VPS13D promotes peroxisome biogenesis

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March 26, 2020

Re: JCB manuscript #202001188

Dr. Richard J Youle  
National Institute of Neurological Disorders and Stroke  
10213 Montgomery Ave  
Kensington, MD 20895

Dear Dr. Youle,

Thank you for submitting your manuscript entitled "VPS13D promotes peroxisome biogenesis" to Journal of Cell Biology. The manuscript has now been assessed by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see the reviewers note that a role for VPS13D in peroxisome biogenesis would be novel but the study does not yet convincingly demonstrate a direct role. The reviewers argue that the observations may be an artefact of cell culture conditions and/or cell viability and that some of the observations or descriptions of protein function contradict current literature concerning peroxisome biology.

Unfortunately I do not have the level of reviewer support that I would need to proceed further with the paper. I do realize that significant further work and expansion might convincingly address some of these issues, but I am hesitant to encourage you to work towards the aim of further consideration at JCB. The level of reviewer criticism makes it impossible for me to guarantee that we will be able to invite resubmission, even after revision. Therefore, it does seem that it will be best for you to consider another journal for this work. Our journal office will transfer your reviewer comments to another journal upon request.

I am sorry our decision is not more positive, but hope that you find the reviews constructive. Of course, this decision does not imply any lack of interest in your work and we look forward to future submissions from your lab.

Thank you for your interest in the Journal of Cell Biology.

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)):

The manuscript "VPS13D promotes peroxisome biogenesis" by Baldwin et al. aims to establish a functional role of human VPS13D in peroxisome biogenesis. The authors describe a stochastic loss of peroxisomes in some but not all VPS13D KO cells. They can exclude defects in peroxisomal inheritance, exclude an enhanced pexophagy and conclude that there might be a direct or indirect role in the PEX19-depentent formation of peroxisomes.

In general, the authors describe some interesting observations and this work gives first indications that VPS13D might have potentially an impact on peroxisome numbers and peroxisome morphology. However, the presented data and chosen argumentation of the study is still too preliminary.

01) p.2/l.20:
The authors claim that "over 30 "PEX" genes" would be involved in peroxisome biogenesis in mammals. However, there are currently 14 known peroxins in mammals. Maybe the quotation marks around the term "PEX" were intended to indicate that the authors also refer to other, non-peroxin proteins that are involved, like the UbcH5 Ub-conjugating enzymes, etc. However, even if this was the intention, the authors should still clearly discriminate between classical peroxins and other proteins linked to peroxisome biogenesis.

02) p.2/l.21.-25:
Nomenclature: The authors describe the function of the proteins PEX1, PEX6, PEX26 and PEX5. However, they write the names in italics, as if it was the corresponding gene name. This is a problem in many parts of the text and the figures. Sometimes the proteins are in major caps in italics, in major caps in regular or only the first letter is in major. Please use one of the last two possibilities consistently throughout the entire manuscript text and figures.

03) p.2/l.25:
PEX5 is best described as matrix protein receptor (and not as matrix protein escort).

04) p.2/l.25:
The correct reference for the role of the AAA complex in export and recycling of PEX5 in mammals is Miyata & Fujiki Mol. Cel. Biol. 2005 (and not Law et al 2017, who described the PEX1-mutation induced peroxisome degradation).

05) P4./l.65:
Nomenclature: The yeast protein is called Vps13p or Vps13 (and not vps13).

06) p.7/l.162:
PEX19 is not localized to the peroxisomal matrix, as described by the authors. The only difference of PEX19 to the other mentioned proteins in the experiment is that these other proteins are PMPs and that PEX19 is a soluble cytosolic protein, which can bind to the cytosolic side of the peroxisomal membrane via PEX3/PEX16.

07) Fig.1C & p5./l.97:
The authors claim that PMP70 "is not detected in VPS13D KO cells". This statement contradicts the data in Fig. 1C, which shows that some cells exhibit no PMP70 signals, while other cells do display them. Therefore, the authors should rephrase their description of the PMP70 localization analysis.
08) Fig. 2A and Fig. 2C:
The data in Fig. 2A and the corresponding model in Fig. 2C show a decline of peroxisome numbers over time. Please explain the experimental setup in more detail. How was the situation at T=0 days defined? Is it supposed to be the day of the gene KO?

09) Fig. 2A:
The authors categorize the cells depending on their peroxisome population in "normal", "partial" and "missing". They use the matrix marker GFP-CAT. However, this partially contradicts their finding in Fig. 1C, where they find that several VPS13D KO cells display cytosolic mislocalization of CAT (catalase). How did this influence the cell counting and categorization in the experiment described in Fig. 2A? Why did the authors not use a membrane marker like PMP70 in this experiment? This might potentially still enable to count import-incompetent peroxisomal structures, even if the matrix marker would be mislocalized to the cytosol.

10) Fig. 2B:
Please explain the data concerning the analysis of peroxisomal inheritance in Fig. 2B better. Why are two pictures shown for VPS13D KO and only one for WT when the statistical analysis finds no difference in peroxisomal inheritance?

11) Fig. 3C to Fig. 3G:
The authors fail to perform a fundamental control experiment, namely the full complementation of the VPS13D KO with a VPS13D-construct in context of the peroxisome phenotype. In Fig. 3C, it is shown that the number of normal cells is slightly increased in presence of the VPS13D-construct, while the number of the more severely affected cells with partial or complete loss of peroxisomes is not altered significantly.
In Fig. 3D to Fig. 3G, the authors describe that the expression of the VPS13D-construct has a dominant negative effect on peroxisome numbers in WT cells. The interesting aspect is that the authors find this effect also when other PMPs are overexpressed, but not in the case of the soluble PEX19. Therefore, the authors should try to find successful ways to detect the subcellular localization of VPS13D. The data could indicate a peroxisomal or peroxisome-relevant ER localization of VPS13D, as it behaves similar to the PMPs in this single aspect. However, the experiments so far do not establish a reliable direct connection of VPS13D to the phenotype of peroxisome loss or to the peroxisomal membrane.

12) Fig. 5
The effect of mutated VPS13D on matrix protein import in patient fibroblasts was analyzed in Fig. 5. The text has the information that cytosolic mislocalization of catalase was "very low (<10%)" in certain patient cell lines and that this was not observed in the parental cell lines. The authors should present a table of these results in Fig. 5 together with the microscopy pictures in order to get an impression of the significance of this observation.

13) Fig. 6 E / p. 9 / l. 248-249
The main conclusion of the authors is the claim that VPS13D "is directly or indirectly involved in peroxisome biogenesis". This is based on the observation that PEX19-constructs can complement peroxisome numbers faster in PEX19 KO-single mutants than in PEX19/VPS13D KO-double mutants. My general concern is - again - the question how peroxisome-specific this observation is. However, even if it was peroxisome-specific, it was not demonstrated that the influence of VPS13D on peroxisome numbers is indeed linked specifically to PMP transport or membrane biogenesis. The problem is that the authors were not able to exclude a direct role of VPS13D in the matrix protein
mechanism or peroxisome fission in previous experiments. Any other factor that has either indirect/pleiotropic effects or a factor that has a direct, but partial redundant role in another part of peroxisomal function could have potentially the same effect in the PEX19 complementation assay, as the VPS13D mutant had.

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

Baldwin and coworkers aimed to study the potential biological roles of mammalian VPS13A-D through the generation and characterization of CRISPR/Cas9 mediated knockouts of each of the four different VSP13 genes in HeLa cells. The same group previously reported that the VPS13D knockout cells exhibit an abnormal mitochondrial morphology and now reported that these cells also show (partial) peroxisomal biogenesis defects.

Major concern
While the experiments are well executed and thoroughly described, including a lengthy materials & methods section, this study primarily concerns a report of microscopical observations without providing any insight (experimental or hypothetical) into a (possible) mechanistic role of VSP13D in peroxisome biogenesis.

Additional comments
1. The fact that part of the VSP13D KO cells not only has catalase in the cytosol but also seem to lack PMP70, is a remarkable finding, as this is normally only observed in PEX3,16 or 19 KO cells. All other PEX mutants, including PEX14 and 26, usually still have peroxisomal membrane proteins present. Remarkably, however, the authors also observed that PEX14 and PEX26 KO cells seemed to have lost PMP70 signal. This should be confirmed by staining for additional peroxisomal membrane proteins.
2. The manuscript contains several inconsistencies and inaccuracies (introduction vs discussion) and sometimes refers to dated information especially concerning peroxisome biology. Eg. line 14-16: classification is not arbitrarily or based on complementation groups, but on the defective PEX genes; Line 20: so far 14 (and not 30) different PEX genes have been implicated in humans; Line 29,30; the interplay between peroxisomes and mitochondria does not involve exchange of lipids; Line 32: Catalase degrades H2O2. etc
3. Peroxisome biogenesis involves not only the formation of the peroxisomal membrane but also import of proteins into the peroxisomal matrix and proliferation of the organelles (eg lines 222, 223)
4. Unclear which and why different VPS13D KO cells (exon 3 vs exon 34 KO) have been used in certain experiments
5. The stochastic peroxisome loss observed in VPS13D-KO cells and the patient's fibroblasts seems to resemble the previously described peroxisome mosaicism observed in Zellweger spectrum cells with less severe mutations. Culturing such cells at elevated temperature often results in increased peroxisome deficiency. Has this been studied in these cells?
6. What could be the role for VSP13D in peroxisome biogenesis and (how) could it be similar as for mitochondria?

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

The Vacuolar Protein Sorting 13 (VPS13) gene family has one member in the yeast Saccharomyces cerevisiae and four members (VPS13A-D) in mammals. VPS13 in yeast has been implicated in sporulation, phospholipid regulation, and mitochondrial integrity. There is limited information on the
roles of VPS13A-D, and especially of VPS13D, although mutation of these genes have been implicated in a variety of neurological disorders (mutation of VPS13D has been implicated in recessive spinocerebellar ataxia) and in protein trafficking defects from membrane-bounded compartments, e.g. mutation in VPS13B compromises protein trafficking from the Golgi complex. In this manuscript, Baldwin and colleagues purport to demonstrate a role for VPS13D in peroxisome biogenesis in mammalian, i.e. human, cells. There are a number of issues with the findings presented in this paper to challenge such a conclusion.

1) Fig. 1C. VPS13D knock out (VPS13D KO) HeLa cells show no evidence of any peroxisomal structures marked by the peroxisomal membrane marker PMP70 in two of the four cells shown. This reviewer has not seen such a scenario except for cells knocked out for PEX3 and PEX19, which act at the ER to initiate pre-peroxisome biogenesis. The result presented here is especially surprising given that the authors conclude by subsequent experiments that VPS13D acts in peroxisome biogenesis downstream of PEX3 and PEX19. The absence of a PMP70 signal in some VPS13D KO cells may be artefactual or evidence of dying cells. That VPS13D KO cells are at different stages on their way to death could also explain the mosaicism in peroxisomal profiles observed in VPS13D KO cells.

2) Catalase (CAT) is targeted to the peroxisomal matrix by a weak peroxisomal targeting signal type 1 (PTS1), and its targeting to the peroxisomal matrix has been shown to be reduced in aging cells or cells exposed to stress. Thus, CAT is not the best marker to use to measure the extent of peroxisomal matrix protein import. Thus, a cytosolic localization of CAT in some VPS13D KO cells as seen in Fig. 1C could again be evidence of stressed cells or cells on the way to death. The same can be concluded for patient LUB1.1 fibroblasts (Fig. 5).

3) Fig. 3G. Overexpression of peroxisomal membrane proteins PEX3, PEX14 and PEX16 leads to the absence of any peroxisomal structures marked by PMP70 in a subset of cells that, quite unexpectedly, seem to be in patches. This reviewer has again not seen such a scenario, which may again reflect a simple artefact of culture or evidence of cells on their way to death.

4) Fig. 3, A and D. Why is GFP-Ub-VPS13D found mostly in nuclei, when VPS13D-i-GFP is found at the Golgi (Fig. S4A)?

5) The quantification of peroxisome absence in PEX26 overexpressing WT and ATG8 6KO cells (Fig. 3F) does not jive with the corresponding microscopic images in Fig. 3G.

6) Fig. 3G. It is important to have the microscopic images for WT and ATG8 KO cells overexpressing PEX19.

7) Fig. 4A. It is unexpected that VPS13D is found at the Golgi complex and not at peroxisomes or at the ER if it has a role in peroxisome biogenesis.

8) Fig. 6. The authors should present a time course of microscopic images in panel D that corresponds to the data in panel E.

ADDITIONAL POINTS:

1) p. 2, lines 20 and 21. That over 30 PEX genes involved in peroxisome biogenesis have been identified in mammals is patently incorrect.
2) p. 4, line 67. "...Ypt35p, Spo71p and Mcp1p...".

3) p. 5, line 98. PEX14 is NOT a tail-anchored protein.

4) p. 7, lines 161 and 162. PEX19 is NOT found in the peroxisomal matrix. It is the PMP receptor found in the cytosol!

5) p. 17. Legend to Fig. 3. Insert '(E)' before 'Manual count of...'.

6) p. 17. Legend to Fig. 4, 2nd line. Remove 'used'.

7) p. 18. Legend to Fig. 6. Asterisks associated with p values do not jive between the legend and the figure. 'Mean number of cells per time point is (x ÷ n) and not (x - n) as in the legend.'

8) p. 19. Legend to Fig. S2. LAMP2 in the legend, LAMP1 in the figure. Correct.

9) Fig. S2. Label the figure with the organelles that the different markers detect.
We thank the reviewers for the thoughtful comments and pointing out our factual errors in the introduction - we have now fixed all of these. We are encouraged to revise the manuscript for JCB because two reviewers called our results “remarkable” and “surprising”, indicating the importance of the findings. In order to address reviewer #3’s most serious concern, that the peroxisomal phenotype is likely due to the cell death or artifacts, we performed several experiments to rule out this possibility. First, we validated the phenotype in different cell types (293T, U2OS and HCT116). Second, we immunostained VPS13D (and PEX14 KO cells as a negative control) with anti-PEX14 antibody and confirmed the loss of peroxisome as PEX14 is mis-localized to mitochondria in cells lacking peroxisomes. We further examined Cytochrome C release (an indicator of apoptosis) in VPS13D KO as well as other PEX KO cells as a recent report showed that significant Cyt.C release is detected in PEX3 and PEX5 KO MEFs cells under normal untreated conditions. We didn’t observe any Cyt.C release in all tested KO cell lines including VPS13D KO cells. In addition, we generated PEX5, PEX14 KO cells and PEX19 KO positive control cells and found that complete peroxisome loss is also observed in the majority of PEX5 and PEX14 KO cells, suggesting that PEX3/16/19 are not the only genes whose deletion leads to complete peroxisome loss. We also expanded on the study of mechanism and addressed almost all the reviewers’ experimental suggestions with new results, which are highlighted in blue in the revised manuscript. Moreover, we also want to point out a BioRxiv paper from the De Camilli lab that reveals more mechanistic links between peroxisomes and VPS13D (bioRxiv 2020.10.07.328906; doi: https://doi.org/10.1101/2020.10.07.328906). Below is our point by point response (in red) to each reviewer’s comments.
Reviewer #1

The manuscript "VPS13D promotes peroxisome biogenesis" by Baldwin et al. aims to establish a functional role of human VPS13D in peroxisome biogenesis. The authors describe a stochastic loss of peroxisomes in some but not all VPS13D KO cells. They can exclude defects in peroxisomal inheritance, exclude an enhanced pexophagy and conclude that there might be a direct or indirect role in the PEX19-depenent formation of peroxisomes.
In general, the authors describe some interesting observations and this work gives first indications that VPS13D might have potentially an impact on peroxisome numbers and peroxisome morphology. However, the presented data and chosen argumentation of the study is still too preliminary.

01) p.2/l.20:
The authors claim that "over 30 "PEX" genes" would be involved in peroxisome biogenesis in mammals. However, there are currently 14 known peroxins in mammals. Maybe the quotation marks around the term "PEX" were intended to indicate that the authors also refer to other, non-peroxin proteins that are involved, like the UbcH5 Ub-conjugating enzymes, etc. However, even if this was the intention, the authors should still clearly discriminate between classical peroxins and other proteins linked to peroxisome biogenesis.
We apologize for this error, which was due to our confusion with the number of yeast PEX genes. (Waterham and Ebberink, 2012, BBA 1822(9)L 1430-1441. Genetics and molecular basis of human peroxisome biogenesis disorders). We have now corrected this mistake.

02) p.2/l.21.-25:
Nomenclature: The authors describe the function of the proteins PEX1, PEX6, PEX26 and PEX5. However, they write the names in italics, as if it was the corresponding gene name. This is a problem in many parts of the text and the figures. Sometimes the proteins are in major caps in italics, in major caps in regular or only the first letter is in major. Please use one of the last two possibilities consistently throughout the entire manuscript text and figures.
Thank you for the correction on nomenclature. While yeast and plants have a clear and strict nomenclature on genes and proteins, the rule is not clear with human genes and proteins. We now made it all consistent in the revised manuscript.
03) p.2/l.25:
PEX5 is best described as matrix protein receptor (and not as matrix protein escort).
Thank you very much – we made that correction.

04) p.2/l.25:
The correct reference for the role of the AAA complex in export and recycling of PEX5 in mammals is Miyata & Fujiki Mol. Cel. Biol. 2005 (and not Law et al 2017, who described the PEX1-mutation induced peroxisome degradation).
We are sorry for this error. Thank you – we made this correction.

05) P4./l.65:
Nomenclature: The yeast protein is called Vps13p or Vps13 (and not vps13).
We again apologize for this incorrect use and our unfamiliarity with the yeast nomenclature. Thank you for this correction.

06) p.7/l.162:
PEX19 is not localized to the peroxisomal matrix, as described by the authors. The only difference of PEX19 to the other mentioned proteins in the experiment is that these other proteins are PMPs and that PEX19 is a soluble cytosolic protein, which can bind to the cytosolic side of the peroxisomal membrane via PEX3/PEX16.
We are sorry for another error. We corrected this.

07) Fig.1C & p5./l.97:
The authors claim that PMP70 "is not detected in VPS13D KO cells". This statement contradicts the data in Fig. 1C, which shows that some cells exhibit no PMP70 signals, while other cells do display them. Therefore, the authors should rephrase their description of the PMP70 localization analysis.
Due to the stochastic nature and incomplete penetrance of Vps13D KO, not every cell loses peroxisomes. We now rephrase the manuscript to “PMP70 is not detected in some VPS13D KO cells”.

08) Fig. 2A and Fig.2C:
The data in Fig.2A and the corresponding model in Fig. 2C show a decline of peroxisome numbers over time. Please explain the experimental setup in more detail. How was the situation at T=0 days defined?
Is it supposed to be the day of the gene KO?

We have now updated the description of Fig 2A: “Each trend line (dashed) represents one well. The lines are colored like a heat map based on the total amount of cells found in the well at the first timepoint (0); timepoint 0 is defined as the first day of imaging (one day after seeding).”

09) Fig. 2A:
The authors categorize the cells depending on their peroxisome population in "normal", "partial" and "missing". They use the matrix marker GFP-CAT. However, this partially contradicts their finding in Fig.1C, where they find that several VPS13D KO cells display cytosolic mislocalization of CAT (catalase). How did this influence the cell counting and categorization in the experiment described in Fig. 2A? Why did the authors not use a membrane marker like PMP70 in this experiment? This might potentially still enable to count import-incompetent peroxisomal structures, even if the matrix marker would be mislocalized to the cytosol,

We are sorry this was unclear. As shown in Fig.1, some VPS13D KO cells have undetectable PMP70 staining and also cytosolic catalase. When peroxisomes are missing, catalase localizes in the cytosol, whereas PMP70 is degraded. Ghost peroxisomes have defects in peroxisome matrix protein import causing catalase to reside in cytosol but PMP70 staining is still apparent. We have stained for PMP70 and catalase many times in the VPS13D KO cells and never detected ghost peroxisomes. Therefore, we think catalase staining truly reflects the peroxisome loss in our Vps13D KO cells. GFP-CAT allows us to do live cell imaging and very reliable counting and quantification. We also added PEX14 staining to further confirm the loss of peroxisomes as unlike catalase and PMP70, PEX14 is mis-localized to mitochondria in the absence of peroxisomes.

10) Fig.2B:
Please explain the data concerning the analysis of peroxisomal inheritance in Fig. 2B better. Why are two pictures shown for VPS13D KO and only one for WT when the statistical analysis finds no difference in peroxisomal inheritance?

Two pictures for VPS13D KO cells were included to show that equal inheritance between VPS13D KO daughter cells was found regardless of whether the parent cell started with normal (top image) or reduced ("partial") (bottom image) number of peroxisomes. We revised the legend text to Fig 2B,
which is now Fig 3B, to explain that as follows. “(B) Wild-type and VPS13D KO cells stained for PMP70, DAPI, and telophase marker- Aurora B. Two representative images of dividing VPS13D KO cells that start with normal (top) or reduced (bottom) number of peroxisomes are included. The difference in peroxisomal number between daughter cells (each dot represents one pair of newly divided daughter cells) is shown on the right. Both WT and VPS13D KO: n=80 images (containing 2 daughter cells each). (C) Model of VPS13D’s heterogenous effect on peroxisome number. Green represents CAT signal (peroxisome-localized or cytosolic). Scale bar = 5 µm.”

11) Fig.3C to Fig. 3G:

The authors fail to perform a fundamental control experiment, namely the full complementation of the VPS13D KO with a VPS13D-construct in context of the peroxisome phenotype. In Fig.3C, it is shown that the number of normal cells is slightly increased in presence of the VPS13D-construct, while the number of the more severely affected cells with partial or complete loss of peroxisomes is not altered significantly.

Overexpression of VPS13D in the VPS13D KO cells only rescues the mitochondrial phenotype but not peroxisomal phenotype. We generated VPS13D stable overexpressing cells and found that that alone induces peroxisome loss (or at least peroxisomal import defect based on cytosolic CAT signal) in wild type cells, further supporting the link between VPS13D and peroxisomes. To further validate this surprising finding, we also generated several PEX gene overexpression stable cell lines and found that OE of PEX14 and PEX16 (membrane proteins) also interferes with peroxisome biogenesis as well. We found a similar effect of PEX14 and PEX16 in ATG8 6KO cells (where autophagy is completely blocked) to show that peroxisome loss caused by PEX14 and PEX16 OE is not due to elevated pexophagy.

In Fig.3D to Fig.3G, the authors describe that the expression of the VPS13D-construct has a dominant negative effect on peroxisome numbers in WT cells. The interesting aspect is that the authors find this effect also when other PMPs are overexpressed, but not in the case of the soluble PEX19. Therefore, the authors should try to find successful ways to detect the subcellular localization of VPS13D. The data could indicate a peroxisomal or peroxisome-relevant ER localization of VPS13D, as it behaves similar to the PMPs in this single aspect. However, the experiments so far do not establish a reliable direct connection of VPS13D to the phenotype of peroxisome loss or to the peroxisomal membrane.
Thank you for this good suggestion. Due to the extremely low expression level of VPS13D (even with transient transfection) and lack of a good antibody, it is very difficult to address this question properly. Like several prior reports in the subcellular localization of other VPS13 genes (VPS13A-C) as well as yeast Vps13, VPS13D is likely also localized to different organelle-organelle contact sites. We performed a subcellular fractionation experiment and found that VPS13D is present in both the heavy membrane (mitochondria and ER-enriched) and light membrane (lysosomes/endosomes/peroxisomes) fractions. In addition, recently the De Camilli lab reported that VPS13D can be recruited to mitochondria and peroxisomes by GTPase Miro (bioRxiv 2020.10.07.328906; doi: https://doi.org/10.1101/2020.10.07.328906). They also found that VPS13D is variably enriched on Golgi complex (as we did) with a weak signal around mitochondria. We have now included mention of the De Camilli group’s findings in the discussion as corroborating evidence.

12) Fig.5
The effect of mutated VPS13D on matrix protein import in patient fibroblasts was analyzed in Fig. 5. The text has the information that cytosolic mislocalization of catalase was "very low (<10%)" in ceratin patient cell lines and that this was not observed in the parental cell lines. The authors should present a table of these results in Fig.5 together with the microscopy pictures in order to get an impression of the significance of this observation.
Figure 7B now includes quantification data.

13) Fig.6 E / p.9/l.248-249
The main conclusion of the authors is the claim that VPS13D "is directly or indirectly involved in peroxisome biogenesis". This is based on the observation that PEX19-constructs can complement peroxisome numbers faster in PEX19 KO-single mutants than in PEX19/VPS13D KO-double mutants My general concern is - again - the question how peroxisome-specific this observation is. However, even if it was peroxisome-specific, it was not demonstrated that the influence of VPS13D on peroxisome numbers is indeed linked specifically to PMP transport or membrane biogenesis. The problem is that the authors were not able to exclude a direct role of VPS13D in the matrix protein mechanism or peroxisome fission in previous experiments. Any other factor that has either indirect/pleiotropic
effects or a factor that has a direct, but partial redundant role in another part of peroxisomal function could have potentially the same effect in the PEX19 complementation assay, as the VPS13D mutant had.

We acknowledge that we do not know how VPS13D affects peroxisome biogenesis. One suggestion based on prior studies linking VPS13D and other proteins such as Atg2 with related N-terminal domains to lipid transport is that it fosters lipid import during peroxisome biogenesis. This would be consistent with the new DeCamilli lab preprint. We cannot rule out indirect effects as pointed out but we note that there is quite striking specificity to peroxisomes and mitochondria as ER, endosomes, lysosomes and Golgi content and morphology are not affected in VPS13D KO cells. Thus, a potential indirect effect of VPS13D would yield a surprising degree of peroxisome specificity. We did seek to better understand the mechanism of VPS13D involvement in PEX3, PEX16 or PEX19 specific pathways. Unfortunately, PEX3 and PEX16 OE also impairs peroxisome import (based on the appearance of cytosolic GFP-SKL), which makes the rescue results in PEX3 KO, PEX16 KO PEX3/VPS13D DKO and PEX16/VPS13D DKO uninterpretable. However, PEX19 OE does not affect peroxisome import. PEX19 initiated peroxisome biogenesis is significantly reduced in PEX19/VPS13D DKO compared to PEX19 KO cells. There are no reports that defects in peroxisome fission lead to peroxisome loss.

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

Baldwin and coworkers aimed to study the potential biological roles of mammalian VPS13A-D through the generation and characterization of CRISPR/Cas9 mediated knockouts of each of the four different VSP13 genes in HeLa cells. The same group previously reported that the VPS13D knockout cells exhibit an abnormal mitochondrial morphology and now reported that these cells also show (partial) peroxisomal biogenesis defects.

Major concern

While the experiments are well executed and thoroughly described, including a lengthy materials & methods section, this study primarily concerns a report of microscopical observations without providing any insight (experimental or hypothetical) into a (possible) mechanistic role of VSP13D in peroxisome biogenesis.

We agree. However, a recent De Camilli preprint mentioned to reviewer #1 yields new insight into
VPS13D and peroxisomes suggesting mechanism. However, that paper does not report any peroxisomal consequences. Together, our manuscript and the De Camilli work yield a phenotype and mechanistic insights and support a previously undescribed, and we feel unanticipated, role of VPS13D in peroxisome biology.

Additional comments

1. The fact that part of the VSP13D KO cells not only has catalase in the cytosol but also seem to lack PMP70, is a remarkable finding, as this is normally only observed in PEX3,16 or 19 KO cells. All other PEX mutants, including PEX14 and 26, usually still have peroxisomal membrane proteins present. Remarkably, however, the authors also observed that PEX14 and PEX26 KO cells seemed to have lost PMP70 signal. This should be confirmed by staining for additional peroxisomal membrane proteins. We appreciate the reviewer noting that the phenotype we have uncovered is remarkable. PMP70 and catalase are the two most commonly used immunofluorescent markers for peroxisome membrane and matrix. We now confirmed our findings with another well known peroxisomal membrane protein marker PEX14. PEX14 is mis-localized to mitochondria in the absence of peroxisomes. We first confirmed the immunostaining is specific to PEX14 signal using PEX14 KO cells (negative staining) and then found that while PEX14 is perfectly localized to peroxisomes in the WT cells, it is completely mis-localized to mitochondria in both VPS13D KO and PEX5 KO cells (Fig 1D, 1G, Fig S4C). We also tried several other peroxisomal membrane proteins, but unfortunately those antibodies did not work for immunostaining.

2. The manuscript contains several inconsistencies and inaccuracies (introduction vs discussion) and sometimes refers to dated information especially concerning peroxisome biology. Eg. line 14-16: classification is not arbitrarily or based on complementation groups, but on the defective PEX genes; Line 20: so far 14 (and not 30) different PEX genes have been implicated in humans; Line 29,30; the interplay between peroxisomes and mitochondria does not involve exchange of lipids; Line 32: Catalase degrades H2O2. etc

We apologize for the many errors and have now corrected the mistakes as also noted specifically to reviewer #1.
3. Peroxisome biogenesis involves not only the formation of the peroxisomal membrane but also import of proteins into the peroxisomal matrix and proliferation of the organelles (eg lines 222, 223)
Thank you for this correction - we have made the proper changes in the text.

4. Unclear which and why different VPS13D KO cells (exon 3 vs exon 34 KO) have been used in certain experiments
We generated two different VPS13D KO clones by CRISPR/gRNAs targeting different exons. Although premature stop codons introduced by the frame-shifting InDels (insertions/deletions) caused by CRISPR often lead to mRNA decay, the consequences of InDels can be dramatically different in terms of how the truncated proteins might be generated. Therefore, targeting different exons will enhance the chance of obtaining null KO clones. In addition, observing similar phenotypes in different independent KO lines helps rule out clonal variation effects. We showed that two independent VPS13D KO clones exhibited similar phenotypes. We now specify which, if not both, KO clone is used in each experiment.

5. The stochastic peroxisome loss observed in VPS13D-KO cells and the patient's fibroblasts seems to resemble the previously described peroxisome mosaicism observed in Zellweger spectrum cells with less severe mutations. Culturing such cells at elevated temperature often results in increased peroxisome deficiency. Has this been studied in these cells?
Thank you for this helpful suggestion. We cultured VPS13D KO cells and human patient fibroblast cells at 40°C but did not observe increased peroxisome deficiency. One prior paper reported the exacerbated aberrant peroxisome phenotype in PEX11β patient fibroblast (Ebberink et al, 2012, J. Med. Genet.) at 40°C is due to a decrease of PEX11γ protein stability. Therefore, such temperature-sensitive phenotypes may be specific to certain proteins.

6. What could be the role for VSP13D in peroxisome biogenesis and (how) could it be similar as for mitochondria?
VPS13D does not affect mitochondria biogenesis but mainly the morphology. Mitochondrial contact sites with other organelles are well studied and it was revealed in yeast that the single Vps13 gene compensates for ERMES loss. We speculate based on structural similarity of VPS13D to Atg2 and interorganelle contact studies that lipid transport may be involved in peroxisome biogenesis and that peroxisomes are more dependent on this process than mitochondria. VPS13D is unique among
mammalian VPS13 isoforms in having a UBA domain, that we now also link to peroxisome biogenesis in the revised manuscript. Thus, the unique role of VPS13D relating to VPS13A-C may involve ubiquitin binding. This is now mentioned in the discussion.

Reviewer #3 (Comments to the Authors (Required)):
The Vacuolar Protein Sorting 13 (VPS13) gene family has one member in the yeast Saccharomyces cerevisiae and four members (VPS13A-D) in mammals. VPS13 in yeast has been implicated in sporulation, phospholipid regulation, and mitochondrial integrity. There is limited information on the roles of VPS13A-D, and especially of VPS13D, although mutation of these genes have been implicated in a variety of neurological disorders (mutation of VPS13D has been implicated in recessive spinocerebellar ataxia) and in protein trafficking defects from membrane-bounded compartments, e.g. mutation in VPS13B compromises protein trafficking from the Golgi complex. In this manuscript, Baldwin and colleagues purport to demonstrate a role for VPS13D in peroxisome biogenesis in mammalian, i.e. human, cells. There are a number of issues with the findings presented in this paper to challenge such a conclusion.

1) Fig. 1C. VPS13D knock out (VPS13D KO) HeLa cells show no evidence of any peroxisomal structures marked by the peroxisomal membrane marker PMP70 in two of the four cells shown. This reviewer has not seen such a scenario except for cells knocked out for PEX3 and PEX19, which act at the ER to initiate pre-peroxisome biogenesis. The result presented here is especially surprising given that the authors conclude by subsequent experiments that VPS13D acts in peroxisome biogenesis downstream of PEX3 and PEX19. The absence of a PMP70 signal in some VPS13D KO cells may be artefactual or evidence of dying cells. That VPS13D KO cells are at different stages on their way to death could also explain the mosaicism in peroxisomal profiles observed in VPS13D KO cells.

We appreciate the reviewer finding our results surprising. As we agree and definitively show this is a robust and clear conclusion not connected to cell death, we are motivated to resubmit a revised manuscript. In context of the reviewers point about the scenario of PEX3 and PEX19 KO cells and the ER, the recent De Camilli preprint on VPS13D fits well with the authors comment. Since PEX3/19 KO cells grow normally with the complete loss of peroxisomes, we don’t think the absence of PMP70 signal in some VPS13D KO cells is evidence of dying cells. We see no difference in cell viability of
VPS13D KO cells compared to WT cells. In addition, our long-term clonogenic assay also failed to detect substantial cell growth defects as a whole in the population directly linked to peroxisomal loss phenotypes. Furthermore, to verify if basal cell death occurs in VPS13D KO cells, we performed Cytochrome C staining and did not observe signs of apoptosis in VPS13D KO cells or in PEX5 KO and PEX19 KO cells.

Live imaging and tracking of dividing cells in the manuscript videos also show cells lacking peroxisomes can divide and thus are not dying. One example of this is shown in Figure R1, below, where we highlight an example of cytosolic GFP-CAT in VPS13D KO cells dividing over a 20-hour period of live imaging (the images are pulled from movies with images taken every 10 min, validating these are dividing cells). Longer-term tracking of GFP-CAT VPS13D KO cells indicates that some VPS13D KO cells without peroxisomes gradually die more often than cells with normal peroxisomes, indicating that peroxisome-less VPS13D KO cells might be more sensitive to environmental stress (See Fig 3A). However, as evidenced by cells with cytosolic catalase dividing, the peroxisome phenotype in VPS13D KO cells is not a consequence of cell death. In contrast to previous reports, we also observed that a large proportion of PEX14 KO cells completely lose peroxisomes instead of displaying a ghost phenotype (See Fig S4C). In addition, if the heterogeneous peroxisomal phenotype is caused by artifacts or cell death, subcloning of the VPS13D KO cells should purify the phenotype or the phenotype should be weakened by long term culturing as the dying peroxisome-less cells should die out of the population. Stochastic peroxisomal phenotypes are also found in our PEX 14 KO cells and also reported in VDAC2 KO MEF cells (Hosoi et al. 2018), showing that the stochasticity is not unique to VPS13D KO cells.
Fig R1. Overnight tracking of GFP-CAT localization in VPS13D KO cells. VPS13D KO (#45) cells stably expressing GFP-CAT were imaged overnight. Shown are selected frames from 0, 560, and 1200 minutes (left to right). Each solid arrow at time 0 indicates an individual cell. The dashed arrows at subsequent timepoints represent the progeny of the parent cell at t0 (parent and daughter cells have same color arrow). The blue and pink arrows each highlight cytosolic catalase cells that divide into daughter cells also lacking peroxisomal CAT signal by time 560 min. These daughter cells stay alive throughout the observed time period. Also note, at time 1200, the yellow asterik points to a cell that appears to have progressively lost peroxisomes from the initial to final timepoints.

2) Catalase (CAT) is targeted to the peroxisomal matrix by a weak peroxisomal targeting signal type 1 (PTS1), and its targeting to the peroxisomal matrix has been shown to be reduced in aging cells or cells exposed to stress. Thus, CAT is not the best marker to use to measure the extent of peroxisomal matrix protein import. Thus, a cytosolic localization of CAT in some VPS13D KO cells as seen in Fig. 1C could again be evidence of stressed cells or cells on the way to death. The same can be concluded for patient LUB1.1 fibroblasts (Fig. 5).

We appreciate this point. We have also done PMP70 staining and consistently found that VPS13D KO cells with cytosolic catalase cells are always PMP70-negative. We also found that PEX14 is mislocalized to mitochondria in VPS13D KO cells lacking peroxisomes, which is additional supporting evidence of peroxisome absence. Therefore, we are confident with the conclusions. In addition, as suggested, we repeated many experiments with PMP70 staining.

3) Fig. 3G. Overexpression of peroxisomal membrane proteins PEX3, PEX14 and PEX16 leads to the absence of any peroxisomal structures marked by PMP70 in a subset of cells that, quite unexpectedly,
seem to be in patches. This reviewer has again not seen such a scenario, which may again reflect a simple artefact of culture or evidence of cells on their way to death.

It is indeed surprising to see complete peroxisome loss in PEX3/14/16 OE cells. We noticed that previous papers only used transient transfection for OE whereas here we generated stable overexpression with virus infection. To avoid the effect of any tagging, we used untagged PEX genes for OE together with GFP-SKL as a reporter. As a control, PEX19 OE does not cause such defects, ruling out the possibility of artefacts in our cultures. We also show these cells are capable dividing (yielding patches or groups of cells as noted by the reviewer), thus they were not on there way to death. In addition, we did the same experiments in 293T and U2OS cells and observed similar phenotypes. Moreover, we did such in ATG8 6KO cells as well to rule out enhanced pexophagy.

4) Fig. 3, A and D. Why is GFP-Ub-VPS13D found mostly in nuclei, when VPS13D-i-GFP is found at the Golgi (Fig. S4A)?

This is now Fig 4A, D. Please notice that GFP-Ub-VPS13D is cleaved by endogenous deubiquitinases to produce GFP-Ub and VPS13D as distinct unconnected proteins. It is a clever way to express an untagged VPS13D but with a GFP-Ub reporter for transfected cells. GFP-Ub can be detected in nuclei and does not reflect the localization of VPS13D. However, VPS13D-i-GFP is a fusion protein where GFP is inserted internally to VPS13D protein (replacing exon 40) and GFP signal will reflect VPS13D localization.

5) The quantification of peroxisome absence in PEX26 overexpressing WT and ATG8 6KO cells (Fig. 3F) does not jive with the corresponding microscopic images in Fig. 3G.

Thank you, we have now corrected this error previously in Fig. 3E,G now Fig5A, 5B.

6) Fig. 3G. It is important to have the microscopic images for WT and ATG8 KO cells overexpressing PEX19.

We have provided the images now in Fig 5A.

7) Fig. 4A. It is unexpected that VPS13D is found at the Golgi complex and not at peroxisomes or at the ER if it has a role in peroxisome biogenesis.

We don’t know how exactly VPS13D regulates peroxisome biogenesis as well as mitochondrial
morphology. The extremely low expression of both endogenous and exogenous VPS13D is a barrier to examine the proper subcellular localization of VPS13D. Another complicating factor is that VPS13D OE interferes with peroxisome biogenesis. Hence, it is possible that VPS13D is indeed localized to peroxisomes but OE prevents it from detection there. In addition, VPS13D sublocalization could be a dynamic process as it is enriched on the Golgi apparatus with variable intensity and mainly in the cytosol. Such subcellular localization pattern on the Golgi apparatus is reported in the latest study from the De Camilli lab. They get better VPS13D expression signal with higher microscopic resolution to show that VPS13D is also weakly around mitochondria as well as ER and can be recruited to mitochondria and peroxisomes by GTPase Miro. We have now discussed their findings in the discussion. Finally, we performed subcellular fractionation experiments and found that VPS13D is present in both heavy membrane (mitochondria and ER-enriched) and light membrane (lysosomes/endosomes/peroxisomes) fractions (Fig. S3B).

8) Fig. 6. The authors should present a time course of microscopic images in panel D that corresponds to the data in panel E.

We agree and improved Fig 8D to include images for all four timepoints

ADDITIONAL POINTS:

1) p. 2, lines 20 and 21. That over 30 PEX genes involved in peroxisome biogenesis have been identified in mammals is patently incorrect.

We are sorry for this mistake, it was an error linked to yeast PEX genes and have now corrected it.

2) p. 4, line 67. "...Ypt35p, Spo71p and Mcp1p...".

No longer relevant as we removed that sentence.

3) p. 5, line 98. PEX14 is NOT a tail-anchored protein.

We have corrected this error. We meant to say PEX14 is a single spanning transmembrane protein.

4) p. 7, lines 161 and 162. PEX19 is NOT found in the peroxisomal matrix. It is the PMP receptor found in the cytosol!

We have corrected this error. See line 279 pg 11.
5) p. 17. Legend to Fig. 3. Insert '(E)' before 'Manual count of...'.
Fixed. Now Fig 5.

6) p. 17. Legend to Fig. 4, 2nd line. Remove 'used'.
Fixed. Now Fig 6.

7) p. 18. Legend to Fig. 6. Asterisks associated with p values do not jive between the legend and the figure. 'Mean number of cells per time point is (x ÷ n) and not (x - n) as in the legend.
Fig 6 is now Fig 8 – We updated Figure legend 8D with the missing p-value. Revised Fig 8D legend and the x/n description is changed to “Total number (over all timepoints) (n) and mean number of cells per timepoint (n,) in each group: PEX19 KO #8 n= 252....”

8) p. 19. Legend to Fig. S2. LAMP2 in the legend, LAMP1 in the figure. Correct.
Yes, thank you, we fixed that error- legend now says LAMP1.

9) Fig. S2. Label the figure with the organelles that the different markers detect.
We labeled the organelles in Fig. S2 accordingly.
February 12, 2021

RE: JCB Manuscript #202001188R-A

Dr. Richard J Youle
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NINDS
35 Convent Drive MSC 3704
Bethesda, MD 20892-3704

Dear Richard,

Thank you for submitting your revised manuscript entitled "VPS13D promotes peroxisome biogenesis". You will see that one reviewer recommended publication, while the other referee still had concerns about the lack of understanding of VPS13D's function and how loss of VPS13D affects peroxisome formation. We do feel that the work will be a significant advance for the field without more mechanism so we are prepared to move forward with publication without analyses of VPS13D function (e.g., in lipid transfer) in the context of peroxisome biogenesis. However, the text edits requested by Rev#2 seem reasonable and would enrich the discussion. In confidential comments to the editors, one referee additionally requested quantifications of the data shown in R1 in the rebuttal, tracking cells for viability. We agree that sharing these data in the paper with more information about their robustness is reasonable.

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Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

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

Reviewer 1:

The authors have tried to address the points that had been raised in the first review. They corrected the mistakes concerning certain published background information, references and nomenclature. They also clarified some methodical aspects that had not been clear before and added some more experimental controls where asked for. However, the main problem remains: In the first manuscript version the authors had found an effect of the VPS13D mutant on peroxisomes by describing that overexpression of VPS13D results in a reduction of peroxisome number, while the loss of VPS13D results in morphologically different peroxisomes. In the revised version the authors still find no indication how this phenotype might be explained or if it is a direct effect at all. They do find that the loss of VPS13D slows down the rescue of peroxisome formation by exogenous PEX19. However, this contradicts the initial phenotypes described in the study and does not show that VPS13D specifically influences PEX19-function. Any
other pleiotropic mutant could have the ability to slow down PEX19-recovery. It would have been interesting to test also for the influence on the recovery time of other peroxins. The authors try to argue that VPS13D might be involved in lipid transfer, because other VPS13-family members have this function at organelle-contact sites. The authors find VPS13D to localize to the Golgi but not peroxisomes. They argue that the signals might be too weak for the detection and cite a paper from the pre-print server BioRxiv, which has not been peer-reviewed yet. In this paper the De Camilli group describes a targeting of VPS13D to mitochondria via Miro and a targeting of VPS13D to peroxisomes, when a splicing variant of Miro is expressed. This group does not comment on the effects on peroxiome biogenesis. However, citing published and non-published data from other groups does not help to fill the gaps in the data set and argumentation of the Youle group manuscript and especially does not help to understand the observed effects on peroxisome number and morphology.

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

The authors addressed most of the concerns of reviewer 2 and 3 adequately and significantly improved the quality of the manuscript.

A few minor comments to consider:
1. re. Reviewer 2 comment 1. De researchers used the mis-localization of PEX14 to mitochondria as a sort of validation for the absence of PMP70, which seems adequate. They mentioned not to have found other PMPs that can be used in IF. For future studies they may want to consider antibodies against ABCD1 and ACBD5, that have been used for IF in previous reports. In particular the latter PMP is very abundant and easily detected.
2. re. Reviewer 2 comment 5. The temperature-sensitive peroxisome mosaicism is not only reported for PEX11B patients, but also a well known phenomenon for hypomorphic PEX mutations. The finding that the stochastic loss in VPS13-D KO cells is insensitive to elevated temperatures may be a relevant observation, if not future clue, for the underlying mechanism and thus should be mentioned in the Discussion.