Experiments that distinguish between enhanced rate of reaction and polyubiquitin chain elongation will be needed to better answer this question.

To provide further insight into Pex22p's mode of action, we have performed additional *in vitro* experiments, following the transfer of ubiquitin from E1 to Pex4p, as well as the rate of transfer of ubiquitin from Pex4p to substrate, both in the presence and absence of Pex22p. Our new data demonstrate that Pex22p binding does not enhance charging of Pex4p by the E1 (Figure 2A) but that it is involved in the conjugation of ubiquitin to a substrate (Figure 2B). As we do not observe ubiquitin chain formation with Pex4p alone, even after 120 min incubation periods (Figure 6B), we can only state that Pex22p binding allows Pex4p to form ubiquitin chains. In the case of the Y172A mutant, it is possible that a transient/weak interaction between Pex4p and Pex22p may still occur, which could account for the stimulation seen with this mutant in the presence of Pex22p. Even in the absence of Tyr 172, an additional 6 residues in Pex4p are capable of forming hydrogen bonds with Pex22p, which may not be sufficient to obtain a detectable interaction using ITC or native gel electrophoresis, but could allow for a low level of Pex22p binding and subsequently, a low level of stimulation. We have discussed this point further in the manuscript.

Another question is whether Pex22p affects the expression of Pex4p in vivo. It would be important to show the levels of Pex4 and mutants in Fig 7.

The expression levels of the different Pex4p constructs have been probed using western blotting but unfortunately, we are unable to detect Pex4p in cells when under control of the PEX4 promoter, using either anti-Pex4p or anti-FLAG antibodies. We have similar problems detecting endogenous Pex4p, probably due to low expression levels. In response to Referee #2's comments concerning the *in vivo* effect of the Y172A mutation, we placed the different PEX4 constructs under control of the catalase promoter, to induce over-expression of the protein. With these constructs, we could clearly show that the Y172A mutant is unable to bind Pex22p *in vivo* (Figure 7B). Although these results do not rule out a possible role for Pex22p in the regulation of Pex4p expression, we feel that the inability of the Y172A mutant to bind Pex22p *in vivo*, together with the loss of Pex5p ubiquitination observed with this mutant correlates well with our *in vitro* data and points towards a more direct role for Pex22p in Pex4p function.

The ubiquitin status of Pex5p is confusing and would require elaboration. The authors should also discuss the difference between the effects of Ubc4p and Pex22p ubiquitination on Pex5p.

As requested, we have elaborated on both the ubiquitination status of Pex5p and the effect of Pex4p- and Ubc4p-dependent ubiquitination on Pex5p in the Introduction and Discussion.

Finally the effect of Pex22p on E2:E3 interaction is not investigated. Although it is interesting that Pex22p could stimulate polyubiquitination of the E2 in the absence of E3, it is not clear whether that is the major function of Pex22p in vivo.

Currently, the identity of the E3 ligase required for Pex4p-dependent ubiquitination of Pex5p is uncertain and therefore, we are unable to address this point at the current time. *In vivo* data suggest that all three RING domain containing proteins (Pex2p, Pex10p and Pex12p) are required for Pex4p dependent ubiquitination of Pex5p (Kragt *et al*., 2005, PMID: 15632140). This observation, coupled with the fact that all three exhibit E3 ligase activity *in vitro* has hindered identification of the actual E3 ligase. It is feasible that the complex of RING proteins acts as a multi-subunit E3 ligase, the mechanism of which remains to be determined. As we have measured the ubiquitin transfer properties of Pex4p in the absence of E3 and Pex5p, we appreciate that ubiquitin conjugation occurs to an artificial substrate in our assays. Nevertheless, we feel that our *in vivo* data confirm the important role Pex22p binding plays in the ubiquitination of Pex5p, verifying the *in vitro* observations. To present a more complete view, we have discussed possible ramifications of Pex22p binding on the Pex4p:E3 interaction in the Discussion.

Comments to Referee #2

In this manuscript, Williams and coworkers determined the crystal structure of the Pex415-183:Pex22S complex from the yeast Saccharomyces cerevisiae. They also demonstrated that Pex22S stimulates Pex4(15-183)'s E2 ubiquitin-conjugating activity in vitro, which helps the formation of additional Ub moieties to make lysine 48 linked Ub chains on Pex415-183. Using in vivo experiments involving Pex4 mutations that fail to interact with Pex22, they conclude that Pex4:Pex22 assembly, but not Pex4 alone, is required for Pex5 ubiquitination in vivo.

The conclusions from the crystal structure and in vitro experiments are clear and justified and represent, in principle, an important advance for the peroxisome biogenesis field. However, while the paper does a fine job on the structure, it could do more on the functional implications of the structure and the conclusions made for the in vivo significance. The authors should be encouraged to revise the MS, as explained below.

It is interesting that in vitro Pex22 appears to stimulate the attachment of multiple Ub (linked via K48) to Pex4. However, in vivo the authors have not focused on whether Pex22 binding allows Pex4 to attach two Ub moieties to the cycling receptor Pex5 as has been shown in vivo by Kragt et al. (2005). Fig 7 only shows mono- ubiquitinated Pex5.

We and others have previously shown that Pex4p attaches two ubiquitin moieties to a cysteine residue in Pex5p. Unfortunately, there is some confusion as to the terms used to describe the different forms of Pex5p ubiquitination. Originally, this modification was referred to as "mono-ubiquitination" (Kragt *et al*., 2005, PMID: 15632140), which suggests the attachment of a single ubiquitin to Pex5p. However, further studies have shown that this modification is in fact the attachment of two ubiquitin moieties to a single site in Pex5p (a di-ubiquitin chain, formed on Pex5p), occurring in a Pex4p-dependent manner (Williams *et al*., 2007, PMID: 17550898) and this is indeed the type of Pex5p ubiquitination probed in Figure 7C. To avoid confusion, we have referred to the two different forms of Pex5p ubiquitination as either Ubc4p- or Pex4p-dependent ubiquitination, depending on the E2 involved. Additionally, we have discussed this point in the text and have included molecular weight markers with the western blots, allowing direct comparison with the published data.

What the authors show nicely is that the Pex4:Pex22 interaction is needed in vitro for charging the E2, Pex4, efficiently, but this is then extrapolated to state that "Pex22 binding is essential for Pex4 to ubiquitinate its target, the Pex5 import receptor in vivo". How can the authors distinguish whether the inefficiency in ubiquitinating Pex5 in vivo is due to one or more of the following - (a) inefficient charging of Pex4 with Ub, (b) inefficient interaction of Pex4 with the appropriate E3 ligase, which they state is still unknown, (c) instability of Pex4, as suggested by the ITC experiments or (d) inefficient ubiquitination of the Pex5 target alone? In other words, what step/s is Pex22 co-activating exactly?

(a) *In vitro* analysis of the transfer of ubiquitin from E1 to the active site cysteine in Pex4p indicates that charging of Pex4p with ubiquitin does not require Pex22p (Figure 2A). Although our attempts to isolate the thioester linked ubiquitin-Pex4p species from yeast have been unsuccessful, very likely due to the low level of the protein present in the cell (see

response to Referee #1's third comment), coupled with the sensitive nature of the linkage, we feel that our *in vitro* data point away from a role for Pex22p in ubiquitin transfer from E1 to Pex4p.

(b) We do envisage a role for Pex22p binding in the Pex4p:E3 ligase interaction and we have commented on this in the Discussion, proposing that Pex22p binding could be involved in the recruitment of the E3 ligase, through an allosteric mechanism. However, a lack of information concerning the E3 ligase operating with Pex4p (see response to Referee #1's last comment) prevents us from being able to address this point experimentally at the current time.

(c) While our ITC data demonstrate the Pex4p:Pex22p complex to be of a highly stable nature, this does not necessarily mean that Pex4p alone is unstable. The CD data suggest that Pex4p adopts a loose structure, which tightens up upon Pex22p binding (see response to Referee #1's first comment). The fact that we cannot detect Pex4p in cells when under the control of its own promoter (see response to Referee #1's third comment) makes addressing *in vivo* stability difficult. However when over-expressed in yeast, both wild type and mutant forms of Pex4p/TM-Pex4p show similar protein levels, suggesting that Pex4p (wild type or mutant) is not fundamentally unstable *in vivo* (Figure 7B).

(d) Our new *in vitro* data show that Pex22p binding enhances the transfer of ubiquitin from Pex4p to a substrate (Figure 2B), which implies a direct role for Pex22p in the ubiquitination of Pex5p. Additionally and thanks to the reviewers comments (see below), we can now clearly demonstrate that the Y172A form of Pex4p, which cannot modify Pex5p *in vivo*, is also unable to bind Pex22p *in vivo* (Figure 7B), providing a direct link between Pex22p binding and the ability of Pex4p to ubiquitinate its substrate in a cellular context.

The authors appear to have the tools to identify the E3 ligase needed for Pex4-dependent ubiquitination of Pex5, and should do this work.

Please see the response to Referee #1's last comment. While this is of course a very interesting and relevant issue and is indeed a future direction for our research, we feel that identifying the E3 ligase functioning with Pex4p goes beyond the scope of the current manuscript.

The conclusions from the in vivo experiments could be more convincing. The major problem is that they did not provide strong data to demonstrate that Pex22 is an E2/co-activator required for Pex5 ubiquitination in vivo. For example, the authors made a mutation of Pex4 (eg. Pex4-Y172A) to detect the effect of the binding site of Pex4 on Pex4's E2 ubiquitin-conjugating activity in vivo, but they did not show any data to demonstrate that Pex22 serves as an E2/co-activator in vivo. To demonstrate that Pex22 is essential for Pex4's E2 ubiquitin-

conjugating activity and functions as an E2/co-activator required for Pex5 ubiquitination in vivo, the authors need to add more experimental data, such as -

(1) What is the effect of mutations in the Pex4 binding site of Pex22 on Pex22-Pex4 interaction in vivo? To prove this, they need to show co-immunoprecipitation of Pex22 (and its binding site mutations) with Pex4, in addition to the effect on Pex5 ubiquitination in vivo.

As suggested, we have performed co-immunoprecipitation experiments with the wild type and mutant forms of Pex4p (Figure 7B) and see quite clearly that the Y172A form of Pex4p/TM-Pex4p is unable to bind Pex22p *in vivo*, confirming our *in vitro* data.

(3) How does Pex22 affect the Pex5 ubiquitination as an E2/co-activator in vivo? To prove this, they need to do some in vivo ubiquitination assays to show how Pex22 (and its binding sites mutations) affects Pex5 ubiquitination in vivo.

Unfortunately, we are unsure as to what the referee is referring to when asking for *in vivo* ubiquitination assays. Using immunoprecipitation analysis of Pex5p, we have assessed the effect disturbing the Pex4p:Pex22p interaction has on Pex4p's ability to ubiquitinate Pex5p *in vivo* (Figure 7C) and would describe this as an *in vivo* ubiquitination assay. By way of explanation, we have added further details concerning interpretation of the data in the Results and Discussion sections.

Other Concerns:

1. On page 3, line 25. I suggest adding Zolman's paper "Identification and functional characterization of Arabidopsis PEROXIN4 and the interacting protein PEROXIN22. Zolman BK, Monroe-Augustus M, Silva ID, Bartel B. Plant Cell. 2005 Dec;17(12):3422-35." as another reference for Pex22.

We have added the suggested reference.

2. On page 7, line 11. What are the roles of the conserved Asp151, Ala165, Gly168 and Ile169 in the function of Pex4 and its interaction with Pex22?

Due to the fact that these residues are conserved, we believe they are involved in complex formation, but that their role is more peripheral than that of Tyr 172. During the course of this study, we have made a number of mutants in the interface, with several of them exhibiting a lower level of Pex22p binding. However, due to the strong nature of the interaction between the two proteins, this reduction in binding does not alter the affect Pex22p exerts on Pex4p's activity, neither *in vitro* nor *in vivo*. Consequently, we have only presented our data on the Y172A mutant.

3. In Fig.5, we can still see some Ub2-Pex4 at T120. It looks like the ubiquitination of Pex4Y172A+Pex22S may just be delayed compared to wild type Pex4+Pex22S. Have longer incubations been attempted?

Please see the response to Referee #1's second comment.

4. In Fig. 7A, how do the authors know that ubiquitination of Pex5 is Ubc4 dependent as stated?

We and others have reported that Pex5p can be modified by, depending on the circumstances, Pex4p and Ubc4p (Platta *et al*., 2004, PMID: 15283676; Kragt *et al*., 2005, PMID: 15632140; Williams *et al*., 2007, PMID: 17550898). Ubc4p-dependent ubiquitination of Pex5p occurs when the recycling process is disturbed; an effect often observed when late acting PEX genes (including PEX4 and PEX22) are knocked out. The pattern of Pex5p ubiquitination seen with our mutants (Figure 7A, second and fourth lanes) corresponds to that seen when the PEX4 gene is knocked out (Figure 7A, last lane) and matches the pattern of Ubc4p-dependent ubiquitination reported in the literature.

5. Please explain residue average B factors (page 6) for general readers.

We have added a description explaining residue average B factors in the Results section.

Comments to Referee #3

This manuscript describes the structure of a complex between Pex4, a ubiquitin E2 enzyme and Pex22, a socalled coactivator and attempts to establish that the complex is the active form of the E2 enzyme. The paper makes the argument that the role of Pex22 is therefore not solely localization to the peroxisome (which was shown before), but also functions to activate the E2.

The paper suffers from a lack of controls as detailed below, but this important point, the dual recruitment and activation function will hopefullly hold up with the proper controls as detailed below and it is a very interesting finding. However, the manuscript does not address the fundamental question whether this should be called a coactivator or an E3 ligase. Clearly Pex22 stimulates intrinsic activity of the E2 and in the usual nomenclature this would be called an E3 enzyme. In this case there are additional RING E3s present in the cellular environment, and I guess that is why the designation 'coactivator' was chosen by the authors. Ideally it would therefore be very interesting to figure out if these are really working simultaneously, since one could also envisage two step models, where Pex22 is required first and then the ring E3s come in. Although that may be too much for this manuscript, it is important to discuss this possibility.

While the function of Pex22p could very well be likened to that of a non-canonical E3 ligase, the presence of three RING E3s at the peroxisomal membrane, coupled with their requirement for the Pex4p-dependent ubiquitination of Pex5p *in vivo* has indeed been one of the reasons why we describe Pex22p as an E2/co-activator. A two step model would certainly be an attractive proposition and would fit in with our data and the literature. We have elaborated on possible mechanisms by which Pex22p performs its function in the Discussion.

In the light of the role as an alternative type of E3 enzyme, the paper should probably discuss the other E3 variants such as RanBP2 and the viral ligases and their mode of action.

We have compared the mode of action of several non-canonical E3 ligases with that of Pex22p and have commented on our findings in the Discussion. We conclude that although several similarities can be seen, particularly with RanBP2, the function of Pex22p is in essence different from that of an E3 ligase.

In addition there are a number of important technical issues in this manuscript that need to be addressed

-The biochemical analysis should show full gels (not cut off at 43 kD), show the free ubiquitin and include controls in the absence of E1 and E2 respectively.

Figures demonstrating *in vitro* ubiquitination activity now show full gels when probed with anti-ubiquitin. As the particular antibody used for ubiquitin staining (clone FK2) recognizes free ubiquitin only poorly (Fujimuro *et al*., 1994, PMID:7519568), we have included a coomassie stained gel (Supplementary Figure 2) showing samples of the *in vitro* ubiquitination reaction presented in Figure 1A. A reaction containing Pex22^{S} alone (control in the absence of E2) is shown in Figure 1B (last lane). Since that all the reactions presented in Figure 1 demonstrate the requirement of ATP for ubiquitin conjugation, we felt that controls lacking the E1 would not add to the interpretation of the data.

-The assay descriptions are missing: e.g. buffer conditions, method of stopping reactions, time points in several different assays.

We have expanded our description of the *in vitro* ubiquitination assays in the Materials and Methods section.

-ITC data should include the titration curve and the fitting to be acceptable for publication. Also ITC can never be used to prove lack of binding, since interaction could be purely entropic and then not be visible either.

As requested, we have added the titration curves and fitting for the ITC data (Supplementary Figure 1). Additionally, we have performed native gel electrophoresis analysis of the Y172A form of Pex4p (Figure 6A) and have confirmed its inability to bind Pex22^{S} *in vitro*.

-The CD data don't add anything to the story. It would be better to remove them or at least move them to the supplemental data.

As suggested, we have moved the CD data to the Supplementary Information.

-Please mention what methods were used to compare the fold to the PDB and give the cutoff that was used to define lack of significance (usually dali/ssm will find some homologs, so z-score cut-off would be appreciated)

To aid interpretation, we have added a table with the top 10 unique hits as provided by PDBeFOLD (Supplementary Table S2), complete with Z-, Q-, and P-scores.

-Please include the Ubc9/importin complex in the comparisons, since it seems to interact in this region also

We have added a comment relating this structure to our own in the Discussion.

In summary, we have been able to greatly improve our manuscript in accordance with the suggestions of the reviewers and we hope that the changes are sufficient to make the manuscript acceptable for publication in *The EMBO Journal*.

Should you require any further information or clarifications, please do not hesitate to contact me.

Yours sincerely

Chris Williams, EMBL-Hamburg

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2nd Editorial Decision 13 October 2011

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees, whose comments are copied below. While referee 1 retains some reservations regarding the in vivo functions of Pex4-Pex22, we feel that in light of the overall improvement of the study, and of the unclear situation regarding Pex5 in vivo ubiquitination and responsible E3 enzymes, these concerns should not further prohibit publication of the study at the current stage. There are some minor points suggested by referee 3 that I would kindly ask you to incorporate in a final round of minor revision; and ideally, showing an additional shorter exposure of the blots in Figure 2 in the supplement, may help to address one of referee 1's lingering concerns.

From an editorial point of view, I was wondering whether somewhat more explicit title could be chosen to appropriately attract the interest of the relevant readership. Minimally, it would help to replace 'E2' in the title with 'ubiquitin-conjugating enzyme'; but I could also imagine a more detailed alternative such as:
'Insights into ubiquitin-conjugating enzyme/coactivator interaction from the structure of the Pex4p-Pex22p complex'
(which may be slightly longer than the normally allowed 100 characters but that shouldn't be a problem)

Following these final modifications, we shall be happy to swiftly proceed with the formal acceptance and production of the paper!

With best regards,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In vitro, Pex22 appears to promote ubiquitin transfer from Pex4, although quantification of the results in Fig 2 is not possible due to overexposure of the unmodified E2 band.

In the absence of expression levels of Pex4, it is difficult to understand the effect of Pex22 in vivo. As the authors have pointed out, the effect of Y172A mutation may still allow transient association sufficient to stimulate E2 activity. On the other hand, expression of Y172A did not rescue effect in vivo. The easiest way to reconcile these differences is that Pex4 levels are affected by Pex22. For example, Cue1 has been shown to stabilize Ubc7 when bound to the E2. The authors could check this by expressing Pex4 with a stronger promoter.

Data on the ubiquitination of Pex5 in vivo remain weak. In the absence of E3 information, it is difficult to understand the roles of Pex22 in vivo.

Referee #3 (Remarks to the Author):

The manuscript has much improved from the previous version, the data are convincing and well-discussed. This paper provides a very interesting novel mechanism of promoting ubiquitin conjugation. Future work that includes E3 ligases will be eagerly awaited.

two minor points:
It would be very nice if the authors could find a different way of describing the binding site on the E2 in stead of 'underside' binding (page 12), since this refers only to their way of orienting the E2 in the figure.
In terms of the analysis of the lack of similar structures, the more extensive explanation of the procedure in the text is welcome, table S2 can be dispensed with, since these are not significant.

2nd Revision - authors' response 18 October 2011

Comments to Referee #1

In vitro, Pex22 appears to promote ubiquitin transfer from Pex4, although quantification of the results in Fig 2 is not possible due to overexposure of the unmodified E2 band.

We have added a shorter exposure of the blots in Figure 2 (Supplementary Figure S2B and C), to aid interpretation of the results.

In the absence of expression levels of Pex4, it is difficult to understand the effect of Pex22 in vivo. As the authors have pointed out, the effect of Y172A mutation may still allow transient association sufficient to stimulate E2 activity. On the other hand, expression of Y172A did not rescue effect in vivo. The easiest way to reconcile these differences is that Pex4 levels are affected by Pex22. For example, Cue1 has been shown to stabilize Ubc7 when bound to the E2. The authors could check this by expressing Pex4 with a stronger promoter.

We have indeed expressed Pex4p under the control of a strong promoter and we do not see differences in protein levels, comparing the wild type and Y172A mutant form of Pex4p, employing both soluble and membrane-anchored versions of Pex4p (Figure 7B). These results suggest that Pex4p levels are not influenced by the ability of the protein to bind Pex22p and that consequently, the phenotype of the Y172A mutant derives from a loss of Pex22p co-activation, rather than lower protein levels.

Data on the ubiquitination of Pex5 in vivo remain weak. In the absence of E3 information, it is difficult to understand the roles of Pex22 in vivo.

Our in vivo results indicate the crucial contribution Pex22p binding plays in Pex4p's function. However, we appreciate that without data on the E3 ligase, the complete story concerning the role of Pex22p in Pex5p ubiquitination is not yet available and have commented on this in the Discussion. Addressing the E3 ligase link is indeed a future direction for our research.

Comments to Referee #3
The manuscript has much improved from the previous version, the data are convincing and well-discussed. This paper provides a very interesting novel mechanism of promoting ubiquitin conjugation. Future work that includes E3 ligases will be eagerly awaited.

We are very pleased by the positive and supportive comments of Reviewer #3.

Two minor points:
It would be very nice if the authors could find a different way of describing the binding site on the E2 instead of 'underside' binding (page 12), since this refers only to their way of orienting the E2 in the figure.

As requested, we have changed our description of the Pex22p binding site. We now refer to this region as the " 3- 4" binding site.

In terms of the analysis of the lack of similar structures, the more extensive explanation of the

procedure in the text is welcome, table S2 can be dispensed with, since these are not significant.

We have removed Table S2 and have added further information on the procedure in the text. Additionally, we have changed the title of the manuscript to "Insights into ubiquitin-conjugating enzyme/co-activator interactions from the structure of the Pex4p:Pex22p complex" and have incorporated your suggested alterations into the abstract. Thank you for your helpful input. Please do not hesitate

