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Recruitment of Peroxin 14 to lipid droplets affects lipid storage in *Drosophila*

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MS TITLE: Recruitment of Peroxin14 to lipid droplets affects triglyceride storage in Drosophila

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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 indicate that the manuscript addresses an important question and explores interesting new cell biology. However, the reviewers raise a number of 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. In particular, reviewer #1 suggests several experiments that would provide additional support regarding the localization of the subcellular localization of Pex proteins. While a complete understanding of the mechanism of regulation of lipolysis is likely out the scope of this manuscript, these additional experiments related to localization seem reasonable. Reviewer 2 provides useful suggestions on the presentation of the manuscript as well as the need for statistics for certain experiments and improved images for specific experiments.

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

This manuscript entitled “Recruitment of Peroxin14 to lipid droplets affects triglyceride storage in Drosophila.” by Anderson-Baron et al. describes the relocalization of peroxisomal proteins to lipid droplets under certain metabolic conditions in Drosophila as well as in mammalian cells. Dual targeting of proteins to distinct organelles is implicated in controlling inter-organelle communication and cellular adaptation to varying nutrient supply. Therefore, this manuscript addresses an important and relevant question that is of potential interest to a broad readership.

This manuscript describes a couple of interesting observations: RNA-SEQ analyses of S2 cells revealed that excess of free fatty acids results in the upregulation of pex14. Elevated Pex14 mRNA levels persisted also under lipolytic conditions. Fat-body-specific knock-down of Pex14 results in less buoyant larvae, less triglyceride storage in lipid droplets and increased lipolysis. These data correlate with a decreased survival rate of larvae that were cultured in presence of excess dietary fat when Pex14 was depleted. The authors claim that Pex14, as well as Pex3 and Pex13, relocalize from peroxisomes to lipid droplets upon oleate treatment of cells. Overexpression of Lsd1 blocks Pex14 (as wells as PEX3 and PEX13) localization to lipid droplets. Overexpression of Pex14 leads to decreased recruitment of the lipase Hsl to lipid droplets. Together, the authors propose a model in which newly synthesized Pex14 is diverted at the ER from the conventional peroxisome biogenesis pathway and targeted to the lipid droplet, where it blocks the recruitment of Hsl and in turn lipolysis of triglycerides. They furthermore suggest that Lsd1 modulates the lipid droplet localization of Pex14.

Comments for the author

The presented observations are interesting and the proposed model is appealing. The manuscript, however, does not address the molecular mechanisms underlying such a pathway. Some of the conclusions are not fully supported by the provided data and the discussion is rather speculative and raises a lot of questions. As outlined below, it would be important to provide additional controls and data to support the current conclusions. Additional experiments would be required to gain mechanistic insight into this phenomenon.

Major points:
1. My main concern about this study is the interpretation of the subcellular localization of Pex14 (and Pex3, Pex13). The data show that Pex14 localizes to non-peroxisomal structures that are in close proximity to lipid droplets when cells were treated with oleate. It would, however, be essential to further analyze this localization. The authors discuss that a direct insertion of the Pex proteins into the phospholipid monolayer of lipid droplets is unlikely because they are supposedly transmembrane proteins. The topologies of Pex14 and Pex13, however, are controversially discussed in the literature and it is important to know in which topology the proteins reside in the structure they observe. Are the proteins integrated into membranes at all or rather peripherally associated with membranes? Alkaline carbonate extraction and protease-protection experiments would shed light on this aspect. Are these structures indeed related to pre-peroxisomal structures as the authors suggest? In such a scenario, PEX14 would not be recruited to LDs as the title states.

A lipid droplet protein marker, instead of a lipid dye, should be used to assess the colocalization of Pex14 with the LD surface. Ideally super-resolution microscopy should be applied to determine the degree as well as the quality of colocalization with the lipid droplet membrane.
In figure 2, the colocalization data of PEX14 with LDs are somewhat confusing, potentially misleading. Figure 2N-P show maximum intensity projections of colocalized pixel. Figure 2Q shows a quantification of the PEX14 colocalization with LDs that is independent of the SKL-signal, meaning those PEX14 signals that do not colocalize with peroxisomes. How is "%" defined/calculated in this graph? From the images shown, it does not appear that up to 100% of PEX14 signals that do not colocalize with peroxisomes do instead colocalize with LDs. There are many PEX14-positive / SKL-negative puncta that also do not colocalize with LDs. Which fraction of total PEX14 pixels is SKL-negative and LD-positive? Is it significantly more than 10%? In the methods section it is stated that random/background colocalization was up to 10%. Panels 2D-F show only maximum intensity projections and no quantifications, which is not meaningful for conclusions about colocalization. Similarly, in Figures 4H-M, the authors only show maximum intensity projection to assess colocalization of different PEX14 truncation mutants with LDs. It is very hard to assess colocalization and whether these few images support their statements and conclusions. Quantifications and a LD protein marker should be used included.
Further colocalization experiments with different organelle markers are necessary to define whether the lipid droplet associated structures may be derived from the ER membrane where also pre-peroxisomes originate. Recent studies have shown that LDs can be enwrapped by ER membrane and that proteins embedded into this ER can be mistaken by LD-localized proteins by fluorescence microscopy. The TEM quality is too poor to allow conclusions on whether the labeling marks the phospholipid monolayer of the lipid droplets or rather additional (ER) membranes that are in close proximity to the lipid droplet surface. Tomography would help to differentiate these possibilities.
On page 14 the authors state "PEX14 was associated with membranes, including those of presumptive LDs or LD-associated vesicles…"", which unfortunately does not help in their interpretation.
The authors also use biochemical fractionation to determine lipid droplet association of Pex14. The Western Blot in Figure 3Q is supposed to show that overexpressed PEX14 resides on isolated LDs. However, the quality of the LD isolation procedure is not demonstrated. Are these fractions free of other contaminating organelles such as ER or peroxisomes? Additional organelle markers should be used in the Western Blot in order to verify that specifically PEX14 is recruited to LDs. This could also provide further insight into which membranes PEX14 is in fact inserted. Does endogenous PEX14 reside in these LD fractions? It is not clear why the authors chose to switch to tagged and overexpressed PEX14. Pex6 and Pex10 should also be included in the analysis of the lipid droplet preparation as a negative control since the authors claim that these proteins do not partition lipid droplets.
The experiment in Figure 3S is of very poor quality. 7 lanes show a signal derived from anti-myc antibodies but only 4 lanes are labeled with text. Are the signals in the other lanes a spill-over? Again, the quality of the LD fractionation was not assessed and it is unclear whether PEX14 indeed specifically co-purifies with LDs.

2. The rationale for many experiments is not clear and some statements appear random.
- For example, on page 11 line 234 "CytC and SKL signal does overlap in some places in the cytosol, but not adjacent to LDs (Figure 4C)". What does this mean and why is it relevant? This panel is derived from PEX19 KO cells, which do not have mature peroxisomes. Why is colocalization of diffuse SKL staining and mitochondria assessed? Similarly, on page 11, line 235: "…in PEX16 RNAi knockdown cells (+oleate) PEX14 does not localize to peroxisomes…." As these cells do not have any peroxisomes also no colocalization with peroxisomes can be assessed.
- In Figure 4F the authors conclude that "PEX14 was largely unassociated with CytC but very strongly localized to LDs". The SKL signal shows a very similar pattern as the PEX14 staining. Does this mean that soluble peroxisomal proteins are specifically recruited to the same structures as PEX14?
- The rationale for the experiment in Figure 3R is not clear. "Pulse-chase radioactive protein labelling showed multiple newly synthesized proteins are recruited to LD in -Oleate cells". What is the conclusion the authors would like to draw from this with respect to PEX14?
3. In order to gain insight into the molecular mechanism on how Pex14 inhibits lipolysis in lipid droplets, additional experiments are required. Does Pex14 directly interact with Lsd1? Which domains of the proteins are involved and would that be in line with the membrane topology of Pex14 (compare also point 1)?

4. Are the observed phenotypes direct and specific for Pex14? In some of the experiments the authors also analyze Pex3 and Pex13 and observe similar relocalization phenotypes as for Pex14. Could these proteins also be involved in the modulation of lipolysis?

Minor points:
1. In Figure 5, the authors conclude that “myc-PEX14 was recruited to LDs even if Bmm or Hsl levels were also elevated”. The PEX14 signals in panels B and D look very different from each other and it is surprising that there should be a similar degree of colocalization between PEX14 and LDs in these cases.
2. Page 10 line 203 “This suggested Pex14 regulation of lipolysis of TGs that was separable from its function at peroxisomes” How do the authors know that this effect is independent of peroxisomes? Upon PEX14 knock-down also the number of peroxisomes is strongly decreased under lipolytic conditions, which could likewise affect TG lipolysis.
3. A more precise and scientific language would improve the manuscript. For example, on page 12 line 258 “The mRNA encoding…(Hsl) was also relatively much higher in S2 cells …” What means “relatively much higher”?
The terms “dependent” and “independent” seem to be inadequately used throughout the manuscript. For example, on page 11 line 230 “… PEX14 surrounding LDs was independent from a mitochondrial marker…”. No dependencies but only colocalizations were assessed in these experiments.
4. The manuscript would strongly benefit from language, grammar and spell checking.
5. In the results section, references to the figures should be checked and corrected (page 10 line 205: “2J” should be “3J”

Reviewer 2

Advance summary and potential significance to field

Anderson-Baron and colleagues investigated the role of Peroxin proteins on lipid storage using Drosophila larvae and cells. Through a small-scale RNAi based screen, they identify few Peroxin proteins whose knock-down affects lipid storage in larvae. In subsequent experiments, they investigate the relation between these Peroxin proteins (focusing on Peroxin14) and the lipid storage organelles, called lipid droplets. As a main conclusion, they present data supporting a function of selected Peroxin proteins in limiting lipolysis.
Overall, the manuscript touches an interesting field of cell biology and provides further information on the interaction between Peroxins and lipid droplet function. The writing of the manuscript as well as the way the data is presented, however, needs to be improved as outlined below to allow for a thorough evaluation.

Comments for the author

Major comments:
- Altogether, the figures are very busy, crowded and complex (with the peak for Fig. 3 with panels labeled up to “X”). The authors only used one supplemental Figure. The authors should, for example, consider moving some/all of the single channel representations to the supplement and only show the merged images in the main figure. While I consider this as not optimal as information gets moved away from the main part, the number of images was at least for me distracting. Further, individual images got very small. As the authors look at small subcellular signals, this makes interpretation more difficult than necessary. The use of insets with zoom-ins to highlight the respective findings would certainly be helpful. Further, the single channel images are sometimes shown in greyscale and sometimes in color (e.g. Fig. 2 / Fig. 4). The authors need to decide for one presentation style (I prefer greyscale images throughout for the individual channels). Further, I think JCS allows 8 Figures, so authors could split Figures apart to make the information more easily accessible.
Lastly, the authors should consider using a color-blind safe palette for the merged channel images.
- In their RNAi screen, the authors experienced varying efficacies in the knock-down of the Pex protein encoding transcripts (Fig. S1). The old rule of thumb is that a knock-down by 70% of the transcript is counted as a successful RNAi. The authors might consider labeling in Fig. 1C which conditions resulted in such a knock-down magnitude and which conditions only reduced transcript

levels to a lower extent. As I am not aware of the similarity of the different Pex proteins (on sequence level) another parameter to consider is the specificity of the knock-down. The authors could add a statement concerning the sequence similarity of the Pex proteins in e.g. the introduction.

- The supplemental tables are very minimalistic. Please add for Table 1 an extra sheet with the explanations of the different work sheets as well as at least the explanation of the most important column headers. Supplemental Table 2 would also benefit from additional information. "[empty field – no data available]" entries present in Supplemental Table 2 are strange and should be explained.
- When I was looking at the sequencing data in Supplemental Tables 1 and 2 (individual gene as well as GO-term level) I was surprised to find basically no lipid metabolism / lipid storage / lipid droplet-associated genes to be regulated. How did the authors perform this experiment? In the main text, they write: "RNA sequencing was used to compare cells cultured in Schneider's medium
+ FBS (Standard) versus +Oleate conditions." and a similar statement is found in the methods section. As is, it is unclear what exactly the RNAseq data is investigating (timeline?). In Fig. 2B also the y-axis label is missing.
- In Fig. 1I no statistics analysis for the growth curves is provided.
This needs to be added.
- The writing should be improved. At the current state, there are a larger number of typos and glitches (such as e.g. missed brackets) present as well as readability could be improved as there are many single statement sentences which are not clearly connected and wordiness examples (e.g. Page 10 line 106:
"..approximate half-life of approximately…"). In the introduction, redundancies need to be removed.
- The quality of the images is different across the figures and should be homogenized & improved. Images in panels V and W of Fig. 3 look distorted. In Fig. 4 the LipidTOX stain is often very ill defined and not clear for the lipid droplets (e.g. panels B, E, I). Fig. 6 panels K and L (EM images) also seem distorted. Please correct / replace.
- In Figure 4, the SKL signal looks very different as compared to other panels (e.g. panels B, C, D in Figure 4 compared to panels A, B, C in Figure 3).
As the authors use this signal to quantify multiple phenotypes, I consider this problematic.
- In Fig. 2 the localization quantification should also be re-checked and/or presented differently. For Pex14, there seems to be more signal in panels O & P. Further, co-localization signals are also present, where no signal can be seen in the original image (e.g. Fig. 2O, leftmost lipid droplet). For example for the shown examples, not only the correlation results, but also the primary segmentation results should be included (supplement?).

Minor comments:

- In Figure 4, the DAPI signal in panels E and F is not mentioned in the text / legend.
- Page 11, line 228: "…were localized to peroxisomes (Figure 2Q)."  this is a plot and subset information can not be deduced?
- In Supplemental Figure 1, the labeling of the Pex3 RNAi clone GD2464 seems incorrect (panel D). Please correct / explain. What do the asterisks in panels I) and T) stand for? I guess thus is the same meaning as in Fig. 1.
Please add.
- On page 7, the authors mention that "Third instar larvae were raised on a chemically defined (holidic, (Piper et al., 2014) diet where the only added lipid was cholesterol as Drosophila are cholesterol auxotrophs. (Vinci et al., 2008)." In principle this is true, but the holidic diet also contains choline which is an important / crucial precursor for e.g. phospholipid synthesis as well as choline also affects lipid metabolism profoundly. Thus, I recommend a different wording.

First revision

Author response to reviewers' comments

Specific responses to Reviewer (1)

(1) My main concern about this study is the interpretation of the subcellular localization of Pex14 (and Pex3, Pex13). The data show that Pex14 localizes to non-peroxisomal structures thatare in close proximity to lipid droplets when cells were treated with oleate. It would, however, be essential to further analyze this localization. The authors discuss that a direct insertion of thePex proteins into the phospholipid monolayer of lipid droplets is unlikely because they are supposedly transmembrane proteins. The topologies of Pex14 and Pex13, however, are controversially discussed in the literature and it is important to know in which topology the proteins reside in the structure they observe. Are the proteins integrated into membranes at all or rather peripherally associated with membranes? Alkaline carbonate extraction and protease-protection experiments would shed light on this aspect. Are these structures indeed related to pre-peroxisomal structures as the authors suggest? In such a scenario, PEX14 would not be recruited to LDs as the title states.

We appreciate this suggestion and have added new data to directly address his point in the revised manuscript. In *Pex19* CRISPR KO cells, superresolution microscopy indicates Pex14 overlaps a second marker that inserts into the LD surface (Livedrop, new Figure 4F). We isolatedLDs from *Pex19*KO cells in isotonic and alkaline carbonate treated conditions and found that Pex14 remains in the LD fraction as does the a large proportion of perilipin Lsd-2 while Cnx99A, (ER) was reduced. Also, CytC (mitochondria) did not co-fractionate with our LDs (new Figure 2I- J). Given that 1) truncation mutations of Pex14 show that a very small region surrounding the transmembrane domain that mediates peroxisome bilayer insertion is sufficient to mediate localization to LDs; 2) RNAi knockdown of *Pex3*, *Pex16* and *Pex19*, and CRISPR knockout of *Pex19*, encoding the key cellular machinery need to insert Pex14 into the peroxisome bilayer membrane enhances LD localziaton of Pex14 and 3) the retention of Pex14 in the LD fraction in alkaline carbonate treated lysates, in the revised manuscript we now favour a model whereby Pex14 is associating with the LD surface directly. We expand on this model in the discussion showing that the Pex14 transmembrane region contains an predicted amphipathic structure (new Figure S5) that could plausibly interact with the LD hemi-membrane. Also, given the relatively small size (71aa) of the Pex14 (76-147)that is clearly sufficient for LD localization, thatprotease protection assays would be relatively uninformative on top of our existing data. The next logical step would be prospective, careful, single amino acid mutation of this predicted amphipathic domain, but given the limited timeframe for revisions we feel strongly that this is outside the reasonable scope for this manuscript and will be addressed in future work that will likely take a year or more to complete.

(1) A lipid droplet protein marker, instead of a lipid dye, should be used to assess the colocalization of Pex14 with the LD surface. Ideally super-resolution microscopy should be applied to determine the degree as well as the quality of colocalization with the lipid dropletmembrane.

We appreciate this suggestion. After 5 months of COVID-interrupted work we have developed stable S2 lines (clonal) that constitutively express Livedrop-eGFP. We have used this protein marker for the LD surface in live cells for our revised/replicated structure-function analysis of known Pex14 domains (new Figure 6) as well as new data showing the effects of knockdown ofother Pex genes (new Figure 7) and a survey of the recruitment of all the conserved DrosophilaPex proteins to the LD surface when cells were cultured in excess oleate (new Figure 8). In addition, in our revised manuscript we now also employ (Monodansylpentane - AUTODOT, Abcepta, Chen et al. Methods in Molecular Biology 1560) that in our hands gave less background than LipidTOX. We have also re-analyzed our localization data avoiding any use ofco-localization with LipidTOX and AUTODOT. We now employ a quantification method that measures signal to a narrow 'shell' outlining the region surrounding the neutral lipid dye signaland reserved co-localization for signals overlapping LiveDrop which directly demarcates the LDmembrane surface.

We thank the reviewer for their suggestion to use superresolution imaging. Although COVID restrictions restricted our access to these facilities, we were able to use STED imaging to show that Pex14 co-localizes with Livedrop and that there is ER-associated (Cnx99A) and independentPex14 surrounding LDs at 30 nm resolution (new Figure 4).

(1) In figure 2, the colocalization data of PEX14 with LDs are somewhat confusing, potentially misleading. Figure 2N-P show maximum intensity projections of colocalized pixel. Figure 2Q shows a quantification of the PEX14 colocalization with LDs that is independent of the SKL- signal, meaning those PEX14 signals that do not colocalize with peroxisomes. How is "%" defined/calculated in this graph? From the images shown, it does not appear that up to 100% of PEX14 signals that do not colocalize with peroxisomes do instead colocalize with LDs. There are many PEX14-positive / SKL-negative puncta that also do not colocalize with LDs. Which fraction of total PEX14 pixels is SKL-negative and LD-positive? Is it significantly more than 10%? In the methods section it is stated that random/background colocalization was up to 10%. Panels 2D-F show only maximum intensity projections and no quantifications, which is not meaningful for conclusions about colocalization.

We now include new data showing co-localization with Livedrop, a LD protein marker. In our revised manuscript we have also re-analysed any data where we marked LDs using neutral lipid dyes. For all our co-localization assays we perform 3-dimensional deconvolution using a calculated PSF as well as calculated (average) background subtraction. The software we use (Huygens) also corrects for uneven lighting (jitter) between stacks and chromatic aberration if present. We acknowledge that our previous estimations of co-localization with a lipid dye wereprobably an underestimate as Pex14 is on the surface of lipid droplets. Thus, in our revised manuscript we employed a revised estimation of 'signal adjacency' which is possible as most lipid droplets are approximately spherical. To calculate adjacency, we segment the LipidTox or AUTODOT stained spheres in a 3-dimensional volume for each LD in the volume. We then expanded the selection by the number of voxels that represented 1% of the smallest dimensionof the volume. In most cases, that was 6 voxels in each direction. The region encompassing thevolume from the original LD border to the expanded selection defined as the 'shell' volume encompassing the region adjacent to the lipid droplet. The percentage proportion of Pex14, Bmm or Hsl positive voxels (signal above local background) within this shell region compared tothe total signal within the entire volume is now reported as "% adjacent". Where appropriate (e.g. old Figure 2 D-F / new Figure 2 E-G) in the revised manuscript we now also include images representing a single plane at the midpoint of the Z-dimension (Mid) of the image stack as well as the maximum intensity projection (MIP).

Similarly, in Figures 4H-M, the authors only show maximum intensity projection to assess colocalization of different PEX14 truncation mutants with LDs. It is very hard to assess colocalization and whether these few images support their statements and conclusions. Quantifications and a LD protein marker should be used included.

The Pex14 truncation mutant experiments were replicated in their entirety in live cells (newFigure 6) comparing them to GFP-tagged Livedrop, a lipid droplet protein marker.
Representative single midpoint plane (Mid) and maximum intensity projections (MIP) images are now provided. The degree of spatial colocalization was assessed using Pearson's correlation coefficient analysis.

(1) Further colocalization experiments with different organelle markers are necessary to define whether the lipid droplet associated structures may be derived from the ER membrane where also pre-peroxisomes originate. Recent studies have shown that LDs can be enwrapped by ER membrane and that proteins embedded into this ER can be mistaken by LD-localized proteins byfluorescence microscopy. The TEM quality is too poor to allow conclusions on whether the labeling marks the phospholipid monolayer of the lipid droplets or rather additional (ER) membranes that are in close proximity to the lipid droplet surface. Tomography would help to differentiate these possibilities. On page 14 the authors state "PEX14 was associated with membranes, including those of presumptive LDs or LD-associated vesicles…"", which unfortunately does not help in their interpretation.
We thank the reviewer for this suggestion and in our revised manuscript we confirmed that the signal from ER marker Cnx99A (ER) is adjacent to LDs in Drosophila fat body (new Figure 1M) and S2 cells (new Figure 4 A-B, E-F). However, using super-resolution imaging as suggested allowed us to show that much of the Pex14 signal is distinct from the ER signal at 30 nm resolution and that this Pex14 signal overlaps markers of the LD-surface (new Figure 4F). EM tomography was attempted but any signal enhancement of the anti-PEX14 nanogold signal ledto high enough background levels that we could not be sufficiently confident in our findings.
Note that this played a large part in our delay in resubmitting our revised manuscript past theinitial

revision due date.

(1) The authors also use biochemical fractionation to determine lipid droplet association of Pex14. The Western Blot in Figure 3Q is supposed to show that overexpressed PEX14 resides on isolated LDs. However, the quality of the LD isolation procedure is not demonstrated. Are these fractions free of other contaminating organelles such as ER or peroxisomes? Additional organelle markers should be used in the Western Blot in order to verify that specifically PEX14 isrecruited to LDs. This could also provide further insight into which membranes PEX14 is in fact inserted. Does endogenous PEX14 reside in these LD fractions? It is not clear why the authors chose to switch to tagged and overexpressed PEX14. Pex6 and Pex10 should also be included in the analysis of the lipid droplet preparation as a negative control since the authors claim that these proteins do not partition lipid droplets

In our revised manuscript we have redone our LD fractionations in Pex19KO cells where peroxisomes were absent (new Figure 4A-B). In our re-done experiment we examined the levelsof endogenous Pex14 rather than tagged protein (new Figure 2I-J). This new data also includes control western blots of LD fractions (and pellet) showing the proportion of co-fraction of Lsd2 (LDs), Cnx99A (ER) and CytC (Mitochondria). *(1) The experiment in Figure 3S is of very poor quality. 7 lanes show a signal derived from anti-myc antibodies but only 4 lanes are labeled with text. Are the signals in the other lanes a spill-over? Again, the quality of the LD fractionation wasnot assessed and it is unclear whether PEX14 indeed specifically co-purifies with LDs.*

The reviewer was quite correct in their assessment that the unlabelled lanes arose from spillover of radiolabelled protein into empty wells. In the revised manuscript we include a new blot derived from samples from the same replicate where spillover is not present (new Figure 3N). In the revised manuscript we now include western blots of our LD fractions probing with ER, lipid droplet and mitochondrial markers to assess the relative quality of our LD fractionationprocedure (new Figure 2J).

2. The rationale for many experiments is not clear and some statements appear random.- For example, on page 11 line 234 "CytC and SKL signal does overlap in some places in the cytosol, but not adjacent to LDs (Figure 4C)". What does this mean and why is it relevant? This panel isderived from PEX19 KO cells, which do not have mature peroxisomes. Why is colocalization of diffuse SKL staining and mitochondria assessed? Similarly, on page 11, line 235: "…in PEX16 RNAi knockdown cells (+oleate) PEX14 does not localize to peroxisomes…." As these cells do not have any peroxisomes also no colocalization with peroxisomes can be assessed.

The reviewer is indeed correct about the misleading statement we made about the existence of functional peroxisomes in *PEX19* KO cells. As for *PEX16* RNAi KD cells, even with relatively robust 2-3 day RNAi treatment of *Pex* genes in S2 cells, in our experience we found that punctate SKL signals indicating peroxisomes can occasionally be seen. We have observed similareffects previously (Mast FD, Li J, Virk MK, Hughes SC, Simmonds AJ, Rachubinski RA. A Drosophila model for the Zellweger spectrum of peroxisome biogenesis disorders. Dis Model Mech. 2011 Sep;4(5):659-72.). The statement highlighted by reviewer #1 was an attempt toacknowledge these observations and to comment on Pex14 relative to these few, residual punctate SKL signals. We have altered the description in our revised manuscript to better describe this event and why it is relevant based on the reviewers' suggestions.

(2) In Figure 4F the authors conclude that "PEX14 was largely unassociated with CytC but very strongly localized to LDs". The SKL signal shows a very similar pattern as the PEX14 staining. Does this mean that soluble peroxisomal proteins are specifically recruited to the same structures as PEX14?
The similar pattern seen with SKL was due to the cross-talk from the Pex14 channel. The imagesin old Figure 4F have been replaced to show that Pex14 is strongly localized to the LDs away from CytC signal in peroxisome deficient cells (*Pex19*KO cell) indicated by cytoplasmic and nuclear mNeonGreen-SKL localization (new Figure 4 C-D).

(2) The rationale for the experiment in Figure 3R is not clear. "Pulse-chase radioactive protein labelling showed multiple newly synthesized proteins are recruited to LD in -Oleate cells". Whatis the conclusion the authors would like to draw from this with respect to PEX14?

The data shown in old Figure 3R-S are meant to be considered together. Old Figure 3R shows the total protein labelled by 35S pulse at the point when cells were transitioned from Standard to +Oleate culture conditions. We included this figure to illustrate that relatively little of the total newly synthesised protein appears in the LD fraction, even with culture in +Oleate conditions. Old Figure 3S represents an autoradiograph of immunoprecipitated Pex14 from the LD fraction showing that a large fraction of Pex14 newly synthesised at the time of transition between culture conditions is in the LD fraction. Due to the way the old Figure 3 was constructed this relationship may have not been sufficiently apparent. Improved replicates of this experiment are now included side by side as new Figure 3 M-N.

3. In order to gain insight into the molecular mechanism on how Pex14 inhibits lipolysis in lipid droplets, additional experiments are required. Does Pex14 directly interact with Lsd1? Which domains of the proteins are involved and would that be in line with the membrane topology of Pex14 (compare also point 1)?

Despite multiple attempts including APEX2 PLA and IP-MS experiments (not included in the revised manuscript) which did identify expected Pex14 interacting proteins like Pex5, we do not see direct interaction between Pex14 and Lsd-1. With our new/revised data (new Figure 2 I-J, Figure 4, and Figure 6) we now discount this model in the discussion in favour of a model whereby the region within the transmembrane domain of Pex14 (aa 124-141) interacts directly with the LD surface.

4. Are the observed phenotypes direct and specific for Pex14? In some of the experiments the authors also analyze Pex3 and Pex13 and observe similar relocalization phenotypes as for Pex14. Could these proteins also be involved in the modulation of lipolysis?

In our expanded screen for the role of Pex14 in fat body lipid storage (new Figure 1) we tested knockdown of multiple *Pex* genes including Pex3 and Pex13. In all cases the strongest effects are linked to *Pex14* knockdown with weaker effects caused by reducing *Pex3*. We also tested RNAi knockdown of the same Pex genes in *Pex19KO* S2 cells and again saw the strongest effects with Pex14 knockdown and aa weaker effect with *Pex13* knockdown, but not with *Pex3* knockdown (new Figure 7J-P). In addition, Pex13 also mitigated LD fragmentation when co- expressed with Hsl under Lipolytic condition (new Figure S4 I-J). Together, this data best supports a model whereby Pex14 is the primary effector of this process with the potential of Pex13 providing a supportive role.

Minor points raised by Reviewer #1

1. In Figure 5, the authors conclude that "myc-PEX14 was recruited to LDs even if Bmm or Hsl levels were also elevated". The PEX14 signals in panels B and D look very different from each other and it is surprising that there should be a similar degree of colocalization between PEX14 and LDs in these cases.

We apologise for this unfortunate issue that stemmed from how the images were converted into a compressed PDF format. We now include separate grayscale images for each channel to better illustrate the relative signal of Pex14 surrounding the LD. In the grayscale images it is clear that Myc-Pex14 surrounds LDs in both conditions.

2. Page 10 line 203 "This suggested Pex14 regulation of lipolysis of TGs that was separable from its function at peroxisomes" How do the authors know that this effect is independent of peroxisomes? Upon PEX14 knock-down also the number of peroxisomes is strongly decreased under lipolytic conditions, which could likewise affect TG lipolysis.

This suggestion was speculation on our part. However, we have now added a new piece of evidence suggesting that Pex14 promotes lipid storage during lipolytic condition in a peroxisome-independent manner in *Pex19* KO cells (new Figure 7 J-P).

3. A more precise and scientific language would improve the manuscript. For example, on page 12 line 258 "The mRNA encoding…(Hsl) was also relatively much higher in S2 cells …" What means "relatively much higher"? The terms "dependent" and "independent" seem to be inadequately used throughout the manuscript. For example, on page 11 line 230 "… PEX14 surrounding LDs was independent from a mitochondrial marker…". No dependencies but only colocalizations were

assessed in these experiments.

We have revised the text to indicate that we are referring to localization changes in terms of markers for these organelles. We have revised the manuscript text to ensure dependencies are described appropriately.

4. The manuscript would strongly benefit from language, grammar and spell checking.

This has been done.

5. In the results section, references to the figures should be checked and corrected (page 10 line 205: "2J" should be "3J"

This has been corrected.

Specific responses to Reviewer #2.

-Altogether, the figures are very busy, crowded and complex (with the peak for Fig. 3 with panels labeled up to "X"). The authors only used one supplemental Figure. The authors should, for example, consider moving some/all of the single channel representations to the supplementand only show the merged images in the main figure. While I consider this as not optimal as information gets moved away from the main part, the number of images was at least for me distracting. Further, individual images got very small. As the authors look at small subcellular signals, this makes interpretation more difficult than necessary. The use of insets with zoom-insto highlight the respective findings would certainly be helpful. Further, the single channel images are sometimes shown in greyscale and sometimes in color (e.g. Fig. 2 / Fig. 4). The authors need to decide for one presentation style (I prefer greyscale images throughout for the individual channels). Further, I think JCS allows 8 Figures, so authors could split Figures apart tomake the information more easily accessible. Lastly, the authors should consider using a color- blind safe palette for the merged channel images.

This manuscript represents the efforts of multiple different individuals and groups, often using different imaging systems. We apologise that in our previous submission we had left the figure formatting as it was provided. We now present the data in 9 figures to increase accessibility. Inthe revised manuscript we have revised all figures to be presented in a common style and where possible have increased the size of the individual panels. In particular, we have split ourconfocal and super-resolution imaging data (new Figure 4) over two pages to ensure that fine detail is not lost in the PDF conversion. We now present all images with individual single channels as greyscale. We have also re-processed all microscopy data and in doing so have converted each to a green/magenta/cyan colourblind friendly colour palette.

-In their RNAi screen, the authors experienced varying efficacies in the knock-down of the Pex protein encoding transcripts (Fig. S1). The old rule of thumb is that a knock-down by 70% of the transcript is counted as a successful RNAi. The authors might consider labeling in Fig. 1C which conditions resulted in such a knock-down magnitude and which conditions only reduced transcript levels to a lower extent. As I am not aware of the similarity of the different Pex proteins (on sequence level) another parameter to consider is the specificity of the knock-down. The authors could add a statement concerning the sequence similarity of the Pex proteins in e.g. the introduction.

In the revised manuscript, note that we have re-done all of the experiments depending on RNAi knockdown. For the animal data shown in Figure 1 and S1 (previously S1), all crosses were repeated with a r4-GAL4 driver line that now includes UAS-Dcr to increase the RNAi efficiency. RNAi knockdown has been verified using qRT-PCR and only those data where the efficiency was >70% is reported. For the cell-specific RNAi treatments, we employ dsRNAs from a master library that has been shown previously to be specific for each Pex gene. In addition we have also generated additional dsRNAs targeting different regions of Pex genes where there was thepossibility of off-target effects.

As I am not aware of the similarity of the different Pex proteins (on sequence level) another parameter to consider is the specificity of the knock-down. The authors could add a statement concerning the sequence similarity of the Pex proteins in e.g. the introduction.

It was unclear if the request was referring to similarity of *Drosophila* Pex proteins to yeast or human or how similar the individual Pex proteins are to each other in terms of sequence? If the former, our previous work shows that Drosophila Pex proteins are highly conserved (Mast, 2011& Baron, 2016). If the latter, then each Pex protein is relatively unique in terms of gene/protein sequence and function. We now include additional references to our previous papers describingthe similarity of Drosophila Pex proteins to other eukaryotes. We also now provide a brief description of the canonical role of the Pex proteins during peroxisome assembly in the introduction. While we were not clear how protein sequence similarity would affect RNAi knockdown, the dsRNAs employed for our work are derived from large (up to 200 base) sequences which generate multiple different targeting siRNAs. For the most part, these sequences were generated from existing libraries used by multiple groups. Similarly, the RNAi knockdown lines used for the animal experiments have been used previously by us and other groups. However, to address the RNAi efficiency, we have repeated and re-validated all of the animal RNAi experiments adding, wherever possible , additional independent dsRNA transgenestargeting different gene regions. Some of the lines used in the previous submission that are predicted to have a potential for off-target effects were left out of the current data.

The supplemental tables are very minimalistic. Please add for Table 1 an extra sheet with the explanations of the different work sheets as well as at least the explanation of the most important column headers.

Supplemental Table 2 would also benefit from additional information. "[empty field - no data available]" entries present in Supplemental Table 2 are strange and should be explained. -WhenI was looking at the sequencing data in Supplemental Tables 1 and 2 (individual gene as well as GO-term level) I was surprised to find basically no lipid metabolism / lipid storage / lipid droplet-associated genes to be regulated. How did the authors perform this experiment? In the main text, they write: "RNA sequencing was used to compare cells cultured in Schneider's medium +FBS (Standard) versus +Oleate conditions." and a similar statement is found in the methods section. As is, it is unclear what exactly the RNAseq data is investigating (timeline?).

A description of the each heading for RNASeq and Gene set enrichment analysis-GSEA tables isnow provided as well as a graphical summary of the top GSEA changes (+/-) for each condition (New Figure 1A-B, S1 and Supplementary Files) is now provided. Our RNASeq data has been expanded with pairwise comparisons of multiple conditions including lipid starved cells and starved cells treated with 3-Amino-1,2,4-triazole (3-AT) which has been shown previously to suppresses ROS metabolism by Catalase in Drosophila cells (S2). In our revised/expanded RNASeq analysis, we now compare S2 cells cultured in all four conditions control-starved, control-starved+3AT, control-oleate, oleate-starved and oleate-starved 3AT.

Notably, our revised GSEA comparison of high-confidence changes ($p<0.05$, padj(FDR)<0.01) byGSEA has several metabolism GO terms including "Cellular response to starvation", "Fatty acid degradation", "Propanoate metabolism", as well as early stages of peroxisome formation "Peroxisome". Notably, within these peroxisome GO clusters, the only *Pex* gene was *Pex14*.

A more descriptive figure (S2)presenting this GSEA analysis is now included. We have carefully curated the data to remove uninformative or redundant GO terms. Comparison of our gene lists with those shown previously to be associated with lipid droplets is now also provided as Supplementary Table S3.

In Fig. 2B also the y-axis label is missing. -In Fig. 1I no statistics analysis for the growth curves is provided. This needs to be added.

Figure 2B has been replaced with a more comprehensive GSEA analysis Figure 2A/B, Figure S2.

Figure 1i has been replaced with a new experiment that included co-expression of Dcr with the same validated dsRNA transgenes to increase the RNAi effect in the fat body as used in Figure

1C/S1. In replicating this experiment (Figure 1D/E), we now show data for 16 RNAi lines including multiple lines for Pex14 and several other Pex genes (where possible) in addition tothe 3 RNAi transgenes tested previously. Rather than showing individual growth curves for 16lines we now present an end-point analysis of % survival of three RNAi-knockdown experimental replicates for, with appropriate statistics and SD bars for each nutritional condition tested

-The writing should be improved. At the current state, there are a larger number of typos and glitches (such as e.g. missed brackets) present as well as readability could be improved as thereare many single statement sentences, which are not clearly connected and wordiness examples(e.g. Page 10 line 106: "..approximate half-life of approximately…"). In the introduction, redundancies need to be removed.

Redundant/wordy statements have been revised. We have taken care to proofread the revised manuscript to eliminate typographical errors and glitches.

The quality of the images is different across the figures and should be homogenized & improved. Images in panels V and W of Fig. 3 look distorted. In Fig. 4 the LipidTOX stain is often very ill defined and not clear for the lipid droplets (e.g. panels B, E, I). Fig. 6 panels K and L (EM images) also seem distorted. Please correct / replace.

The distorted images in this old Figure 3 have been replaced with new data as new Figure 5M-N.

The Pex14 structure/function experiment shown previously as Figure 4 where the LipidTOX stain was weak and have been replaced by a new experiment Livedrop fused to GFP to marklipid droplets in live S2 cells (Figure 6 B-I).

In Figure 4, the SKL signal looks very different as compared to other panels (e.g. panels B, C, D in Figure 4 compared to panels A, B, C in Figure 3). As the authors use this signal to quantify multiple phenotypes, I consider this problematic.

The pattern of punctate SKL indicating peroxisomes shown as old Figure 3A-D is typical for *Drosophila* S2 cells (Baron, 2016, Faust 2016). The data presented as old Figure 4B-D represented cells where key genes for the early steps peroxisome assembly (*Pex16* and *Pex19*) are RANi suppressed or null. In these cells peroxisome assembly would be expected to be defective with little to no import of SKL even if the pre-peroxisome vesicles were present. The cells labelled Pex19KO shown in Figure 4B-C are a CRISPR derived precise *Pex19* deletion and the diffuse mNeon-GreenSKL pattern would be typical for cells where PTS1(SKL) proteins wouldbe diffuse and not concentrated via import via Pex13/Pex14 into pre-peroxisome vesicles.
Based on the conservation of Pex16, a similar lack of puncate SKL signal indicating mature peroxisome would be expected for old Figure 4D. We also include images of Pex14 localization relative to Abcd3/ABCD3, an alternative marker of the peroxisome membrane. This PMP wouldbe incorporated into the membrane of non-functional peroxisomes (ghosts) that cannot importSKL.

-In Fig. 2 the localization quantification should also be re-checked and/or presented differently. For Pex14, there seems to be more signal in panels O & P. Further, co-localization signals are also present, where no signal can be seen in the original image (e.g. Fig. 2O, leftmost lipid droplet). For example, for the shown examples, not only the correlation results, but also theprimary segmentation results should be included (supplement?).

This figure attempted to demonstrate our observations that the Pex14 signal seen at lipid droplets was independent of peroxisome membrane. From the comments the oversimplifiedformat presented in the original manuscript clearly did not achieve that goal. Taking the reviewers suggestion, the data is now presented more extensively including single plane images/segmentation data as Supplementary figure 2. This additional space provided by moving the data to a supplementary figure eliminates the artificial appearance of what appeared as signal differences in Pex14 between panels in the original figure.

Minor points raised by Reviewer #2.

In Figure 4, the DAPI signal in panels E and F is not mentioned in the text / legend.

This has been added.

-Page 11, line 228: "…were localized to peroxisomes (Figure 2Q)." à this is a plot and subset information can not be deduced?

This has been corrected to refer to the proper figure.

-In Supplemental Figure 1, the labeling of the Pex3 RNAi clone GD2464 seems incorrect (panel D). Please correct / explain. What do the asterisks in panels I) and T) stand for? I guess thus is the same meaning as in Fig. 1. Please add.

This experiment has been replicated in its entirety to address concerns related to RNAi knockdown efficiency. All experiments shown in FigureS1 have been re-done with UAS-Dcr andobtaining new/additional validated RNAi lines. The asterisks in the previous version of the figure referred to heterozygous lines that we used because they were available to us during COVID-19 related shutdowns of the Drosophila stock centers as well as our University. With thelifting of COVID restrictions, we obtained additional new homozygous lines, which were used togenerate new data. Note that these new experiments replicated our previous findings, but we include only the improved experiments in our revised manuscript. We now refer to each individual line by their Bloomington (BDSC) or Vienna (VDRC) Stock Centre number to unambiguously define each UAS-RNAi line.

-On page 7, the authors mention that "Third instar larvae were raised on a chemically defined (holidic, (Piper et al., 2014) diet where the only added lipid was cholesterol as Drosophila are cholesterol auxotrophs. (Vinci et al., 2008)." In principle this is true, but the holidic diet also contains choline, which is an important / crucial precursor for e.g. phospholipid synthesis as well as choline also affects lipid metabolism profoundly. Thus, I recommend a different wording.

We also replicated this experiment entirely with the improved UAS-Dicer RNAi driver. We also changed the Holidic food used in this replicated experiment to the version with minimal lipids. We now refer to this formulation as 'lipid reduced' due to the presence of choline and other lipids.

Second decision letter

MS ID#: JOCES/2021/259092

MS TITLE: Recruitment of Peroxin14 to lipid droplets affects lipid storage in Drosophila

AUTHORS: Kazuki Ueda, Matthew Anderson-Baron, Julie Haskins, Sarah C Hughes, and Andrew Simmonds
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.