The methanol sensor Wsc1 and MAPK Mpk1 suppress degradation of methanol-induced peroxisomes in methylotrophic yeast

Shin Ohsawa, Koichi Inoue, Takahiro Isoda, Masahide Oku, Hiroya Yurimoto and Yasuyoshi Sakai
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MS TITLE: Methanol sensor Wsc1 and downstream MAP kinase suppress degradation of methanol-induced peroxisomes in yeast

AUTHORS: Yasuyoshi Sakai, Shin Ohsawa, Takahiro Isoda, Koichi Inoue, Masahide Oku, and Hiroya Yurimoto
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

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

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

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

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

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

Reviewer 1

Advance summary and potential significance to field

In this study, Ohsawa et al. revealed the mechanism by which the methylotrophic yeast Komagataella phaffi suppresses pexophagy (autophagic degradation of peroxisomes) in the presence of methanol. The authors found that whereas wild-type cells induce pexophagy when they use up methanol in medium, cells lacking the methanol sensor Wsc1 do so much earlier in the presence of a high concentration of methanol. The authors further showed that the pexophagy adaptor Atg30 abnormally accumulates in the phosphorylated forms in WSC1 KO cells, and this is likely to be caused by defects in transmitting the methanol signal to the downstream pathway involving the MAPK cascade, the transcription factor Rlm1, and the protein phosphatase Ptp2A. Thus, this study advances our understanding of pexophagy regulation in conjunction with methanol-driven peroxisome biogenesis. However, the authors should address the following issues to convincingly draw their conclusions.

Comments for the author

(1) Fig. 1B: A double mutant for Wsc1 and Atg30 should also be examined to confirm that early Pex11 degradation is mediated by Atg30-dependent pexophagy.

(2) Line 143: Fig. 1D should be Fig. 1B.

(3) Fig. 2C should contain a control strain normal for Atg30 phosphorylation.

(4) Fig. 3A: To see whether the accumulation of phosphorylated Atg30 in WSC1 KO cells can be explained by Rlm1 inactivation alone, WSC1 KO cells and WSC1 RLM1 double KO cells should be included in this experiment.

(5) The authors should examine whether RLM1 KO cells and PTP2A KO cells also show an earlier onset of pexophagy.

(6) Fig. 3C: The effect of PTP2A KO on Atg30 phosphorylation looks weaker than RLM1 KO. Does additional knockout of PTP2B or MSG5 have an effect on the level of phosphorylated Atg30?

Reviewer 2

Advance summary and potential significance to field

This paper shows that both peroxisome biogenesis and pexophagy are regulated by the methanol sensing plasma membrane protein, Wsc1, in the methylotrophic K. phaffi. This occurs via activation of a MAP kinase pathway and its downstream effectors. KpWsc1 negatively regulates pexophagy at methanol concentrations greater than 0.15% by phosphoregulation of the pexophagy receptor, Atg30. While not being necessary for peroxisome biogenesis, KpMpk1, KpRlm1 and a phosphatase (likely KpPTP2A) were found to suppress pexophagy by controlling the overall phosphorylation level of KpAtg30. The paper is a nice example of how the opposing processes of peroxisome biogenesis and turnover are coordinated.

The data presented in this paper elucidate the signaling pathway involved in the negative regulation of pexophagy in K. phaffi in response to methanol and it represents an advance to the field. I would like to recommend publication after the following questions and suggestions are addressed.

Comments for the author

Major or requiring some experiments
1. In Fig. 1B, it would be helpful if the authors quantitate their claim that the ratio of YFP/Pex11-YFP is higher in the Kpwsc1 mutant.

2. It would be important to show the site of KpPTP2A action, if possible, and also if it acts directly on KpAtg30. Since KpAtg30 has over a dozen phosphosites of which two are known to be important at S71 and S112 for binding Atg8 and Atg11, respectively, are these sites dephosphorylated by the phosphatase?

3. Is this mechanism of pexophagy inhibition by KpWsc1 peculiar to methanol. For example, is the activation of macropexophagy affected in the Kpwsc1 mutant?

4. Lines 219-222 – is the regulation of the level of PTP2a at the transcriptional level?
Minor or easily addressed

a. In the title "yeast" should be replaced by "yeast, K. phaffi" or "methylotrophic yeast".

b. I strongly suggest the use of the phrase "pexophagy receptor" rather than pexophagy adaptor for Atg30. I view adaptor as some intermediary protein that is not the primary recipient of the signal triggering the act of pexophagy.

c. Line 57 – KpAtg30 mediates its effects through Pex3, Pex14 and Atg37 (PMID: 24535825, 29260977).

d. In Fig. S2A, the legend says Wsc1-Flag and Wsc3-Flag were used, but these are labeled as Wsc1-YFP and Wsc3-YFP in the figure. This should be made consistent.

First revision

Author response to reviewers' comments

KYOTO UNIVERSITY
Graduate School of Agriculture
Division of Applied Life Sciences
Laboratory of Microbial Biotechnology
Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan

Prof. Tamotsu Yoshimori
Editor
Journal of Cell Science

Dear Prof. Tamotsu Yoshimori,

Thank you very much for your editorial effort on our manuscript , informing us of your favorable comments regarding our manuscript, "Methanol sensor Wsc1 and downstream MAP kinase suppress degradation of methanol-induced peroxisomes in yeast" (JOCES/2020/254714) by Ohsawa *et al.*, along with the comments from two reviewers. **We have changed title of the manuscript to**

“Methanol sensor Wsc1 and MAP kinase suppress degradation of methanol-induced peroxisomes in methylotrophic yeast”, and have responded to all of the comments from the reviewers as in the attached sheets and revised the figures and manuscript following their suggestions.

In addition to these correspondences, several parts of the original manuscript have been edited as follows:

i) Koichi Inoue has taken and shared the 1st co-authorship with Shin Ohsawa, and Takashi Isoda has been placed as the 3rd author (p.1, line 7 & 23). These changes have been permitted from all of the authors.

ii) Involvement of KpWsc3 in methanol-sensing has been described in Introduction of the revised manuscript (p. 5, line 7).

iii) As to regulation of selection autophagy
We have changed the description as to negative regulation of pexophagy (p.6, line 4-5; p.15, line 2 from the bottom – p16, line 1).

iv) Construction of new *msg5*-deletion plasmid has been added to Materials and Methods section (p.19, line 7-15).

v) As to western blots, we used white background images.
The original images have been uploaded as a ppt file “western_fullblot.pptx ”.

All of the edited parts are marked with yellow in the revised Ms and Supplementary file.

Finally, we thank you and the reviewers again for kind suggestions, comments and patience. We sincerely hope that this revised manuscript is now acceptable for publication in your *Journal of Cell Science*.

Sincerely yours,
Yasuyoshi Sakai, Ph.D. Professor
Division of Applied Life Sciences
Graduate School of Agriculture
Kyoto University
Kitashirakawa-Oiwake, Sakyo-ku
Kyoto 606-8502, Japan

Reviewer 1 Advance Summary and Potential Significance to Field:
In this study, Ohsawa et al. revealed the mechanism by which the methylotrophic yeast Komagataella phaffi suppresses pexophagy (autophagic degradation of peroxisomes) in the presence of methanol. The authors found that whereas wild- type cells induce pexophagy when they use up methanol in medium, cells lacking the methanol sensor Wsc1 do so much earlier in the presence of a high concentration of methanol. The authors further showed that the pexophagy adaptor Atg30 abnormally accumulates in the phosphorylated forms in WSC1 KO cells, and this is likely to be caused by defects in transmitting the methanol signal to the downstream pathway involving the MAPK cascade, the transcription factor Rlm1, and the protein phosphatase Ptp2A. Thus, this study advances our understanding of pexophagy regulation in conjunction with methanol-driven peroxisome biogenesis. However, the authors should address the following issues to convincingly draw their conclusions.

Thank you for your favorable comments to improve our manuscript.
All of the edited parts are marked with yellow in the revised Ms and Supplementary file.

Reviewer 1 Comments for the Author:
(1) Fig. 1B: A double mutant for Wsc1 and Atg30 should also be examined to confirm that early Pex11 degradation is mediated by Atg30-dependent pexophagy.

(4) Fig. 3A: To see whether the accumulation of phosphorylated Atg30 in WSC1 KO cells can be

explained by Rlm1 inactivation alone, WSC1 KO cells and WSC1 RLM1 double KO cells should be included in this experiment.

New Sup. Fig. 1B indicates clearly that early Pex11 degradation is Atg30-dependent. The original data was uploaded as a ppt file "western full blot".

In a preliminary experiment, we noticed that peroxisome assembly was retarded in the *wsc1Δ* strain after 9 h of methanol induction but almost comparable to WT strain after 12 h of methanol induction, in which KpWsc3 supports peroxisome assembly.
Therefore, we used 12-h methanol-induced cells for pexophagy assay throughout this manuscript. The peroxisome assembly of *atg30Δ* strain was comparable to WT.

According to your comment, we have derived the *wsc1Δatg30Δ* strain. However, this double knockout strain did not give efficient peroxisome induction even after 12-h of methanol induction. The reason is not clear. These situation made difficult to evaluate regulatory function of another gene, e.g. Atg30, Rlm1, *etc*, in relation to Wsc1, at this time point.

(2) Line 143: Fig. 1D should be Fig. 1B.
We have corrected to Fig. 1B. (p.8, line 6 from the bottom).

(3) Fig. 2C should contain a control strain normal for Atg30 phosphorylation.
We have replaced Fig. 2C with a new Fig 2C including data with a control strain.

(5) The authors should examine whether RLM1 KO cells and PTP2A KO cells also show an earlier onset of pexophagy.

We have performed Pex11-YFP cleavage assay with WT and *Kprlm1Δ*, as newly added Fig. S4. In *Kprlm1Δ*, cleavage of YFP/Pex11-YFP at 18 and 22 was enhanced in the *Kprlm1Δ* strain where methanol was still remained in the medium (Fig. S4). This was described in p.11, line 3-2 from the bottom.

(6) Fig. 3C: The effect of PTP2A KO on Atg30 phosphorylation looks weaker than RLM1 KO. Does additional knockout of PTP2B or MSG5 have an effect on the level of phosphorylated Atg30?

We have re-examined phosphorylation levels of Atg30-HA in *Kpmsg5Δptp2AΔpex11Δ* strain under the condition where methanol is still remained highly in the medium.
As shown in new Fig. 4, we observed enhancement of phosphorylation in the *ptp2AΔ* strain. And further enhancement of phosphorylation could be pbserved in *Kpmsg5Δptp2AΔ* strain compared with single mutant of *msg5Δ* or *ptp2AΔ*. The result has been described in p12, line 9-15.

Reviewer 2 Comments for the Author:

Reviewer 2 Advance Summary and Potential Significance to Field:
This paper shows that both peroxisome biogenesis and pexophagy are regulated by the methanol sensing plasma membrane protein, Wsc1, in the methylotrophic K. phaffi. This occurs via activation of a MAP kinase pathway and its downstream effectors. KpWsc1 negatively regulates pexophagy at methanol concentrations greater than 0.15% by phosphoregulation of the pexophagy receptor, Atg30. While not being necessary for peroxisome biogenesis, KpMpk1, KpRlm1 and a phosphatase (likely KpPTP2A) were found to suppress pexophagy by controlling the overall phosphorylation level of KpAtg30. The paper is a nice example of how the opposing processes of peroxisome biogenesis and turnover are coordinated.

The data presented in this paper elucidate the signaling pathway involved in the negative regulation of pexophagy in K. phaffi in response to methanol and it represents an advance to the field. I would like to recommend publication after the following questions and suggestions are addressed.

Thank you for your favorable comments to improve our manuscript.
All of the edited parts are marked with yellow in the revised Ms and Supplementary file.

Major or requiring some experiments
1. In Fig. 1B, it would be helpful if the authors quantitate their claim that the ratio of YFP/Pex11-YFP is higher in the Kpwsc1 mutant.

We have added new Figure in Fig 1B right panel, which shows quantitative data from the density of GFP-band on Western (left panel) . We also edited the legend to this figure.

2. It would be important to show the site of KpPTP2A action, if possible, and also if it acts directly on KpAtg30. Since KpAtg30 has over a dozen phosphosites of which two are known to be important at S71 and S112 for binding Atg8 and Atg11, respectively, are these sites dephosphorylated by the phosphatase?

Based on sequence similarity, both Ptp2A and Msg5 are assumed to be tyrosine phosphatase. And our preliminary experiments suggested PTP2A phosphatase did not act on KpAtg30 directly. These have been described in the text (p12, line 3-1 from the bottom).

3. the activation of macropexophagy affected in the Kpwsc1 mutant?

We performed Atg8-YFP cleavage assay with *Kpwsc1Δ* and Wt cells under macropexophagic condition by shifting methanol-induced cells to ethanol medium. As newly added Sup. Fig.2, we did not observe enhancement of macropexophagy in the *wsc1Δ* mutant. And our previous study also showed that ethanol repression of peroxisome induction and pexophagy involved ethanol metabolism yielding acetyl-CoA, which was distinct from methanol-signaling (Ohsawa et al., 2018 *Sci Rep*. DOI: https://doi.org/10.1038/s41598-018-36732-2). The results have been described in p.8, line 3 from the bottom – p.9, line 2.

4. Lines 219-222 – is the regulation of the level of PTP2a at the transcriptional level?

Yes, it is. We have changed the phrase "the expression levels" to "the transcript levels" to avoid misreading (p.12, line 4-5).

Minor or easily addressed

a. In the title "yeast" should be replaced by "yeast, K. phaffi" or "methylotrophic yeast".

According to the suggestion and considering limitation of the title length (120 characters), we have changed the title to "Methanol sensor Wsc1 and MAP kinase suppress degradation of methanol-induced peroxisomes in methylotrophic yeast" (112 characters) (p1, line 1-3, 112).

b. I strongly suggest the use of the phrase "pexophagy receptor" rather than pexophagy adaptor for Atg30. I view adaptor as some intermediary protein that is not the primary recipient of the signal triggering the act of pexophagy.

We have changed the pharase "pexophagy adaptor" to "pexophagy receptor" throughout the manuscript.

c. Line 57 – KpAtg30 mediates its effects through Pex3, Pex14 and Atg37 (PMID: 24535825, 29260977).

According to the suggestion, we have added Atg37 and reference (PMID: 24535825). (p.4, line 10-12; p.27, line 4-6).

d. In Fig. S2A, the legend says Wsc1-Flag and Wsc3-Flag were used, but these are labeled as Wsc1-YFP and Wsc3-YFP in the figure. This should be made consistent.

We have corrected Wsc1-YFP and Wsc3-YFP to Wsc1-FLAG and Wsc3-FLAG, respectively, in new Fig. S3A.

Second decision letter

MS ID#: JOCES/2020/254714

MS TITLE: Methanol sensor Wsc1 and MAP kinase suppress degradation of methanol-induced peroxisomes in methylotrophic yeast

AUTHORS: Shin Ohsawa, Koichi Inoue, Takahiro Isoda, Masahide Oku, Hiroya Yurimoto, and Yasuyoshi Sakai
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

I find the revised manuscript is now suitable for publication in JCS.

Comments for the author

I find the revised manuscript is now suitable for publication in JCS.

Reviewer 2

Advance summary and potential significance to field

See first review

Comments for the author

The authors have addressed all of my comments satisfactorily and the paper is ready for acceptance into JCS