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Spastin tethers lipid droplets to peroxisomes and directs fatty acid trafficking through ESCRT-III

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March 3, 2019

Re: JCB manuscript #201902061

Dr. Jennifer Lippincott-Schwartz
Janelia Research Campus
19700 Helix Dr.
Ashburn, VA 20147

Dear Dr. Lippincott-Schwartz,

Thank you for submitting your manuscript entitled "Spastin tethers lipid droplets to peroxisomes and directs fatty acid trafficking through ESCRT-III". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that while the reviewers find the description of a novel mechanism of FA transfer from LDs to peroxisomes potentially intriguing, further experimental evidence is required to substantiate that Spastin acts as a tether between LDs and peroxisomes for FA transfer.

In revising, please address these three main points experimentally:

1. Further evidence for and a more detailed examination of the function of endogenous Spastin is needed (reviewer #1 points 2, 3, 9, reviewer #2 points 2, 5).

2. Strengthen the evidence to rule out the microtubule severing activity of Spastin (reviewer #1 points 1, 4).

3. Additional data to demonstrate that Spastin promotes FA transfer to peroxisomes is required (reviewer #1 points 7, 8, reviewer #2 point 6, reviewer #3 point 3). Providing substantially stronger evidence for FA transfer is the most crucial of these three points and is essential to proceed with publication in JCB. In particular, I find the experiment requested by reviewer #3 point 3 vital.

Regarding the requests from reviewer #2 for more mechanistic insight (points 4, 7) I agree this would be very interesting, and mechanistic insight would of course help provide more evidence for the role of Spastin in FA transfer. While we would welcome such experiments, they are not required for resubmission to JCB, and can be the subject of separate follow-up studies. Please ensure however that in your model you are balanced in your description of which aspects still need to be experimentally validated (reviewer #2 point 7).

Finally, we hope that you will be able to address all of the remaining reviewer comments in your revised manuscript.

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

GENERAL GUIDELINES:

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

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

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

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

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

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

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

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

Sincerely,

William Prinz, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

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

This study uses a variety of state-of-the-art microscopy techniques and makes many interesting observations. Chang and colleagues show that Spastin over-expression promotes LD-peroxisome

contacts in tissue culture cells, and this depends on a functional AAA ATPase domain of Spastin. They demonstrate that a hydrophobic inserting motif in the N-terminal region of Spastin targets it to LDs, and the LD targeting is sufficient to recruit ESCRT-III proteins IST1 and CHMP1B to LDs. Using fluorescent fatty acids probes, they also show data suggesting that Spastin-driven LD-peroxisome contacts promote fatty acid transfer between LDs and peroxisomes.

In general, this is an interesting study. However, numerous experiments appear over-interpreted, and key controls are missing. The initial half of the paper, which focuses on structure-function dissection of Spastin is more developed. The latter half that uses fluorescent fatty acids and interrogates the involvement of the ESCRTs in LD-peroxisome lipid exchange appears more preliminary. The major concern is that the classical role of Spastin as a microtubule severing protein is not ruled out to explain many of the phenotypes observed. This limits the impact of the new proposed model for Spastin as an LD-peroxisome tether. There are several interesting avenues of investigation here, but these feel incomplete and not thoroughly developed. The study feels too premature to warrant publication in its current state. Here are a list of specific issues which need to be addressed to improve the work:

Major concerns:

1. General concern: It is unclear if the effects generated from Spastin M1 over-expression are due to its putative role as a LD-peroxisome tether, or rather an indirect affect from its role as a severing protein of microtubules. One way to better dissect this is to see if the Spastin-OE driven LD-peroxisome contacts require proper MT dynamics. Are the LD-peroxisome contacts observed in Spastin-OE suppressed in nocodazole treatment, or affected in taxol treatment? Establishing this early in the study seems essential.

2. In Figure 1, enhanced LD-peroxisome contacts are beautifully observed via several methodologies (CLEM, FIB-SEM, and using photo-activatable fluorescence). However these experiments are all in Spastin-OE conditions. It would be helpful to compare these directly to control WT conditions with Spastin at endogenous levels. This would be particularly helpful for the PA-GFP-SKL experiments.

3. Figure 1J: this siRNA experiment indicates that partial depletion of Spastin (both M1 and M87 variants) reduces LD-peroxisome contacts, but the number of contacts is very low in these cells to begin with. This change is so minimal it is hard to make strong conclusions. Were these cells in this experiment treated with OA, or is there a way to induce more abundant LD-peroxisome contacts for this experiment? A related issue is that both Spastin isoforms are depleted in this experiment, and this may have numerous affects to cell homeostasis and MT homeostasis that complicates the interpretation.

4. Figure 3: Through fragment dissection the C-terminal region of Spastin is proposed to have a peroxisome interacting (PXI) region. Again, it is not clear if the PXI region promotes direct LD-peroxisome tethering, or alternatively affects the MT network. The M1(1-92)+197-328 appears to localize to MTs (Figure 3E). Does this construct affect MT stability? Similarly, does the DsRed2 tagged construct still promote LD-peroxisome contacts in the presence of nocodazole?

5. Does the PXI region biochemically purify with peroxisomes? Some additional biochemistry in addition to imaging may help.

6. Figure 4: ABCD1 is primarily known as a peroxisomal fatty acid importer, so its knock-down may

affect peroxisome stability or abundance in the cell. The peroxisomes in the knock-down look smaller and more dispersed, so could this be the reason why there is less contact with LDs?

7. Figure 5: It's a bit unclear why BODIPY-C12 is used as a FA probe for LD-peroxisome exchange, since peroxisomes are thought to import very long chain FAs and this is a very short chain one. It is unclear if the import of this probe is through bone vide FA peroxisome import, or rather just permissive diffusion if LDs and peroxisomes are docked. A good control: Is the BODIPY-C12 peroxisome import affected in an ABCD1 knock-down, or in another condition that blocks peroxisome FA import? A good baseline control for this assay seems necessary, especially given the mild reductions observed in the mutant backgrounds.

8. One expectation if spastin is an LD-pex tether is that loss of the tether may increase cellular VLCFAs. Are these elevated in the Spastin siRNA?

9. Does the localization of mEmerald-IST1 to LDs rely on the over-expression of M1-spastin? The localization of IST1/CHMP1B to LDs is potentially exciting but would be more substantiated if it was observed without ectopic expression of Spastin, or if endogenous IST1/CHMP1B were detected on LDs. Do other ESCRT-III subunits (CHMP3?) also localize to LDs in this condition, or is it just these two?

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

This study by Chang et al. reports a novel mechanism of fatty acid delivery from lipid droplets (LD) to peroxisomes. The paper purports to show that: a) the HSP protein M1 spastin localizes to the LD, b) this requires a hairpin sequence in the first 91 amino acids of M1 spastin, c) overexpression of M1 spastin promotes increased association of LD with peroxisomes, d) this increased association requires the ATPase domain and a peroxisomal targeting domain that co-IP with the peroxisomal protein ABCD1, e) this association promotes transfer of labeled fatty acids to peroxisomes, f) spastin via its MIT domain recruits ESCRT-II proteins IST1 and CHMP1B to the contact site and these are required for fatty acid transfer, and g) this complex and process is required for transfer of oxidized lipids to peroxisomes.

1. This paper is very well done and presented in a logical manner. Most of the data on key points are from experiments of high quality with good controls and thorough quantifications.
2. A major weakness of the paper is most of the interpretations result from experiments that use overexpression of the M1 spastin protein, and it is not clear if this isoform and its dissected functions are relevant under normal expression and cellular conditions. It would have been stronger to generate a knockout cell line and reconstitute this with different isoforms and mutants of the spastin protein. Some attempts should be made to determine how much of the M1 form of the protein is normally expressed and involved in this process. If the M1 was selectively lost via a knockdown, is there a functional consequence to cells for fatty acid trafficking or oxidation, or for oxidized fatty acids? The process has been very well dissected, but it remains unclear how important it is in the context of a cell that does not overexpress this protein.
3. Effects observed for the knockdown of spastin on the peroxisome-LD overlap, while significant, appear small and it is hard to see that this is meaningful. Are there growth conditions where peroxisome-LD contacts are naturally enhanced, which could be used to probe for effects of spastin knockdown?
4. The role of ABCD1 seems unclear. Is the LD-peroxisome overlap affected by ABCD1 knockdown

without overexpression of spastin?
5. CHMP1B and IST1 localize to LD upon spastin overexpression. Does this also occur under endogenous expression levels of spastin, CHMP1B and IST1?
6. The duration of the initial pulse-chase experiment using NBD-labeled C12 appears very long and thus not necessarily allows the conclusion that there is direct delivery from LD to peroxisomes.
7. The model, while intriguing and novel, appears to be overinterpreted. Do the authors observe ESCRT-III mediated membrane deformation specifically at the site of LD-peroxisome contact in their EM-data? There is not much mechanistic data on how this would work. Would IST1 and CHMP1B provide access for lipases? Are the effects of lipase inhibitors and IST1 knockdown additive, or does either block the pathway?

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

Chang et al. present data supporting the idea that the hereditary spastic paraplegia protein spastin on lipid droplets promotes FA transfer to peroxisomes via interaction with the tethering protein ABCD1 and and by recruiting IST1 and CHMP1B to LDs via a specific MIT domain. In addition, these proteins "relieve" LDs of peroxidated lipids. The authors use HeLa cells (confirmed in U2 OS osteosarcoma cells and MRC-5 fibroblasts) and a variety of fluorescently labeled proteins and lipids as well as sophisticated imaging. A few questions remain:
1. In an extensive proximity labeling study of lipid droplets in U2OS and Huh7 cells, none of these proteins was identified. The authors should reference PMID: 29275994 and comment on the discrepancy.
2. In another proximity labeling study, the related proteins spartin and ABCD3 were identified. Might these have functions similar to those of spastin? PMID: 30190326
3. The fluorescently labeled NBD-C12 was used to show movement from the LD to the peroxisomes. Because this is the primary functional point of the associated proteins examined in this paper, the authors should show by TLC (or mass spec) that the incorporated NBD-C12 is actually esterified to a TG molecule, that after the chase, it is recovered as a free FA in the peroxisomes, and that with knockdown of either spartin or ABCD1, this does not occur. These changes could be semi-quantified at least by intensity of TG versus free FA. Inhibition of the "transfer" by the lipase inhibitor DEUP is too indirect. The authors should also show that the amount of fluorescently labeled TG remains unchanged.
4. Page 14 Discussion. "complex is critical" is too strong a statement since lack of the various interacting proteins does not totally block the putative FA transfer.
Other points
1. Page 15 "...we found that spastin OVER-expression..."
2. Figs 5, S5, and 6. Suggest using contrasting colors for arrows: magenta and red are too similar

We would like to thank all the reviewers for their comments and suggestions. We have conducted new experiments, modified the figures, and edited the manuscript (highlighted in yellow) extensively to address all of the expressed concerns. These are presented in a point-by-point response (in blue) below following each of reviewers' comments.

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

This study uses a variety of state-of-the-art microscopy techniques and makes many interesting observations. Chang and colleagues show that Spastin over-expression promotes LD-peroxisome contacts in tissue culture cells, and this depends on a functional AAA ATPase domain of Spastin. They demonstrate that a hydrophobic inserting motif in the N-terminal region of Spastin targets it to LDs, and the LD targeting is sufficient to recruit ESCRT-III proteins IST1 and CHMP1B to LDs. Using fluorescent fatty acids probes, they also show data suggesting that Spastin-driven LD-peroxisome contacts promote fatty acid transfer between LDs and peroxisomes.

In general, this is an interesting study. However, numerous experiments appear over-interpreted, and key controls are missing. The initial half of the paper, which focuses on structure-function dissection of Spastin is more developed. The latter half that uses fluorescent fatty acids and interrogates the involvement of the ESCRTs in LD-peroxisome lipid exchange appears more preliminary. The major concern is that the classical role of Spastin as a microtubule severing protein is not ruled out to explain many of the phenotypes observed. This limits the impact of the new proposed model for Spastin as an LD-peroxisome tether. There are several interesting avenues of investigation here, but these feel incomplete and not thoroughly developed. The study feels too premature to warrant publication in its current state. Here are a list of specific issues which need to be addressed to improve the work:

Major concerns:

1. General concern: It is unclear if the effects generated from Spastin M1 over-expression are due to its putative role as a LD-peroxisome tether, or rather an indirect affect from its role as a severing protein of microtubules. One way to better dissect this is to see if the Spastin-OE driven LD-peroxisome contacts require proper MT dynamics. Are the LD-peroxisome contacts observed in Spastin-OE suppressed in nocodazole treatment, or affected in taxol treatment? Establishing this early in the study seems essential.
We have examined LD-peroxisome contacts in M1 Spastin-overexpressing cells with nocodazole treatment to disrupt microtubule network. As shown in Figure 1E, nocodazole treatment had no effect on LD-peroxisome contact formation mediated by M1 Spastin overexpression. Similarly, overexpression M87 Spastin, which resides in the cytosol and severs microtubules, failed to enhance LD-peroxisome contacts (Figure S1C-S1E). Together, these data ruled out the possibility that the effect of M1 Spastin in enhancing LD-peroxisome contacts was due to its role in affecting microtubule dynamics.

2. In Figure 1, enhanced LD-peroxisome contacts are beautifully observed via several methodologies (CLEM, FIB-SEM, and using photo-activatable fluorescence). However these

experiments are all in Spastin-OE conditions. It would be helpful to compare these directly to control WT conditions with Spastin at endogenous levels. This would be particularly helpful for the PA-GFP-SKL experiments.

We thank reviewer for pointing this out as it is a very good idea. We have performed the suggested experiment and found that both stable and transient LD-peroxisome contacts exist throughout the 10-min imaging period (Figure 1I). The existence of stable contacts in control cells further suggests endogenous tethering mechanisms. We have quantified the relative duration time of LDs contacting peroxisomes and found that M1 Spastin overexpression indeed extends the contacting time of these organelles (Figure 1J). We also applied this approach to show that endogenous Spastin appears to have a role in regulating the contacting time between LDs and peroxisomes (see below).

3. Figure 1J: this siRNA experiment indicates that partial depletion of Spastin (both M1 and M87 variants) reduces LD-peroxisome contacts, but the number of contacts is very low in these cells to begin with. This change is so minimal it is hard to make strong conclusions. Were these cells in this experiment treated with OA, or is there a way to induce more abundant LD-peroxisome contacts for this experiment? A related issue is that both Spastin isoforms are depleted in this experiment, and this may have numerous affects to cell homeostasis and MT homeostasis that complicates the interpretation.

We agree with these comments. In the original siRNA experiment, cells were not treated with OA. Our preliminary results showed that OA treatment only slightly increases LD-peroxisome contacts (not shown). Nonetheless, we found a ~2-fold increase in LD-peroxisome contacts in cells treated with cumene hydroperoxide (Cumyl-OOH) or sodium arsenite (As^{3+}) (Figures 2A and 2B), which induces oxidative stress and lipid peroxidation. We further found that siSpastin transfection led to a significant reduction in LD-peroxisome contacts in these conditions (Figure 2C). Moreover, siSpastin also significantly decreased the relative duration of LD-peroxisome contacts as demonstrated by photoactivation experiments using PA-GFP-SKL to monitor peroxisomes (Figure 2D). Given that M87 Spastin overexpression had a minimal effect on LD-peroxisome contacts (Figure S1E), these observations suggest that endogenous M1 Spastin plays a role in tethering LDs to peroxisomes. These results are described in a new section "**Endogenous Spastin is required for LD-peroxisome contact formation**".

4. Figure 3: Through fragment dissection the C-terminal region of Spastin is proposed to have a peroxisome interacting (PXI) region. Again, it is not clear if the PXI region promotes direct LD-peroxisome tethering, or alternatively affects the MT network. The M1(1-92)+197-328 appears to localize to MTs (Figure 3E). Does this construct affect MT stability? Similarly, does the DsRed2 tagged construct still promote LD-peroxisome contacts in the presence of nocodazole?

We cannot rule out the effects of $M1^{1-92}$-197-328 on microtubule dynamics. Nonetheless, disrupting the microtubule network by nocodazole treatment had minimal effects on LD-peroxisome contacts mediated by DsRed- $M1^{1-92}$-197-328 overexpression (Figure 4G). Our IP data (Figures 5C-5E) is also consistent with microtubules being dispensable for M1 Spastin or PXI to interact with peroxisomes because microtubules are mostly disrupted in cell lysates during immunoprecipitation at 4°C.

5. Does the PXI region biochemically purify with peroxisomes? Some additional biochemistry in addition to imaging may help.
We addressed this question by purifying peroxisomes in mApple-C1 (control) or in mApple-PXI-expressing cells. We were concerned that mApple-M1$^{1-92}$-PXI overexpression would generate LD-peroxisome contacts, which might affect proper peroxisome purification. Thus, we engineered mApple-PXI that does not contain a LD-targeting motif and is localized in the cytoplasm (Figure 4H). We found that mApple-PXI, not mApple, co-purified with peroxisome (Figure 4I), suggesting that the PXI region indeed has affinity with peroxisomes.

6. Figure 4: ABCD1 is primarily known as a peroxisomal fatty acid importer, so its knock-down may affect peroxisome stability or abundance in the cell. The peroxisomes in the knock-down look smaller and more dispersed, so could this be the reason why there is less contact with LDs?
We thank reviewer for pointing this out. We have quantified the number of peroxisomes in siCtrl and siABCD1-transfected cells and found no significant changes in peroxisome number (Figure S5A). In addition, knockdown of ABCD1 reduced LD-peroxisome contacts under oxidative stress (Figure S5B). Together, these data suggest that ABCD1 has a role in LD-peroxisome tethering, which is not due to its effects on peroxisome abundance.

7. Figure 5: It's a bit unclear why BODIPY-C12 is used as a FA probe for LD-peroxisome exchange, since peroxisomes are thought to import very long chain FAs and this is a very short chain one. It is unclear if the import of this probe is through bone vide FA peroxisome import, or rather just permissive diffusion if LDs and peroxisomes are docked. A good control: Is the BODIPY-C12 peroxisome import affected in an ABCD1 knock-down, or in another condition that blocks peroxisome FA import? A good baseline control for this assay seems necessary, especially given the mild reductions observed in the mutant backgrounds.
For clarification, we used NBD-C12, not BODIPY-C12, to monitor LD-to-peroxisome FA trafficking. We agree that VLCFAs are exclusively imported to peroxisomes. Nonetheless, peroxisomes also import other FAs for a variety of lipid metabolic pathways. To our knowledge, the exact repertoire of FAs being imported to peroxisomes and its underlying mechanisms are not completely clear. We speculate that NBD-C12, a modified C12 FA, is somehow recognized by a peroxisomal FA import machinery. To demonstrate this, we performed thin layer chromatography (TLC) experiments in siCtrl and siABCD1 cells following NBD-C12 pulse-chase (Figure 6A). We found that siABCD1 transfection resulted in a substantial reduction in free C12 coinciding with an accumulation of esterified C12 as compared with siCtrl-treated cells (Figure 6E). This results suggested that NBD-C12 was indeed transported from LDs into peroxisome as free FA.

8. One expectation if spastin is an LD-pex tether is that loss of the tether may increase cellular VLCFAs. Are these elevated in the Spastin siRNA?
This is a good suggestion and we also expect this is the case. This will be one of our future directions as a continuation of this project. To test this idea, we need to perform lipidomics analysis, which requires a significant amount of work and time. Due to the length of the current

manuscript that already covers multiple aspects of LD-peroxisome contacts and their functional impacts, we decided not to include lipidomics analysis in our current manuscript. We hope the reviewer accepts this decision.

9. Does the localization of mEmerald-IST1 to LDs rely on the over-expression of M1-spastin? The localization of IST1/CHMP1B to LDs is potentially exciting but would be more substantiated if it was observed without ectopic expression of Spastin, or if endogenous IST1/CHMP1B were detected on LDs. Do other ESCRT-III subunits (CHMP3?) also localize to LDs in this condition, or is it just these two?

We visualized endogenous IST1 by immunostaining and found its partial co-localization with overexpressed M1 Spastin. This observation suggested that M1 Spastin is capable of recruiting endogenous IST1 to LDs. We did not detect IST1 or CHMP1B, either endogenous or ectopically expressed, localize to LDs without M1 Spastin overexpression. We speculate that these ESCRT-III proteins are dynamically recruited to LDs. Thus, it is difficult to detect ESCRT-III on LDs without M1 Spastin overexpression to stabilize the interaction.

It is unlikely that other ESCRT-III subunits can be recruited to LDs by M1 Spastin overexpression because the original yeast two-hybrid screening only identify the interaction of IST1 and CHMP1B with Spastin's MIT domain. Nonetheless, we do not exclude the possibility that other ESCRT-III components can be recruited to LDs via additional mechanisms.

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

This study by Chang et al. reports a novel mechanism of fatty acid delivery from lipid droplets (LD) to peroxisomes. The paper purports to show that: a) the HSP protein M1 spastin localizes to the LD, b) this requires a hairpin sequence in the first 91 amino acids of M1 spastin, c) overexpression of M1 spastin promotes increased association of LD with peroxisomes, d) this increased association requires the ATPase domain and a peroxisomal targeting domain that co-IP with the peroxisomal protein ABCD1, e) this association promotes transfer of labeled fatty acids to peroxisomes, f) spastin via its MIT domain recruits ESCRT-II proteins IST1 and CHMP1B to the contact site and these are required for fatty acid transfer, and g) this complex and process is required for transfer of oxidized lipids to peroxisomes.

1. This paper is very well done and presented in a logical manner. Most of the data on key points are from experiments of high quality with good controls and thorough quantifications.

We thank reviewer for recognizing the quality of our work.

2. A major weakness of the paper is most of the interpretations result from experiments that use overexpression of the M1 spastin protein, and it is not clear if this isoform and its dissected functions are relevant under normal expression and cellular conditions. It would have been stronger to generate a knockout cell line and reconstitute this with different isoforms and mutants of the spastin protein. Some attempts should be made to determine how much of the M1 form of the protein is normally expressed and involved in this process. If the M1 was selectively lost via a knockdown, is there a functional consequence to cells for fatty acid trafficking or oxidation, or for oxidized fatty acids? The process has been very well dissected,

but it remains unclear how important it is in the context of a cell that does not overexpress this protein.

We appreciate this concern. To address this, we first established that overexpression of M87 Spastin is incapable of enhancing LD-peroxisome contact formation (Figure S1E), suggesting a primary role of M1 Spastin in this process. We then extensively examined LD-peroxisome contacts in siSpastin-treated cells under various conditions to strengthen the requirement of endogenous Spastin in tethering LDs to peroxisome (Figure 2; response 3). Given that M87 Spastin overexpression had minimal effect on LD-peroxisome contacts (Figure S1E), these observations suggest endogenous M1 Spastin plays a major role in LD-peroxisome tethering.

3. Effects observed for the knockdown of spastin on the peroxisome-LD overlap, while significant, appear small and it is hard to see that this is meaningful. Are there growth conditions where peroxisome-LD contacts are naturally enhanced, which could be used to probe for effects of spastin knockdown?

We agree with this concern. We have conducted a series of experiment to examine the effect of endogenous Spastin on LD-peroxisome contacts (Figure 2). We found a ~2-fold increase in LD-peroxisome contacts in cells treated with cumene hydroperoxide (Cumyl-OOH) or sodium arsenite (As3+) (Figures 2A and 2B), which induces oxidative stress and lipid peroxidation. We further found that siSpastin transfection led to a significant reduction in LD-peroxisome contacts in these conditions (Figure 2C). Moreover, siSpastin also significantly decreased the relative duration of LD-peroxisome contacts as shown by photoactivation experiment (Figure 2D). These results are described in a new section "**Endogenous Spastin is required for LD-peroxisome contact formation**".

4. The role of ABCD1 seems unclear. Is the LD-peroxisome overlap affected by ABCD1 knockdown without overexpression of spastin?

We indeed observed a reduction of LD-peroxisome overlap by ABCD1 knockdown in oxidative stress condition without M1 Spastin overexpression (Figure S5B). This result further supports the idea that ABCD1 plays a role in tethering peroxisomes to LDs.

5. CHMP1B and IST1 localize to LD upon spastin overexpression. Does this also occur under endogenous expression levels of spastin, CHMP1B and IST1?

We visualized endogenous IST1 by immunostaining and found its partial co-localization with overexpressed M1 Spastin (Figure 8D). This observation suggested that M1 Spastin is capable of recruiting endogenous IST1 to LDs. We did not detect IST1 or CHMP1B, either endogenous or ectopically expressed, localize to LDs without M1 Spastin overexpression. We speculate that these ESCRT-III proteins are dynamically recruited to LDs. Thus, it is difficult to detect ESCRT-III on LDs without M1 Spastin overexpression to stabilize the interaction.

6. The duration of the initial pulse-chase experiment using NBD-labeled C12 appears very long and thus not necessarily allows the conclusion that there is direct delivery from LD to peroxisomes.

We agree with this concern. In our transient pulse-chase experiment, we observed NBD-C12 signal in peroxisomes following ~2-h chase (Figure 7D). We further performed thin layer

chromatography (TLC) experiments and observed a substantial reduction in free NBD-C12 in ABCD1 knockdown cells (Figure 6E). These TLC data suggested that the presence of NBD-C12 in peroxisomes is dependent on an active peroxisomal import mechanism and is not due to permissive diffusion. Based on these observations, we speculate the existence of a direct LD-to-peroxisome FA delivery pathway. Nonetheless, we understand that it is almost impossible to demonstrate direct FA/lipid trafficking at membrane contact sites in intact cells. To avoid confusion and over interpretation, we have toned down our statement to a more suggestive notion.

7. The model, while intriguing and novel, appears to be overinterpreted. Do the authors observe ESCRT-III mediated membrane deformation specifically at the site of LD-peroxisome contact in their EM-data? There is not much mechanistic data on how this would work. Would IST1 and CHMP1B provide access for lipases? Are the effects of lipase inhibitors and IST1 knockdown additive, or does either block the pathway?
We thank the review for pointing out this concern. We did not observe ESCRT-III mediated membrane deformation at LD-peroxisome contacts in our EM data. We believe it is difficult to visualize membrane deformation at the contacts due to the close membrane association. Nonetheless, based on the previous study showing IST1 and CHMP1B positively curving the membrane bilayer, it is plausible to speculate similar membrane morphology at LD-peroxisome contacts. To avoid overinterpretation, we have added “Speculation” to the right boxed panel of our model figure (Figure 10). We have also carefully modified our discussion to make clear that our description of the boxed panel is merely a speculation.

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

Chang et al. present data supporting the idea that the hereditary spastic paraplegia protein spastin on lipid droplets promotes FA transfer to peroxisomes via interaction with the tethering protein ABCD1 and and by recruiting IST1 and CHMP1B to LDs via a specific MIT domain. In addition, these proteins "relieve" LDs of peroxidated lipids. The authors use HeLa cells (confirmed in U2 OS osteosarcoma cells and MRC-5 fibroblasts) and a variety of fluorescently labeled proteins and lipids as well as sophisticated imaging. A few questions remain:

1. In an extensive proximity labeling study of lipid droplets in U2OS and Huh7 cells, none of these proteins was identified. The authors should reference PMID: 29275994 and comment on the discrepancy.
2. In another proximity labeling study, the related proteins spartin and ABCD3 were identified. Might these have functions similar to those of spastin? PMID: 30190326
We thank the reviewer for bringing these two papers to our attention. To address the reviewer’s concerns, we have added the following paragraph to our discussion.

“Despite these findings, a recent proximity labeling study of LDs in U-2 OS and Huh7 cells did not identify Spastin or ABCD1 (Bersuker et al., 2018). It is possible that endogenous Spastin or ABCD1 was expressed at too low levels in these cells to be detected by proximity labeling or that other tethering complexes maintained LD-peroxisome contacts in these cells. Related to the latter possibility, other proteomic studies have identified a series of potential

tethers between LDs and peroxisomes, including a HSP-related protein called Spartin and ABCD3 (Pu et al., 2011; Young et al., 2018).”

3. The fluorescently labeled NBD-C12 was used to show movement from the LD to the peroxisomes. Because this is the primary functional point of the associated proteins examined in this paper, the authors should show by TLC (or mass spec) that the incorporated NBD-C12 is actually esterified to a TG molecule, that after the chase, it is recovered as a free FA in the peroxisomes, and that with knockdown of either spartin or ABCD1, this does not occur. These changes could be semi-quantified at least by intensity of TG versus free FA. Inhibition of the "transfer" by the lipase inhibitor DEUP is too indirect. The authors should also show that the amount of fluorescently labeled TG remains unchanged.
We thank the reviewer for making this suggestion. We have included TLC data in Figure 6. In control cells, both esterified C12 and free C12 were detected following pulse-chase (Figure 6D). Nonetheless, in DEUP-treated cells, an accumulation of esterified C12 coinciding with a reduction in free C12 was observed (Figure 6D). These results indicate NBD-C12 was indeed incorporated into TG and then released as free C12 by lipase activity. We further demonstrated that knockdown of ABCD1 led to a substantial reduction in free C12, suggesting that free C12 was indeed imported into peroxisomes (Figure 6I). We have generated a new paragraph in our results section “**Monitoring LD-to-peroxisome FA trafficking using NBD-C12**” to describe these observations together with an imaging-based pulse-chase experiment.

4. Page 14 Discussion. "complex is critical" is too strong a statement since lack of the various interacting proteins does not totally block the putative FA transfer.
We thank reviewer to point this out. We have modified the sentence to “In this study, we identify a protein complex that tethers LDs to peroxisomes to support FA trafficking between these organelles.”

Other points

1. Page 15 "...we found that spastin OVER-expression..."
We have modified the text accordingly.

2. Figs 5, S5, and 6. Suggest using contrasting colors for arrows: magenta and red are too similar
We have change the magenta arrow heads to open magenta arrow heads.

May 16, 2019

RE: JCB Manuscript #201902061R

Dr. Jennifer Lippincott-Schwartz
Janelia Research Campus
19700 Helix Dr.
Ashburn, VA 20147

Dear Dr. Lippincott-Schwartz:

Thank you for submitting your revised manuscript entitled "Spastin tethers lipid droplets to peroxisomes and directs fatty acid trafficking through ESCRT-III". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

As you will see, reviewer #1 still had two remaining issues. Regarding the TLC experiments, as the band labeled "esterifed" seems certainly correct I do not find it essential to know what the particular esterified species are, and quantifications do not seems necessary to support your conclusions. Providing a number of chase times instead of just 7h would I agree strengthen your story, though is not required for publication. Finally, the second concern also does not seem essential to address experimentally given the level of additional insight that would be gained.

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

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

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

3) * Figure formatting: Scale bars must be present on all microscopy images, * including inset magnifications (e.g. ID, F, G; 4B, F; 5A; 7E; 8C, D, E; 9D, F, G; S1; S3; S4). Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments

(either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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

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

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

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

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

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

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

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

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

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

B. FINAL FILES:

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

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

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

Sincerely,

William Prinz, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

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

This study demonstrates that Spastin contributes to LD-peroxisome tethering and suggests a role in fatty acid exchange between these two organelles. The revised manuscript has addressed most of the concerns raised. The authors nicely show that M1 but not M87 Spastin over-expression can induce LD-peroxisome contacts (Fig 1), and that nocodazole treatment does not perturb this (suggesting it is not an indirect effect of MT alterations) (Fig 1). They also show that endogenous Spastin contributes to LD-peroxisome contacts (Fig 2), and that the PXI domain of Spastin is sufficient to be targeted to peroxisomes via biochemical fractionation (Fig 4). These new experiments are well conducted and conclusive. Modulation of Spastin expression clearly affects LD-peroxisome contacts.

There are two remaining issues:

1) The new TLC experiments examining NBD-C12 processing (Fig 6D,E) are hard to interpret. NBD-C12 is labeled as either 'free' or 'esterified', but it is unclear what this 'esterified' NBD-C12 species is. Is NBD-C12 incorporated into TG or another neutral lipid, or modified in some other way? A standard for TG or cholesterol esters would help interpret this data. Also, these experiments are conducted after a 7-hour chase, so it is unclear how they aligns with the 18-hr chase experiments in this figure. Is some of the NBD-C12 not yet esterified, or is the free pool from lipolysis? The DEUP experiment is also hard to interpret here. There is much more NBD-C12 in the origin than the Ctrl. Is NBD-C12 esterification also perturbed in these cells when treated with DEUP? The experiments in Panel Fig 6E are also hard to interpret as the data quality is not good here. These TLC experiments need to be repeated several times and their averages quantified, as there may be significant variability between experiments.

2) The endogenous IST1 immuno-staining in Fig 8 is also perplexing. The authors clearly demonstrate that over-expression of an MIT domain containing Spastin can recruit essentially all mEmerald-IST1 to LDs, but the endogenous IST1 observed in Fig 8D suggests that most IST1 is not on Spastin-positive LDs. Can this endogenous IST1-LD association be quantified relative to non-Spastin OE cells? Alternatively, can the LDs be purified to show that IST1 is actually associating with them and not just sitting on endosome near the LDs?

In summary, the manuscript is substantially improved and demonstrates a role for Spastin in LD-peroxisome crosstalk, but the later half of the manuscript still appears preliminary and requires some final experiments to be suitable for publication.

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

The authors have addressed my concerns.

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

My questions and suggestions have been dealt with appropriately.