JCB

Mitochondrial fragmentation enables localized signaling required for cell repair

Adam Horn, Shreya Raavicharla, Sonna Shah, Dan Cox, and Jyoti Jaiswal

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November 7, 2019

Re: JCB manuscript #201909154

Dr. Jyoti K Jaiswal
Children's National Health Systems
111 Michigan Av NW
Washington, DC 20010

Dear Dr. Jaiswal,

Thank you for submitting your manuscript entitled "Mitochondrial fission enables localized signaling required for cell repair". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. You will see that the reviewers find your study interesting as it opens up questions regarding the spatial regulation of mitochondrial dynamics. However, both reviewers raise valid points that will have to be addressed with further experimentation to be considered for publication as detailed below.

Reviewer 1 asks for additional experiments directed at characterizing how mitochondrial fission and RhoA activity is selectively regulated proximal to the plasma membrane and, in particular, for experiments that more directly assess the role of mitochondrial Ca2+ in the plasma membrane damage response. Reviewer 2's comments are also in part focused on a better understanding of the specificity of spatial aspects of this putative pathway, as well as a better temporal resolution of the response. All of these points, as well as requests for additional controls, are valid and will improve the manuscript. If you choose to submit a revised manuscript, we will make every effort to have the revision re-evaluated by the same reviewers.

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

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Text limits: Character count for a Report is < 20,000, 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: Reports may have up to 5 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.

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

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

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

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

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief

Andrea L. Marat, Ph.D.
Scientific Editor

Journal of Cell Biology

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

Plasma membrane injury can lead to neurodegeneration, myositis, or neuromuscular diseases. It has previously been reported that in neurodegeneration and during injury of the plasma membrane of myotubes mitochondria migrate to the site of injury. In this study authors assess mitochondrial dynamics during focal plasma membrane injury of MEFs. The authors clearly show that focal plasma membrane injury causes increased mitochondrial calcium uptake, ROS production, and mitochondrial fragmentation in proximal area of injury (roughly 30% percent of the cells mitochondria). Additionally Ca2+ signaling is required for plasma membrane repair as EGTA treated media (lacking Ca2+) is unable to elicit plasma membrane injury repair. Plasma membrane repair is facilitated by Ca2+ induced mitochondrial ROS production activating F-actin assembly presumably through the redox regulation of RhoA-GTPases. This is shown to occur in a Drp1 and Mid49 dependent manner as when mitochondria are unable to undergo fission, ROS production is attenuated, and F-actin assembly is not observed so cells undergo plasma membrane injury repair at a slower rate and to a lesser extent. Interestingly, already fragmented mitochondria (MFNdKO) are able to recover from plasma membrane injury.

Novelty: Focal plasma membrane injury fragments mitochondria proximal to the site of injury. DRP knockout cells do not undergo localized mitochondrial fragmentation and cells exhibit plasma membrane injury repair to a lesser extent.

Major Concerns:

More direct causation between the signaling networks is needed.
• Does blocking mCa2+ uptake prevent plasma membrane tissue repair via localized Drp1 mediated mitochondrial fission? Authors could treat cells with Ru360 and see if they observe mitochondrial fission in proximal mitochondria following plasma membrane injury.

Is ROS production and actin assembly localized to the area where fragmented mitochondria are observed? If possible,
• Please report mitoSOX fluorescence in distal and proximal mitochondria before and after injury.
• Please report Lifeact mCherry fluorescent activity in distal and proximal mitochondria before and after injury.

Could the authors assess if RhoA is actually activated in areas of fragmented mitochondria? The FRET based RhoA sensor, RhoA flare could be used.

Figure 2. In MFN dKO cells, fragmented mitochondria still have the ability to facilitate plasma membrane repair to levels similar to WT cells. This suggests that plasma membrane repair is not dependent on Drp1 mediated fission, but is possibly due to the phenotype of fragmented mitochondria. It should be assessed if already fragmented mitochondria (MFN dKO cells) display differences in mCa2+ uptake, ROS production, or F-actin assembly during plasma membrane repair. This would show whether Drp1 driven mitochondrial fragmentation is the sole reason for plasma membrane injury or just the result of the phenotype of fragmented mitochondria. In the MFN dKO cells please measure mCa2+ uptake, ROS production, and F actin assembly +/- EGTA.

Fig 3. Authors should assess if MiD49 patient MEFs have any difference in cCa2+ changes, mCa2+ uptake and ROS production following plasma membrane injury compared to WT. Please evaluate in proximal and distal mitochondria. Does this reflect what is seen in Drp1 KO MEFs?

Fig 4. The peak amplitude of mCa2+ uptake in proximal and distal mitochondria is ~50 percent lower in Drp1 KO cells. This could be due to less Ca2+ entering the cell or less cytosolic Ca2+ mobilization (thus less mCa2+ uptake being observed). The authors must assess cytosolic Ca2+ levels using cytosolic calcium sensors. Examples: Fura-FF, Fluo-4, GECOs, GCaMPs. The peak amplitude (F/F0) should be calculated and included in Fig 4. It is particularly important to assess this as stimulated skeletal muscle Drp1 knockout cells have recently been shown to have reduced cytosolic Ca2+ transients. (Favaro, Nature Communications, 2019). Time to maximum, time to 50 percent decay, and area under the curve can be placed in a supplemental figure.

In Fig 4. mCa2+ uptake is ~50 percent reduced in Drp1 KO mitochondria proximal and distal to the site of injury. In Supp. Fig 3A authors show that global mCa2+ uptake is not significantly different between WT and Drp1 KO cells. How is this possible? For this to be true, distal Drp1 KO mitochondria would be expected to have increased mCa2+ uptake which is not seen in Fig 4D. This is another reason cytosolic Ca2+ levels must be assessed.

Fig 4E. Please include data of maximum fluorescence of both proximal and distal mitochondria for WT and Drp1 knockouts. Are there significant differences?

Fig 4F-H. Graphs should be moved to a supplemental figure.

Please indicate how cytosolic Ca2+ levels increase following plasma membrane injury. Is this a result of the plasma membrane injury itself, or is there intracellular Ca2+ mobilization of the SR or a combination of both.

Minor issues:

Fig 1 and Methods. In the methods please describe how the authors determined failure to repair. At what time point post laser ablation is this assessed? What happens to cells that fail to repair, do they undergo apoptosis or is repair eventually initiated? If these cells undergo apoptosis please assess at least the percentage of apoptotic cells due to plasma membrane injury in all experiments (Annexin-V staining). WT MEFs +/- EGTA. WT and MFNdKO MEFs. WT and Mid49 deficient MEFs. WT and Drp1 KO MEFs.

Fig 2H. MiD49 patient MEFs have a negative decrease in Lifeact mCherry fluorescence intensity following plasma membrane injury. What could account for this?

Fig 4B and D. Please merge these graphs as it would highlight the fact that Drp1 KO mitochondria exhibit half the amount of mCa2+ uptake compared to WT.

Fig 4F-H. Graphs should be moved to a supplemental figure.

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This manuscript presents an extension of previous work from the lab documenting the contribution of calcium-induced mitochondrial ROS generation upon laser-mediated damage of the PM. They showed that this ROS burst was critical to activate RhoA and the generation of an F-actin zone essential for PM repair. The current study now demonstrates a transient, and spatially restricted, fragmentation of mitochondria is required to generate this ROS burst. Noting that patients carrying mutations in the Drp1 receptors MID49 present with muscle pathology similar to those with errors in membrane repair, they used patient derived fibroblast cells to first show the inhibition of membrane repair. Loss of MID49 completely ablated the burst of F-actin (seen with LifeAct, Fig 2E). Similar observations were seen in cells lacking Drp1. Loss of the mitofusins, leading to persistant mitochondrial fragmentation, were fully capable of mediating PM repair. Mitochondrial calcium transients measured with the mCAR-GECO1 probe showed a loss in the transient calcium uptake into hyperfused mitochondria, which they had previously shown as requisite for the ROS burst and RhoA mediated F-actin polymerization. Drp1KO cells are fully capable of taking up calcium and generating ROS in response to other mitochondrial toxins, but not the 10msec laser induced damage. The primary data are very clear, and there are two major questions that emerge: 1) why fission is required for calcium uptake at the site of injury and 2) how does the injury signal Drp1 recruitment and activation. There have been a number of studies linking F-actin to Drp1 recruitment and fission, particularly from the Higgs (PMID:
27559132) and Holzbaur labs (PMID:27686185), and this group previously suggested a feed-forward loop of calcium transients driving ROS that could further amplify RhoA activation and F-actin accumulation (Horn 2017). The major novelty in the present study is the spatial restriction of mitochondrial division that plays a key role in driving plasma membrane repair. I have three points of clarification to better establish the order of events they propose.

1. The use of lasers to damage the plasma membrane is rather acute. I assume laser injury also directly damages the underlying mitochondria? Is the signaling for Drp1 recruitment to mitochondria initiated from the events at the PM, or are there 2 different things damaged? Photodamage to mitochondria has long been known to be significant, where the electrochemical potential is lost through transient openings of the permeability transition pore. This, to me, seems difficult to disentangle with the model of damage used in this study. Is there another way to acutely damage the PM without the use of lasers?

2. It would be very informative to visualize these events in rapid time lapse imaging post-injury. The use of TMRE potentiometric dyes to follow transient depolarization, with LifeAct to monitor the swarms of F-actin more carefully. Where do they form? Where exactly is the block when mitochondria are hyperfused? The images from the Horn 2017 paper and in Fig 5D show the F-actin as rather a cloud without much definition near the wound site. Am I seeing it on the ER in the Drp1KO? Given the study by the Holzbaur group showing F-actin "circling" mitochondria that drove their fragmentation, is F-actin is directly recruited to this population of mitochondria, or the underlying ER (where calcium fluxes would presumably exit). Does F-actin accumulate at mitochondria or ER in the Mfn KO cells? Do the Mfn KO mitochondria fragment further, or are they just "able" to facilitate the same calcium transients and ROS generation as the wt cells? It would add to our understanding of the spatio-temporal events that are being described here if we could analyze them in a time-series. And when does the mitochondrial morphology return to tubular following repair?

3. Is RhoA activity required for Drp1 recruitment and mitochondrial fragmentation, or is Drp1 recruitment initiated soley due to the calcium burst upon injury (or local mitochondrial damage)? Can the RhoA mediated actin polymerization be separated from the fission event? Blocking ROS and calcium uptake was shown before to block the RhoA mediated repair, but mitochondrial morphology was not examined in that 2017 Horn paper. Presumably there would be no fission without the RhoA activity, but it should be directly shown. Loss of Drp1 blocked the calcium uptake, and Lifeact cloud, suggesting that fission is upstream of the actin, but a few experiments seem to be missing to confirm this feed-forward loop between calcium, fission, RhoA, and actin. Ultimately the GEF for RhoA must be activated by the ROS signal, so where/how do the authors envision this?

1st Revision - Authors' Response to Reviewers: February 5, 2020

We thank the reviewers for their general enthusiasm for this work and very helpful comments. As outlined in the pointwise response below, we have addressed all of their comments by way of clarification, rewriting and additional experiments that has resulted new data being added to the main and supplemental figures. We find these edits have further enhanced the impact of our findings and thank the reviewers for that.

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

Plasma membrane injury can lead to neurodegeneration, myositis, or neuromuscular diseases. It has previously been reported that in neurodegeneration and during injury of the plasma membrane of myotubes mitochondria migrate to the site of injury. In this study authors assess mitochondrial dynamics during focal plasma membrane injury of MEFs. The authors clearly show that focal plasma membrane injury causes increased mitochondrial calcium uptake, ROS production, and mitochondrial fragmentation in proximal area of injury (roughly 30% percent of the cells mitochondria). Additionally Ca2+ signaling is required for plasma membrane repair as EGTA treated media (lacking Ca2+) is unable to elicit plasma membrane injury repair. Plasma membrane repair is facilitated by Ca2+ induced mitochondrial ROS production activating F-actin assembly presumably through the redox regulation of RhoA-GTPases. This is shown to occur in a Drp1 and Mid49 dependent manner as when mitochondria are unable to undergo fission, ROS production is attenuated, and F-actin assembly is not observed so cells undergo plasma membrane injury repair at a slower rate and to a lesser extent. Interestingly, already fragmented mitochondria (MFNdKO) are able to recover from plasma membrane injury.

Novelty: Focal plasma membrane injury fragments mitochondria proximal to the site of injury. DRP knockout cells do not undergo localized mitochondrial fragmentation and cells exhibit plasma membrane injury repair to a lesser extent.

Major Concerns:
More direct causation between the signaling networks is needed.
1. Does blocking mCa2+ uptake prevent plasma membrane tissue repair via localized Drp1 mediated mitochondrial fission? Authors could treat cells with Ru360 and see if they observe mitochondrial fission in proximal mitochondria following plasma membrane injury.

Response: We addressed this concern by treating cells with Ruthenium Red, which we previously demonstrated to prevent mitochondrial calcium uptake after injury and cause poor repair (Horn et al., 2017). We found that treatment with Ruthenium Red does not prevent fragmentation of injury-proximal mitochondria, demonstrating that injury-triggered fission of mitochondria is independent of calcium uptake. This data has now been included in revised Figure 1.

2. Is ROS production and actin assembly localized to the area where fragmented mitochondria are observed? If possible,
- *Please report mitoSOX fluorescence in distal and proximal mitochondria before and after injury.*
- *Please report Lifeact mCherry fluorescent activity in distal and proximal mitochondria before and after injury.*

Response: We have now included quantification of both, mitochondrial ROS production (mitoSOX) and actin accumulation (Lifeact) at regions distal and proximal to the site of injury (in revised Figure 5). We found that mitochondrial ROS production in the mitochondria distal to the site of injury was significantly less than the levels proximal to the injury site. Further, while we observed greater F-actin levels at the injury-proximal sites, we did not observe any increase in F-actin in the injury-distal regions. These data support the model in which mitochondrial fragmentation establishes polarity with respect to calcium increase, with higher levels in the injury proximal regions, leading to localized ROS signaling and actin accumulation in this region.

3. Could the authors assess if RhoA is actually activated in areas of fragmented mitochondria? The FRET based RhoA sensor, RhoA flare could be used.

Response: We examined RhoA to determine if the increased RhoA activity is localized to the (injury-proximal) region with mitochondrial fragmentation. RhoA activity increases immediately and consistently in this region. In contrast, the injury distal region where mitochondria do not fragment showed the RhoA activity stays at the baseline level even after 90s post injury. This data is now included in revised Figure 5.

4. Figure 2. In MFN dKO cells, fragmented mitochondria still have the ability to facilitate plasma membrane repair to levels similar to WT cells. This suggests that plasma membrane repair is not dependent on Drp1 mediated fission, but is possibly due to the phenotype of fragmented mitochondria. It should be assessed if already fragmented mitochondria (MFN dKO cells) display differences in mCa2+ uptake, ROS production, or F-actin assembly during plasma membrane repair. This would show whether Drp1 driven mitochondrial fragmentation is the sole reason for plasma membrane injury or just the result of the phenotype of fragmented mitochondria. In the MFN dKO cells please measure mCa2+ uptake, ROS production, and F actin assembly +/- EGTA.

Response: We performed new experiments to address whether the existence of pre-fragmented mitochondria in MFN dKO cells still results in polarized repair signaling as in healthy cells that undergo mitochondrial fragmentation at the time of injury. We find that MFN dKO cells establish a polarized mCa^{2+} response similar to WT cells, such that injury-proximal mitochondria retain significantly higher calcium than the injury-distal mitochondria (revised Figure 4). This indicates that it is the presence of fragmented mitochondria that is required for generating the calcium polarity after injury. Consistent with this localized high mCa^{2+} level, we found that in the MFN dKO cells, injury caused an increase in F-actin level at the injury-proximal regions similar to WT cells (revised Figure 2). This validates the reviewer's comment regarding mitochondrial morphology, which is driven by Drp1 mediated fission, being the reason for plasma membrane repair.

5. Fig 3. Authors should assess if MiD49 patient MEFs have any difference in cCa2+ changes, mCa2+ uptake and ROS production following plasma membrane injury compared to WT. Please evaluate in proximal and distal mitochondria. Does this reflect what is seen in Drp1 KO MEFs?

Response: To determine whether the failure of MiD49 patient fibroblast mitochondria to fragment results in lack of repair signaling, as in Drp1 KO cells, we measured mCa2+ following injury of MiD49 patient fibroblasts. Similar to the WT mouse fibroblasts, control human fibroblasts establish a proximal-distal polarity of mCa2+. However, similar to Drp1 KO cells, MiD49 patient cells failed to establish a proximal/distal polarity in injury-triggered increase in mCa2+. Further, MiD49 patient cells show a trend toward reduced calcium uptake and time to half maximum compared to control human fibroblasts (data added to Supplemental Figure 3). Thus, failure of mitochondrial fission dysregulates repair signaling in MiD49 patient cells similar to the Drp1 KO cells.

6. Fig 4. The peak amplitude of mCa2+ uptake in proximal and distal mitochondria is ~50 percent lower in Drp1 KO cells. This could be due to less Ca2+ entering the cell or less cytosolic Ca2+ mobilization (thus less mCa2+ uptake being observed). The authors must assess cytosolic Ca2+ levels using cytosolic calcium sensors. Examples: Fura-FF, Fluo-4, GECOs, GCaMPs. The peak amplitude (F/F0) should be calculated and included in Fig 4. It is particularly important to assess this as stimulated skeletal muscle Drp1 knockout cells have recently been shown to have reduced cytosolic Ca2+ transients. (Favaro, Nature Communications, 2019). Time to maximum, time to 50 percent decay, and area under the curve can be placed in a supplemental figure.

Response: As per reviewer suggestions we have added the peak amplitude in Figure 4. Regarding the effect of Drp1 KO on mitochondrial calcium uptake, we find that Drp1 KO causes decreased mCa^{2+} uptake of cytosolic Ca^{2+} in response to injury. This is in contrast with the Favaro et al finding that Drp1 KO causes

increased mCa^{2+} uptake during muscle contraction. Favaro et al attribute this finding to increased levels of MCU complex (Favaro et al., 2019). Recently we have shown that altering MCU complex by selective knockout of the MCU regulatory protein MICU1 lowers the mCa^{2+} increase without affecting the cytosolic Ca^{2+} level (Debattisti, 2019). This stands to reason since unlike muscle contraction, where cytosolic Ca^{2+} increase is due to release from ER, increase in cytosolic Ca^{2+} following membrane injury is caused by the influx of millimolar amount of extracellular Ca^{2+}, the level of which cannot be controlled by change in mCa^{2+} uptake.

7. In Fig 4. mCa2+ uptake is ~50 percent reduced in Drp1 KO mitochondria proximal and distal to the site of injury. In Supp. Fig 3A authors show that global mCa2+ uptake is not significantly different between WT and Drp1 KO cells. How is this possible? For this to be true, distal Drp1 KO mitochondria would be expected to have increased mCa2+ uptake which is not seen in Fig 4D. This is another reason cytosolic Ca2+ levels must be assessed.

Response: We thank the reviewer for raising this valid point. We have re-examined this data and found that despite the global mCa^{2+} trending toward a lower value, it was not statistically significant due to inclusion of data from cells that had such low level expression of the mCa^{2+} reporter that the signal went minimally above the background. To address this we have now reanalyzed this data and included only those cells that robustly expressed the mCa^{2+} reporter. Doing so shows that the difference is indeed significant, and we have now included the revised data (Supplemental Figure 3) and revised the interpretation accordingly.

8. Fig 4E. Please include data of maximum fluorescence of both proximal and distal mitochondria for WT and Drp1 knockouts. Are there significant differences?

Response: This data is now included and it shows that the difference in maximal fluorescence between injury proximal and distal mitochondria is significant for WT and Mfn DKO cells, but not for Drp1 KO cells.

9. Fig 4F-H. Graphs should be moved to a supplemental figure.

Response: We have removed plots that describe the time to maximal calcium and area under the curve for injury-proximal regions and instead describe these findings in the text. However, with the addition of new data to Figure 4, including new data using MFN dKO, we decided to keep the plots highlighting the statistical evaluation of maximal Ca^{2+} uptake and time of Ca^{2+} decay to half maximum in the main figure since these measurements are important for the interpretation of our findings.

10. Please indicate how cytosolic Ca2+ levels increase following plasma membrane injury. Is this a result of the plasma membrane injury itself, or is there intracellular Ca2+ mobilization of the SR or a combination of both.

Response: A number of previous studies have already established the importance of extracellular Ca^{2+} influx for membrane repair (Koerdt and Gerke, 2017; Mellgren et al., 2009; Steinhardt et al., 1994). Our finding that chelation of extracellular calcium by EGTA prevents mitochondrial fragmentation and membrane repair, further supports this and leads us to infer that it is the extracellular calcium influx into the cytosol that is the primary contributor for triggering plasma membrane signaling pathway we have identified.

Minor issues:

11. Fig 1 and Methods. In the methods please describe how the authors determined failure to repair. At what time point post laser ablation is this assessed? What happens to cells that fail to repair, do they undergo apoptosis or is repair eventually initiated? If these cells undergo apoptosis please assess at least the percentage of apoptotic cells due to plasma membrane injury in all experiments (Annexin-V staining). WT MEFs +/- EGTA. WT and MFNdKO MEFs. WT and Mid49 deficient MEFs. WT and Drp1 KO MEFs.

Response: For experiments quantifying the ability of cells to undergo plasma membrane repair, we used the lipophilic FM 1-43 dye, which is a well-established reagent for this purpose (Demonbreun et al., 2016; Howard et al., 2011; Koerdt and Gerke, 2017). By using this dye, we can examine both the kinetics and success of plasma membrane repair. To determine whether cells successfully repaired or failed to repair, we assessed dye entry out to four minutes post-injury. This length of time was chosen as it is significantly longer than the time necessary for healthy cells to undergo repair, which occurs within the first one to two minutes post-injury in mammalian cells (Demonbreun et al., 2016; Howard et al., 2011; Koerdt and Gerke, 2017; Mellgren et al., 2009). Using this method, failure to repair is indicated by continuously increasing FM 1-43 fluorescence in the cell over the entire four-minute period post-injury, with no plateau, which would indicate closure of the plasma membrane wound. The cells that fail to close the wound typically swell and undergo necrosis, thus apoptotic assays are not pertinent.

12. Fig 2H. MiD49 patient MEFs have a negative decrease in Lifeact mCherry fluorescence intensity following plasma membrane injury. What could account for this?

Response: Plasma membrane injury causes a rapid F-actin depolymerization in all cells, which results in a local drop in LifeAct fluorescence as can be seen from the control (black) trace in Figure 2J as well as traces in Figure 5H. However, when the repair signaling occurs normally, the F-actin starts to build up leading to increased LifeAct fluorescence. In case of MiD49 deficient patient cells this buildup of F-actin is very slow and weak, causing the return of LifeAct fluorescence to take longer and even then barely return to the pre-injury baseline.

13. Fig 4B and D. Please merge these graphs as it would highlight the fact that Drp1 KO mitochondria exhibit half the amount of mCa2+ uptake compared to WT.

Response: To address this, we have now changed the y-axis of the Drp1 KO plot to match the WT. This, along with placing the plots adjacent to each other (rather than stacked vertically) highlights that Drp1 KO mitochondria take up less calcium compared to WT. With the addition of data from MFN dKO cells, merging all graphs would make the plot too busy to interpret effectively.

14. Fig 4F-H. Graphs should be moved to a supplemental figure.

Response: See response to comment #9 above.

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

This manuscript presents an extension of previous work from the lab documenting the contribution of calcium-induced mitochondrial ROS generation upon laser-mediated damage of the PM. They showed that this ROS burst was critical to activate RhoA and the generation of an F-actin zone essential for PM repair. The current study now demonstrates a transient, and spatially restricted, fragmentation of mitochondria is required to generate this ROS burst. Noting that patients carrying mutations in the Drp1 receptors MID49 present with muscle pathology similar to those with errors in membrane repair, they used patient derived fibroblast cells to first show the inhibition of membrane repair. Loss of MID49 completely ablated the burst of F-actin (seen with LifeAct, Fig 2E). Similar observations were seen in cells lacking Drp1. Loss of the mitofusins, leading to persistant mitochondrial fragmentation, were fully capable of mediating PM repair. Mitochondrial calcium transients measured with the mCAR-GECO1 probe showed a loss in the transient calcium uptake into hyperfused mitochondria, which they had previously shown as requisite for the ROS burst and RhoA mediated F-actin polymerization. Drp1KO cells are fully capable of taking up calcium and generating ROS in response to other mitochondrial toxins, but not the 10msec laser induced damage. The primary data are very clear, and there are two major questions that emerge: 1) why fission is required for calcium uptake at the site of injury and 2) how does the injury signal Drp1 recruitment and activation. There have been a number of studies linking F-actin to Drp1 recruitment and fission, particularly from the Higgs (PMID: 27559132) and Holzbaur labs (PMID:27686185), and this group previously suggested a feed-forward loop of calcium transients driving ROS that could further amplify RhoA activation and F-actin accumulation (Horn 2017). The major novelty in the present study is the spatial restriction of mitochondrial division that plays a key role in driving plasma membrane repair. I have three points of clarification to better establish the order of events they propose.

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Response: To address this concern we made use of mechanical injury using glass beads – an approach we have previously described for this purpose (Defour et al., 2014), and have shown requires mitochondrial function for repair (Horn et al., 2017). We observed that following such an injury to the plasma membrane, mitochondria at the injury proximal site fragmented. Therefore, injury-induced fragmentation of mitochondria is independent of the nature of the focal plasma membrane injury. This data is included in Supplemental Figure 1.

2. It would be very informative to visualize these events in rapid time lapse imaging post-injury. The use of TMRE potentiometric dyes to follow transient depolarization, with LifeAct to monitor the swarms of F-actin more carefully. Where do they form? Where exactly is the block when mitochondria are hyperfused? The images from the Horn 2017 paper and in Fig 5D show the F-actin as rather a cloud without much definition near the wound site. Am I seeing it on the ER in the Drp1KO? Given the study by the Holzbaur group showing F-actin "circling" mitochondria that drove their fragmentation, is F-actin is directly recruited to this population of mitochondria, or the underlying ER (where calcium fluxes would presumably exit). Does F-actin accumulate at mitochondria or ER in the Mfn KO cells? Do the Mfn KO mitochondria fragment further, or are they just "able" to facilitate the same calcium transients and ROS generation as the wt cells? It would add to our understanding of the spatio-temporal events that are being described here if we could analyze them in a time-series. And when does the mitochondrial morphology return to tubular following repair?

Response: As described in response to the query by Reviewer 1 (comment #12) and in our previous study, injury leads to rapid F-actin depolymerization and concomitant fragmentation of the injury-proximal mitochondria. This precludes the F-actin polymerization-induced mitochondrial fragmentation. Rather F-actin buildup is a process that occurs following mitochondrial fragmentation and downstream signaling to activate RhoA. We have also recently demonstrated that plasma membrane injury leads to ER fragmentation (Chandra et al., 2019). In light of all these observations it is clear that the F-actin "circling" is quite distinct from what occurs following plasma membrane injury. Excess calcium uptake by mitochondria is known to cause drop in mitochondrial membrane potential (Duchen et al., 1998). To examine if the rapid calcium uptake following injury also causes mitochondrial depolarization we used the potentiometric dye TMRE. This showed that rapid loss of membrane potential in injury proximal mitochondria (but not in the distal mitochondria) occurs concurrently with mitochondrial calcium uptake and precedes fragmentation (revised Figure 1F-H). Moreover, it also showed that these injury-proximal mitochondria can then repolarize fairly rapidly with a time course that coincides with the return of cytosolic calcium to baseline (by 60 seconds post-injury). This shows that mitochondria proximal to the injury site are able to restore themselves to their pre-injury state relatively quickly, even if their morphology is still fragmented. We have observed, regardless of cell type that mitochondria remain fragmented and do not become tubular at least until 30 minutes post-injury. However, the finding that these mitochondria are able to rapidly repolarize suggests their function may be restored independently of their morphology. Finally, using our ability to resolve mitochondrial shape, we have not been able to obtain any evidence of mitochondrial fragmentation in the Mfn dKO cells, leading to the conclusion that presence of mitochondria at the injury site that are fragmented after or prior to injury, is needed to generate the localized signaling required for repair.

3. Is RhoA activity required for Drp1 recruitment and mitochondrial fragmentation, or is Drp1 recruitment initiated soley due to the calcium burst upon injury (or local mitochondrial damage)? Can the RhoA mediated actin polymerization be separated from the fission event? Blocking ROS and calcium uptake was shown before to block the RhoA mediated repair, but mitochondrial morphology was not examined in that 2017 Horn paper. Presumably there would be no fission without the RhoA activity, but it should be directly shown. Loss of Drp1 blocked the calcium uptake, and Lifeact cloud, suggesting that fission is upstream of the actin, but a few experiments seem to be missing to confirm this feed-forward loop between calcium, fission, RhoA, and actin. Ultimately the GEF for RhoA must be activated by the ROS signal, so where/how do the authors envision this?

Response: To address whether RhoA activity, which is induced by the plasma membrane injury, is needed to regulate mitochondrial fragmentation, we treated cells with the mitochondria-targeted antioxidant MitoTEMPO. We previously showed that mitochondrial redox signaling activates local RhoA and that MitoTEMPO treatment reduces RhoA activity after injury by preventing mitochondrial ROS buildup (Horn et al., 2017). Contrary to our initial expectation, we found that injury-triggered increase in RhoA activity was not necessary for mitochondrial fragmentation. This is also supported by our finding that Ruthenium Red treatment is unable to prevent mitochondrial fragmentation (revised Figure 1I, J). These findings demonstrate that the mitochondrial signaling pathway needed for repair (dependent on calcium uptake and ROS production) is not needed to regulate the fragmentation of mitochondria. This suggests that Drp1 recruitment instead depends upon cytosolic calcium increase. We have included an updated discussion, which reflects our new findings.

References

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February 28, 2020

RE: JCB Manuscript #201909154R

Dr. Jyoti K Jaiswal
Children's National Health Systems
111 Michigan Av NW
Washington, DC 20010

Dear Dr. Jaiswal:

Thank you for submitting your revised manuscript entitled "Mitochondrial fission enables localized signaling required for cell repair". 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.

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Sincerely,

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

Andrea L. Marat, Ph.D.
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Journal of Cell Biology

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

In this revised version, authors addressed all of the comments raised by the reviewers. My only minor suggestion is to change the word "fission" in the title with "fragmentation" as the former suggests to the reader well versed with the mitochondrial dynamics field a Drp1 (and its adaptors)

specific effect.

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

This study demonstrated a requirement for mitochondrial fission in the process of plasma membrane repair. The main concern from both reviews focused around obtaining a better map of the sequence of events linking the wound, ER flux, Rho activation, Drp1 recruitment, loss of potential and fragmentation. The authors had assays to quantify and monitor these things, but there were some suggestions to improve the resolution of these events. The authors responded to our suggestions and the work, while there are many remaining questions for future studies, represents a solid contribution that will advance the field and be of interest to the varied audience of JCB.