JCB

A fluorogenic complementation tool kit for interrogating lipid droplet-organelle interaction

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December 26, 2023

Re: JCB manuscript #202311126

Dr. Chi-Lun Chang
St. Jude Children's Research Hospital
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262 Danny Thomas Pl.
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Memphis, TN 38105

Dear Dr. Chang,

Thank you for submitting your manuscript entitled "A fluorogenic complementation tool kit for interrogating lipid droplet-organelle interaction." The 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 all three reviewers found your new tool kit promising but raise several issues, which all need to be addressed. The most important is point #1 from of Reviewer #2, who asks for a better demonstration that your system does not itself induce contacts. This reviewer asked you to "...benchmark FABCON in relation to unperturbed control cells (or cells expressing the same organelle markers but in the absence of splitFAST moiety)." This is critical. The reviewer suggests using EM, which is still the gold standard for quantitatively measuring contact sites and would be best. However, FRET could be an acceptable alternative. It is not necessary to verify all the results, but a second method should be used to make sure that LD wrapping is not being induced. The reviewer's concerns about the effects of ligand washout are also important.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

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, and acknowledgments. Count does not include materials and methods, figure legends, 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, https://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.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. If your paper will include cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots

should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

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 at cellbio@rockefeller.edu.

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

Sincerely,

William Prinz, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

In their manuscript "A fluorogenic complementation tool kit for interrogating lipid droplet-organelle interaction", Li and colleagues establish tools to detect membrane contact site dynamics in cell lines. Their reporters are based on fluorogen-activated bimolecular complementation at contact sites, named FABCON (making use of the previously established low affinity split protein splitFAST). The authors center their manuscript around membrane contact sites established by lipid droplets. To this end, they establish a LD-anchored NFAST protein with sufficiently low affinity to prevent artificial tethering to the CFAST-half anchored to another organelle (here, they use ER, mitochondria and peroxisomes). As a proof-of-principle, the authors then mainly focus on LD-ER contacts and extensively assess LD-ER contact site changes in response to different metabolic transitions as well as upon deletion of previously established regulators of this contact site. In addition, they also provide a substantial amount of data for LD-mitochondria contacts and present some results for LD-peroxisome contacts. Finally, the authors show that these FABCON constructs can also be applied to visualize ER-mitochondria contact sites. In addition, they develop an automated line-scanning analysis pipeline for unbiased contact site mapping (which they name COSIMA) and apply this on the ER-LD FABCON to show that this pipeline can be used to quantitatively asses contact site size and coverage. While this pipeline seems robust to me, I do not have the competence to thoroughly evaluate this part of the study (Figure 6) and will thus base my evaluation on the remaining part of the manuscript.

The manuscript is well-written and the data is of high quality. The tools that are established come very timely and will likely be of high interest to the scientific community. This new tool largely eliminates some of the problems with previously available tools for the detection of membrane contact sites, in particular the caveat that most of these tools not only detect but also induce/enforce contact formation. Overall, the reporters have been thoroughly characterized, and the proof-of-principle presented for LD-ER and LD-mito contact sites is very interesting and not only recapitulates what has previously been shown for this contact site (e.g. the dependency of LD-ER contact on VAB) but also extends this to dynamic changes upon metabolic cues. Presented data is convincing and the experiments are thoroughly executed. I only have a few comments, mainly in respect to additional control experiments to complement presented data, that should be addressed:

Major:
1. The authors engineer a synthetic LD targeting motif (6xHp) based on Spastin's Hp motif (1xHp) and characterize its localisation in detail, showing that 6xHp is almost exclusively localized to LD, while a large part of 1xHp is still found in the ER. Figures 2D-H are all dedicated to validate that 6xHp is mainly/almost exclusively localized to LDs, and these data in principle are convincing. Still, Fig. 2E indicates a residual ER localisation also for 6xHp. A sucrose gradient was done for 1xHp, showing substantial ER localisation in addition to LD localization (in Fig. S1A). Please add similar experiments for 6xHp to provide a more quantitative evaluation of what fraction of 6xHp remains in the ER (microscopy suggests "negligible ER localization")

2. Figure 2I: the authors address the concern that protein crowding on LDs upon expression of 6xHp might affect localisation of other proteins: please add as a necessary control also cells not expressing 6xHp to ensure that PLN2 localisation is not affected. Simple showing PLN2 in cells expressing 6xHp does not allow any conclusion.

3. According to the immunoblots shown in Figure S5C, the PLIN5 knockdown seems rather inefficient. Is there indeed a decrease in protein levels? This is not clearly visible. For these minor differences, a quantification of several experiments will be needed.

4. Table 2: Why was coverage not quantified for the ER-LD contact site in HeLa cells but for all other combinations shown in this table? The respective microscopy for ER-LD in HeLa cells is shown in Fig. 4A, please add the respect quantifications to Table 2.

5. The author show that NFASThigh on LD induces contact site formation, but NFASTlow does not. These are very important experiments to support that the tools that have been developed indeed overcome previous limitations. Sufficient controls are included for:
-the LD-Pex site (Figure S2, where the authors also assess Halo-6xHp and PMP34-RedFP without the fusion to CFAST and NFASThigh/low, and provide a quantification of co-localisation between LD and peroxisomes).
-the LD-mito site (Fig. 3E and F, containing a control in absence of CFAST and corresponding quantification of co-localisation).

However, for the LD-ER site, a respective control is missing in Fig. 3A, C. Please include respective control (e.g. using Halo-ER without CFAST) and provide corresponding quantification of LD - ER colocalization as done in Figs. 3F (LD-mito) and S2B (LD-Pex).

6. I am a bit puzzled by the extent of contact formation, relating both to the % of LDs in contact with other organelles as well as to the amount of "full coverage". Quantifications presented in Table 2 indicate that in U2OS cells, 96% of LDs make contact with the ER, 94% make contact with mitochondria and 66% make contact with peroxisomes. This indicates that actually almost all LDs simultaneously make contact with the ER and mitochondria (and a large part in addition also with peroxisomes), which is of course highly interesting. At the same time, the amount of "coverage" is very high, e.g. 54% of the LDs are complete covered in ER-LD contact sites, and about 24% are complete covered in Mito-LD contact sites. The rest shows partial coverage. Does this indicate e.g. that 54% of the LDs are fully wrapped in ER, while still almost all (94%) LDs make substantial contact with the mitochondria? Could the authors please comment on this and clarify?

Minor:

1. Please correct "following inhibition of glycolysis inhibition" at the end of the introduction

2. Figure 1 is already described at the end of the introduction and not in the results section, which is a bit confusing. The authors might consider to restructure this to indeed describe also Figure 1 in the results, not the introduction.

3. Please briefly explain which targeting motif was used for CFAST-ER when introduced for the first time (signal sequence/TMD/ER retention signal?)

4. For readers not familiar with the APEX system, please add a brief explanatory sentence in respect to the APEX-6xHp/H2O2 used for EM.

5. Please correct N-terminal to N-terminus in:
"We tagged high-affinity NFAST to the N-terminal of mApple-6xHp (NFASThigh-LD) and CFAST to the N-terminal of Halo-ER (CFAST-ER) to detect the effect of NFASThigh-CFAST interaction on these organelles."
6. The authors might consider to include representative micrographs for the analysis of ER-LD contacts in control and VAP DKO HeLa cells (corresponding to the quantification shown in 4D) and for the oleate treatment (0 h versus 1 h of oleate treatment), quantified in Fig. 4E.

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

Response to "A fluorogenic complementation tool kit for interrogating lipid droplet-organelle interaction".
Summary: This paper develops a new tool to visualize and study Lipid droplet (and other organelle) contact sites, based on a reversible and low affinity split fluorescent reporter. The authors convincingly validate a new marker of the lipid droplet membrane, 6xHp derived from spastin. They use this marker in their split fluorescence system to show interaction of the lipid droplets with the ER, mitochondria, and peroxisomes. This system was used to monitor the dynamics of ER-LDs and Mito-LDs contacts under various conditions. Moreover, the authors describe a pipeline to enable the analysis of membrane contact sites on large scale. Overall the development of new tools for the analysis of membrane contact sites would be of interest to community. However, there are a number of concerns (detailed below) and a general trend for overstating results and not

explaining data clearly within the main text.
Major points:
1) The uniqueness of the system described by the authors are the reversibility and "minimal perturbation to organelle interaction". In my opinion neither of these features is convincingly demonstrated. Although the system is demonstrably better than the high-affinity FAST system, this does not demonstrate that it isn't inducing contact sites on its own. It would be important to benchmark FABCON in relation to unperturbed control cells (or cells expressing the same organelle markers but in the absence of splitFAST moiety). This would involve careful analysis of contact sites in both cells types, ideally including electron microscopy. The almost complete wrapping of LDs by ER and in some cases also mitochondria is worrying and suggests that the FABCON system is not neutral to membrane contact sites.
The authors show that the fluorescent ligand can be quickly washed out (Fig S3). However, the data presented is not informative in relation to the contact site itself. Further characterization of the contact sites in absence, presence and after ligand wash out would be important. Perhaps the FRAP assay described in Figure 3B/D would be a good starting point (although it would be good to include more details on how the experiment was performed).
2) The genetic analysis undertaken is largely devoid of biological context. While reducing the data to a single number is attractive, it fails to provide the complexity of later analysis (like in figure 6). This reviewer would like to know why the 'relative ER-LD contact sites' might change: fewer lipid droplets, a change in contact site intensity, altered lipid droplet size etc which may all impact this readout.
Minor points:
1) Several experiments should be explained better in the text (what's expected, what the analysis is, and how this fits into the context): e.g. Fig. 2H, Fig. 4E.
2) As this paper focuses on LD contact sites with the ER and mitochondria, it would be good to see all of these in a single image, e.g. by adding mitotracker to ER-NFAST/LD-CFAST expressing cells.
3) Describing changes in LDs by quantifying Bodipy intensity (for example Figure 1J/K, S1 etc) is not very informative. Changes in paramenters like LD size and number are a standard in the field.
4) In several places oleic acid is used at higher and lower amounts - it would be informative if a justification for the amount of OA used was provided.
5) In Figure S4A-C, CFAST HALO LD looks very different in the various cells. Why?
6) There are a few typing errors throughout the text that the authors should see to. For example:
the acronym LM is not explained (I assume it is light microscopy).
Page 4, second paragraph, line 8, "incubation" should be "following incubation". Page 8, second paragraph, line 5, "moderately" should be "moderate".6) Figure 4G is not referred to in the text.
The reference "Scorrano et al."is incomplete.

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

Split-FAST is a clever idea, and applying it to various contacts must be the way to go. However, a decent amount of scoping is needed before everyone piles in and any misconceptions arise. Many aspects of this study are excellent; including as examples: (i) the demonstration that NFAST with a low endogenous affinity of the split partner (Kd =200 μM) has very little effects on endogenous organelles; (ii) modulation of mito-LD contacts with physiological change over periods of ~60 minutes

One issue is that SplitFAST may be fast but not fast enough: we don't a priori know what the time scale is for altering organelle physiology. Fig S3C shows development of contact at 30 seconds, and then the amount of contact increases by ~40% in the next 2-3 minutes. Are the traces here individual contact sites? If so, given the movement is the movement of contact as shown in Fig 5B one reason for variability amount the traces? Is the drop after 3 minutes caused by bleaching? (NB values with more time resolution between 0 and 30 seconds for both wash-in and wash-out would help, maybe in separate experiments to avoid bleaching effects). Does the 93% of LDs with contacts reported (Table 2) occur at 30 seconds or later? This paper on SplitFAST at contacts is the ideal place to show if this proportion changes over the next 20+ minutes to provide further evidence to support the statements here that it is OK to apply the fluorogen for up to 30 minutes.

Colours for merge images:
The choice of colour pairs is a long way from ideal. For the ~8% of men with minority forms of colour vision (mainly deuteranomaly), purple vs turquoise is perhaps the least helpful combination. Advice can be gained from web sources such as https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2667680/.

Minor: too much text in Introduction reads like results. However, Fig 1A is unnecessary and Fig 1B is confusing: one set of double headed arrows is gone and one still there. What can that mean? The diagram does not explain the heterodimer+fluorogen combination - see Fig 1A of Tebo and Gautier for the kind of thing needed.

Figure S2. Low affinity splitFAST affects PEX: quite a strong effect with NFAST-high and in the image provided a considerable effect with NFAST-low (not "minimal")

Typos: about glycolysis in Intro.; citations without years

Writing: a bit over-interpretative "faithfully reports the expected changes" (p6): one cannot be faithful to something that has not been observed before but is just expected.

Spurious accuracy: 4 significant figures for ER-LD contact (p7) are ?2 too many.

Other stats: median less than mean (p7): this is almost universal for a value where low readings are likely to be limited, here by resolution and dminess. So this specific aspect is not "Interestingly. An alternative would be to test the distribution for bimodlaity.

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

In their manuscript "A fluorogenic complementation tool kit for interrogating lipid droplet-organelle interaction", Li and colleagues establish tools to detect membrane contact site dynamics in cell lines. Their reporters are based on fluorogen-activated bimolecular complementation at contact sites, named FABCON (making use of the previously established low affinity split protein splitFAST). The authors center their manuscript around membrane contact sites established by lipid droplets. To this end, they establish a LD-anchored NFAST protein with sufficiently low affinity to prevent artificial tethering to the CFAST-half anchored to another organelle (here, they use ER, mitochondria and peroxisomes). As a proof-of-principle, the authors then mainly focus on LD-ER contacts and extensively assess LD-ER contact site changes in response to different metabolic transitions as well as upon deletion of previously established regulators of this contact site. In addition, they also provide a substantial amount of data for LD-mitochondria contacts and present some results for LD-peroxisome contacts. Finally, the authors show that these FABCON constructs can also be applied to visualize ER-mitochondria contact sites. In addition, they develop an automated line-scanning analysis pipeline for unbiased contact site mapping (which they name COSIMA) and apply this on the ER-LD FABCON to show that this pipeline can be used to quantitatively asses contact site size and coverage. While this pipeline seems robust to me, I do not have the competence to thoroughly evaluate this part of the study (Figure 6) and will thus base my evaluation on the remaining part of the manuscript.

The manuscript is well-written and the data is of high quality. The tools that are established come very timely and will likely be of high interest to the scientific community. This new tool largely eliminates some of the problems with previously available tools for the detection of membrane contact sites, in particular the caveat that most of these tools not only detect but also induce/enforce contact formation. Overall, the reporters have been thoroughly characterized, and the proof-of-principle presented for LD-ER and LD-mito contact sites is very interesting and not only recapitulates what has previously been shown for this contact site (e.g. the dependency of LD-ER contact on VAB) but also extends this to dynamic changes upon metabolic cues. Presented data is convincing and the experiments are thoroughly executed. I only have a few comments, mainly in respect to additional control experiments to complement presented data, that should be addressed:

Major:

1. The authors engineer a synthetic LD targeting motif (6xHp) based on Spastin's Hp motif (1xHp) and characterize its localisation in detail, showing that 6xHp is almost exclusively localized to LD, while a large part of 1xHp is still found in the ER. Figures 2D-H are all dedicated to validate that 6xHp is mainly/almost exclusively localized to LDs, and these data in principle are convincing. Still, Fig. 2E indicates a residual ER localisation also for 6xHp. A sucrose gradient was done for 1xHp, showing substantial ER localisation in addition to LD localization (in Fig. S1A). Please add similar experiments for 6xHp to provide a more quantitative evaluation of what fraction of 6xHp remains in the ER (microscopy suggests "negligible ER localization")

Authors' response:

In the revised manuscript, we have included the sucrose gradient data for 6xHp. To do this, we first generated the mApple-6xHp lentivirus and transduced the HepG2 cells. We then performed sucrose gradient fractionation as described in our methods. The sucrose gradient fractionation clearly shows that 6xHp is highly enriched in lipid droplets (LDs) relative to the pellet fraction while 1xHp was indistinguishably distributed across both LDs and the pellet (Figures 2D–F). This fractionation data supports our microscopy data, which demonstrates a significant enrichment of 6xHp in LDs with relative low ER localization (Figures 2B and 2C).

2. Figure 2I: the authors address the concern that protein crowding on LDs upon expression of 6xHp might affect localisation of other proteins: please add as a necessary control also cells not expressing 6xHp to ensure that PLN2 localisation is not affected. Simple showing PLN2 in cells expressing 6xHp does not allow any conclusion.

Authors' response:

To address this concern, we have included cells that do not express the 6xHp as a control. The updated data can be found in Figure 3A, which now demonstrates that the localization of endogenous PLIN2 is similar between non-transfected cells and cells overexpressing 6xHp. With this control, we can conclude that the overexpression of 6xHp does not affect PLIN2's ability to access LDs.

3. According to the immunoblots shown in Figure S5C, the PLIN5 knockdown seems rather inefficient. Is there indeed a decrease in protein levels? This is not clearly visible. For these minor differences, a quantification of several experiments will be needed.

Authors' response:

We agree with the reviewer's comment. To address this point, we performed three independent PLIN 5 knockdown experiments. Densitometry analysis showed that PLIN 5 knockdown was ~60%. The new data is now included in Figure 6C and 6D of the revised manuscript.

4. Table 2: Why was coverage not quantified for the ER-LD contact site in HeLa cells but for all other combinations shown in this table? The respective microscopy for ER-LD in HeLa cells is shown in Fig. 4A, please add the respect quantifications to Table 2.

Authors' response:

We have now added the quantification data to Table 2 in the revised manuscript. Our measurements of ER-LD contact sites in HeLa cells revealed that 44% of LDs had complete coverage of the ER-LD contact site, while 56% had partial coverage.

5. The author show that NFASThigh on LD induces contact site formation, but NFASTlow does not. These are very important experiments to support that the tools that have been developed indeed overcome previous limitations. Sufficient controls are included for:

- the LD-Pex site (Figure S2, where the authors also assess Halo-6xHp and PMP34-RedFP without the fusion to CFAST and NFASThigh/low, and provide a quantification of co-localisation between LD and peroxisomes).
- the LD-mito site (Fig. 3E and F, containing a control in absence of CFAST and corresponding quantification of co-localisation).

However, for the LD-ER site, a respective control is missing in Fig. 3A, C. Please include respective control (e.g. using Halo-ER without CFAST) and provide corresponding quantification of LD - ER colocalization as done in Figs. 3F (LD-mito) and S2B (LD-Pex).

Authors' response:

We appreciate the reviewer's suggestion. To address this, we conducted control experiments by using constructs that do not contain splitFAST. Specifically, we co-transfected the constructs Halo-ER (an ER marker) with mApple-6xHp (an LD marker) and performed FRAP experiments. Our imaging data did not show colocalization between Halo-ER and mApple-6xHp. Additionally, the Halo-ER FRAP in regions near LD was comparable to those in the ER. This additional data is now presented in Figure 4A and 4B.

6. I am a bit puzzled by the extent of contact formation, relating both to the % of LDs in contact with other organelles as well as to the amount of "full coverage". Quantifications presented in Table 2

indicate that in U2OS cells, 96% of LDs make contact with the ER, 94% make contact with mitochondria and 66% make contact with peroxisomes. This indicates that actually almost all LDs simultaneously make contact with the ER and mitochondria (and a large part in addition also with peroxisomes), which is of course highly interesting. At the same time, the amount of "coverage" is very high, e.g. 54% of the LDs are complete covered in ER-LD contact sites, and about 24% are complete covered in Mito-LD contact sites. The rest shows partial coverage. Does this indicate e.g. that 54% of the LDs are fully wrapped in ER, while still almost all (94%) LDs make substantial contact with the mitochondria? Could the authors please comment on this and clarify?

Authors' response:

Thank you for your thoughtful question. We have now incorporated a brief discussion in the Result section of our manuscript (page 6, lines 3–5) about the limitations of confocal light microscopy regarding capturing the coverage of contact sites using our FABCON reporters. Our microscope's lateral resolution is 200–300 nm, and its axial resolution is approximately 500 nm, which means that the contact site coverage cannot be resolved perfectly if the size of the organelle contact site is smaller than the microscope's resolution or adjacent contact sites are too close to be resolved. Thus, we cannot rule out the possibility of overestimating our measurement of contact site coverage due to these limitations.

Minor:

1. Please correct "following inhibition of glycolysis inhibition" at the end of the introduction

Authors' response:

Thank you for pointing this out. This is now corrected in the revised manuscript (page 3, line 20).

2. Figure 1 is already described at the end of the introduction and not in the results section, which is a bit confusing. The authors might consider reshaping this to describe Figure 1 in the results, not the introduction.

Authors' response:

We appreciate this feedback. Figure 1 is no longer cited in the Introduction of our revised manuscript. We have incorporated the citation of Figure 1 into the first paragraph of the Results section (page 3, line 29).

3. Please briefly explain which targeting motif was used for CFAST-ER when introduced for the first time (signal sequence/TMD/ER retention signal?)

Authors' response:

Cytochrome b5 ER membrane domain or Sec61β were used for the targeting motif for CFAST-ER. This information has been added to the revised manuscript (page 4, line 32).

4. For readers not familiar with the APEX system, please add a brief explanatory sentence in respect to the APEX-6xHp/H2O2 used for EM.

Authors' response:

In our revised manuscript, we have described the APEX system to improve readability. Thank you for the suggestion (page 4, lines 10–12).

5. Please correct N-terminal to N-terminus in:
"We tagged high-affinity NFAST to the N-terminal of mApple-6xHp (NFASThigh-LD) and CFAST to the N-terminal of Halo-ER (CFAST-ER) to detect the effect of NFASThigh-CFAST interaction on these organelles."

Authors' response:

Thank you for bringing this to our attention. We have made the necessary corrections to the revised manuscript.

6. The authors might consider to include representative micrographs for the analysis of ER-LD contacts in control and VAP DKO HeLa cells (corresponding to the quantification shown in 4D) and for the oleate treatment (0 h versus 1 h of oleate treatment), quantified in Fig. 4E.

Authors' response:

Thank you for your suggestions. We have included representative images for the analysis of ER-LD contacts in the control and VAP DKO HeLa cells (Figure 5E); we also updated the images for WT and Seipin knockout cells to better represent the analysis (Figures 5I and 5J).

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

Response to "A fluorogenic complementation tool kit for interrogating lipid droplet-organelle interaction".

Summary: This paper develops a new tool to visualize and study Lipid droplet (and other organelle) contact sites, based on a reversible and low affinity split fluorescent reporter. The authors convincingly validate a new marker of the lipid droplet membrane, 6xHp derived from spastin. They use this marker in their split fluorescence system to show interaction of the lipid droplets with the ER, mitochondria, and peroxisomes. This system was used to monitor the dynamics of ER-LDs and Mito-LDs contacts under various conditions. Moreover, the authors describe a pipeline to enable the analysis of membrane contact sites on large scale. Overall the development of new tools for the analysis of membrane contact sites would be of interest to community. However, there are a number of concerns (detailed below) and a general trend for overstating results and not explaining data clearly within the main text.

Major points:

1) The uniqueness of the system described by the authors are the reversibility and "minimal perturbation to organelle interaction". In my opinion neither of these features is convincingly demonstrated. Although the system is demonstrably better than the high-affinity FAST system, this does not demonstrate that it isn't inducing contact sites on its own. It would be important to benchmark FABCON in relation to unperturbed control cells (or cells expressing the same organelle markers but in the absence of splitFAST moiety). This would involve careful analysis of contact sites in both cells types, ideally including electron microscopy.

Authors' response:

We appreciate the reviewer's critical feedback. We have addressed the concern of whether FABCON induces contact sites on its own by imaging experiments via light and electron microscopy. At the whole organelle level, we performed colocalization experiments between three pairs of organelles: ER-LD, mito-LD, and PX-LD. Across all pairs, we consistently found low-affinity splitFAST had a minimal effect on organelle colocalization while high-affinity splitFAST significantly enhanced it (Figures 4A, 4C–E, and S1A and S1B). At the contact site level, we used FRAP analysis as a surrogate readout for the affinity of organelle interaction. We observed that low-affinity splitFAST did not affect organelle interaction at contact sites whereas high-affinity splitFAST significantly strengthened this interaction (Figure 4B). Finally, we imaged wild-type HeLa cells and cells that expressed the FABCON$^{mito\text{-}LD}$ marker with a scanning transmission electron microscope. We measured the length of contact sites between mitochondria and LDs and found that the lengths of mito-LD contact sites in cells expressing the FABCON marker were similar to the control, suggesting that FABCON was not enhancing native membrane contact sites (Figure 4F). In conclusion, FABCON implemented with low-affinity splitFAST did not enhance contact sites formation within our experimental set-ups.

The almost complete wrapping of LDs by ER and in some cases also mitochondria is worrying and suggests that the FABCON system is not neutral to membrane contact sites.

Authors' response:

As we noted in response to a similar comment from reviewer 1, we believe that this is due to the resolution limit of the confocal microscope, which has a lateral resolution of 250 nm and an axial resolution of 500 nm. Therefore, if the size of contact sites or the distance between adjacent contact sites is smaller than the resolution limit, the confocal microscope cannot resolve them accurately, giving an overestimated coverage of contact sites. We have included a description about the resolution limitation in the revision (page 6, lines 3–5).

The authors show that the fluorescent ligand can be quickly washed out (Fig S3). However, the data presented is not informative in relation to the contact site itself. Further characterization of the contact sites in absence, presence and after ligand wash out would be important. Perhaps the FRAP assay described in Figure 3B/D would be a good starting point (although it would be good to include more details on how the experiment was performed).

Authors' response:

As suggested by the reviewer, we have characterized the contact sites between mitochondria and LDs in cells expressing the FABCON$^{mito\text{-}LD}$ in the absence, presence, and after ligand washout using electron microscopy. As shown in Figure 4F, none of these conditions significantly altered the contact site lengths between mitochondria and LDs (page 6. lines 24–26). This data further confirms that the FABCON system does not induce contact sites but rather reports the existence of them.

2) The genetic analysis undertaken is largely devoid of biological context. While reducing the data to a single number is attractive, it fails to provide the complexity of later analysis (like in figure 6). This reviewer would like to know why the 'relative ER-LD contact sites' might change: fewer lipid droplets, a change in contact site intensity, altered lipid droplet size etc which may all impact this readout.

Authors' response:

We appreciate the reviewer's feedback. The focus of this manuscript is to demonstrate FABCON's utility to study contact sites. The purpose of analyzing genetically modified cells is to provide biological validation that the FABCON tool can report the expected changes in contact sites upon manipulation using established systems, such as in the VAPA/B knockout and PLIN 5 knockdown cells. Once this is established, we and others in the field will use this tool kit to investigate new biological questions about organelle contact sites with confidence. We have provided background information about the genetic analysis and included additional discussion about their possible biological context in the revision. For these reasons, we hope that the reviewer can understand why we did not dig deep into the biology in the current study and appreciate the importance of this FABCON tool kit.

Quantification of individual contact sites is technically challenging in HeLa and SUM159 cells with clusters of small lipid droplets due to resolution limitation of confocal microscopy (page 6, lines 3–5). Thus, we developed the simplified, relative contact site analysis, in which the extent of contact sites was quantified by normalizing the intensity of the dye HBR-2,5DOM from a cell to that of the CFAST-Halo-LD fluorescence to account for variations in FABCON expression levels. This measurement was not expected to correlate with the size and number of LDs because of the Halo-LD normalization. We further quantified the number and size of LDs during biogenesis in the revised manuscript (Figures S3A and S3B) and found no correlation of these factors to the dynamic regulation of ER-LD contact sites; ER-LD contact sites showed a peaked increase after 1 hour of biogenesis while the number of LDs increased steadily at both 1 and 4 hours and LD size only increase after 4 hours (Figures 5G, S3A, and S3B). Similarly, we did not observe a correlation between either size or number of LDs with mito-LD contact sites during the oleic acid (OA) withdrawal experiment (Figures 6F, S3C, and S3D). Therefore, the relative contact site

intensity seems to be not decided by number or size of LDs, but most likely by how the two organelles interact with each other.

Minor points:
1) Several experiments should be explained better in the text (what's expected, what the analysis is, and how this fits into the context): e.g. Fig. 2H, Fig. 4E.
Authors' response:
Modifications in the text have been made as suggested by the reviewer (new Figure 2K and Figure 5G).

2) As this paper focuses on LD contact sites with the ER and mitochondria, it would be good to see all of these in a single image, e.g. by adding mitotracker to ER-NFAST/LD-CFAST expressing cells.
Authors' response:
While we agree that it would be nice to see both contacts sites of LDs with the ER and mitochondria in one image, it requires two orthogonal pairs of split reporters to achieve this. We discussed the possibility of using split HaloTag or ddFP as orthogonal reporter in the future in the Discussion section (page 11, line 9–12). Adding mitotracker to FABCON$^{ER\text{-}LD}$-expressing cells would simply reveal mitochondria in proximity with LDs/ER-LD contact sites; however, that would not necessarily mean they are contact sites with LDs due to the resolution limit of confocal microscope. Therefore, we did not include this experiment in our revised manuscript. We hope the reviewer can accept this decision.

3) Describing changes in LDs by quantifying Bodipy intensity (for example Figure 1J/K, S1 etc) is not very informative. Changes in parameters like LD size and number are a standard in the field.
Authors' response:
As suggested by the reviewer, quantification of the size and number of LDs in control and 6xHp-expressing cells has been provided in the revision (Figures 3B–E). We believe our data indicates the 6xHp does not significantly affect either the size or the number of LDs during biogenesis and breakdown.

4) In several places oleic acid is used at higher and lower amounts - it would be informative if a justification for the amount of OA used was provided.
Authors' response:
In general, concentrations over 100 μM of OA were intended to induce a large number of LDs. The variation in concentration was based on the cell lines used and time of incubation. For example, 500 μM OA was used for rapid LD formation in HeLa cells while 200 μM OA was sufficient to induce LD formation in HepG2 hepatocytes overnight for the fractionation experiment. Without any addition of OA, the number of LDs varies substantially across the different cell populations. We used 20 μM OA to establish a homogenous cell population with a low amount of LDs, mimicking the resting state to facilitate our analysis in this study.

5) In Figure S4A-C, CFAST HALO LD looks very different in the various cells. Why?
Authors' response:
The CFAST-Halo-LD is a marker for LDs in Figures S2A–C. LDs are commonly heterogeneous from cell to cell, and we believe the LDs shown in these figures are within the normal range of variation in terms of size, number, and distribution. However, to avoid any possible confusion for the readers, we replaced these images in the revised manuscript.

6) There are a few typing errors throughout the text that the authors should see to. For example:
the acronym LM is not explained (I assume it is light microscopy).
Page 4, second paragraph, line 8, "incubation" should be "following incubation". Page 8, second

paragraph, line 5, "moderately" should be "moderate".6) Figure 4G is not referred to in the text. The reference "Scorrano et al."is incomplete.

Authors' response:

We apologize for the typos and have corrected them in the revised manuscript.

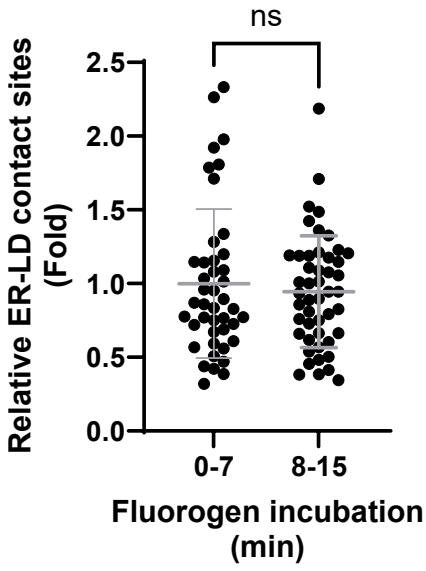
Reviewer #3 (Comments to the Authors (Required)):

Split-FAST is a clever idea, and applying it to various contacts must be the way to go. However, a decent amount of scoping is needed before everyone piles in and any misconceptions arise. Many aspects of this study are excellent; including as examples: (i) the demonstration that NFAST with a low endogenous affinity of the split partner (Kd =200 µM) has very little effects on endogenous organelles; (ii) modulation of mito-LD contacts with physiological change over periods of ~60 minutes

One issue is that SplitFAST may be fast but not fast enough: we don't a priori know what the time scale is for altering organelle physiology. Fig S3C shows development of contact at 30 seconds, and then the amount of contact increases by ~40% in the next 2-3 minutes. Are the traces here individual contact sites? If so, given the movement is the movement of contact as shown in Fig 5B one reason for variability amount the traces? Is the drop after 3 minutes caused by bleaching? (NB values with more time resolution between 0 and 30 seconds for both wash-in and wash-out would help, maybe in separate experiments to avoid bleaching effects). Does the 93% of LDs with contacts reported (Table 2) occur at 30 seconds or later? This paper on SplitFAST at contacts is the ideal place to show if this proportion changes over the next 20+ minutes to provide further evidence to support the statements here that it is OK to apply the fluorogen for up to 30 minutes.

Authors' response:

We apologize if this was not clear in our initial manuscript. We first validated that FABCON reports the existence of contact sites with the fluorogen but does not induce contact formation under our experimental set-ups (see Figures 4 and S1 A and S1B). The purpose of the experiment shown in Figures S1E and S1F (S3C in original manuscript) was to demonstrate the kinetics of reversible splitFAST-fluorogen interaction during addition and washout. We believe that the appearance of fluorescence signal upon the addition of fluorogen simply reflects splitFAST-dye complementation at pre-existing contact sites (page 5, lines 21–29). During the entire addition-washout process, ER-LD contact sites were not expected to be altered. This is further supported by our new EM data showing that mito-LD contact sites remained similar in size during dye addition and washout (Figure 4F). The individual trace in Figure S1F represents relative fold changes in fluorogen intensity from each cell during our addition-washout experiment.



All our data were acquired within 15 min after dye incubation except for the time-lapse images in Figures 5B and 6B (~25 min). Within this 15-min period, relative contact sites remained similar between 0–7 and 8–15 min (see right panel). For the biogenesis (Figure 5G) and OA withdrawal (Figure 6F) experiments, we prepared replicated samples for each time point and data from each sample was obtained within 15-min of dye addition. There is a chance that the dye would begin to enhance contact formation with longer incubation periods. Therefore, quantification of experiments with dye incubation longer than 15–25 min needs to be further validated. Alternatively, due to the

reversibility of the system, the same samples can be imaged for a longer period of time provided that the dye is washed out after each imaging time point.

Colours for merge images:
The choice of colour pairs is a long way from ideal. For the ~8% of men with minority forms of colour vision (mainly deuteranomaly), purple vs turquoise is perhaps the least helpful combination. Advice can be gained from web sources such as https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2667680/.
Authors' response:
We thank the reviewer for this suggestion. We did try to cater to individuals with different forms of color vision and purposefully avoided a green-red combination. We consulted with a colleague with deuteranomaly and found that purple vs turquoise/cyan was accessible to him; we chose this color combination for this reason. Additionally, as the reviewer suggested in the reference above, all the images from different channels were shown separately in greyscale and only merged images in colors. Based on these reasons, we did not change the color schemes. We hope the reviewer can accept our decision.

Minor: too much text in Introduction reads like results. However, Fig 1A is unnecessary and Fig 1B is confusing: one set of double headed arrows is gone and one still there. What can that mean? The diagram does not explain the heterodimer+fluorogen combination - see Fig 1A of Tebo and Gautier for the kind of thing needed.
Authors' response:
We apologize for the confusion. We have now moved the text related to Figure 1 in the Introduction to the first paragraph of the Results section (page 3, line 29). We made corresponding changes to the new Figure 1B.

Figure S2. Low affinity splitFAST affects PEX: quite a strong effect with NFAST-high and in the image provided a considerable effect with NFAST-low (not "minimal")
Authors' response:
We did not notice a significant effect of the NFAST-low on peroxisomes from either the images or the quantification, but to avoid confusion, we have replaced the image with a new one in the revised manuscript.

Typos: about glycolysis in Intro.; citations without years
Authors' response:
Thank you for pointing these issues out. They have been corrected in the revised manuscript.

Writing: a bit over-interpretative "faithfully reports the expected changes" (p6): one cannot be faithful to something that has not been observed before but is just expected.
Authors' response:
We have removed the word 'faithfully'.

Spurious accuracy: 4 significant figures for ER-LD contact (p7) are ?2 too many.
Authors' response:
We thank the reviewer for pointing this out. The corresponding correction has been made.

Other stats: median less than mean (p7): this is almost universal for a value where low readings are likely to be limited, here by resolution and dminess. So this specific aspect is not "Interestingly. An alternative would be to test the distribution for bimodlaity.

We thank the reviewer for this suggestion. We have fitted the population distribution data to bimodal distribution using Gaussian mixture model analysis (Figures 7D and 7E). The description of the bimodal distribution can be found in page 8, lines 14–21.

May 20, 2024

RE: JCB Manuscript #202311126R

Dr. Chi-Lun Chang
St. Jude Children's Research Hospital
Cell and Molecular Biology
262 Danny Thomas Pl.
M4426
Memphis, TN 38105

Dear Dr. Chang,

Thank you for submitting your revised manuscript entitled "A fluorogenic complementation tool kit for interrogating lipid droplet-organelle interaction". We would be happy to publish your paper in JCB pending the minor changes recommended by the reviewers as well as 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 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.

2) Figure formatting: Tools may have up to 10 main text figures. 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. Please add scale bars to inset magnifications in Figures 2B, 3A, 4A, & S1A.

Also, please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

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. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments). If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

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, 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."

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) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial, please add a reference citation if possible.

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. JCB formatting does not allow for supplemental references, please remove these and add any non-duplicate references to the main reference list.

8) Supplemental materials: Tools may have up to 5 supplemental figures and 10 videos. 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. Please include one brief sentence per item.

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. Please note that ORCID IDs are required for all authors. At resubmission of your final files, please be sure to provide your ORCID ID and those of all co-authors.

13) JCB requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

14) Journal of Cell Biology now requires a data availability statement for all research article submissions. These statements will be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).

B. FINAL FILES:

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

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

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

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

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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If you need an extension for whatever reason, please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions at cellbio@rockefeller.edu.

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

Sincerely,

William Prinz, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

In the revised version of the manuscript, the authors have included a substantial amount of new data. The additional experiments support and strengthen previous results, e.g. the sucrose gradient added for 6xHp provides convincing support for the strong enrichment of 6xHp on LDs and confirms negligible ER localization, and new data support that the FABCON system (CFAST combined with NFASTlow) can indeed be used to observe contact site dynamics without inducing tethering (a main caveat of most of the tools available so far to asses membrane contact site dynamics). I am still a bit puzzled about the high percentage of "coverage rate" for all of the different contact sites assessed, but the additional paragraph added by the authors to discuss this provides at least some possible explanation (possible overestimation of contact site coverage due to limited resolution). In sum, my concerns have been addressed and I am supportive of this study.

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

The revised manuscript by Li and colleagues has improved. In particular, the new data in Figure 4 supports the notion that expression of the NFASTlow-CFAST system has only a marginal effect on organelle contacts. The analysis of ER-LD contacts by SEM was also an important addition. However, only quantified data was shown. I would suggest that the authors include some representative examples.

The extent of the ER-LD contacts reported in Figure 5 is still surprising, even considering the resolution limitations of conventional light microscopy. In Figure 5A-B, what is the size distribution of LDs? I wonder if there is an inverse correlation between LD size and extent of coverage.

We appreciate that all reviewers took this affinity issue seriously. We completely agree with the reviewers and believe this is the foundation of engineering split reporter-based tools for interrogating dynamic organelle interaction and beyond. Therefore, we invested much time and effort to validate our tool kit and provide feasible and detailed experimental procedures to address this issue (page 5, lines 29-31; page 9, lines 41-46; *FABCON imaging* in Methods). Based on our data, we are confident that FABCON did not expand contact sites under these experimental set-ups. To fundamentally resolve this issue in the future, we propose engineering the next-gen FABCON using the latest developed splitFAST-fluorogen pairs of different affinities (page 10, lines 1-5) and/or reversible split HaloTag and ligands (page 11, lines 10-13) as discussed in the Discussion section.

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

In the revised version of the manuscript, the authors have included a substantial amount of new data. The additional experiments support and strengthen previous results, e.g. the sucrose gradient added for 6xHp provides convincing support for the strong enrichment of 6xHp on LDs and confirms negligible ER localization, and new data support that the FABCON system (CFAST combined with NFASTlow) can indeed be used to observe contact site dynamics without inducing tethering (a main caveat of most of the tools available so far to asses membrane contact site dynamics). I am still a bit puzzled about the high percentage of "coverage rate" for all of the different contact sites assessed, but the additional paragraph added by the authors to discuss this provides at least some possible explanation (possible overestimation of contact site coverage due to limited resolution). In sum, my concerns have been addressed and I am supportive of this study.

Authors' response:

We thank the reviewer for recognizing our efforts to address the concerns.

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

The revised manuscript by Li and colleagues has improved. In particular, the new data in Figure 4 supports the notion that expression of the NFASTlow-CFAST system has only a marginal effect on organelle contacts. The analysis of ER-LD contacts by SEM was also an important addition. However, only quantified data was shown. I would suggest that the authors include some representative examples.

Authors' response:

We thank the reviewer for recognizing our efforts to address the concerns. We now include representative electron micrographs for mito-LD contact sites in Figure 4G to further strengthen the data that FAB$^{mito\text{-}LD}$, either by itself or with fluorogen, did not substantially enhance contact sites formation.

The extent of the ER-LD contacts reported in Figure 5 is still surprising, even considering the resolution limitations of conventional light microscopy. In Figure 5A-B, what is the size distribution of LDs? I wonder if there is an inverse correlation between LD size and extent of coverage.

Authors' response:

The size of LD in HeLa cells is ~1-2 μm^2 (Figure S3) while that in U2OS cells ranges from 2 to 10 μm^2 (calculated from COSIMA data, Figure 7B). Therefore, the size distribution of LDs in 5A (HeLa cells) differs greatly than that in 5B (U2OS cells); yet the difference in the extent of ER-LD and mito-LD contact sites coverage appears to be subtle (Table 2). This result suggests minimal correlation between the LD size and contact site coverage, which is consistent with the data of ER-LD in Figures 5G and S3B, and mito-LD in Figures 6F and S3D.