Simultaneous deletion of TRPV3 and $Ca_V3.2$ T-type channels in mice undermines fertility and Ca^{2+} homeostasis in oocytes and eggs

Aujan Mehregan, Golii Ardestani, Hiroki Akizawa, Ingrid Carvacho and Rafael Fissore
DOI: 10.1242/jcs.257956

Editor: John Heath

Review timeline
Original submission: 6 December 2020
Editorial decision: 25 January 2021
First revision received: 7 May 2021
Accepted: 17 May 2021

Original submission

First decision letter

MS ID#: JOCES/2020/257956

MS TITLE: Simultaneous deletion of TRPV3 and CaV3.2 T-type calcium channels in mice undermines fertility and Ca2+ homeostasis in oocytes and eggs

AUTHORS: Aujan Mehregan, Golii Ardestani, Ingrid Carvacho, and Rafael A Fissore
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This paper describes experiments to investigate the role of the TrpV3 and CaV3.2 Ca2+ influx channels on Ca2+ stores and Ca2+ oscillations in mouse eggs. It makes extensive use of the double KO mice lacking both TrpV3 and CaV3.2 and channels. The study provides clear evidence that both channels contribute the Ca2+ influx and maintaining total Ca2+ store content in mouse eggs. The data also suggests that TRPM7 channels provide a third mechanism for both Ca2+ and Sr2+ influx into mouse eggs during the occurrence of Ca2+ oscillations. It is a well conducted study with appropriate controls, and it adds to our knowledge of Ca2+ homeostasis in mammalian eggs.

Comments for the author

Specific comments on Mehregan et al. 2020.

1. Evidence is presented that the total Ca2+ store content is reduced in dKO eggs mouse eggs. However, it is not known whether the resting cytosolic Ca2+ level has also changed. IP3R opening and PLCzeta activity are both sensitive to the cytosolic Ca2+ and so this could also affect the frequency of Ca2+ oscillations. It would be helpful if the authors analyzed the fura2 340/380 fluorescence ratios in WT and dKO eggs before any stimuli to see if there is any sign of a significant change in resting cytosolic Ca2+ levels.

2. The total amount of Ca2+ in stores is assessed in dKO vs WT mice using ionomycin release, but it would interesting to know whether the free Ca2+ within the ER has altered. This point is critical to the discussion about the way in which reduced Ca2+ influx affects the frequency of Ca2+ oscillations via Ca2+ leakage through IP3Rs (lines 413-414). Previous work from this group used a FRET probe of Ca2+ concentration store levels. It would helpful if this probe were used in WT and dKO eggs.

3. In the Discussion (lines 402-425) various ideas are discussed for how Ca2+ influx or Ca2+ stores could alter the frequency of Ca2+ oscillations. The authors suggest that the data of Matsu-ra et al. (2019) invalidates the idea of Ca2+ induced stimulation of PLCz playing are role in the rise of each Ca2+ transient. They say that *'This possibility is however less likely to serve as the trigger of the next Ca2+ rise because besides being of small magnitude compared to the ambient [IP3] concentrations in the zygote, the [IP3] peaks occur after each Ca2+ rise (Matsu-ura et al., 2019).'* This paper did not establish whether they were measuring ambient (resting levels of) IP3. Furthermore, the fact that the IP3 peak lags the Ca2+ peak is not decisive. The issue for the positive Ca2+-IP3 feedback model is whether the IP3 increase leads the Ca2+ increase during the rising phase. Fig.4C in Matsu-ura et al. (2019) clearly suggests that the rise in IP3 occurs before the Ca2+ rise hence their data is consistent with the model presented by Sanders et al. (2018).

4. Fig 6 shows Ca2+ oscillation in eggs triggered by PLCz and/or Sr2+. Fig 6G shows the effect of PLCz, plus Sr2+, in Mg2+ free medium. Hence this combines two strong stimuli for oscillations with Mg2+ free medium. I would expect eggs to oscillate with high frequency from the very start, but the trace in Fig 6G shows no oscillations for over an hour. Was this a consistent result? Does this imply that PLCzeta and Sr2+ somehow negate each other's effects?

5. The data on Fig 7D-F is not clear. Thimerosal may stimulate TRM7 channels, but it also causes Ca2+ release and so could be acting indirectly. Also, the effects of the Ni2+ influx look marginal. These experiments could be repeated with BAPTA loaded eggs to prevent Ca2+ release. Alternatively, these experiments could be omitted because the data in Fig.7A-C already provides good evidence for the upregulation of TRPM7 channels.

6. One lines 430-433 the authors suggest that some downstream products of PLCz may stimulate

Ca2+ influx. There is already evidence that PKC activation by DAG stimulates Ca2+ influx in mouse eggs (Halet et al. 2004 J.Cell Biol. 164 p1033).

7. Line 7. I assume the authors mean ‘PLCz mRNA injection’.

Reviewer 2

Advance summary and potential significance to field

The authors indicated that the transient receptor potential vanilloid member3 (TRPV3) and T-type channel(Cav3.2) contribute to filling of Ca2+ store and ca2+ influx in mice oocytes and eggs in the manuscript that entitled "Simultaneous deletion of TRPV3 and Cav3.2 T-type calucium channel in mice undermines fertility and ca2+ homeostasis in oocytes and eggs". The paper is very interesting in interaction between Ca2+ homeostasis and calcium channels on plasma membrane of oocytes and eggs, therefore I hope that the authors should improve the manuscript.

Comments for the author

Major revision;
In Primer set A of Fig. S1, the authors should draw the picture that the upper band in the heterozygous mice lane of Primer set A is clear as same as Primer set B.
The authors should show that there are, or are not, TRPV3 and Cav3.2 in the WT oocytes/eggs and the dKO oocytes/eggs with the western blot or imunostainning.

In Fig. 3, authors should observe the dimension, quantity, and distribution of ER in the in the WT eggs and the dKO eggs with ER-specific stainning (e.g. Imunostaining and ER-tracker), in order to indicate that ER in the dKO oocytes/eggs are normal.

Minor revision;
The authors should describe about TRPM7 in Introduction, because of the important subject.
The authors are better to rethink the short name of T-type knockout. "tKO" may mistakes to "triple KO".

In P10, L219, authors should rewrite the reference from number (33) to name.

In Fig. S5-A, the authors should erase ")" on Y-axis. (340/380)/min")".

First revision

Author response to reviewers' comments

Responses to reviewer’s specific comments, Mehregan et al. 2020.
Comments by Reviewer #1.

1. Evidence is presented that the total Ca^{2+} store content is reduced in dKO eggs mouse eggs. However, it is not known whether the resting cytosolic Ca^{2+} level has also changed. IP3R opening and PLCzeta activity are both sensitive to the cytosolic Ca^{2+} and so this could also affect the frequency of Ca^{2+} oscillations. It would be helpful if the authors analyzed the fura2 340/380 fluorescence ratios in WT and dKO eggs before any stimuli to see if there is any sign of a significant change in resting cytosolic Ca^{2+} levels.

- We have analyzed the baseline Ca^{2+} levels as suggested by the reviewer. We were unable to observe any differences in these values. We have added this information on Page 9, Lines 184 to 186.

2. The total amount of Ca^{2+} in stores is assessed in dKO vs WT mice using ionomycin release, but it would interesting to know whether the free Ca^{2+} within the ER has altered. This point is critical to the discussion about the way in which reduced Ca2+ influx affects the frequency of Ca^{2+} oscillations via Ca^{2+} leakage through IP3Rs (lines 413-414). Previous work from this group used a FRET probe of Ca^{2+} concentration store levels. It would helpful if this probe were used in WT and dKO eggs.

- We have used the Cameleon D1ER used in our previous study cited by the reviewer that was provided by the Tsien lab and adapted by us for use in eggs (Wakai et al., 2013). To estimate basal $[Ca^{2+}]_{ER}$ levels, we first examined YFP/CFP ratios. Although the YFP/CFP baseline ratios were higher in WT than in dKO eggs, the difference was not significant due to the wide range in the collected values for dKO eggs. Importantly, after the addition of IO, YFP/CFP ratios underwent a rapid decrease, and the changes were faster and greater in dKOs than in WT eggs ($p < 0.05$). These data are presented on Page 10, Lines 225-236 and Fig. 3H-J. Further, after IO stimulation, the basal YFP/CFP ratios were higher in WT than in dKO eggs and remain so throughout the monitoring. Collectively, these results suggest that dKO eggs efflux a greater proportion of Ca^{2+} from the ER than WT eggs, and filling and/or re-filling of the internal Ca^{2+} stores require the participating of TRPV3 and CaV3.2 channels.

3. In the Discussion (lines 402-425) various ideas are discussed for how Ca^{2+} influx or Ca^{2+} stores could alter the frequency of Ca^{2+} oscillations. The authors suggest that the data of Matsu-ra et al. (2019) invalidates the idea of Ca^{2+} induced stimulation of PLCz playing are role in the rise of each Ca^{2+} transient. They say that 'This possibility is however less likely to serve as the trigger of the next Ca^{2+} rise because besides being of small magnitude compared to the ambient [IP3] concentrations in the zygote, the [IP3] peaks occur after each Ca^{2+} rise (Matsu-ura et al., 2019).' This paper did not establish whether they were measuring ambient (resting levels of) IP3. Furthermore, the fact that the IP3 peak lags the Ca^{2+} peak is not decisive. The issue for the positive Ca^{2+} -IP3 feedback model is whether the IP3 increase leads the Ca^{2+} increase during the rising phase. Fig.4C in Matsu-ura et al. (2019) clearly suggests that the rise in IP3 occurs before the Ca^{2+} rise hence their data is consistent with the model presented by Sanders et al. (2018).

- The reviewer raises an interesting point. As noted in the manuscript and by the reviewer, we stated "… This possibility is however less likely to serve as the trigger…", and it "does not invalidate the idea of Ca^{2+} induced stimulation of PLCz playing a role in the rise of each Ca^{2+} transient." We still believe that our results align more closely with the overall interpretation of the data by the authors of the cited manuscript. The authors (Matsu-ura et al. (2019)) noted, *"Our data suggest that the mechanism elicits the rising phase of Ca^{2+} spikes in fertilized mouse eggs is more complex. Initially, CICR dominantly works as the positive feedback loop, and Ca^{2+}-induced IP3 production gradually participates to produce Ca^{2+} spikes cooperatively with CICR in the later phase of Ca^{2+} oscillations. Ca^{2+} -induced IP3 production through PLC produces [IP3] rises at each Ca^{2+} spike to help keeping [IP3] over the basal level, which results in long lasting Ca^{2+} oscillations in fertilized eggs."* In this sentence, the authors argue against a major role of Ca^{2+}-induced IP_3 production as the trigger of the abrupt Ca^{2+} increase that accompanies the rising phase of Ca^{2+} rises. They indicate that the initial Ca^{2+} rises are not accompanied by IP_3 increases and even at later stages, the IP_3 increases appear to contribute to maintaining [IP3] levels above basal concentrations rather than lead to the upstroke of Ca^{2+} during the rise. We have modified this sentence to accommodate this detail. Importantly, our new results with D1ER suggest a pivotal role for ER Ca^{2+} levels in contributing to the frequency of the oscillations, as stimulated dKO eggs lose a greater portion of their Ca^{2+} content, they do it faster, and Ca^{2+} ER levels seem to stay persistently lower than in WT eggs.

4. Fig 6 shows Ca^{2+} oscillation in eggs triggered by PLCz and/or Sr^{2+}. Fig 6G shows the effect

of PLCz, plus Sr^{2+} in Mg^{2+} free medium. Hence this combines two strong stimuli for oscillations with Mg^{2+} free medium. I would expect eggs to oscillate with high frequency from the very start, but the trace in Fig 6G shows no oscillations for over an hour. Was this a consistent result? Does this imply that PLCzeta and Sr^{2+} somehow negate each other's effects?

- We were surprised by this observation as well. We have repeated this one more time, and the data are consistent. There is a delay in the initiation of Sr^{2+} oscillations in WT eggs following the expression of PLCz cRNA. Nevertheless, we are unaware of the mechanism, and because it did not impact the interpretation of the point we are making in this particular figure, we have not explored this phenomenon further.

5. The data on Fig 7D-F is not clear. Thimerosal may stimulate TRM7 channels, but it also causes Ca^{2+} release and so could be acting indirectly. Also, the effects of the Ni^{2+} influx look marginal. These experiments could be repeated with BAPTA loaded eggs to prevent Ca^{2+} release. Alternatively, these experiments could be omitted because the data in Fig.7A-C already provides good evidence for the upregulation of TRPM7 channels.

- We appreciate the reviewer's comments and agree that the addition of thimerosal introduces some variables that obscure the point we were trying to make. To avoid this issue, we carried out the studies in the absence of thimerosal and directly monitored the rate of Mg-Fura2 quenching following the addition of Ni^{2+}. The addition of Ni^{2+} into MII WT failed to induced marked changes in fluorescence of Mg-Fura2, whereas a clear inflection was seen in dKO eggs (Fig. 7E; $p<0.05$). Given the electrophysiological results and the inhibition of the current by NS8593, we proposed the active channel that allows enhanced Ni^{2+} influx in dKO eggs is TRMP7.

6. On lines 430-433 the authors suggest that some downstream products of PLCz may stimulate Ca^{2+} influx. There is already evidence that PKC activation by DAG stimulates Ca2+ influx in mouse eggs (Halet et al. 2004 J. Cell Biol. 164 p1033).

- Thank for you bringing this up. We have added the reference, and also noted that the channel(s) targeted by PKC is unknown and that whether PKC plays a role in Ca^{2+} influx during fertilization remains to be demonstrated, as the isoform tested in the Halet et al manuscript are not the ones highly expressed in mouse eggs. The reference and comment have been added on Page 18, Lines 488-494.

7. Line 7. I assume the authors mean 'PLCz mRNA injection'.

- Thank you for pointing this out. It has now been corrected.

Reviewer #2.

The authors indicated that the transient receptor potential vanilloid member3 (TRPV3) and T-type channel(Cav3.2) contribute to filling of Ca^{2+} store and Ca^{2+} influx in mice oocytes and eggs in the manuscript that entitled "Simultaneous deletion of TRPV3 and Cav3.2 T-type calcium channel in mice undermines fertility and Ca^{2+} homeostasis in oocytes and eggs". The paper is very interesting in interaction between Ca^{2+} homeostasis and calcium channels on plasma membrane of oocytes and eggs, therefore I hope that the authors should improve the manuscript.

Major Revision:

1. In Primer set A of Fig. S1, the authors should draw the picture that the upper band in the heterozygous mice lane of Primer set A is clear as same as Primer set B.

- Thank you for pointing this out. We have left the figure as originally presented, as primers sets produced different intensity, and it is clear for Primer set A that the upper band in the heterozygous lane in the WT band.

2. The authors should show that there are, or are not, TRPV3 and Cav3.2 in the WT oocytes/eggs and the dKO oocytes/eggs with the western blot or imunostainning.

- We demonstrate that there are no functional TRPV3 or Cav3.2 channels on the plasma membrane of dKO eggs by performing electrophysiology. The patch-clamp technique used is a very sensitive tool to test the identity, and confirm the functional presence of ion channels on the plasma membrane (Hamill O.P. *et al.*, Pflügers Arch, 1981; Hille, 2001). Please note that *each channel's current is like a fingerprint of the functional expression of a channel, and therefore the absence of the current indicates the absence of a particular channel(s)*. Further, both of these KOs are whole-body KO animals, and gametes and full KO males and females are used to maintain the null lines.

We have already published evidence demonstrating the lack of expression of these channels in KO eggs. Regarding TRPV3, in **Figure 2A** in the present manuscript we show a cationic outwardly rectifying current elicited by 200 μM 2-APB. We and others have previously reported this current is exclusively mediated by TRPV3, which was confirmed here by being absent in *TrpV3* KO eggs (**Figure 2B),** consistent with our previous publication (Carvacho *et al.*, *Cell Reports*, 2013). The line of mice used in the present study is the same line that we used in the previous study. Please note that measurement of net current is a direct measure of functional ion channel density at the plasma membrane, and in our experience, is more specific than Western blots, as while a Western can detect the presence of a protein it does not inform anything about the function of the protein; electrophysiology does both. Importantly, the commercially available anti-TRPV3 antibodies are not specific, and when we attempted to test protein levels in GV and MII, we obtained many non-specific bands. However, as shown in **Figure R1,** TRPV3 protein distribution in WT eggs (*unpublished data*) can be detected using the anti-TrpV3 cation channel antibody, Neuromab, clone N15/4 (mouse monoclonal IgG2a), catalog number: 75-043, which we used previously in Carvacho *et al.*, Cell Reports, 2013 for immunostaining of TRPV3 WT eggs, which was not detected in KO eggs.

Figure R1. TRPV3 protein expression in mouse MII zona-free eggs. A. Cortical granules stained with Lens culinaris Agglutinin (LCA)-FITC. **B**. Differential Interference Contrast (DIC) image. **C**. TRPV3 protein. Scale bar: 10 μm. [NOTE: We have removed a figure which was provided for the referees in confidence.]

For Cav3.2, we have used KO animals also used in Bernhardt *et al.*, (J. of Cell Science, 2015), which were obtained from the Jackson laboratories. Here and in previous studies, the Cav3.2 current in mouse eggs was recorded by whole-cell patch clamp (Kang *et al.*, Biochem and Biophys Research Comm, 2007; Bernardt *et al.*, J. of Cell Science, 2015) using a voltage step protocol in the presence of 20 mM external Calcium (Peres A., J. Physiol, 1987). This protocol gives a characteristic IV curve corresponding to a Voltage-gated Calcium Channel that belongs to the T-type subfamily (Hille, 2001). These currents were not present in Cav3.2 KO eggs (current **Figure 2C**., red trace), just as it was not present in other reports of Cav3.2 KO mice (Bernhardt *et al.*, J. of Cell Science, 2015). The Cav3.2 channel has particular biophysical characteristics (Barium permeability, Nickel blockade, inactivation kinetics, between others) that were all tested, in mouse eggs by us (*unpublished*), and that confirmed that the Cav3.2 is absent in our eggs. We tried WB using a commercial antibody (Alomone labs., ACC- 025); however, we did not get any signal, most likely because of the low amount of Cav3.2 protein in eggs, confirming that electrophysiology is a more sensitive technique to detect functional channels at the plasma membrane of mouse eggs. Below are the references that support the above statements.

1. Improved patch- clamp techniques for High Resolution Current Recording from Cells and Cell-Free membrane patches. Hamill O.P., Marty A, Neher E., Sakmann B., Sigworth F.J. Pflügers Arch (1981) 391:85-100.
2. Ion channels of excitable membranes, Hille B. SINAUER ASSOCIATES INC.,U.S. (2001).
3. Carvacho, I., Lee, H. C., Fissore, R. A. & Clapham, D. E. TRPV3 channels mediate strontium-induced mouse-egg activation. *Cell Rep* **5**, 1375–86 (2013).
4. Bernhardt, M. L. *et al.* CaV3.2 T-type channels mediate $Ca(^{2+})$ entry during oocyte maturation and following fertilization. *J Cell Sci* **128**, 4442–52 (2015).
5. Kang, D., Hur, C. G., Park, J. Y., Han, J., Hong, S. G. Acetylcholine increases Ca^{2+} influx by activation of CaMKII in mouse oocytes. *Biochem Biophys Res Commun* **360**, 476–82 (2007).
6. Peres, A. The calcium current of mouse egg measured in physiological calcium and temperature conditions. J. Physiol. Oct; 391:573–88.

3. In Fig. 3, authors should observe the dimension, quantity, and distribution of ER in the in the WT eggs and the dKO eggs with ER-specific staining (e.g., Imunostaining and ER-tracker), in order to indicate that ER in the dKO oocytes/eggs are normal.

- To answer this request, we used the same D1ER construct that we used previously (Wakai et al., 2013) and here (Fig. 3G-J) to measure intro ER-Ca^{2+} store levels. We have added these data to Supplementary Fig. 4. We did not see major differences in the distribution and organization of the ER in WT vs. dKO GVs or eggs.

Minor revision;
The authors should describe about TRPM7 in Introduction, because of the important subject.

- We introduced the role of TRPM7 in the introduction as suggested. Page 6, Lines96 to 98.

The authors are better to rethink the short name of T-type knockout. "tKO" may mistakes to "triple KO".

- For triple KO, we use the abbreviation of TrplKO, which is very different than tKO.

In P10, L219, authors should rewrite the reference from number (33) to name.

- Thank you. This was done.

In Fig. S5-A, the authors should erase ")" on Y-axis. (340/380)/min")".

- Thank you. This is done.

Second decision letter

MS ID#: JOCES/2020/257956

MS TITLE: Simultaneous deletion of TRPV3 and CaV3.2 T-type calcium channels in mice undermines fertility and Ca2+ homeostasis in oocytes and eggs

AUTHORS: Aujan Mehregan, Golii Ardestani, Hiroki Akizawa, Ingrid Carvacho, and Rafael A Fissore
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.
Reviewer 1

Advance summary and potential significance to field

This paper describes experiments to investigate the role of the TrpV3 and CaV3.2 Ca2+ influx channels on Ca2+ stores and Ca2+ oscillations in mouse eggs. It makes extensive use of the double KO mice lacking both TrpV3 and CaV3.2 and channels. The study provides clear evidence that both channels contribute the Ca2+ influx and maintaining total Ca2+ store content in mouse eggs. The data also suggests that TRPM7 channels provide a third mechanism for both Ca2+ and Sr2+ influx into mouse eggs during the occurrence of Ca2+ oscillations. It is a well conducted study with appropriate controls, and it adds to our knowledge of Ca2+ homeostasis in mammalian eggs.

Comments for the author

I am satisfied with all the new data and changes made in the revised manuscript. I still have some difference of opinion about interpreting the mechanmism of InsP3 driven Ca2+ oscillations (lines 443-45), but this is not the place to discuss this further.

Reviewer 2

Advance summary and potential significance to field

This manuscript shows that TRPV3 and Cav3.2 (Ca2+ channels) are important for the Ca2+ homeostasis (including the Ca2+ oscillation) at the oocyte maturation and fertilization of mammal. These findings support that we understand the mechanism of the Ca2+ homeostasis in oocytes and eggs.

Comments for the author

This study is interesting, and data in the manuscript is sufficient. Thus, I think that this manuscript is suitable for the criteria of JCS.