JCB

Mitotic phosphorylation of Pex14p regulates peroxisomal import machinery

Koichiro Yamashita, Shigehiko Tamura, Masanori Honsho, Hiroto Yada, Yuichi Yagita, Hidetaka Kosako, and Yukio Fujiki

Corresponding Author(s): Yukio Fujiki, Kyushu University and Shigehiko Tamura, Kyushu University

Review Timeline:

Submission Date:	2020-01-02
Editorial Decision:	2020-02-04
Revision Received:	2020-04-28
Editorial Decision:	2020-06-24
Revision Received:	2020-07-10

Monitoring Editor: John Aitchison

Scientific Editor: Andrea Marat

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.202001003

February 4, 2020

Re: JCB manuscript #202001003

Prof. Yukio Fujiki
Kyushu University
Medical Institute of Bioregulation,
3-1-1 Maidashi,
Higashi-ku
Fukuoka 812-8582
Japan

Dear Prof. Fujiki,

Thank you for submitting your manuscript entitled "Mitotic phosphorylation of Pex14p regulates peroxisomal import machinery in mammals". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

In particular, it is critical to address Rev#1's specific technical comments and concerns about the data quality and to provide a clearer picture of the mechanism by which Pex14 phosphorylation affects peroxisome import with more substantive experimental support, as recommended by Rev#2. In addition, we consider that knockdown of Cdk1 would be important to support the hypothesis that Cdk1 is the kinase responsible for mitotic phosphorylation of Pex14.

Although the manuscript would be appropriate for JCB if revised to address the reviewers' comments and concerns, our primary concern is that the current work does not address the biological implications for reduced import of peroxisomal matrix proteins at mitosis. While some plausible hypotheses, including preservation of ATP or relocalization of catalase to the cytosol to protect DNA from oxidative damage, are proposed, there is no support for these suggestions. An ideal revision of this manuscript would include an experimentally validated explanation of the advantage conferred upon cells by mitotic inhibition of matrix import. However, considering the challenges of addressing this question experimentally, references supporting plausible hypotheses are minimally required.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation,

http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

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

Sincerely,

John Aitchison, Ph.D.
Monitoring Editor

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

Journal of Cell Biology

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

Post-translational modification of proteins by phosphorylation can modulate protein function either positively or negatively. This applies to many proteins involved in many cellular processes, including proteins involved in protein import into organelles, protein trafficking in the cell or in organellar dynamics. For example, phosphorylation of the p97 ATPase during mitosis promotes mitotic Golgi disassembly in mammalian cells, while phosphorylation of Tom6 in mitosis in yeast promotes the import of proteins into mitochondria and leads to increased respiratory activity by mitochondria. Although a number of proteins required for peroxisome biogenesis, or Pex proteins, have been shown to be phosphorylated primarily by large screens, little is known about how phosphorylation affects the activity of Pex proteins, especially those involved in import of proteins into the peroxisomal matrix. In this manuscript Yamashita and colleagues investigate the effects of

phosphorylation of mammalian Pex14, a membrane protein that is a major component of the peroxisomal importomer involved in the import of soluble proteins into the peroxisomal matrix. The authors show that Pex14 is phosphorylated at Ser232 in mitotically arrested HeLa cells, and using phosphomimetic forms of Pex14, the authors show that phosphorylation of Pex14 at Ser232 results in reduced peroxisomal matrix protein import. The authors provide evidence that this reduced peroxisomal matrix import by phosphorylated Pex14 is probably due to reduced export of the peroxisomal matrix protein targeting receptor Pex5, with which Pex14 interacts, from the peroxisome matrix to the cytosol for additional rounds of import of matrix protein cargo into the peroxisome. The authors provide additional evidence that phosphorylation of Pex14 induces a conformational change in Pex14, increasing Pex14-Pex14 interaction and thereby reducing Pex14's capacity to bind Pex5 in the peroxisomal lumen and facilitate its export to the cytosol for another round of import of matrix cargo by Pex5. The authors conclude that Pex14 undergoes specific phosphorylation in a cell-cycle-dependent fashion to modulate its interaction with the peroxisomal matrix protein targeting receptor Pex5 in the peroxisomal lumen, consequently reducing recycling of Pex5 and, as a consequence, matrix protein import.

Issues that the authors should address:

1) In Figs. 6 and 7, the authors designate so called non-specific bands by an asterisk (*), but they provide no evidence showing that these bands are indeed non-specific. The authors must provide evidence for the non-specificity of these bands.

2) Fig. 6B. The Pex14(156-376) SD mutant tagged with FLAG shows increased homodimerization with Pex14(156-376) tagged with HA. The authors should report what is observed with full-length Pex14.

3) Figs. 6C and 6D. The authors state that Pex14(200-376)S232D is more compact than WT Pex14(200-376) or Pex14(200-376)S232A by BN-PAGE (Fig. 6D). But this is not similarly observed the larger constructs using Pex14(156-176). Why is there this inconsistency?

4) p. 7, first paragraph. Results of Fig. 3C. Treatment with phosphatase will of course dephosphorylate many proteins, not only Pex14. Therefore, the last sentence of this paragraph should be toned down in its conclusion, e.g. remove 'most likely'.

5) p. 9, third sentence. What is meant by 'sufficiently recovered'. Remove 'sufficiently'.

6) p. 9, first paragraph. (Fig. 6C, right panel) NOT (Fig. 6C, bottom panel).

7) p. 10, first paragraph, second to last sentence. I'm not exactly sure what the authors are trying to say here, but I think it is that structural changes in the C-terminus of Pex14 translates to changes in the N-terminus of Pex14, which is the part of Pex14 that binds Pex5, leading to reduced Pex5 binding, reduced Pex5 export from the peroxisomal matrix to the cytosol, and thus overall reduced peroxisomal matrix protein import. The authors should rephrase to improve clarity.

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

The peroxide Pex14 forms a major component of the peroxisomal import machinery, whereby proteins carrying peroxisomal targeting signal sequences (PTSs) are actively and specifically imported into the peroxisome; in the case of PTS1 type sequences, this is via a mechanism that

involves shuttling of the cargo receptor Pex5 across the peroxisomal membrane. Here, the authors show that Pex14 in mammalian cells is phosphorylated in vivo, agreeing with extensive earlier data from several model yeast systems. Having shown that Pex14 appears to be a major mitotic-specific phoshorylation target in mammalian cells in vivo, MS and molecular genetics studies neatly demonstrate that Pex14 is phosphorylated predominantly on Ser232 and that specific substitution of an Ala there largely abolishes Pex14 mitotic phosphorylation in vivo. The authors then targeted the mitosis-specific kinase argeted Cdk1 as the likely source of mitotic Pex14 phosphorylation, and the use of a Cdk1-specific inhibitor confirmed Cdk1 as the primary kinase for Pex14. A phosphomimetic mutant of Pex14 on the major phosphorylation site Ser232 (but not, as an important control, the minor site Ser334) specifically reduces peroxisomal import, and immunoprecipitation studies show that phosphorylation does not appear to alter the interaction of Pex14 with Pex5, the PTS1 signal sequence interacting factor. Use of a clarified cell-free peroxisomal transport system led to the determination that Pex5 export, but not import, is suppressed in mitotic peroxisomes. Limited proteolysis assays indicated that phosphorylation at S232 induces a conformational change in Pex14's coiled-coil domain, leading to stabilization of the Pex14 homo-oligomeric state and an alteration of the N-terminal portion of Pex14p, resulting in the strong reduction of Pex5-mediated PTS1 cargo export.

The study represents an elegant preliminary set of experiments implicating phosphorylation as a major regulator of mitotic peroxisomal transport via alteration of the binding of the shuttling factor Pex14 to the N-terminal domain of Pex14. However, I worry that it may be too preliminary for a full article in JCB, as the precise mechanism by which phosphorylation affects transport - is it direct to prevent Pex5 docking to the N-terminus of Pex14, or is it via the interaction of Pex14 with an intermediary factor, for example - is lacking. Perhaps it may be better for the shorter report format?

There are significant grammatical issues throughout the manuscript, and the authors would be advised to persuade a native reader to proofread it.

April 27, 2020

John Aitchison, Ph.D., Monitoring Editor,
Marie Anne O'Donnell, Ph.D., Scientific Editor,
Journal of Cell Biology
950 Third Avenue, 2nd Floor
New York, NY 10022
U.S.A.
Tel: +1-212-327-8581
E-mail: jcellbiol@rockefeller.edu

Re: Revision of JCB Ms #202001003
Title: Mitotic phosphorylation of Pex14p regulates peroxisomal import machinery in mammals
Authors: Koichiro Yamashita, Shigehiko Tamura, Masanori Honsho, Hiroto Yada, Yuichi Yagita, Hidetaka Kosako, and Yukio Fujiki

Drs. Aitchison and O'Donnell,

Thank you very much for your decision letter of February 5, 2020, together with the two Reviewers' comments. We are very glad to hear that our manuscript JCB Ms #202001003 would be rated acceptable after adequate revision. We thank both of you and the reviewers for the time taken to review our manuscript and the helpful comments and suggestions. By taking their comments into account, we have revised the manuscript with several additional data. Attached please find the revised version of our manuscript JCB Ms #202001003, where the revised parts are marked in red. One copy of the non-red marked manuscript is also attached.

We agree to accept equal responsibilities for accuracy of the contents of the revised manuscript. Repair was made (see a separate sheet and point-to-point replies to the Reviewers), on the line to the comments made by the Reviewers. We wish that the revised version were now a subject to the acceptance for publication in the *Journal of Cell Biology*. This manuscript has not been submitted elsewhere and the data have not been reported elsewhere in any form of language.

Best wishes.
Faithfully yours,

Yukio Fujiki, Ph.D.
Professor
Medical Institute of Bioregulation
Kyushu University
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I. To the Monitoring and Scientific Editors:

In particular, it is critical to address Rev#1's specific technical comments and concerns about the data quality and to provide a clearer picture of the mechanism by which Pex14 phosphorylation affects peroxisome import with more substantive experimental support, as recommended by Rev#2. In addition, we consider that knockdown of Cdk1 would be important to support the hypothesis that Cdk1 is the kinase responsible for mitotic phosphorylation of Pex14.
Authors' reply:
As suggested, we evaluated the effect of Cdk1 knockdown on mitotic phosphorylation of Pex14p. Cdk1 knockdown significantly suppressed phosphorylation of Pex14p in HeLa cells treated with nocodazole, suggesting that Cdk1 is the kinase responsible for mitotic phosphorylation of Pex14p.

Although the manuscript would be appropriate for JCB if revised to address the reviewers' comments and concerns, our primary concern is that the current work does not address the biological implications for reduced import of peroxisomal matrix proteins at mitosis. While some plausible hypotheses, including preservation of ATP or relocalization of catalase to the cytosol to protect DNA from oxidative damage, are proposed, there is no support for these suggestions. An ideal revision of this manuscript would include an experimentally validated explanation of the advantage conferred upon cells by mitotic inhibition of matrix import. However, considering the challenges of addressing this question experimentally, references supporting plausible hypotheses are minimally required.
Authors' reply:
We revised text and additionally cited a recent reference for the significance of cytosolic catalase involved in DNA damage response (new reference Dubreuil et al., 2020). Dubreuil et. al. reported that non-localized catalase is most protective in cells treated with H_2O_2, suggesting that the localization of catalase in the cell determines its efficiency in ameliorating oxidative stress. Further studies would be required to experimentally and fully understand the biological implications for reduced import of peroxisomal matrix proteins including catalase at mitosis.

II. Main revised points:

1) To address the issues raised by the editors and reviewers, we performed additional experiments as follows:
   (a) Cdk1 knockdown to assess the phosphorylation of Pex14p in HeLa cells synchronized in mitosis with nocodazole (newly added Fig. 2G, authors' reply to the editors).
   (b) assessing the specificities of antibodies to Pex14p N- and C-terminal region (newly added Fig. S3, authors' reply to Reviewer 1).
2) We modified the text and Figures (Fig. 2G and Fig. S3) according to the suggestions made by the editors and reviewers.

III. Replies to the Reviewers:
Reviewer #1 (Comments to the Authors (Required)):

Post-translational modification of proteins by phosphorylation can modulate protein function either positively or negatively. This applies to many proteins involved in many cellular processes, including proteins involved in protein import into organelles, protein trafficking in the cell or in organellar dynamics. For example, phosphorylation of the p97 ATPase during mitosis promotes mitotic Golgi disassembly in mammalian cells, while phosphorylation of Tom6 in mitosis in yeast promotes the import of proteins into mitochondria and leads to increased respiratory activity by mitochondria. Although a number of proteins required for peroxisome biogenesis, or Pex proteins, have been shown to be phosphorylated primarily by large screens, little is known about how phosphorylation affects the activity of Pex proteins,

especially those involved in import of proteins into the peroxisomal matrix. In this manuscript Yamashita and colleagues investigate the effects of phosphorylation of mammalian Pex14, a membrane protein that is a major component of the peroxisomal importomer involved in the import of soluble proteins into the peroxisomal matrix. The authors show that Pex14 is phosphorylated at Ser232 in mitotically arrested HeLa cells, and using phosphomimetic forms of Pex14, the authors show that phosphorylation of Pex14 at Ser232 results in reduced peroxisomal matrix protein import. The authors provide evidence that this reduced peroxisomal matrix import by phosphorylated Pex14 is probably due to reduced export of the peroxisomal matrix protein targeting receptor Pex5, with which Pex14 interacts, from the peroxisome matrix to the cytosol for additional rounds of import of matrix protein cargo into the peroxisome. The authors provide additional evidence that phosphorylation of Pex14 induces a conformational change in Pex14, increasing Pex14-Pex14 interaction and thereby reducing Pex14's capacity to bind Pex5 in the peroxisomal lumen and facilitate its export to the cytosol for another round of import of matrix cargo by Pex5. The authors conclude that Pex14 undergoes specific phosphorylation in a cell-cycle-dependent fashion to modulate its interaction with the peroxisomal matrix protein targeting receptor Pex5 in the peroxisomal lumen, consequently reducing recycling of Pex5 and, as a consequence, matrix protein import.

Issues that the authors should address:

1) In Figs. 6 and 7, the authors designate so called non-specific bands by an asterisk (*), but they provide no evidence showing that these bands are indeed non-specific. The authors must provide evidence for the non-specificity of these bands.
Authors' reply:
In Figs. 6 and 7, immunoblot analyses were performed by using antibodies to Pex14p N- or C-terminal region. We added Supplementary Fig. S3 describing specificities of these antibodies. PNS fractions each from HeLa and *pex14* HeLa cells were analyzed by immunoblotting. The bands designated by asterisk (*) in Fig. 7A right panel were detected in PNS fractions from both HeLa and *pex14* HeLa cells, showing that these bands shown in Fig. 7A right panel were likely to be non-specific bands. (supplementary Fig. 3) On the other hands, Pex14p and the lower band designated by asterisk in Fig. 7A left panel were not detected in PNS fraction from *pex14* HeLa cells, suggesting the lower band is degradation product derived from Pex14p. Therefore, we revised asterisk (*) in Fig. 7A left panel to dot (●) designating degradation product. Moreover, after partial digestion with Proteinase K, anti-Pex14pC antibody-crossreactive, slower-migrating protein was detected as an unknown band. This unknown band is indicated by solid arrowhead.

2) Fig. 6B. The Pex14(156-376) SD mutant tagged with FLAG shows increased homodimerization with Pex14(156-376) tagged with HA. The authors should report what is observed with full-length Pex14.
Authors' reply:
We revised and added the text in Result section on page 9 as follows.
"In regard to FLAG- and HA- tagged full length Pex14p with or without mutations, SD mutation did not enhance homo-interaction apparently, suggesting that the effect of phosphorylation is restricted to region proximal to S232 of Pex14p."

3) Figs. 6C and 6D. The authors state that Pex14(200-376)S232D is more compact than WT Pex14(200-376) or Pex14(200-376)S232A by BN-PAGE (Fig. 6D). But this is not similarly observed the larger constructs using Pex14(156-176). Why is there this inconsistency?
Authors' reply:
As you suggested, it is not clear to show the effect of S232 phosphorylation by use of Pex14p(156-376) harboring a coiled-coil domain. In contrast, the conformational change of homo-oligomer is more readily observed upon deletion of a coiled-coil domain as shown in Fig. 6D. These results also suggest that the effect of phosphorylation is restricted to the region

proximal to S232 of Pex14p. Further structural analyses on Pex14p C-terminal domain would be required to understand the conformational change modulated by S232 phosphorylation.

4) p. 7, first paragraph. Results of Fig. 3C. Treatment with phosphatase will of course dephosphorylate many proteins, not only Pex14. Therefore, the last sentence of this paragraph should be toned down in its conclusion, e.g. remove 'most likely'.
Authors' reply:
We revised the text as you suggested in Result section on page 7, lines 13. The word "most" was removed.

5) p. 9, third sentence. What is meant by 'sufficiently recovered'. Remove 'sufficiently'.
Authors' reply:
We revised the text as pointed out in the Results section on page 9, line 3.

6) p. 9, first paragraph. (Fig. 6C, right panel) NOT (Fig. 6C, bottom panel).
Authors' reply:
We revised it to the Fig. 6C, right panel.

7) p. 10, first paragraph, second to last sentence. I'm not exactly sure what the authors are trying to say here, but I think it is that structural changes in the C-terminus of Pex14 translates to changes in the N-terminus of Pex14, which is the part of Pex14 that binds Pex5, leading to reduced Pex5 binding, reduced Pex5 export from the peroxisomal matrix to the cytosol, and thus overall reduced peroxisomal matrix protein import. The authors should rephrase to improve clarity.
Authors' reply:
We revised the text in Result section, p. 10, as suggested.

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

The peroxide Pex14 forms a major component of the peroxisomal import machinery, whereby proteins carrying peroxisomal targeting signal sequences (PTSs) are actively and specifically imported into the peroxisome; in the case of PTS1 type sequences, this is via a mechanism that involves shuttling of the cargo receptor Pex5 across the peroxisomal membrane. Here, the authors show that Pex14 in mammalian cells is phosphorylated in vivo, agreeing with extensive earlier data from several model yeast systems. Having shown that Pex14 appears to be a major mitotic-specific phosphorylation target in mammalian cells in vivo, MS and molecular genetics studies neatly demonstrate that Pex14 is phosphorylated predominantly on Ser232 and that specific substitution of an Ala there largely abolishes Pex14 mitotic phosphorylation in vivo. The authors then targeted the mitosis-specific kinase argeted Cdk1 as the likely source of mitotic Pex14 phosphorylation, and the use of a Cdk1-specific inhibitor confirmed Cdk1 as the primary kinase for Pex14. A phosphomimetic mutant of Pex14 on the major phosphorylation site Ser232 (but not, as an important control, the minor site Ser334) specifically reduces peroxisomal import, and immunoprecipitation studies show that phosphorylation does not appear to alter the interaction of Pex14 with Pex5, the PTS1 signal sequence interacting factor. Use of a clarified cell-free peroxisomal transport system led to the determination that Pex5 export, but not import, is suppressed in mitotic peroxisomes. Limited proteolysis assays indicated that phosphorylation at S232 induces a conformational change in Pex14's coiled-coil domain, leading to stabilization of the Pex14 homo-oligomeric state and an alteration of the N-terminal portion of Pex14p, resulting in the strong reduction of Pex5-mediated PTS1 cargo export.

The study represents an elegant preliminary set of experiments implicating phosphorylation as a major regulator of mitotic peroxisomal transport via alteration of the binding of the

shuttling factor Pex14 to the N-terminal domain of Pex14. However, I worry that it may be too preliminary for a full article in JCB, as the precise mechanism by which phosphorylation affects transport - is it direct to prevent Pex5 docking to the N-terminus of Pex14, or is it via the interaction of Pex14 with an intermediary factor, for example - is lacking. Perhaps it may be better for the shorter report format?
Authors' reply:
To understand the precise mechanism by which phosphorylation affects transport, structural analyses on Pex14p C-terminal domain would be required to understand the conformational change modulated by S232 phosphorylation. This issue would be clarified in the future.

There are significant grammatical issues throughout the manuscript, and the authors would be advised to persuade a native reader to proofread it.
Authors' reply:
We tried our best, thank you.

June 24, 2020

RE: JCB Manuscript #202001003R

Prof. Yukio Fujiki
Kyushu University
Medical Institute of Bioregulation,
3-1-1 Maidashi,
Higashi-ku
Fukuoka 812-8582
Japan

Dear Prof. Fujiki:

Thank you for submitting your revised manuscript entitled "Mitotic phosphorylation of Pex14p regulates peroxisomal import machinery in mammals". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In addition, in your final version the data comparing the extent of dimerization of full-length wild-type Pex14 compared to that of full-length Pex14SD needs to be included as part of a figure.

Furthermore, in your text, the proposed reason for Pex14 phosphorylation should be discussed in a clear and consistent manner. For example it is unclear what is meant in abstract when you say phosphorylation of Pex14 synchronizes mitotic responses with other organelles. In the discussion you propose that the phosphorylation and consequent redistribution of catalase is a mechanism of protecting DNA - presumably as the DNA is exposed to the cytoplasm upon NE breakdown at mitosis. This should be conveyed in the abstract as well. This message could also include the weaker PTS on catalase as the reason its distribution is more impacted by the change in Pex14. Finally, your text will be professionally copy-edited during production, though having it proofread by a colleague beforehand would be useful.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel

electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

* We suggest a slightly edited title: Mitotic phosphorylation of Pex14p regulates the peroxisomal import machinery

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

John Aitchison, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

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

No additional comments.

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

There are numerous grammatical issues throughout the manuscript, as I discussed in my prior review; these still have not been fixed. Here are the first three sentences of the manuscript, with suggested grammatical corrections in square parentheses:
"Peroxisome ~~is [are] ubiquitous, single-membrane-bounded organelle with a large variety of metabolic functions such as ß-oxidation of very long chain fatty acids and biosynthesis of plasmalogens (Fujiki, 1997; Lazarow and Moser, 1995). [The]Pperoxisomal[e's] metabolism depends on the import of nuclear-encoded proteins from [the] cytosol into [each] peroxisomes (Lazarow and Fujiki, 1985). The majority of matrix proteins is[are] destined for [import into] the peroxisomal matrix by a distinct dynamic system involving peroxins such as Pex1p, Pex2p. Pex5p, Pex6p, Pex7p, Pex10p, Pex12p, Pex13p, Pex14p, and Pex26p (Fujiki et al., 2006; Platta and Erdmann, 2007)." There are far too many examples for me to correct. One of the authors is now at the MRC-LMB, and can thus surely ask a colleague there to correct the manuscript's grammar.~~

~~My concerns regarding the suitability of this manuscript as a full article in JCB remain. Experimentally, the authors have now performed siRNA on Cdk1 to show that in knockdown Cdk1, Pex14 phosphorylation but not protein level was massively reduced. Moreover, they checked the specificities of their antibodies to the Pex14p N- and C-terminal regions. While laudable, these do not strongly impact my overall statements.~~
~~The revisions do not experimentally clarify the precise mechanism by which phosphorylation affects transport, but rather revise the textual discussion. Thus, I still leave it to the editors whether such a revised manuscript is better suited for the shorter report format.~~

2nd Revision - Authors' Response to Reviewers: July 10, 2020

July 3, 2020

John Aitchison, Ph.D., Monitoring Editor,
Andrea L. Marat, Ph.D., Scientific Editor,
Journal of Cell Biology
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Re: Revision of JCB Ms #202001003R
Title: Mitotic phosphorylation of Pex14p regulates peroxisomal import machinery
Authors: Koichiro Yamashita, Shigehiko Tamura, Masanori Honsho, Hiroto Yada, Yuichi Yagita, Hidetaka Kosako, and Yukio Fujiki

Dear Drs. Aitchison and Marat,

Thank you very much for your decision letter of June 24, 2020, together with the two Reviewers' comments. We are very glad to hear that our manuscript JCB Ms #202001003R would be published after adequate revision including the adjustment with the formatting guidelines. We thank both of you and the reviewers for the time took to review our revised manuscript and the helpful comments. By taking your comments into account, we have revised the manuscript with revision mainly in Abstract and Figure 6B. Attached please find the revised version of our manuscript JCB Ms #202001003R, where the revised parts are marked in red. One copy of the non-red marked manuscript is also attached.

We agree to accept equal responsibilities for accuracy of the contents of the revised manuscript. Repair was made (see a separate sheet and point-to-point replies to the Reviewers). We wish that the revised version were now a subject to the publication in the *Journal of Cell Biology*. This manuscript has not been submitted elsewhere and the data have not been reported elsewhere in any form of language.

Best regards,

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I. To the Monitoring and Scientific Editors:

1. *Your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). In addition, in your final version the data comparing the extent of dimerization of full-length wild-type Pex14 compared to that of full-length Pex14SD needs to be included as part of a figure.*

Authors' reply:
As suggested, we assessed the revised manuscript in accordance with all the items in the JCB formatting guidelines. In regard to the dimerization of the full-length wild-type Pex14 in comparison to the full-length Pex14SD, we added the data in Fig. 6B and revised the text on page 9 in Results section.

2. *Furthermore, in your text, the proposed reason for Pex14 phosphorylation should be discussed in a clear and consistent manner.*

Authors' reply:
We revised such points in Abstract, lines 13-14, in a consistent manner with those in Discussion.

3. *Proofreading the text by a native speaker.*

Authors' reply:
As in the reply to the Reviewer #2 (see below), we have tried our best as found in the revised text.

II. Replies to the Reviewers:
Reviewer #1 (Comments to the Authors (Required)):
No additional comments.

Authors' reply:
Thank you very much for the wonderful comment.

Reviewer #2 (Comments to the Authors (Required)):
There are significant grammatical issues throughout the manuscript, and the authors would be advised to persuade a native reader to proofread it.

Authors' reply:
As suggested, we asked for a chance to have a native English speaker at the MRC-LMB proofread the manuscript. However, unfortunately, people in the lab are very busy because the "Lockdown" from the COVID-19 pandemic was ceased just a few days ago, making everyone need to work hard to start up the experiments, submitting manuscripts and grants, etc. So, we have tried our best, as you could find in the revised the text. Thank you.