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FIT2 organizes lipid droplet biogenesis with ER tubule-forming proteins and septins

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September 3, 2019

Re: JCB manuscript #201907183

Dr. Junjie Hu
Chinese Academy of Sciences
15 Datun Rd. Chaoyang District
Beijing, Beijing 100101
China

Dear Dr. Hu,

Thank you for submitting your manuscript entitled "FIT2 organizes lipid droplet biogenesis with ER tubule-forming proteins and septins". Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that there is a fair amount of interest in the potential regulation of lipid droplet biogenesis by the interaction of FIT2 with septins and ER-shaping proteins, but the reviewers raise a number of significant issues that should be addressed substantively. All the reviewers had good suggestions that will improve the manuscript and it is particularly important to address their points thoroughly as further mechanistic insight into how FIT2 and septins control lipid droplet biogenesis, and confirmation that this is a function of endogenous septins, were key issues raised in the editorial decision letter for a previous version of this study. For example, it would be necessary to include imaging using endogenous tagged proteins.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. We would encourage you to provide a detailed revision plan and rebuttal at an early stage to avoid spending time on experimental work that may not be sufficient for re-review. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count 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: Your manuscript may have up to 10 main text figures. To avoid delays in production, 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. Your manuscript 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.

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, 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. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Elizabeth Miller, Ph.D.
Monitoring Editor

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

Journal of Cell Biology

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

Chen et al. show that FIT2 interacts with ER tubule forming proteins and septins to promote lipid droplet (LD) formation. From a screen for ER morphology mutations in C. elegans, they observed that mutations in the FIT2 homolog FITM-2 caused ER sheet expansion. Depletion of FIT2 in COS-7 cells similarly expanded ER sheets. Lack of FIT2 function resulted in loss of LDs, while overexpression of FIT2 resulted in formation of bubble-like structures that were positive for FIT2 but lacked neutral lipids and were therefore not bona fide LDs. These bubbles were decorated with Rtn4a and REEP5 and other ER tubule forming proteins, as well as the septins. FIT2 was tagged endogenously with HA and immunoprecipitates contained endogenous Rtn4 and REEP5 but not control ER proteins. Using peptides of cytosolic portions of FIT2, they were able to pull down recombinant septin complexes with the N terminal and second cytosolic loop, indicating a direct interaction. Depletion of FIT2, Rtn4a, REEP5 or knockout of the septins resulted in decreased numbers and sizes of LDs. These effects were seen in differentiated 3T3-L1 adipocytes and in larval C. elegans mutants. Finally, time course experiments revealed the appearance of FIT2 at 85% of sites were nascent LDs formed. Similar results were obtained with REEP5 and Rtn4a, the latter much less frequently, though. Septins also appeared to transiently mark these sites.

Overall this is a well-written and clearly presented story that provides insight into LD formation. The association with the tubule promoting ER proteins and the septins would not have been expected and the results compellingly support their contributions to lipid storage. I have two minor criticisms that the authors should address to improve the manuscript:

1. On the 4th page of results, the authors conclude that since they cannot see co-immunoprecipitation of FIT2 with Rtn4a when they are expressed in separate cells and mixed, that they must interact by their transmembrane domains. I do not agree with this conclusion as this could simply reflect the possibility that each protein is already assembled in stable complexes with their untagged endogenous binding partners in each lysate, so they are unable to interact after mixing. It does not tell you the portions of the proteins that are interacting, only that they are not able to.

2. In quantifying the effects of knockdown or knockout the authors have analyzed the data differently for the tubule forming proteins (Imaris) and the septins (ImageJ) and presented them differently. The Imaris results appear more robust and the presentation is clearer with diameters of droplets rather than arbitrary units. All of the data should be presented that way to allow comparisons. As well, this may explain the difference in numbers of LDs in control cells between figure 5A or B and figure 5C of D.

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The manuscript is well written and appears to be technically well-done with the authors using a variety of systems and cell types, and a combination of cell biological and biochemical approaches. Conceptually and mechanistically, however, the manuscript falls short of determining the function of FIT2 and septins in LD biogenesis. At the very least, I think FIT2 function and role should be defined a little better than presently, especially with regard to other factors involved in LD biogenesis (e.g., seipin), its phosphatase activity and the budding vs. maturation stages of LD biogenesis (see below in major comments 3 & 4).

My understanding is that the role of FIT2 in LD biogenesis has already been established by previous studies, so it appears that the main advance of this study is the interaction of FIT2 with ER tubule proteins and septins, and its potential role in LD biogenesis from peripheral ER tubules. The latter aspect needs more strengthening (see comment 4) and placing the new interactions in a mechanistic context in terms of LD biogenesis/maturation would make the paper more suitable for JCB.

Major Comments:

1. Co-immunoprecipitations of FIT2 with ER tubule-enriched proteins (Rtn4a, REEP5) and septins

have been performed with over-expressed HA-tagged FIT2. Did the authors attempt to co-IP endogenous FIT2 with these proteins? Co-IP of endogenous FIT2 with endogenous Rtn4a, REEP5 and septins will further boost the confidence level in these interactions.

2. In previous work, SEPT9 was the septin that was determined to function in LD growth. The authors only looked at the SEPT2/6/7 complex. Does SEPT9 co-IP with FIT2 as well? Does SEPT9 or other septin subunits interact directly with FIT2?

3. To enhance the functional and mechanistic aspect of the manuscript, can the authors determine the spatio-temporal order/hierarchy of FIT2, Rtn4a, REEP5 and septin localization during LD formation with time-lapse imaging and determine whether FIT2 localization on budding LDs depends on Rtn4a and REEP5?

Does FIT2 function/localize to budding LDs with seipin? Is the role/function of FIT2 upstream or down-stream of seipin? How does knock-down of seipin impact FIT2/septin localization and vice versa?

Does the interaction FIT2 with Rtn41, REEP5 and/or septins depends on its phosphatase activity? Previous studies have shown that FIT2 mutants with defective phosphatase activity were not able to restore LD budding in FIT deficient cells. Perhaps, these mutants can be utilized to determine whether FIT2 activity is required for the interaction with septins.

4. The authors claim that FIT2 and FIT2-interacting proteins mark peripheral LD formation sites (Figure 7). This has interesting implications for the spatial control of LD formation in sub-domains of the ER. However, the claim is not very strongly supported by the data. The authors should make an effort to address the following:
i) The population of peripheral LDs should be expressed as percentage of total LDs and that percentage should be quantified relative to control in cells knocked down for FIT2 and Sept2. Peripheral localization of LDs should be standardized (in terms of a specific radius/distance from the center or edge of the cell).
ii) Can the authors determine whether there is a preferential localization of FIT2 and/or septin 2 to peripheral ER tubules? This should not be so hard to determine and quantify by staining for marker of tubular ER and FIT2/septin2. If indeed FIT2 and septins localize preferentially to peripheral ER tubules, this will boost a role of FIT2 in the spatial control of LD biogenesis/maturation from subdomains of the ER.

Minor Comments:

- The authors should avoid using the terms "septin cytoskeleton" and "septin filaments" throughout the manuscript. It is unclear whether the LD membrane associated septins are indeed filaments and most likely these septins are not cytoskeletal elements like actin and microtubules, which do not appear to influence septin-FIT2 association.

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This study proposes that FIT2 interacts with several ER shaping proteins as well as septin cytoskeletal proteins, and together these regulate LD biogenesis off the ER surface. FIT2 is found to interact with ER tubule localizing proteins Rtn4a and REEP5, as well as septin cytoskeletal components. The study is well conducted and strengths include highly quantitative imaging, thorough protein-protein interaction studies using co-immunoprecipitations, and some timelapse microscopy of nascent LD biogenesis. Cell work is also conducted in both C elegans and tissue culture cells, adding to the broadness of the study. Overall is the study is informative and adds valuable new information regarding the function of FIT2 proteins in LD biogenesis and ER shape/homeostasis.

There are several general concerns and some minor issues:

1) It is still not completely clear if the septin effects on FIT2 and LD production are direct or indirect. Septins are first observed to colocalize with FIT2 only when FIT2 is over-expressed to generate ER bubbles (Fig 2D). There does not appear to be any endogenous co-localization experiments indicating septin can localize with native FIT2 under non over-expressed conditions. To this point, the septin2-FIT2 co-ip data appears to be with over-expressed FIT2-HA as well (Fig 4). Is there any detected endogenous septin at the ER when FIT2 is not over-expressed?

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3) There is not much analysis of the ER bubbles that are generated by FIT2 over-expression. Are they ER bilayers or monolayer bulges that are devoid of neutral lipids? Some higher resolution imaging or electron microscopy data would greatly add to this study.

4) Fig 7 uses time lapse imaging to monitor nascent LD biogenesis. This work is particularly exciting but not well quantified. Can the number of septin positive LiveDrop puncta be quantified? Same with Reep5?

Minor comments:

1) Several image panels are quite dark and should be set to gray scale. Fig 7 red and magenta channels are particularly dark and should be gray.

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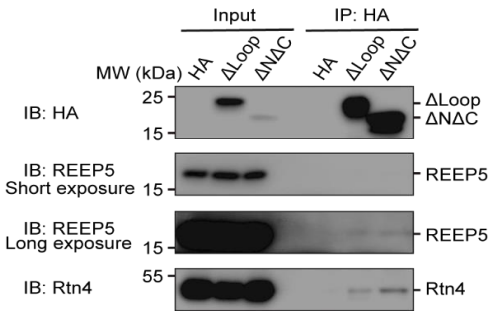
We reason that detergent-isolated and -shielded TM regions are less likely to engage other TMs during the co-immunoprecipitation. Therefore, if two integral membrane proteins interact through their TM regions, they would fail to interact if they are isolated from different membranes. In contrast, if they interact through their soluble regions, they should still be able to interact upon mixing of the lysates, even if they were isolated from different membranes, because their soluble regions would be exposed and available. Consistent with our reasoning, loop-replaced or truncated FIT2 mutants still interact with endogenous REEP5 or Rtn4 (shown below). To avoid confusion, we softened the conclusion here. If helpful, we could add the panel below.



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We repeated the analysis for septins with Imaris (**Fig. 5C,D and Fig. S4K**).

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

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We performed co-IP of endogenous FIT2 and Rtn4/REEP5 (**Fig. 3B**). The HA-tagged FIT2 was from a CRISPR/Cas9-based knock-in of the HA tag. We also added co-IP of endogenous FIT2-HA and septins (**Fig. 4D**).

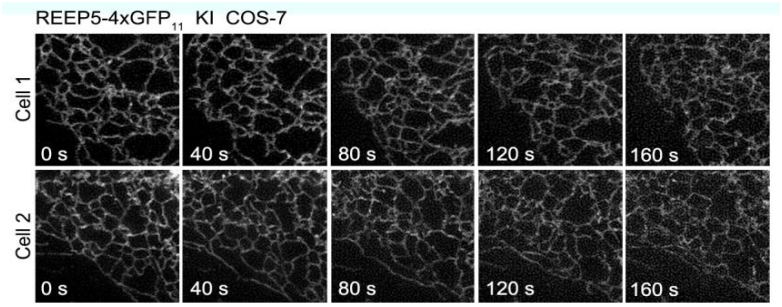
2. In previous work, SEPT9 was the septin that was determined to function in LD growth. The authors only looked at the SEPT2/6/7 complex. Does SEPT9 co-IP with FIT2 as well? Does SEPT9 or other septin subunits interact directly with FIT2?

We performed co-flotation of FIT2 and purified SEPT9, finding no interaction (**Fig. 4F**). We also purified SEPT2/6/7 individually and found that SEPT7 interacts with FIT2 directly, whereas SEPT2 only weakly engages FIT2 (**Fig. 4F**). We previously deleted SEPT2 and found

that both LD size and number were decreased compared to WT cells (**Fig. 5C,D**). We depleted other septins individually and observed similar defects (**Fig. S4H-K**). These results suggest that FIT2 recruits septins mainly through binding to SEPT7, and septins act as polymers in facilitating LD formation.

3. To enhance the functional and mechanistic aspect of the manuscript, can the authors determine the spatio-temporal order/hierarchy of FIT2, Rtn4a, REEP5 and septin localization during LD formation with time-lapse imaging and determine whether FIT2 localization on budding LDs depends on Rtn4a and REEP5?

We understand that these experiments are very important for strengthening our argument. However, they are technically very challenging. To tackle this problem, we generated the knock-in cell lines: FIT2-HA, FIT2-mCherry, FIT2-4xGFP$_{11}$, REEP5-mCherry, REEP5-4xGFP$_{11}$, etc. Due to low abundance of the endogenous proteins, we failed to detect fluorescent signals with fixed FIT2-HA KI cells by IF, or with FIT2-mCherry, FIT2-4xGFP$_{11}$, or REEP5-mCherry KI cells by live cell imaging or IF. The REEP5-4xGFP$_{11}$ KI cells (co-expressing GFP$_{1-10}$) were barely visible under GI-SIM, and the signals decayed too quickly to attempt time-lapse imaging (see images shown below). Therefore, we fixed wt COS-7 cells stably expressing LiveDrop, performed antibody staining for endogenous SEPT2, and visualized co-localization of nascent LDs and septins using SIM microscopy. We found that the SEPT2 puncta had a significant overlap with LiveDrop puncta, and the degree of overlap decreased when FIT2 was depleted in these cells (**Fig. 8C-E** and **S5E**).
In live cell imaging experiments, in which FIT2-mCherry, REEP5-mCherry, and SEPT7-mCherry were individually co-expressed with LiveDrop, we observed transient clustering of FIT2 and REEP5 near LiveDrop-positive sites (**Fig. 8A,B**). Similarly, SEPT7 puncta transiently overlapped with LiveDrop puncta (**Fig. S5C**). Interestingly, the formation of both FIT2 and REEP5 puncta preceded that of LiveDrop puncta. In contrast, SEPT7 puncta only approached LiveDrop when LiveDrop signals were readily punctate. These results suggest a spatiotemporal order/hierarchy of FIT2-mediated nascent LD formation in which FIT2 and ER tubule-forming proteins gather in early stages and septins join as a handrail when nascent LD has grown to a certain size (**Fig. 8F**). We added these new experiments and adjusted the discussion accordingly.



Does FIT2 function/localize to budding LDs with seipin? Is the role/function of FIT2 upstream or down-stream of seipin? How does knock-down of seipin impact FIT2/septin localization and vice versa?

We previously tested the interactions between seipin and FIT2 and found a very weak association, even when both are overexpressed. We reason that seipin and FIT2 act independently. First, when seipin was depleted in FIT2-HA KI cells, interactions between endogenous FIT2 and Rtn4/REEP5 were not altered (**Fig. S3I**). Second, when seipin was depleted in LiveDrop-expressing COS-7 cells, the degree of overlap between LiveDrop and SEPT2 was not affected (**Fig. S5F,G**). These results support the notion that FIT2-mediated nascent LD formation is not influenced by seipin. We added these new experiments and adjusted the discussion accordingly.
As suggested, we attempted to transfect COS-7 cells with FIT2-halo and septin7-mCherry while depleting seipin. These cells became rather sick, and we failed to obtain any usable time-lapse data.

Does the interaction FIT2 with Rtn41, REEP5 and/or septins depends on its phosphatase activity? Previous studies have shown that FIT2 mutants with defective phosphatase activity were not able to restore LD budding in FIT deficient cells. Perhaps, these mutants can be utilized to determine whether FIT2 activity is required for the interaction with septins.

Based on the predicted site of action, which is close to the lumen side, we suspect that its enzymatic activity does not likely affect its interactions with Rtn4/REEP5/septins. We performed co-IP experiments with FIT2 H155A/H214A and endogenous Rtn4/REEP5 or SEPT2/SEPT7, finding no changes compared to wt FIT2 (**Fig. S2J** and **S2N**).

4. The authors claim that FIT2 and FIT2-interacting proteins mark peripheral LD formation sites (Figure 7). This has interesting implications for the spatial control of LD formation in sub-domains of the ER. However, the claim is not very strongly supported by the data. The authors should make an effort to address the following:
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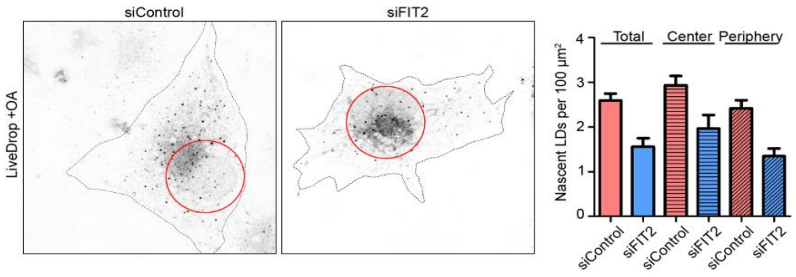
As suggested, we counted nascent LDs marked by LiveDrop and compared their distribution between the perinuclear region (cell center) and cell periphery. We defined the center region by drawing a circle with a radius of 17.5 μm from the center of the nucleus (red lines in images shown below). We observed an equivalent density of nascent LDs in these two regions. In addition, when FIT2 was depleted, the numbers of nascent LDs were decreased in both regions to a similar extent. Due to limitations in live cell imaging by GI-SIM, we were only able to monitor nascent LD formation in the cell periphery as defined above and, therefore, could only address the spatiotemporal organization of FIT2-mediated nascent LD biogenesis in the cell periphery. We have clarified this point in the text.



ii) Can the authors determine whether there is a preferential localization of FIT2 and/or septin 2 to peripheral ER tubules? This should not be so hard to determine and quantify by staining for marker of tubular ER and FIT2/septin2. If indeed FIT2 and septins localize preferentially to peripheral ER tubules, this will boost a role of FIT2 in the spatial control of LD biogenesis/maturation from subdomains of the ER.

We thank the reviewer for the suggestion but find it challenging to do so. As mentioned above in response to point 3, we failed to detect a signal for endogenous FIT2 in FIT2-mCherry or FIT2-4xGFP$_{11}$ KI cells. When FIT2 was overexpressed, the ER morphology was distorted. In the cell periphery, almost all LiveDrop puncta occurred in ER tubules. However, it is difficult to test the perinuclear region due to the high density of ER structures there, including both tubules and sheets (there is almost always a cluster of ER tubules in the perinuclear region). Notably, ER sheets have high membrane curvature similar to the tubules at the edges, and ER tubule-forming proteins (i.e., curvature stabilizing proteins) localize to the edges of sheets. In theory, nascent LD could also be generated in ER sheets, even if curvature and curvature-stabilizing proteins are needed. We have clarified this point in the text.

Minor Comments:

- The authors should avoid using the terms "septin cytoskeleton" and "septin filaments" throughout the manuscript. It is unclear whether the LD membrane associated septins are indeed filaments and most likely these septins are not cytoskeletal elements like actin and microtubules, which do not appear to influence septin-FIT2 association.

We have made the suggested changes.

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There are several general concerns and some minor issues:

1) It is still not completely clear if the septin effects on FIT2 and LD production are direct or indirect. Septins are first observed to colocalize with FIT2 only when FIT2 is over-expressed to generate ER bubbles (Fig 2D). There does not appear to be any endogenous co-localization experiments indicating septin can localize with native FIT2 under non over-expressed conditions. To this point, the septin2-FIT2 co-ip data appears to be with over-expressed FIT2-HA as well (Fig 4). Is there any detected endogenous septin at the ER when FIT2 is not over-expressed?

As mentioned above in response to reviewer #2, we performed endogenous co-IP with FIT2-HA (knocked-in) and septins (**Fig. 4D**). The interactions between FIT2 and septin 7 were only detected during OA-induced LD formation after starvation and with chemical crosslinking. Co-flotation experiments indicate that purified FIT2 and septin 7, but not other septins, have direct interactions.

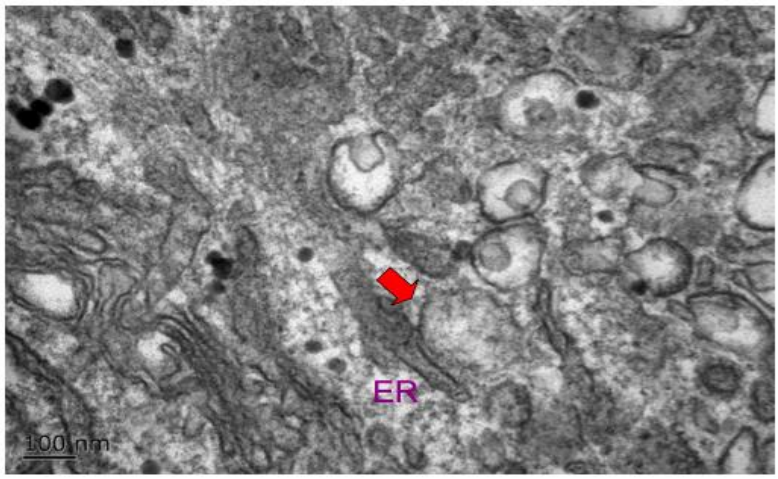
2) Septin is thought to be recruited to FIT2 bubbles via protein-protein interactions, but an alternative possibility is that septins are attracted to the bubble membrane itself independent of FIT2. Some Septins have been reported to encode amphipathic helices that may target to LDs or LD like compartments, so perhaps this is targeting septin to the bubbles? Can the region of septin that targets to FIT2 be further defined?

As mentioned above, we now show that FIT2 directly interacts with septin 7. The C-terminal amphipathic helix was found in septin2/6/9 but not septin 7. Therefore, we find it less likely that septins are recruited to FIT2 through indirect association with LD membranes. Having said this, we believe that membrane association of septins would contribute to their roles in LD biogenesis and propose that, once FIT2 engages septin 7-containing septin polymers, other septins attach to the phospholipid surface of the nascent LDs, serving as a handrail (**Fig. 8F**). As shown previously and now confirmed by bio-layer interferometry (BLI), septin 7 interacts mostly with the N-terminal cytosolic loop of FIT2 (**Fig. 4G-I**). To narrow down the regions of septin 7 that interact with FIT2, we purified the C-terminal coiled coil (CC) region of septin 7 and a truncated septin 7 that lacks the CC domain. We showed that SEPT7-ΔCC had similar

interactions with the FIT2-NTL peptide as wt SEPT7. Consistently, the SEPT7 CC had a much weaker interaction with the peptide (**Fig. 4I**). These results suggest that FIT2 interacts mostly with the GTPase domain of SEPT7.

3) There is not much analysis of the ER bubbles that are generated by FIT2 over-expression. Are they ER bilayers or monolayer bulges that are devoid of neutral lipids? Some higher resolution imaging or electron microscopy data would greatly add to this study.

We previously attempted CLEM analysis of the bubbles, but the results were inconclusive (see below for an example). The overexpression of FIT2 greatly altered the ER morphology. Because the bubbles are generated artificially, we were afraid that detailed interpretation of their nature would lack physiological context.



4) Fig 7 uses time lapse imaging to monitor nascent LD biogenesis. This work is particularly exciting but not well quantified. Can the number of septin positive LiveDrop puncta be quantified? Same with Reep5?

We added the quantification as suggested (**Fig. S5C**). In addition, we analyzed the degree of overlap between LiveDrop puncta and endogenous septin 2 (**Fig. 8C-E**). We found that the overlap was affected by the depletion of FIT2, but not seipin (**Fig. S5F,G**).

Minor comments:

1) Several image panels are quite dark and should be set to gray scale. Fig 7 red and magenta channels are particularly dark and should be gray.

We have made changes as appropriate.

January 25, 2021

RE: JCB Manuscript #201907183R

Dr. Junjie Hu
Institute of Biophysics
15 Datun Rd. Chaoyang District
Beijing, Beijing 100101
China

Dear Dr. Hu:

Thank you for submitting your revised manuscript entitled "FIT2 organizes lipid droplet biogenesis with ER tubule-forming proteins and septins". The paper has now been seen again by the original reviewers, both of whom recommend acceptance. Thus, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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, https://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 and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends. You are currently below this limit but please bear it in mind when revising.

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

3) 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 (both in the figure legend itself and 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, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) 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 (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) 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.

6) 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.).

7) 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.

8) There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
Please note that we do not allow additional reference sections in the supplemental materials. Thus, you will need to delete the supplemental references section and include any non-duplicated references in the main reference section.
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.

9) 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. It should begin with "First author name(s) et al..." to match our preferred style.

10) 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."

11) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

12) 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.

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).

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

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

Sincerely,

Elizabeth Miller, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor

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

The authors have demonstrated that FIT2 cooperates with septins and ER tubule forming proteins to organize lipid droplet formation. The current version of the manuscript is significantly improved from the original submission and I have no further concerns about its suitability for publication.

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

The revisions address the majority of concerns raised. More protein-protein interaction work has been conducted, and use of endogenously-tagged FIT2 strengthens the conclusions of this section.
There is also new work supporting the claim that FIT2 interacts with septins, and potentially specifically interacts with SEPT7.
Quantification of LD spatial positioning in the cell periphery has also been improved.
The text has also been adjusted, and conclusions are generally in line with the experimental evidence.