Impaired Cx43 gap junction endocytosis causes morphological and functional defects in zebrafish

Caitlin Hyland, Michael Mfarej, Giorgos Hiotis, Sabrina Lancaster, Noelle Novak, Mary Kathryn Iovine, and Matthias Falk

Corresponding author(s): Matthias Falk, Lehigh University

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Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E20-12-0797
TITLE: Impaired Cx43 gap junction endocytosis causes cardiovascular defects in zebrafish

Dear Dr. Falk,

First, please accept my apologies for the delay in responding.

Second, two reviewers have given your manuscript a careful reading. Both find your work potentially valuable and appropriate for Molecular Biology of the Cell. After sifting through their comments, I feel that the requested revisions are sufficiently significant that your manuscript will require re-review.

Both Reviewers 1 and 2 mention the need for better vetting of your deletion. Reviewer 1 notes that there are significant concerns about the Cx43 (256-289) deletion in terms of controlling for off-target effects. Reviewer 1 is unclear (at least to me) about where the issue related to cultured cells or fish, or both, but this is an issue that you should address. Reviewer 2 is more explicit regarding the fish work and the need for additional evidence that your interpretation of what you believe you have done in generating the deletion line is correct.

Reviewer 2 also has concerns about interpretation of your results, given the increase in mRNA in the mutant Cx43 line and the relevance of increased protein levels given that transcriptional backdrop. While your interpretation about endocytosis may account for the data, dealing with alternative hypotheses that are less interesting seems important. Reviewer 1 hints that additional experimental support in this area would also improve the paper.

In addition to these issues, in your Response to Reviewers, please respond to each of the points the Reviewers raise regarding data analysis and other specific concerns.

Thanks for submitting your work to MBOC. Again, my apologies for the delay.

Best regards,
Jeff Hardin
Monitoring Editor

Molecular Biology of the Cell

Dear Prof. Falk,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

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Specific comments:
No control was used to account for the potential off-target effects of the Cx43 (256-289) deletion process. The WT groups may not serve as adequate controls in this respect. The authors should note this and other limitations of approach and on interpretation in the discussion.

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5. What is the endogenous levels of zebrafish atrial and ventricular Cx43 relative to other connexin proteins? It is highly unusual to see significant atrial expression of Cx43, approaching that of ventricular expression.
6. Patch clamping pairs of cells containing WT and mutant Cx43 will help establish and quantify increased intercellular conductance relative to increase plaque density.
7. The authors previously used Gap27 to inhibit gap junction formation and GJIC, and reverse mutant induced zebrafish phenotypes (Bhattacharya et al, 2020). A similar cardiovascular rescue in this study would considerably strengthen the results.

Point-by-point response to Manuscript #E20-12-0797:

We thank the reviewers and the Monitoring Editor for their verbatim criticism and the time they invested to carefully evaluate our manuscript. We are pleased, that both reviewers found our study to be important and interesting. We have addressed all concerns as detailed below. In response to the critique, four main changes have been introduced to the revised manuscript:

(1) In response to the critique, we repeated qRT-PCR analyses, this time by pooling 15 embryos per biological replicate instead of using just one embryo as done in the previous analyses. We are aware that the previous analysis was not optimal as phenotype severity is quite heterogeneous and thus individual embryos may not represent the average, however at that time, we did not have enough homozygote mutant embryos to perform a large enough study. Importantly, this modification now shows that mRNA levels of *cx43* in the mutant are not increased, and indeed are essentially identical to WT. Thus, the observed increase in Cx43 protein and gap junction levels is not due to upregulated *cx43* expression, but impaired gap junction turnover. Indeed, with this new data fueling stronger confidence, we now express clearer that impaired gap junction turnover causes all sorts of problems, as demonstrated in detail here for vasculature morphology and function.

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(4) Finally, we present in much more detail how the *cx43*^{lh10} mutant zebrafish line was generated, what we did to prevent off-target side effects, and show verification of the mutant via DNA sequencing data as suggested by Reviewer 2 (Supplemental Table 1, and Supplemental Figures 1, 2).

Edits have been performed throughout the manuscript and major changes, including in response to reviewer critique, have been highlighted in yellow. Our substantial additions and revisions certainly have generated a much-improved manuscript that much better supports our important novel finding that regulated gap junction turnover is essential for gap junction function; and we hope our revised manuscript is now suitable for publication in *MBoC*. Please see detailed responses below:

Reviewer #1 (Remarks to the Author):

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Figure 7C: Missing errors bars
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7. The authors previously used Gap27 to inhibit gap junction formation and GJIC, and reverse mutant induced zebrafish phenotypes (Bhattacharya et al, 2020). A similar cardiovascular rescue in this study would considerably strengthen the results.
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RE: Manuscript #E20-12-0797R
TITLE: "Impaired Cx43 gap junction endocytosis causes morphological and functional defects in zebrafish"

Dear Dr. Falk,
Thanks for submitting your revised manuscript. The crucial qRT-PCR data, the GAP27 epxeirment, and the additional information on your CRISPR allele seem to address the most significnat comments from the Reviewers. As a result, I am pleased to say that I think you paper is now acceptable for publication in Molecular Biology of the Cell.

Please address the following small issues in your final manuscript:
Line 125: "Potential effects on cx40.8, a cx43-like gene in zebrafish are discussed." There seems to be a word or phrase missing. "discussed below"? "dealt with in the Discussion section"?
Lines 364-375: There may be some inconsistency in capitalization of "Cx43". Please check carefully.
Line 393: "heterozygote mutations" should be "heterozygous for mutations"
Line 442: cell guiding" should be "cell guidance"
Line 994-995: "Note, that the heart rate of embryos injected with Gap27 was restored closely to WT levels" should be ""Note, that the heart rate of embryos injected with Gap27 was restored to close to WT levels"

Then I think your manuscript should be ready for publication.

Many thanks for submitting your work to MBoC!

Best regards,
Jeff Hardin
Monitoring Editor

Dear Prof. Falk,

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To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are

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Thanks again for choosing MBoC.

Best regards,
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Dear Prof. Falk:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
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