Localized TPC1-mediated Ca^{2+} release from endolysosomes contributes to myoseptal junction development in zebrafish

Keira Lee Rice, Sarah E. Webb and Andrew L. Miller
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MS TITLE: Localized TPC1-mediated Ca^{2+} release from endolysosomes contributes to myoseptal junction formation and maintenance in zebrafish

AUTHORS: Keira Lee Rice, Sarah E Webb, and Andrew L Miller
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 gave favourable reports but raised some critical points that will require amendments to your manuscript, particularly changes has been suggested in manuscript text and data presentation. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from 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 study provides evidence that TPC1 found in endolysosomes and lysosomes influences myoseptal junction formation in zebrafish. Overall, the manuscript is well written and the experiments appear well designed. While the conclusions related to the relationship between TPC1 and the myoseptal are not completely novel, the authors provide significant data that lends confidence to the previously proposed relationship.

Comments for the author

(1) The authors should further explain the significance of the aquaporin measurements and provide more background information regarding MC-generated Ca^{2+} signaling. Related to this, the authors should better explain the delay in SP1 compared to WT. Could the authors provide a mechanistic explanation for this finding?
(2) The authors provide evidence that TPC1 localizes to endosomes and to a lesser extent, lysosomes using a lysotracker. Does loss of TPC1 change the lifetime of endosomes before conversion to lysosomes and/or the steady-state ratio of the two?
(3) The readability of the manuscript could be improved by providing an introductory figure diagramming the muscle that is described in the Introduction. Refering to this figure later in the Results and Discussion sections would help in understanding the conclusions of the study.

Reviewer 2

Advance summary and potential significance to field

This is a significant advance identifying a role for the endo-lysosomal ion channel TPC1 in maintaining skeletal muscle architecture. Key findings are reported *in vivo* using the zebrafish. And as is typical of this lab, the imaging is beautiful. It builds on work in mammalian systems showing TPC1 regulates organellar contacts sites.

Comments for the author

Overall, the morphology analysis is very well done. I have only minor comments.
Abstract. Needs more introduction. As written, it 'jumps in'. Please use fewer abbreviations.
Introduction. Again, this is not written in the most accessible way. Be gentler to the novice! The authors should cite primary literature identifying contact sites between lysosomes and the ER from the Patel and Evans lab. The latter is particularly relevant given the focus on muscle Figure 4. Why go *in vitro*? Consider adding movies as supplemental data to support Figure 4.
Figure 5. These data are convincing. But did the authors analyze acidic organelles in the mutants or with bafilomycin A1 (see next comment)? This is not essential. But it would make for a more consistent analysis.
Figure 6. There is no observable effect of Ned-19 in the example shown. Nor do the statistics show any difference. I would therefore recommend removing these data. Again, do the authors have any genetic data here to support the AMO and bafilomycin results?
Figure 8. These functional data are the weakest in my opinion. More information is needed to qualify signal changes as an 'event' and how movement was taken into account. There is no evidence that the signal is localized apart from the assumption that the low affinity of the indicator will insulate it from bulk changes in the cytosol. The BAPTA data is a start. But there is no BAPTA-FF and EGTA control. The authors should better characterize the probe or substantially tone down conclusions as to what is going on here.

Reviewer 3

Advance summary and potential significance to field

The purpose of this study appears to be to investigate the role of TPC1 on slow muscle structure in the zebrafish model. The data that are presented are on average of very high quality with clear controls and lots of methodological details, giving confidence in rigor and reproducibility. This is the first study I'm aware of to investigate impacts of protein trafficking on

Comments for the author

My major issues with the manuscript are related to it's presentation because the data are compelling. The introduction is confusing and doesn't provide organized rationale for undertaking these studies. The results are a list of experiments done and results. While discussion should not overlap with the results section, it would be very helpful for the reader if there were one sentence introductions and conclusions explaining why experiments were done. The discussion includes a lot of this rationale and the impact, but should be divided into titled subsections to facilitate reading.

Major:
1. It is likely important to begin the abstract with a more broadly compelling rationale: either coming from the perspective of endolysosomes are critical for cell function yet regulation is unknown, attachment of muscle to the MJs is critical yet not understood….. As it reads right now the abstract is very detailed without much attempt at communicating to non specialists. For example TPC1 is not defined, highly localized non-propogating is a lot of adjectives to include prior to the subject of the first sentence, myoseptal junctions are not defined and likely not understood by anyone who doesn't work on fish…..
2. This same sort of comment applies to the introduction. The authors would produce a much more compelling, widely read and cited paper if they framed their story more logically – as it is there seems to be some jumping between Ca^{2+} and TPCs and it is somewhat confusing.
3. Somite boundaries are reported to be important for stopping fibers from crossing MJs later in development. The slow fibers crossing at 24hpf – did those somite boundaries form or are the fibers crossing because the boundaries didn't form? When is TPC expressed during development? Is it expressed in forming somites? The authors show a phenotype in heterozygous fish at 48hpf. Are they viable? Are there impacts on swimming?

Minor:
1. Line 44 – The authors note that there are slow and fast muscles in the zebrafish trunk but they are focusing only on slow. Is TPC expressed in the fast muscles and are there impacts of the morpholinos/crispants on fast muscle? It seems a bit odd to only focus on slow because they are at the periphery, is there a physiological reason? The authors focus on how calcium transients are known in SMCs so maybe that could be a rationale for studying them and not the fast.
2. Line 56 – The authors are clearly very careful with their experimental methods and focusing on specific myotomes at particular time points, but this detail detracts from the introduction and would be better suited for the results section.
3. Line 43: is the DAPC the only complex linking ECM to sarcomeres or do Integrins play a role?
4. Line 196: It would be helpful to define Rab5c, Rab11ba, Lamp1, or Rab32b and explain why they were used.

First revision

Author response to reviewers' comments

Response to Reviewers' Comments

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We have conducted literature searches using the following combinations of keywords: 'two pore channel 1', 'TPC1' or 'TPC' and 'myoseptal' or 'muscle' using NCBI PubMed and Google and did not come across any previous papers (articles or reviews) reporting a relationship between TPC1 and the myoseptal junction. Some work has been published on the role of **TPC1** on Ca^{2+} changes in smooth muscle cells (Pereira et al., 2014; Jiang *et al.*, 2013, 2018; Trufanov et al., 2019; Hu et al., 2021) and cardiomyocytes (Davidson et al., 2015; García-Rúa *et al.*, 2016), but not in skeletal muscle cells. There are several papers that describe the role of **TPC2**-mediated Ca^{2+} signalling in skeletal muscle formation or function (Lin et al., 2015; Kelu et al., 2015, 2017). Indeed, two of these are from our laboratory (Kelu et al., 2015, 2017). For this reason, we strongly believe that our findings regarding **TPC1** and the **myoseptal junction** are novel and will therefore be of interest to the J. Cell Sci. readership as well as the scientific community in general.

Reviewer 1 Comments for the Author...

(1) The authors should further explain the significance of the aquaporin measurements and provide more background information regarding MC-generated Ca^{2+} signaling. Related to this, the authors should better explain the delay in SP1 compared to WT. Could the authors provide a mechanistic explanation for this finding?

We now briefly describe what aequorin (not aquaporin) is, the first time it is mentioned (**see lines 297- 302**). We also provide more information in the Discussion to suggest what might cause the SP1 delay in morphants compared with WT embryos (**see lines 487-493**).

(2) The authors provide evidence that TPC1 localizes to endosomes and to a lesser extent, lysosomes using a lysotracker. Does loss of TPC1 change the lifetime of endosomes before conversion to lysosomes and/or the steady-state ratio of the two?

While we observed that the disruption of *tpcn1* expression led to the altered morphology of lysotracker-stained puncta in whole embryos (Fig. 5), we did not design our experiments to study the effect of attenuated TPC1 expression on the lifetime of endolysosomes or the steady-state ratio between different vesicles. Interesting as these question are, they were not the focus of our study. Keeping in mind that lysotracker stains all acidic organelles, including late endosomes, further investigations are needed to find out whether the large puncta shown in Fig 5 are acidic endosomes or lysosomes, and how *tpcn1* knockdown might affect the equilibrium between the two.

(3) The readability of the manuscript could be improved by providing an introductory figure diagramming the muscle that is described in the Introduction. Referring to this figure later in the Results and Discussion sections would help in understanding the conclusions of the study.

We have added several schematics to show the arrangement of skeletal muscle in the trunk of zebrafish embryos – please **see new Figs 1A and S1**. We refer to these figures in various places throughout the text as suggested. We have also rewritten the Abstract and Introduction (as suggested by Reviewers 2 and 3) to improve the readability of the manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field...

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We have modified the Abstract as requested.
Introduction. Again, this is not written in the most accessible way. Be gentler to the novice! The authors should cite primary literature identifying contact sites between lysosomes and the ER from the Patel and Evans lab. The latter is particularly relevant given the focus on muscle.
We have modified the Introduction as requested and hope that it is now more understandable to the novice. We have also cited primary literature from the Evans (Kinnear et al., 2004) and Patel (Kilpatrick et al., 2013) labs – **see line 98**.

Figure 4. Why go in vitro? Consider adding movies as supplemental data to support Figure 4.
SMC primary cultures were used for some experiments as they have a better fluorescent signal-to- noise ratio than intact embryos. This information has been added to the main text – **see lines 225-227**. We have also submitted two short movies (**Supplemental Movies 1 and 2**) to accompany figures 3D and 4D.

Figure 5. These data are convincing. But did the authors analyze acidic organelles in the mutants or with bafilomycin A1 (see next comment)? This is not essential. But it would make for a more consistent analysis.
We have now included acidic organelle data for homozygous mutants (and compared these with wild- type controls). Please see our **revised Fig. 5**.

Figure 6. There is no observable effect of Ned-19 in the example shown. Nor do the statistics show any difference. I would therefore recommend removing these data. Again, do the authors have any genetic data here to support the AMO and bafilomycin results?
We have removed the *trans*-Ned-19 data as recommended and have added new data from *tpcn1* E8 crispants and Cas9 controls. Please see our **revised Fig.6**.

Figure 8. These functional data are the weakest in my opinion. More information is needed to qualify signal changes as an 'event' and how movement was taken into account. There is no evidence that the signal is localized apart from the assumption that the low affinity of the indicator will insulate it from bulk changes in the cytosol. The BAPTA data is a start. But there is no BAPTA-FF and EGTA control. The authors should better characterize the probe or substantially tone down conclusions as to what is going on here.
We suggest that Fig. 8 panels B, and Cb to Cd, do show the localized nature of Ca^{2+} signalling events (in the absence of any introduced cytosolic Ca^{2+} buffer) – and that they are generated by Ca^{2+} release via TPC1 resident in the membranes of Lamp 1-decorated vesicles – as recorded by TPC1-GG.
With regards to the quite reasonable suggestion that the low affinity of G-GECO might insulate it from bulk changes in $[Ca^{2+}]_i$, we have, as suggested, now included an additional Ca^{2+} buffer control (Fig. 8D). However, we suggest that 5,5' difluoro-BAPTA might not the best control as it has been previously shown that in *Pelvetia* eggs, the inhibitory buffer concentration is very similar to that of BAPTA due to very similar K_Ds (i.e., 0.72 μM *versus* 0.70 μM; Pethig et al., 1989; Speksnijder et al., 1989). Therefore, we selected 5,5' dimethyl-BAPTA-AM (DMB-AM), which has a K_D of 0.44 μM (Pethig et al., 1989), and which we have previously demonstrated in intact zebrafish to be a good control for BAPTA-AM due to their differences in K_D (Guo et al., 2020). In our extended TPC1-GG experiments, we used BAPTA-AM and DMB-AM at the same concentration, and they both resulted in a decrease in the percentage of TPC1-GECO puncta at the ends of SMCs, and the amount of attenuation was related to the K_D of the buffer as demonstrated by Speksnijder et al. (1989) and Guo et al. (2020). Please see **revised Fig.8**.

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We have updated the Introduction. We have also divided the Discussion into titled subsections (as suggested), which we agree helps to facilitate reading.

Major:
1. It is likely important to begin the abstract with a more broadly compelling rationale: either coming from the perspective of endolysosomes are critical for cell function yet regulation is unknown, attachment of muscle to the MJs is critical yet not understood….. As it reads right now the abstract is very detailed without much attempt at communicating to non specialists. For example, TPC1 is not defined, highly localized non-propagating is a lot of adjectives to include prior to the subject of the first sentence, myoseptal junctions are not defined and likely not understood by anyone who doesn't work on fish…..
We have updated the Abstract and hope that it is now understandable to non-specialists.

2. This same sort of comment applies to the introduction. The authors would produce a much more compelling, widely read and cited paper if they framed their story more logically – as it is there seems to be some jumping between Ca^{2+} and TPCs and it is somewhat confusing.
We have also updated the Introduction as recommended.

3. Somite boundaries are reported to be important for stopping fibers from crossing MJs later in development. The slow fibers crossing at 24 hpf – did those somite boundaries form or are the fibers crossing because the boundaries didn't form?
We did not observe the boundary crossing phenotype at 24 hpf but it was a prominent feature by 48 hpf (Fig. 1). We have now included a new figure (**see Fig. S6A**) and added the following section to the Discussion (**lines 378-391**) to address this point:
"The vertical myosepta form from the early somite boundaries, which develop in the paraxial mesoderm during the segmentation stage (Kimmel et al., 1995; Henry et al., 2000). We found that these somite boundaries still developed in the *tpcn1* morphants (Fig. S6A). These observations are supported by a previous report showing that no regular Ca^{2+} transients were generated during the formation of the rostral/caudal somite boundary (Leung et al., 2009). Furthermore, the local uncaging of a photolabile Ca^{2+} buffer (Diazo-2) in the paraxial mesoderm just prior to the onset of somite formation did not inhibit the formation of the rostral/caudal somite boundary although the extension of the lateral somite boundary was abnormal when compared with the controls (Leung et al., 2009). These data suggest that rostral/caudal somite boundary formation does not require a Ca^{2+} transient generated by release via any Ca^{2+} channel (including TPC1). We suggest, therefore, that the myotome boundary crossing phenotype we observed at 48 hpf when *tpcn1* expression was attenuated, is not due to the absence of a rostral/caudal somite boundary. Rather, we propose that the lack of capture of SMCs is due to the absence of an essential MJ component (or components) that are delivered to the forming MJ in a TPC1-dependent manner".

When is TPC expressed during development? Is it expressed in forming somites?
We have now included data to show that *tpcn1* is expressed from 16-48 hpf, when much of muscle development occurs (**see updated Fig. S3Ba**). There is also evidence in the Expression Atlas (https://www.ebi.ac.uk/gxa/home), that *tpcn1* (ensdarg00000062362) is expressed as early as the zygote and cleavage stages in zebrafish. TPC1 is expressed in the somites. We have also included two panels showing the localisation of TPC1 in embryos at ~24 hpf (see **updated Fig. S3Bb,Bc**). We also describe this in our revised text (**see the legend for Fig. S3**).

The authors show a phenotype in heterozygous fish at 48hpf. Are they viable?

We found that ~40% of the heterozygous embryos remained viable to adulthood, and so could be used for breeding purposes. This information has now been added to the **legend for Fig. S4**.

Are there impacts on swimming?
We don't have information about swimming *per se* following *tpcn1* knockout or knockdown. However, we present new data demonstrating the effect of *tpcn1* knockdown (and rescue) on the onset and frequency of the spontaneous coiling behaviour, an activity dependent primarily on SMC excitation- contraction coupling (**see new Fig. S5**).

Minor:
1. Line 44 – The authors note that there are slow and fast muscles in the zebrafish trunk but they are focusing only on slow. Is TPC expressed in the fast muscles and are there impacts of the morpholinos/crispants on fast muscle? It seems a bit odd to only focus on slow because they are at the periphery, is there a physiological reason? The authors focus on how calcium transients are known in SMCs so maybe that could be a rationale for studying them and not the fast.
To address this point, in our updated Introduction, we now state that:
"The bulk of the Ca^{2+} generating these early myogenic transients is released mainly from the sarcoplasmic reticulum of SMCs via a combination of inositol 1,4,5-trisphosphate receptors and ryanodine receptors (Brennan et al., 2005; Cheung et al., 2011). However, it was recently reported that these Ca^{2+} signals are initiated via the action of two-pore channel (TPC) type 2 (TPC2) (Kelu et al., 2015; 2017), perhaps via a proposed triggering mechanism (Zhu et al., 2010a). Indeed, the knockdown or pharmacological inhibition of TPC2 completely eliminated both phases of the propagating Ca^{2+} transients in SMCs and resulted in a disruption of myofibrillogenesis and subsequent motility (Kelu et al., 2017)". **(See lines 80-87)**
AND
"As TPC-related isoform-specific roles have previously been reported from experiments using *Xenopus* oocytes and cultured mammalian cells (Lin-Moshier et al., 2014), we hypothesized that Ca^{2+} release from TPC1 might have independent and distinct developmental/regulatory functions from TPC2, during myogenesis in SMCs". (**See lines 108-111**)

2. Line 56 – The authors are clearly very careful with their experimental methods and focusing on specific myotomes at particular time points, but this detail detracts from the introduction and would be better suited for the results section.

In our revised Introduction, we removed the information about the specific myotomes and particular time points selected, and the information is now in the Results section – **see lines 147-148**.

3. Line 43: is the DAPC the only complex linking ECM to sarcomeres or do Integrins play a role?
It has been reported that there are at least two ECM/sarcolemmal linkage systems in MJs. In addition to the DAPC there is also integrin α7 (and in a supporting role, integrin α6), which are reported to be crucial for maintaining skeletal muscle integrity and attachments (Goody et al., 2012). While it would be of interest to explore the respective roles (and potential differences) between these linkage systems in zebrafish SMCs, this wasn't the focus of this manuscript. We have, however, included information to describe this in the Discussion. **See lines 362-364.**

4. Line 196: It would be helpful to define Rab5c, Rab11ba, Lamp1, or Rab32b and explain why they were used.
We have now defined Rabs and Lamp1 and have explained why we used them - **see lines 216-222.**

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Second decision letter

MS ID#: JOCES/2021/259564

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AUTHORS: Keira Lee Rice, Sarah E Webb, and Andrew L Miller
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

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The authors have addressed all of my concerns and I recommend publication.

Comments for the author

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Comments for the author

The authors have done a good job responding to reviews.