JCB

Endoplasmic reticulum maintains ion homeostasis required for plasma membrane repair

Goutam Chandra, Sen Sreetama, Davi Mazala, Karine Charton, Jack vanderMeulen, Isabelle Richard, and Jyoti Jaiswal

Corresponding Author(s): Jyoti Jaiswal, Children's National Hospital

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August 13, 2020

Re: JCB manuscript #202006035

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 "Endoplasmic reticulum maintains ion homeostasis required for plasma membrane repair" and for your patience as it took longer than typical to provide you with a decision on your study. The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers agree that your study addresses an interesting topic that is in principle suitable for the readership of JCB. However, reviewer 1 in particular finds that additional experimental controls are necessary to ensure that your data fully supports your conclusions. Specifically, it is essential to determine if mitochondria may be a critical agent in PMR (rev 1 points 4, 5, 6), and to validate your FRET probe (point 3). In addition, you need to show the relative expression of ANO5 in muscle compared with other tissues (rev 1 point 1) as this information will inform your model.

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

GENERAL GUIDELINES:
Text limits: Character count for 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.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 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.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on 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.

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.
Senior Scientific Editor

Journal of Cell Biology

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

The authors here explain the contribution of ER Ca2+ homeostasis- ANO5-in the PMR process. The topic is potentially interesting, but in my opinion this work needs additional experimental controls.
1) In wt mice, is ANO5 specifically expressed in muscle? This would be expected if considering the main tissue affected in the disease mentioned by the authors.
2) Whether changes in the expression levels of other Ca2+ homeostasis players characterize the models used in this work has not been explored. This could change the interpretation of the whole story; for example: PMCA, MCU, STIM/ORAI, PLN.
3) Most importantly, being composed of YFP and Turquoise, T1ER fluorescence and consequently the FRET signal could also be affected by anion levels. How could the authors exclude that the results obtained by using this probe are not biased due to YFP anion sensitivity?
Correct citation of the T1ER probe is: Parallel adaptive feedback enhances reliability of the Ca2+ signaling system; Ellen Abell, Robert Ahrends, Samuel Bandara, Byung Ouk Park, Mary N. Teruel Proceedings of the National Academy of Sciences Aug 2011, 108 (35) 14485-14490; DOI: 10.1073/pnas.1018266108

4) The authors conclude that: "These results identify that ER Ca2+ imbalance in the ANO5 patient cells disrupts multiple Ca2+-dependent PMR processes". However, the consequentiality of the events is not completely clear if looking at the provided dataset. In particular, I am not sure the authors can exclude that what they observe is a mitochondria-mediated PMR. Indeed, it was shown that NPPB uncouples mitochondria (see The chloride channel blocker 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) uncouples mitochondria and increases the proton permeability of the plasma membrane in phagocytic cells (1991)). Indeed the authors find that mitochondria are depolarized in patients fibroblasts.
5) The authors state: "this force loss was due to greater myofiber damage as shown by enhanced entry of membrane impermeant dye (procion orange) in the EDL myofibers from LC-injured ANO5-KO muscle". However, they did not verify here that the steady state ER Ca2+ content is different in comparison to wt, and did not discuss the potential contribution of the latter.
6) The fact that ATP failed to increase in [Ca2+]ER in the patient cells is very interesting but somehow suggest that the overall calcium dyshomeostasis is affected: could the defective PMR be a consequence of the overall Ca2+ handling disruption? And in particular, of Mitochondrial Ca2+ dyshomeostasis?
7) In the methods section: represented with intervals of five frames, this means frames/seconds?
8) In the figure legends, the authors provide numbers for the cells or samples. (e.g. N=...) Do the authors refer to the number of cells, or to independent experiments? Could the authors specify n. of cells, n. of technical replicates, n. of individual experiments or n. of mice?

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

Title: Endoplasmic reticulum maintains ion homeostasis required for plasma membrane repair.

This manuscript addresses an interesting point: The effect of activity of the endoplasmic reticulum in maintaining calcium homeostasis on repair of plasma membranes after damage. At each step in the advancement of our knowledge of membrane repair, the field reaches a consensus of, "We know the mechanism". And then, a new mechanism, a new regulation, is found. Often the insights come from the study of human disease. Inevitably, the pathology gives insights that are important to the basic cell biology.

There are many experiments in this paper that are extremely well done, more so than most work in this field. There is a clear demonstration that disruption specific proteins (known to mutated in some disease), compromise the ability of the ER to sequester calcium and there is a loss of membrane repair. This alone would be a correlation: It could be the same mutation is affecting other physiological processes in addition to sequestering calcium. However, the authors then add BAPTA to sequester the calcium and show that this normalizes plasma membrane repair.

While I am impressed with the paper, there are two things that I think should be addressed and a third, which I acknowledge may be hard to address experimentally, perhaps the authors can address with speculation.

What should be added:
1. The writing. There are a few places where I there are claims made without reference or places where I think the wording is awkward. These latter points I leave to the editor. (details below)
2. The figures. I am not a fan of showing images with two or more pseudocolored images. I think that each channel should be shown as an independent black and white image, and then the

pseudocolored image can be presented next to it. For me this is the fairest way to show the data. I realize that this may take more "space" (a strange word when most people get the paper electronically). So again, this may be an editors choice. If the black and white images cannot be presented in the main text, maybe they can be included in the supplement

What would be nice: Speculation on why an disruption of the time course of calcium results in lack of repair. One might have thought that with a greater calcium influx then there would be greater fusion of membranes to heal the site. Clearly, in this case, extending the calcium influx actually blocks the process. What do they think is the mechanism?

Minor points:
Introduction "There is a thousand-fold calcium (Ca2+) gradient".
Perhaps say an approximately thousand fold calcium gradient since the magnitude of the gradient varies between organisms.

Paragraph 2: "major reservoir for intracellular calcium".
- How much of the total calcium is in the ER and how much in the mitochondria? Is this refer to freely available or bound calcium or...?

Paragraph 2: "SERCA, the pump responsible for Ca2+ import into the ER, is not sensitive to the cytosolic Ca2+ level."
What is the basis for this claim?

Reword: "showed that they show" in the introduction.

Last paragraph of introduction:
"In view of the role of ANO5 as calcium-regulated ion channel at the ER and the inability of ANO5 deficient cells to repair we have also examined if ER-mediated calcium homeostasis may play a role in repair of these cells."

The first 2/3 of the sentence is just a repeat of the material from before. Either delete it from earlier, or just say, "We also examined if ER-mediated calcium homeostasis may play a role in repair of these cells."

Results:
First paragraph: "the repair did not occur in the absence of extracellular calcium". Was it buffered outside? How low? How does it vary with calcium level? Would it be possible to use a rapid flow of calcium in and out to see how it varies with the magnitude and the time course? Although this might be best saved for a separate study.

"PM injury of vehicle treated cells led to anion uptake induced rapid quenching of YFPER fluorescence, decreasing YFPER/RFPER ratio (Fig. 2A, 2B). As expected, this was prevented in NPPB treated cells."
Why does the NPPB signal keep increasing? Does it not reach a steady state?

On page 6:
"To examine if injured ANO5 patient fibroblasts exhibit altered ER calcium homeostasis, we monitored ER calcium level following injury of ANO5 patient fibroblasts."
This result demonstrates a very clear lack of increase of calcium in the AN05 patient fibroblasts in response to injury. However, do they have an alteration in the steady state level of calcium? Does

this affect other cellular activities?

On page 7:

"In contrast to the healthy myoblasts where PM injury caused the mitochondrial potential to transiently increase before returning to pre-injury level within the next minute, mitochondria in the patient cells were increasingly depolarized following injury (Fig. 3O)."
It is difficult to follow this with the colored image. Please provide a black& white image for the TMRE and the YFP-TOM separately and then, if you wish, the superimposed pseudocolored image. I can pick out the TMRE going down in the patient more so than the normal, but in the normal it looks like the TMRE goes down as well. It seems as if there is a greater effect on the YFP-Tom in the control than in the patient. That is why it would help to see the two separately.

Page7:
"ANO5-KO myofibers allowed significantly higher FM1-43 dye entry and nearly twice as many ANO5-KO myofibers failed to repair as compared to the WT myofibers (Fig. 4A-C)."

The assumption is that the ANO5-KO and wt are both injured to the same degree and the difference in the FM1-43 dye entry is due to a delayed repair. I agree that this is the most parsimonious view, since the same injury conditions are used for each. However, sometimes nature is malicious. Is it possible that the ANO5-KO fibers are more susceptible to injury and thus, they take longer to repair? Is there a way to quantify the magnitude of the instantaneous injury and show it is the same?

Figure 4D:
Please clarify what the arrow head is point out? Also, the figure in the lower left quadrant of figure 4D has the letter "f" in black on the figure and the figure in the lower right quadrant of figure 4D has the letter "g" in black on the figure.

It is not clear to me if the changes in the mitochondrial number and function is an epi-phenomena from the disruption of the regulation of ER calcium or if it is directly involved in the membrane repair. The authors write: "in ANO5 deficient cells, cytosolic Ca2+ overload resulted in excessive mitochondrial Ca2+ accumulation, resulting in loss of mitochondrial membrane potential and to poor PMR." I agree that in the ANO5 deficient cells there is both:
- Excessive mitochondrial Calcium accumulation
- Loss of mitochondrial membrane potential
- Poor PMR.

It is not clear if the first two are correlate with or causal of the 3rd.

Response to comments from Reviewer #1:

The authors here explain the contribution of ER Ca2+ homeostasis- ANO5-in the PMR process. The topic is potentially interesting, but in my opinion this work needs additional experimental controls.

1) In wt mice, is ANO5 specifically expressed in muscle? This would be expected if considering the main tissue affected in the disease mentioned by the authors.

Response: Tissue-specific and developmental expression of ANO5 gene has been described in the literature (Mizuta et al., 2007; Tsutsumi et al., 2005; Xu et al., 2015). These studies support the reviewer's expectation of higher expression in muscle and bone. However, in response to the reviewer's comment we examined ANO5 expression in multiple organs, including additional skeletal muscles (**Figure panel A below** and **Supplemental figure 3**), which shows higher expression of ANO5 in skeletal muscles.

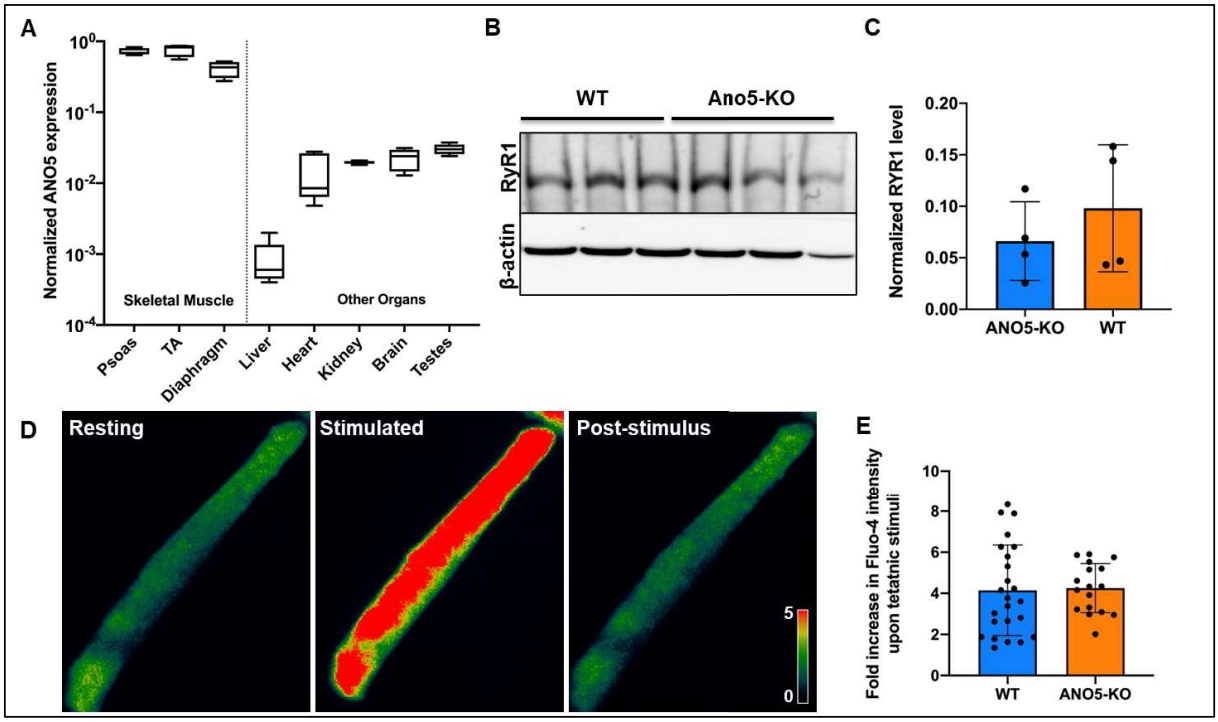

Figure: ANO5 expression and effect of its deficit on ER Ca^{2+} handling machinery: *(A) Quantitative PCR analysis of ANO5 mRNA expression in various mouse tissues. (B, C) Western Blot showing (B) a representative blot and (C) quantification of RYR1 levels in tibialis anterior muscles from WT and ANO5-KO mice. (D) Pseudocolored images and (E) Plot showing increase in FDB myofiber Ca^{2+} level in WT and ANO5-KO mice using the Fluo-4 dye.*

2) Whether changes in the expression levels of other Ca2+ homeostasis players characterize the models used in this work has not been explored. This could change the interpretation of the whole story; for example: PMCA, MCU, STIM/ORAI, PLN.

Response: To address the issue of calcium homeostatic machinery A) we examined the level of SR calcium release channel RYR1 and B) due to the calcium homeostasis role of the proteins listed by the reviewer during excitation-contraction (EC) coupling, instead of merely measuring the level of these proteins, we took to functionally measuring their activity by monitoring calcium homeostasis during EC coupling. These experiments showed that the RYR1 levels are unaltered in ANO5 KO muscle (**Figure panels B, C**). Monitoring Ca^{2+} handling during excitation contraction coupling showed that ANO5-KO myofibers exhibit similar levels of contraction-induced increase in Ca^{2+} level as the WT myofibers (**Figure panels D, E**). Together, these data, establish normal Ca^{2+} homeostasis in the ANO5-KO myofibers at rest and during physiological myofiber contraction, identifying normal functioning of calcium handling machinery under these conditions. These results are included in **Supplemental figure 3**.

3) Most importantly, being composed of YFP and Turquoise, T1ER fluorescence and consequently the FRET signal could also be affected by anion levels. How could the authors exclude that the results obtained by using this probe are not biased due to YFP anion sensitivity?
Correct citation of the T1ER probe is: Parallel adaptive feedback enhances reliability of the Ca2+

signaling system; Ellen Abell, Robert Ahrends, Samuel Bandara, Byung Ouk Park, Mary N. Teruel Proceedings of the National Academy of Sciences Aug 2011, 108 (35) 14485-14490; DOI: 10.1073/pnas.1018266108

Response: We thank the reviewer for pointing out this citation, which we have rectified. We recognize that the anion sensitivity of YFP could be expected to affect T1ER readout, but aside from the original report demonstrating that the T1ER FRET signal is strongly Ca^{2+} sensitive, following data included in our study independently confirms that the FRET signal of T1ER is affected by Ca^{2+} level.

1) *Use of the channel blocker NPPB to prevent anion entry* - If changes in anion level appreciably effects the T1ER signal by quenching of YFP then, in the cells not treated with NPPB, anions will enter the ER and quench the T1ER signal, while in NPPB treated cells T1ER signal will increase due to the failure of anion entry into the ER. Contrary to this expectation, NPPB treated cells show low T1ER signal and untreated cells show high T1ER signal (Fig. 2C). This identifies that anion quenching of YFP does not appreciably alter T1ER signal observed in our study.

2) *Chelation of calcium and selective inhibition of ER Ca^{2+} uptake* – Extracellular calcium entry into the injured cell raises the cellular Ca^{2+}. We find that chelating extracellular Ca^{2+}- (-) Ca^{2+}. which prevents cytosolic Ca^{2+} increase (reported by Fluo-4, Fig. 1A), also prevents increase in T1ER signal (Fig. 1F, G). Further, as the increase in ER Ca^{2+} is caused by SERCA pump, we have used this feature to regulate Ca^{2+} entry into the ER by using SERCA inhibitor Thapsigargin. Unlike the increase in T1ER FRET signal seen in injured control (Healthy) cells, the FRET signal failed to increase when the SERCA calcium pump was inhibited by thapsigargin - (+) Thapsigargin (Fig. 1F, G). Together, these direct measurements of FRET signal of T1ER, demonstrate that the changes in T1ER signal reported in our experiments selectively report on the changes in the ER Ca^{2+} level.

4) The authors conclude that: "These results identify that ER Ca2+ imbalance in the ANO5 patient cells disrupts multiple Ca2+-dependent PMR processes". However, the consequentiality of the events is not completely clear if looking at the provided dataset. In particular, I am not sure the authors can exclude that what they observe is a mitochondria-mediated PMR. Indeed, it was shown that NPPB uncouples mitochondria (see The chloride channel blocker 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) uncouples mitochondria and increases the proton permeability of the plasma membrane in phagocytic cells (1991)). Indeed the authors find that mitochondria are depolarized in patients fibroblasts.

Response: We agree with the reviewer that mitochondria-mediated PMR contributes to the repair deficit we observe here. In support of this view, in the discussion section of the manuscript we had stated “In ANO5 deficient cells, cytosolic Ca^{2+} overload resulted in excessive mitochondrial Ca^{2+} accumulation, resulting in loss of mitochondrial membrane potential and to poor PMR.” We have now elaborated this statement further by adding the following section (on page 9, second paragraph) to more explicitly indicate the involvement and relevance of mitochondria in PMR deficit in absence of ANO5. “In ANO5 deficient cells, cytosolic Ca^{2+} overload leads to excessive mitochondrial Ca^{2+} accumulation, resulting in loss of mitochondrial membrane potential in injured cells, which affects multiple calcium dependent PMR processes. Key amongst them is the mitochondria, which gets overloaded with Ca^{2+} in injured ANO5 patient cells, losing membrane potential. Mitochondrial depolarization and dysregulated calcium homeostasis lead to altered mitochondrial redox signaling required for actin-mediated PM wound closure and this mitochondria-mediated PMR response is compromised in several diseases (Boehler et al., 2019; Debattisti et al., 2019; Horn et al., 2020). As mitochondrial pathology in ANO5 deficient cells is downstream of primary deficit in ER calcium handling by the injured cells, it raises the possibility that addressing mitochondrial deficit may offer therapeutic benefits.”

5) The authors state: "this force loss was due to greater myofiber damage as shown by enhanced entry of membrane impermeant dye (procion orange) in the EDL myofibers from LC-injured ANO5-KO muscle". However, they did not verify here that the steady state ER Ca2+ content is different in comparison to wt, and did not discuss the potential contribution of the latter.

Response: Loss of muscle contractile force due to repeated eccentric contraction has been identified to be caused by mechanical injury to sarcomere and sarcolemma (Lieber and Friden, 1999). This fact is validated in our previous studies with muscular dystrophies associated with greater myofiber damage using the approach we have employed here (Defour et al., 2017; Vila et al., 2017). This damage-induced muscle force loss is distinct from muscle force loss due to isometric contractions, where calcium imbalance leads to loss of contractile force. As shown above (in response to comments #1 and 2), we show that the calcium regulatory machinery and calcium homeostasis during myofiber contraction are unperturbed in ANO5 deficient myofibers. In light of these, we have followed up on the reviewer suggestion and discussed this issue as follows “To assess if greater LC-induced damage is due to SR Ca^{2+} imbalance during excitation

contraction coupling. Neither the level of the SR Ca^{2+} release channel RYR1, nor Ca^{2+} homeostasis during myofiber contraction showed any dysregulation in ANO5-KO mice (**Supplementary Figure 3**). Together, above findings indicate that the force loss following LC contraction was due to greater myofiber damage. This was confirmed by the enhanced entry of membrane impermeant dye (procion orange) in the EDL myofibers from LC-injured ANO5-KO muscle as compared to the WT muscles (**Fig. 4G, H**)."

6) The fact that ATP failed to increase in [Ca2+]ER in the patient cells is very interesting but somehow suggest that the overall calcium dyshomeostasis is affected: could the defective PMR be a consequence of the overall Ca2+ handling disruption? And in particular, of Mitochondrial Ca2+ dyshomeostasis?

Response: We agree with the reviewers' suggestion, which is in full agreement with the interpretation of our findings with the ANO5 deficient cells. In a previous study we had observed that ANO5 deficient patient cells show altered cytosolic calcium handling following sarcolemmal injury and addressing this deficit restores the repair ability of the patient cells (Chandra et al., 2019). In the present study, we find that this is true even for the mature skeletal muscle fiber lacking ANO5. Our finding that ER calcium handling is disrupted following membrane injury and affects both Annexin and mitochondria-mediated repair of the injured plasma membrane. We have stated this in the discussion section "Using ANO5 deficient patient myoblasts and fibroblasts as models for genetic deficit of ER-resident CaCC, we find that failure of ER calcium uptake in these cells disturbs Annexin reparative response following injury and also causes $[Ca^{2+}]$m overload, together both these deficits lead to excessive mitochondrial depolarization and dysfunction"

7) In the methods section: represented with intervals of five frames, this means frames/seconds?

Response: We have now expanded this statement as follows to clarify the frame rate – "Cells were imaged using IX81 microscope (Olympus America, Center Valley, PA, USA). Change in FM dye intensity (ΔF/F where F is the intensity at the start of imaging) was used to quantify cell membrane repair kinetics. Images were acquired at 2 s intervals and data shown in the plots represent frames that are 10 s apart."

8) ***In the figure legends, the authors provide numbers for the cells or samples. (e.g. N=...) Do the authors refer to the number of cells, or to independent experiments? Could the authors specify n. of cells, n. of technical replicates, n. of individual experiments or n. of mice?***

Response: In the revised manuscript, we have stated the number of technical replicates and mice replicates in the 'Laser injury assays' subsection of the methods section.

Response to comments from Reviewer #2:

Title: Endoplasmic reticulum maintains ion homeostasis required for plasma membrane repair.
This manuscript addresses an interesting point: The effect of activity of the endoplasmic reticulum in maintaining calcium homeostasis on repair of plasma membranes after damage. At each step in the advancement of our knowledge of membrane repair, the field reaches a consensus of, "We know the mechanism". And then, a new mechanism, a new regulation, is found. Often the insights come from the study of human disease. Inevitably, the pathology gives insights that are important to the basic cell biology.
There are many experiments in this paper that are extremely well done, more so than most work in this field. There is a clear demonstration that disruption specific proteins (known to mutated in some disease), compromise the ability of the ER to sequester calcium and there is a loss of membrane repair. This alone would be a correlation: It could be the same mutation is affecting other physiological processes in addition to sequestering calcium. However, the authors then add BAPTA to sequester the calcium and show that this normalizes plasma membrane repair.
Response: We thank the reviewer for the encouraging comments.

While I am impressed with the paper, there are two things that I think should be addressed and a third, which I acknowledge may be hard to address experimentally, perhaps the authors can address with speculation.
What should be added:
1. The writing. There are a few places where I there are claims made without reference or places where I think the wording is awkward. These latter points I leave to the editor. (details below)
Response: We thank the reviewer for their careful read based on which we have revised the manuscript to improve wording and include additional references.

2. The figures. I am not a fan of showing images with two or more pseudocolored images. I think that each channel should be shown as an independent black and white image, and then the pseudocolored image can be presented next to it. For me this is the fairest way to show the data. I realize that this may take more "space" (a strange word when most people get the paper electronically). So again, this may be an editors choice. If the black and white images cannot be presented in the main text, maybe they can be included in the supplement
Response: We have now included **Supplemental figure 2** to present the black and white images for experiments involving dual channel imaging.

What would be nice: Speculation on why an disruption of the time course of calcium results in lack of repair. One might have thought that with a greater calcium influx then there would be greater fusion of membranes to heal the site. Clearly, in this case, extending the calcium influx actually blocks the process. What do they think is the mechanism?
Response: We thank the reviewer for asking a very pertinent question that we had not fully explained in the original draft of the manuscript. To address this, we have included the following discussion on page 9, third paragraph. “Calcium influx triggered by the PM injury is a widely recognized prerequisite for the activation of PMR. However, our study highlights the Goldilocks-like nature of calcium during PMR, such that not just too little increase in cytosolic calcium, but also too much increase in cytosolic calcium is detrimental to PMR. We previously observed that excessive cytosolic calcium increase causes excessive ER fragmentation (Chandra et al., 2019). Here we find that this calcium excess also dysregulates the vesicle-mediated PMR machinery involving Annexins and cytoskeleton-mediated PMR machinery involving mitochondria. Thus, while the lack of increase in calcium fails to trigger PMR response, extended and excessive increase in calcium leads to protracted and excessive response by Annexins and mitochondria, which represent the PMR machineries involved in membrane and cytoskeletal remodelling. These findings also help explain the reports of mitochondrial abnormalities (Griffin et al., 2016), and annexin response (Foltz et al., 2020) in skeletal muscles in an independent ANO5 KO mouse model.”

Minor points:
Introduction "There is a thousand-fold calcium (Ca2+) gradient". Perhaps say an approximately thousand fold calcium gradient since the magnitude of the gradient varies between organisms.
Response: We have modified the wordings as per the reviewer’s suggestion.

Paragraph 2: "major reservoir for intracellular calcium".
- How much of the total calcium is in the ER and how much in the mitochondria? Is this refer to

freely available or bound calcium or...?
Calcium concentration in the ER ranges between 100 to 400 μM (Samtleben et al., 2013; Verkhratsky, 2005), while mitochondrial matrix calcium ranges from 50-300 nM (Palmer et al., 2006), and the resting cytosolic calcium concentration is 50-100 nM (Palmer et al., 2006; Verkhratsky, 2005). With the highest calcium capacity, ER is the Ca^{2+} reservoir most equipped to buffer the calcium onslaught from millimolar amounts of extracellular calcium influx. The calcium reporters used in our study measure the free Ca^{2+}, which is now clarified in the methods subsection 'Ca^{2+} and Cl^- measurement assays' (page 11).

Paragraph 2: "SERCA, the pump responsible for Ca2+ import into the ER, is not sensitive to the cytosolic Ca2+ level." What is the basis for this claim?
Response: The basis of this statement is the extensive literature on the regulation of SERCA activity e.g. reviewed in (Chen et al., 2020; Stammers et al., 2015) that attribute changes in SERCA activity on the abundance of SERCA channel and other small proteins such as sarcolipin and phospholamban that bind SERCA to modulate its activity. In response to the reviewer question we note the potential ambiguity of this statement and have revised it as follows – "SERCA, the pump responsible for Ca^{2+} import into the ER, binds Ca^{2+} for its import. But, instead of cytosolic Ca^{2+} level, the SERCA pump activity is regulated by binding to multiple small molecular weight proteins such as sarcolipin and phospholamban Chen et al., 2020; Stammers et al., 2015)."

Reword: "showed that they show" in the introduction.
Response: We have now corrected the typo in this sentence.

Last paragraph of introduction: "In view of the role of ANO5 as calcium-regulated ion channel at the ER and the inability of ANO5 deficient cells to repair we have also examined if ER-mediated calcium homeostasis may play a role in repair of these cells." The first 2/3 of the sentence is just a repeat of the material from before. Either delete it from earlier, or just say, "We also examined if ER-mediated calcium homeostasis may play a role in repair of these cells."
Response: We have reworded the sentence in revised manuscript.

Results: First paragraph: "the repair did not occur in the absence of extracellular calcium". Was it buffered outside? How low? How does it vary with calcium level? Would it be possible to use a rapid flow of calcium in and out to see how it varies with the magnitude and the time course? Although this might be best saved for a separate study.
Response: Calcium sensitivity of the membrane repair machinery was investigated in the seminal work that identified this process and reported that maximal repair of mammalian cells occurred at extracellular Ca^{2+} concentration >0.8mM and was lacking at Ca^{2+} levels <0.1mM (Steinhardt et al., 1994). In all of our assays, extracellular Ca^{2+} concentration was maintained at 2mM, while for experiments in absence of extracellular Ca^{2+}, calcium was buffered using 0.5mM calcium chelator - EGTA. We and others have previously confirmed the validity of these extracellular Ca^{2+} in regulating PMR (Chandra et al., 2019; Horn et al., 2020; Potez et al., 2011; Sreetama et al., 2016; Wu et al., 2020). Further, we agree with the reviewer's suggestion that a separate study in the future using Ca^{2+} stop flow approach to monitor till what point post injury is calcium influx helpful and at what point it starts to compromise cell health would be interesting.

"PM injury of vehicle treated cells led to anion uptake induced rapid quenching of YFPER fluorescence, decreasing YFPER/RFPER ratio (Fig. 2A, 2B). As expected, this was prevented in NPPB treated cells." Why does the NPPB signal keep increasing? Does it not reach a steady state?
Response: Decrease in the YFP_{ER}/RFP_{ER} ratio in injured control cells is due to CaCC activity, which facilitates anions to enter the ER and quench the YFP signal. However, in NPPB treated cells, lack of the CaCC activity will prevent no addition anion entry, causing Ca^{2+} that enters the ER following injury to bind any free anion still available in the ER and this will cause a continual reduction of free anions in the ER and this continual increase in YFP_{ER}/RFP_{ER} ratio. We too expect that this signal should reach a plateau, but cannot offer a speculation for why it did not plateau over this time course.

On page 6: "To examine if injured ANO5 patient fibroblasts exhibit altered ER calcium homeostasis, we monitored ER calcium level following injury of ANO5 patient fibroblasts." This result demonstrates a very clear lack of increase of calcium in the AN05 patient fibroblasts in response to injury. However, do they have an alteration in the steady state level of calcium? Does this affect other cellular activities?
Response: We examined the basal ER Ca^{2+} levels by monitoring T1ER FRET values in uninjured cells and found this is also different between healthy (1.68 ± 0.20) and patient 1 (1.10 ± 0.15) or patient 2 (0.72 ±

0.10). This information is now included in the revised text. These differences may or may not affect other cellular activities, but working out if and of so, what these may be is beyond the scope of this study focused on the consequences on plasma membrane repair.

On page 7: "In contrast to the healthy myoblasts where PM injury caused the mitochondrial potential to transiently increase before returning to pre-injury level within the next minute, mitochondria in the patient cells were increasingly depolarized following injury (Fig. 3O)." It is difficult to follow this with the colored image. Please provide a black& white image for the TMRE and the YFP-TOM separately and then, if you wish, the superimposed pseudocolored image. I can pick out the TMRE going down in the patient more so than the normal, but in the normal it looks like the TMRE goes down as well. It seems as if there is a greater effect on the YFP-Tom in the control than in the patient. That is why it would help to see the two separately.
Response: We have provided the raw black and white images of TMRE and TOMM20-YFP in the revised manuscript (**Supplemental figure 2C**).

Page7: "ANO5-KO myofibers allowed significantly higher FM1-43 dye entry and nearly twice as many ANO5-KO myofibers failed to repair as compared to the WT myofibers (Fig. 4A-C)." The assumption is that the ANO5-KO and wt are both injured to the same degree and the difference in the FM1-43 dye entry is due to a delayed repair. I agree that this is the most parsimonious view, since the same injury conditions are used for each. However, sometimes nature is malicious. Is it possible that the ANO5-KO fibers are more susceptible to injury and thus, they take longer to repair? Is there a way to quantify the magnitude of the instantaneous injury and show it is the same?
Response: This is a fair comment and we were concerned to this issue. It is thus, that we used several complimentary approaches for controlled myofiber injury – Laser injury of isolated myofibers (Fig. 4A-E), laser injury of intact muscle (Fig. 4D, E), and mechanical injury (eccentric contraction F, G), all of which yielded the same outcome. We acknowledge that while not immune to nature's malice, these findings are amongst the most thorough experimental injury approaches to guard against it. Thus, we suggest that poor repair ability of ANO5 deficient fibers is the appropriate interpretation of our results.

Figure 4D: Please clarify what the arrow head is point out? Also, the figure in the lower left quadrant of figure 4D has the letter "f" in black on the figure and the figure in the lower right quadrant of figure 4D has the letter "g" in black on the figure.
Response: These arrowheads indicate the sites of injury, which is now stated in the legend, and we have removed the unnecessary lettering in Fig 4D.

It is not clear to me if the changes in the mitochondrial number and function is an epi-phenomena from the disruption of the regulation of ER calcium or if it is directly involved in the membrane repair. The authors write: "in ANO5 deficient cells, cytosolic Ca2+ overload resulted in excessive mitochondrial Ca2+ accumulation, resulting in loss of mitochondrial membrane potential and to poor PMR." I agree that in the ANO5 deficient cells there is both:
- Excessive mitochondrial Calcium accumulation
- Loss of mitochondrial membrane potential
- Poor PMR.
It is not clear if the first two are correlate with or causal of the 3rd.
Response: Since our discovery of the role of mitochondria in plasma membrane repair (Sharma et al., 2012), we have reported that dysregulation of calcium handling (Debattisti et al., 2019; Horn et al., 2020) and loss of mitochondrial membrane potential (Boehler et al., 2019; Vila et al., 2017) compromise mitochondria-mediated PMR and result in muscle diseases. Mitochondria mediates PMR through calcium uptake-triggered acute and focal increase in mitochondrial ROS, which activates local actin accumulation to close the wound (Horn et al., 2020; Horn et al., 2017). If the mitochondria are depolarized, or if excessive calcium enters the mitochondria, then mtROS signalling and actin-mediated PM wound closure is compromised. In the revised manuscript we discuss this "In ANO5 deficient cells, cytosolic Ca^{2+} overload leads to excessive mitochondrial Ca^{2+} accumulation, resulting in loss of mitochondrial membrane potential in injured cells, which affects multiple calcium dependent PMR processes. Key amongst them is the mitochondria, which gets overloaded with Ca^{2+} in injured ANO5 patient cells, losing membrane potential. Mitochondrial depolarization and dysregulated calcium homeostasis lead to altered mitochondrial redox signaling required for actin-mediated PM wound closure and this mitochondria-mediated PMR response is compromised in several diseases (Boehler et al., 2019; Debattisti et al., 2019; Horn et al., 2020)."

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January 5, 2021

RE: JCB Manuscript #202006035R

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

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