



# Citrullination regulates wound responses and tissue regeneration in zebrafish

Netta Golenberg, Jayne Squirrell, David Bennin, Julie Rindy, Paige Pistono, Kevin Eliceiri, Miriam Shelef, Junsu Kang, and Anna Huttenlocher

*Corresponding Author(s): Anna Huttenlocher, University of Wisconsin Madison*

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## Review Timeline:

Submission Date:	2019-08-20
Editorial Decision:	2019-10-05
Revision Received:	2019-12-17
Editorial Decision:	2020-01-24
Revision Received:	2020-01-29

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*Monitoring Editor: Jodi Nunnari*

*Scientific Editor: Andrea Marat*

## 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.)

**DOI: <https://doi.org/10.1083/jcb.201908164>**

October 5, 2019

Re: JCB manuscript #201908164

Dr. Anna Huttenlocher  
University of Wisconsin Madison  
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Dear Anna,

Thank you for submitting your manuscript entitled "Citrullination regulates wound responses and tissue regeneration in zebrafish". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We sincerely apologize for the delay in sending our decision to you. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that all reviewers find the results interesting. We share the reviewers' interest in presenting the importance of citrullination in wound healing in vivo and characterizing the role of Padi2 in these processes. The work is a good fit for the Report format as it opens up new avenues of research into the contribution of the machinery controlling citrullination to development and wound healing/regeneration and the impact of the modification in vivo. Therefore, we find it reasonable that there would be open mechanistic questions, in particular how Padi2-mediated histone modification influences regeneration/inflammation. However, for further consideration at the journal, the key conclusions of the Report need to be definitive. We agree with Revs#1-2 that more evidence is required to definitively conclude that Padi2 is not needed for early zebrafish development and plays a role in proliferation control during wound healing through citrullination of histones in particular cells. We suggest you focus experimental efforts in revision to tackle the reviewers' points as follows:

- Reviewer #1 makes constructive suggestions to strengthen the core observations. We editorially find that their points are valid and relevant and should be addressed in full. They request more data to bolster the claim that citrullination does not affect zebrafish early development (Rev#1, point #2), that citrullination of histones in the notochord is essential for proliferation control during wound healing (Rev#1, point #3d), and that Padi2 is important for wound healing (Rev#1, #3a, c; see also Rev#2 points #6-7-8). Both Revs#1 and #2 want to know more about the cells showing the citrullinated histone signal (Rev#1 #3d, Rev#2 #9) and we find this point valid given the scope of the analyses and importance of these results to your model for Padi2 function in wound healing.
- In addition, it will be important to respond to Reviewer #2's point #3 and strengthen the phenotypic characterizations (Rev#2 #6-7-8). The reviewers request more antibody validation (Rev#1 #1) and more clarity on the isoforms of padi2 (Rev#2, #2; Rev#1 #1 and minor points, transcript nomenclature) and these points should be addressed as well.
- On the other hand, Rev#2's suggestion to assess myelinated axon counts in Padi2 mutant fish

(#5) to strengthen the comparison of its role in development between mice and fish, given past published studies in mice, seems more peripheral and less essential to us. This would not be required for publication.

- Rev#2 highlights that wound healing and regeneration are distinct concepts. Our view is that, if you were to refocus on wound healing, you wouldn't need to test regeneration in the adult as Rev#3 suggested, which is not essential to support the current set of conclusions, but certainly interesting.

Please let us know if you anticipate any issues addressing these points or have any questions. We would be happy to discuss the revisions further.

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 and 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. 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/fora.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.\*\*\*

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

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

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

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

Sincerely,

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

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

The manuscript by Golenberg et al., describes a zebrafish loss of function for Padi2, a Ca-dependent citrullinating enzyme in development. The authors demonstrate an unexpected lack of obvious developmental phenotype and argue for the lack of general role for citrullination in early development in zebrafish. Intriguingly, they uncover a role in wound-healing specifically affecting H4 citrullination in a subpopulation of notochord and surrounding cells and dissect the role of Padi2 in wound healing by various transgene imaging techniques and cell proliferation assays. They conclude that the defect in proliferation in wound healing is independent from the normal growth of caudal fin fold originating from the ventral mesenchyme. This ms has important novel findings, which however need verification and or more details of cause of effect to strengthen the conclusions.

Main Points

1. Antibody specificity: There are 4 bands in the Western blots of wt and Padi2 mutant zebrafish, 2 stronger ones and 2 weaker. Which of them are considered to be specific and are they expected to represent products from the two transcripts 001 and 002? If this is the case then the statement: "this antibody did not detect a protein of equivalent size to the predicted short transcript" refer to is somewhat confusing as from the transcript analysis one could expect 3 protein sizes (2 confirmed by direct RNA analysis). The associated figure and transcript-derived protein detection needs to be more clearly explained.

Additionally, overexpression of Padi2 from synthetic mRNA would be helpful, not only to verify antibody specificity in Westerns, but also for proving the phenotypes are indeed due to Padi2 protein loss in the injected mutant embryos.

2. Role of citrullination in development: The authors suggest that Padi2 is the only citrullinating enzyme active during zebrafish development and that citrullination is not essential for 'broadly normal' zebrafish development. This claim is not satisfactorily proven. If there is no early (e.g. pluripotency) effects, suggesting major difference from mammalian functions, this would indeed be an important finding, however, this possibility is not yet fully verified. Citrullination was only measured in one stage of development (referred to as 48 hpf, correctly: long pec stage). Secondly, this experiment does not answer whether there is citrullination happening in early development and whether early citrullination was affected in the mutant. If there was no effect on early citrullination, then, this could be due to compensation by maternal Padi2 (or other PAD paralog) protein present in the early embryos, which are diluted by the time the authors checked Padi2 by Western blot. If there was no maternal protein, there could still be other Padi proteins, which may be responsible for early (before long pec) citrullination, not tested by the authors. To clarify these options, which are necessary for one of the conclusions of this manuscript, early phenotyping (early embryonic citrullination defects) and analysis of the expression profile of Padi2 variants and Padi2 paralogs is necessary. Is Padi2 zygotic or maternal zygotic expressed gene? Is the loss of protein in zygotic mutants observable throughout development or only in later stages? If the gene was maternal (or there are other citrullinating proteins which are maternal), the interpretation of the mutant phenotype will be affected and potentially indicate partial (e.g. delayed) loss of citrullination in development, with an untested residual (e.g. subfunctionalised paralog or maternal variant Padi2) activity from the mother potentially complementing zygotic loss of Padi2 at early stages, which are

unexplored in the manuscript.

### 3. Wound healing effects:

- a. These are interesting sets of experiments, revealing wound healing roles for Padi2, but it is unclear how were the mutant larvae which were analysed identified (genotyped).
- b. The absence of PAD4 ortholog (btw what is the data used as evidence for lack of?) suggested by the authors begs for the question whether Padi2 of zebrafish is nuclear localised, as was seen suggested for Padi4, the only PAD in mammals with nuclear localisation signal. This needs to be verified by Western blot on nuclear extracts, but would also be helpful if the antibody was tested by immunostaining. Alternatively, binding of Padi2 to chromatin/H4 ought to be tested.
- c. In addition to nuclear localisation of the protein another key piece of evidence for direct effect by Padi2 on wound healing is to show that padi2 mRNA is expressed in wt and/or regenerating fin fold and notochord. ZFIN expression data suggests highly specific expression of padi2 RNA in epidermis, pectoral fin etc.
- d. The proposed effects in proliferation upon wound healing and in normal caudal fin mesenchyme are intriguing, but are not sufficiently linked to the observed effects in citrullination of histones in the notochord. The key question is whether the observed effects are due to H4 citrullination in the notochord. While this may not be possible to fully answer within the scope of the ms, it is expected that additional lines of evidence strengthen the proposed link. Among others, a possible set of experiments could include dissecting cell autonomy of Padi2 function in relation to the observed phenotype by strategies used in the zebrafish genetics field, such as blastomere transplantation to generate mosaics (see e.g. PUBMED:17597528) or mosaic expression of padi2 mRNA in single blastomere injected at cleavage stage wt and mutant embryos to monitor cell specific/cell autonomy of defects. Such experiments could help in tracking of those cells emerging from the notochord bead, which are expected either to be directly affected by the mutation and or are interacting with fin fold mesenchyme cells, the proliferation of which was suggested to be affected. Is there evidence for citrullination of H4 in tissues where padi2 is expressed in wt embryos and larvae?

### Minor points

1. Manuscript organisation could be revised to help the reviewer:
  - a. add page numbers and line numbers;
  - b. figure panels should have their numbering on the figure;
  - c. ideally the legend should also be printed on the bottom of figures, which can be reduced to 80% print size without losing important information, for ease of navigating the manuscript.
2. Switch order of transcripts 001 and 2 in Suppl. Fig 1a to fit with panel B. refer to transcripts by their annotated names in the ms text.
3. The new variant described by the authors needs to be clearly indicated as new and a distinguishing transcript name used.
4. Western blot in Fig S1 C needs caption of expected size markers (kD) to identify the bands (presumably the two strong ones) attributed to Padi2.
5. Figure legends for Fig 1E,F refers to larvae, while Methods indicate 2 days old embryos were used. Please clarify the accurate stage of embryos and larvae using ZFIN anatomy nomenclature throughout the manuscript for all experiments and indicate them in the legend of figures.
6. Fig. 3B needs addition of untreated controls at the relevant stages, as well as moving Fig. S3F to the main Figure.

The manuscript by Golenberg et al., identifies histone citrullination in a subset of cells within the regenerative notochord bead. In the Padi2 mutant where citrullination does not occur, the authors show that leucocytes dynamics is altered, proliferation is perturbed and regeneration is less efficient.

The findings reported in this manuscript proposes Padi2 (citrullination) as an intermediary between calcium and regenerative mechanisms.

In my view there are several points that need to be addressed for clarity.

Just a note, there are no pages in the manuscript and the figures are not labelled.

#1 - Wound healing/repair is different from regeneration. Wound healing/repair is in fact an early stage of the regenerative process. Wound healing is characterized by the formation of a wound epithelium and the expression of *dlx5a* for example; is then followed by the formation of a blastema characterized by the expression of genes like *msxc* and *msxe*; and from here there is an outgrowth phase.

The authors use the terms wound healing/repair and regeneration throughout the manuscript as if they were the same. The authors should revise these concepts in the entire manuscript and be more accurate with what they actually mean.

#2 - The terms full-length and short-length to name the alternative transcripts is a bit confusing because then the authors talk about a full-length exon 10. I would propose to name longer and shorter transcripts. Bioinformatically there are 4 alternative transcripts. The authors say that they identified only 2 splice variants, but they do not say how these were identified?

#3 - The authors should explain why did they target exon 7 to generate the Padi2 mutant.

#4 - "These observations provide the first evidence that citrullination is not necessary for broadly normal development of a vertebrate." The author should re-phrase this sentence because citrullination in mammals were shown to have an impact on development. This paragraph should be re written and the precise Padi genes should be mentioned, because actually none of the papers cited in this paragraph are about Padi2.

#5 - The authors refer that "The mammalian PAD2 is the predominant isozyme in skeletal muscle and nervous system" and they went on to show that in zebrafish Padi2 mutants the skeletal fibers have a normal morphology with more neuromuscular junctions. What about the number of myelinated axons, as these were shown to be less in mice mutant for Padi2?

#6 - The regenerate fin fold length in Padi2 mutants is smaller when compared with control wt larvae (Fig. 2B). These measures were done at 3 days post excision. As it takes 2-4 days for complete regrowth, the authors should quantify fin fold regenerate size at later stages. Only then it will be possible to claim that regeneration is impaired and not simply delayed.

#7 - In Fig.2B, it is possible to see that fin fold in Padi2 mutants are slightly bigger when compared with control wt larvae. These measures were done at 5 days post fertilization. Are the differences in size seen also at earlier stages, namely at the stages when the measurements of the regenerate size were done (i.e. 3 dpf)? Are these size differences seen only in the fin fold or are the larvae bigger as a whole?

#8 - In Fig. 2F, the authors claim that Padi2-deficient larvae have "... impaired neutrophil resolution from the wound at 48 hpw. I would argue that Padi2 mutants have more leucocytes in the wound to

start with, and so the degree of decrease is in the same order as in the wt. In addition an increase in leucocytes is not necessarily negative, as it was shown that this early inflammation is important for successful regeneration in adult zebrafish. These issues should be discussed.

#9 - What is the cellular nature of the subpopulation with citrullinated histones? Are these notochord cells or are these some kind of blastema cells? Do these cells share notochord or blastema markers? Are these cells leucocytes? The authors should be careful with the term pluripotency because in the fins of zebrafish regeneration is not really done using bona fide stem cells but by mechanisms a de-differentiation. And as far as I know the level of pluripotency exhibited by these de-differentiated cells is no really pluripotency, actually they have a very narrow differentiation potential.

Reviewer #3 (Comments to the Authors (Required)):

Golenberg et al., here characterized the effects of knockout of a  $\text{Ca}^{2+}$  dependent histone modifier on injury-induced leukocyte recruitment and larval regeneration.

For what it tries to show, this is a well-executed/controlled zebrafish gene KO study for an interesting enzyme that could transduce some of the physiological effects of wound-induced calcium signals. Notably, the authors have confirmed their gene KO approach, with orthogonal morpholino-mediated knockdown (in the supplement). I also really like how the authors characterize padi2's enzymatic activity (or lack thereof in the mutant). All the necessary controls for the presented experiments seem to be there. I have little technical concerns with the presented data.

It would be interesting to know whether the observed (moderate?) tail fin regeneration defects in larvae translate to notable adult tail fin regeneration defects. I think such an experiment would not be too difficult to do and could consolidate the idea that padi2 is important during regeneration, which most people still define as adult regeneration. Mechanistically, there remain of course many unanswered questions, some of them pointed out by the authors in their discussion. What are these padi2 positive cells in the notochord bead, and what do they do? How does padi2 mediated histone modification influence regeneration/inflammation? Etc. Whether there remain too many open mechanistic questions for this particular journal is an editorial call.

Regardless, the study touches on interesting biological aspects, i.e., how rapid wound signals are stored to inform long-term responses such as regeneration by activation of a less known histone modifier. So, I enjoyed reading the paper, which is also well-written. Maybe I would add to the discussion that there are many ways by which  $\text{Ca}^{2+}$  can influence wound healing/regeneration given the very many wound relevant,  $\text{Ca}^{2+}$  dependent pathways. Padi2 is one interesting possibility, but by far not the only one.

**We are pleased to submit our revised manuscript “Citrullination regulates wound responses and tissue regeneration in zebrafish”. We appreciate the reviews of our original manuscript and below provide a point by point response to the reviews. The revisions have improved the manuscript and we thank the reviewers for their comments.**

**Thank you for your consideration of our revised manuscript.**

Editor:

You will see that all reviewers find the results interesting. We share the reviewers' interest in presenting the importance of citrullination in wound healing in vivo and characterizing the role of Padi2 in these processes. The work is a good fit for the Report format as it opens up new avenues of research into the contribution of the machinery controlling citrullination to development and wound healing/regeneration and the impact of the modification in vivo. Therefore, we find it reasonable that there would be open mechanistic questions, in particular how Padi2-mediated histone modification influences regeneration/inflammation. However, for further consideration at the journal, the key conclusions of the Report need to be definitive. We agree with Revs#1-2 that more evidence is required to definitively conclude that Padi2 is not needed for early zebrafish development and plays a role in proliferation control during wound healing through citrullination of histones in particular cells. We suggest you focus experimental efforts in revision to tackle the reviewers' points as follows:

**We have focused on the points raised by the editor and these are addressed below in the response to reviewers.**

- Reviewer #1 makes constructive suggestions to strengthen the core observations. We editorially find that their points are valid and relevant and should be addressed in full. They request more data to bolster the claim that citrullination does not affect zebrafish early development (Rev#1, point #2), that citrullination of histones in the notochord is essential for proliferation control during wound healing (Rev#1, point #3d), and that Padi2 is important for wound healing (Rev#1, #3a, c; see also Rev#2 points #6-7-8). Both Revs#1 and #2 want to know more about the cells showing the citrullinated histone signal (Rev#1 #3d, Rev#2 #9) and we find this point valid given the scope of the analyses and importance of these results to your model for Padi2 function in wound healing.

- In addition, it will be important to respond to Reviewer #2's point #3 and strengthen the phenotypic characterizations (Rev#2 #6-7-8). The reviewers request more antibody validation (Rev#1 #1) and more clarity on the isoforms of padi2 (Rev#2, #2; Rev#1 #1 and minor points, transcript nomenclature) and these points should be addressed as well.

- On the other hand, Rev#2's suggestion to assess myelinated axon counts in Padi2

mutant fish (#5) to strengthen the comparison of its role in development between mice and fish, given past published studies in mice, seems more peripheral and less essential to us. This would not be required for publication.

- Rev#2 highlights that wound healing and regeneration are distinct concepts. Our view is that, if you were to refocus on wound healing, you wouldn't need to test regeneration in the adult as Rev#3 suggested, which is not essential to support the current set of conclusions, but certainly interesting.

Please let us know if you anticipate any issues addressing these points or have any questions. We would be happy to discuss the revisions further.

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.

**We thank the editor for their comments and provide a point-by-point response below.**

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

The manuscript by Golenberg et al., describes a zebrafish loss of function for Padi2, a Ca-dependent citrullinating enzyme in development. The authors demonstrate an unexpected lack of obvious developmental phenotype and argue for the lack of general role for citrullination in early development in zebrafish. Intriguingly, they uncover a role in wound-healing specifically affecting H4 citrullination in a subpopulation of notochord and surrounding cells and dissect the role of Padi2 in wound healing by various transgene imaging techniques and cell proliferation assays. They conclude that the defect in proliferation in wound healing is independent from the normal growth of caudal fin fold originating from the ventral mesenchyme. This ms has important novel findings, which however need verification and or more details of cause of effect to strengthen the conclusions.

#### Main Points

1. Antibody specificity: There are 4 bands in the Western blots of wt and Padi2 mutant zebrafish, 2 stronger ones and 2 weaker. Which of them are considered to be specific and are they expected to represent products from the two transcripts 001 and 002? If this is the case then the statement: "this antibody did not detect a protein of equivalent size to the predicted short transcript" refer to is somewhat confusing as from the transcript analysis one could expect 3 protein sizes (2 confirmed by direct RNA analysis). The associated figure and transcript-derived protein detection needs to be more clearly explained.

**We thank the reviewer for this comment. To clarify we have modified the text and figure to more clearly represent the findings. We have included arrows in the revised figure highlighting the two 16-exon protein products in figure S1 C to clarify the confusion. We are unclear as to which bands the reviewer is referring to as the weaker bands. A band at ~200 kDa can be observed in the wild-type protein lane and absent in the *padi2* mutant protein lane, marked by an asterisk. A protein of this size would not be expected on a denaturing gel (as we are using). Other bands appear to be nonspecific as they are still present in the *padi2* mutant lane and in our pre-immune blot (Fig S1 C and D).**

Additionally, overexpression of Padi2 from synthetic mRNA would be helpful, not only to verify antibody specificity in Westerns, but also for proving the phenotypes are indeed due to Padi2 protein loss in the injected mutant embryos.

**We have also confirmed the specificity of the antibody with re-expression of Padi2 RNA, confirming that the antibody recognizes zebrafish Padi2. We have included this in the revised manuscript in Fig S1 E. The expected Padi2 doublet is specifically absent in the mutant providing further evidence of the specificity of the antibody.**

**Due to the low expression of the injected Padi2 RNA (~12% of wild-type expression), we were unable to use these embryos for phenotypic rescue studies.**

2. Role of citrullination in development: The authors suggest that Padi2 is the only citrullinating enzyme active during zebrafish development and that citrullination is not essential for 'broadly normal' zebrafish development. This claim is not satisfactorily proven. If there is no early (e.g. pluripotency) effects, suggesting major difference from mammalian functions, this would indeed be an important finding, however, this possibility is not yet fully verified. Citrullination was only measured in one stage of development (referred to as 48 hpf, correctly: long pec stage).

**We thank the reviewer and think this is an important point. We now show citrullination activity early in development (2 and 7 hours post fertilization (hpf)) that is absent in the mutant. This is exciting new data that strengthens the manuscript significantly. We agree that this is an important finding and it has been included in the manuscript in figure 1 I.**

Secondly, this experiment does not answer whether there is citrullination happening in early development and whether early citrullination was affected in the mutant. If there was no effect on early citrullination, then, this could be due to compensation by maternal Padi2 (or other PAD paralog) protein present in the early embryos, which are diluted by the time the authors checked Padi2 by Western blot. If there was no maternal protein, there could still be other Padi proteins, which may be responsible for early (before long pec) citrullination, not tested by the authors.

**As indicated above, we have included new data that shows that early citrullination is happening and that it is absent in the mutant. In addition, there is no concern about maternal effect since these mutant progeny are a result of a homozygous adult mother crossed to a homozygous mutant male, eliminating the possibility of maternal contribution during early development. While a Padi paralogue is a possibility, our citrullination assay demonstrates an absence of citrullination in the *padi2* mutants. As this assay is testing for citrullination rather than Padi2 activity, we can conclude that there is a loss of citrullination activity in the mutant zebrafish and no citrullination activity contribution by another paralogue in the *padi2* mutant larvae, at the stage tested.**

To clarify these options, which are necessary for one of the conclusions of this manuscript, early phenotyping (early embryonic citrullination defects) and analysis of the expression profile of Padi2 variants and Padi2 paralogs is necessary.

**This issue has now been addressed in the revised manuscript (see above).**

If the gene was maternal (or there are other citrullinating proteins which are maternal), the interpretation of the mutant phenotype will be affected and potentially indicate partial (e.g. delayed) loss of citrullination in development, with an untested residual (e.g. subfunctionalised paralog or maternal variant Padi2) activity from the mother potentially complementing zygotic loss of Padi2 at early stages, which are unexplored in the manuscript.

**See above. The issue of maternal effect is addressed in the revised manuscript.**

Is Padi2 zygotic or maternal zygotic expressed gene? Is the loss of protein in zygotic mutants observable throughout development or only in later stages?

**Padi2 is expressed both maternally and zygotically. By looking at both citrullination activity and Padi2 expression by western, we observed expression at 2 hpf before MTZ and this expression persisted at 7 hpf which is within the expected time of MTZ (6-10 hpf). We believe this is an important piece of data when introducing this zebrafish as a model to study citrullination and have included this data in figure 1 I.**

3. Wound healing effects:

a. These are interesting sets of experiments, revealing wound healing roles for Padi2, but it is unclear how were the mutant larvae which were analysed identified (genotyped).

**We have now clarified this point in the revised text's materials and methods section. Thank you for raising this issue.**

b. The absence of PADI4 ortholog (btw what is the data used as evidence for lack of?) suggested by the authors begs for the question whether Padi2 of zebrafish is nuclear localised, as was seen suggested for Padi4, the only PAD in mammals with nuclear localisation signal. This needs to be verified by Western blot on nuclear extracts, but would also be helpful if the antibody was tested by immunostaining. Alternatively, binding of Padi2 to chromatin/H4 ought to be tested.

**The evidence for no PADI4 ortholog is based on data base searching and the absence of citrullination in the larval period in the *padi2* mutant. The best evidence for zebrafish Padi2 having a nuclear role is the requirement of Padi2 for histone citrullination since it is absent in the mutant by immunostaining and western blotting. We attempted the nuclear extract on whole larvae but had contamination making the data difficult to interpret. Additionally, reports of nuclear localized Padi2 have been published (Zheng, L., et al. 2019; Cherrington, B., et al. 2012). However, we have modified the text to raise the points above which support Padi2 as having nuclear activity based on its requirement for histone citrullination.**

c. In addition to nuclear localisation of the protein another key piece of evidence for direct effect by Padi2 on wound healing is to show that *padi2* mRNA is expressed in wt and/or regenerating fin fold and notochord. ZFIN expression data suggests highly specific expression of *padi2* RNA in epidermis, pectoral fin etc.

**We include data showing that the mRNA is indeed expressed in the fin based on qRT-PCR in figure S3 A. This was confirmed as well by RNA sequencing data in epithelial cells (data not shown). We did not see evidence of differential expression with wounding but rather show it is differentially activated after wounding (based on the appearance of the citrullinated histones near the blastema).**

d. The proposed effects in proliferation upon wound healing and in normal caudal fin mesenchyme are intriguing, but are not sufficiently linked to the observed effects in citrullination of histones in the notochord. The key question is whether the observed effects are due to H4 citrullination in the notochord. While this may not be possible to fully answer within the scope of the ms, it is expected that additional lines of evidence strengthen the proposed link. Among others, a possible set of experiments could include dissecting cell autonomy of Padi2 function in relation to the observed phenotype by strategies used in the zebrafish genetics field, such as blastomere transplantation to generate mosaics (see e.g. PUBMED:17597528) or mosaic expression of *padi2* mRNA in single blastomere injected at cleavage stage wt and mutant embryos to monitor cell specific/cell autonomy of defects. Such experiments could help in tracking of those cells emerging from the notochord bead, which are expected either to be directly affected by the mutation and or are interacting with fin fold mesenchyme cells, the proliferation of which was suggested to be affected. Is there evidence for citrullination of H4 in tissues where *padi2* is expressed in wt embryos and larvae?

**We agree with the reviewer. This is an important question but we also agree that it is likely beyond the scope of the current manuscript based on the new data included with this revision. We do not have the experience with the blastomere transplantation as suggested and therefore would not be able to pursue these experiments in a timely response to review.**

Minor points:

1. Manuscript organisation could be revised to help the reviewer:

- a. add page numbers and line numbers;
- b. figure panels should have their numbering on the figure;
- c. ideally the legend should also be printed on the bottom of figures, which can be reduced to 80% print size without losing important information, for ease of navigating the manuscript.

**These have been modified within the manuscript and figures.**

2. Switch order of transcripts 001 and 2 in Suppl. Fig 1a to fit with panel B. refer to transcripts by their annotated names in the ms text.

**This has been modified within the manuscript, figures, and materials and methods.**

3. The new variant described by the authors needs to be clearly indicated as new and a distinguishing transcript name used.

**This has been modified within the manuscript, figures, and materials and methods.**

4. Western blot in Fig S1 C needs caption of expected size markers (kD) to identify the bands (presumably the two strong ones) attributed to Padi2.

**Fig S1 C and its figure legend have been modified to indicate the expected Padi2 doublet.**

5. Figure legends for Fig 1E,F refers to larvae, while Methods indicate 2 days old embryos were used. Please clarify the accurate stage of embryos and larvae using ZFIN anatomy nomenclature throughout the manuscript for all experiments and indicate them in the legend of figures.

**We elected to use the nomenclature used in larval zebrafish regeneration literature.**

6. Fig. 3B needs addition of untreated controls at the relevant stages, as well as moving Fig. S3F to the main Figure.

**It is unclear what the reviewer means by untreated controls. We have done the secondary only control on 24 hpw wounded larvae (data not shown) to confirm that this H4cit3 signal is not due to nonspecific binding. We are electing to keep figure S3 G in supplement as the conclusion from the figure is that this is a wound-specific process with the non-wounded fins in supplement acting as a**

**control.**

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

The manuscript by Golenberg et al., identifies histone citrullination in a subset of cells within the regenerative notochord bead. In the Padi2 mutant where citrullination does not occur, the authors show that leucocytes dynamics is altered, proliferation is perturbed and regeneration is less efficient.

The findings reported in this manuscript proposes Padi2 (citrullination) as an intermediary between calcium and regenerative mechanisms.

In my view there are several points that need to be addressed for clarity.

Just a note, there are no pages in the manuscript and the figures are not labelled.

#1 - Wound healing/repair is different from regeneration. Wound healing/repair is in fact an early stage of the regenerative process. Wound healing is characterized by the formation of a wound epithelium and the expression of *dlx5a* for example; is then followed by the formation of a blastema characterized by the expression of genes like *msxc* and *msxe*; and from here there is an outgrowth phase.

The authors use the terms wound healing/repair and regeneration throughout the manuscript as if they were the same. The authors should revise these concepts in the entire manuscript and be more accurate with what they actually mean.

**We agree with the reviewer. For clarity and as also suggested by the editor we now refer to this stage of repair as wound healing in the revised text. Wound healing was also used when referring to mammalian healing to distinguish that these models do not fully regenerate.**

#2 - The terms full-length and short-length to name the alternative transcripts is a bit confusing because then the authors talk about a full-length exon 10. I would propose to name longer and shorter transcripts. Bioinformatically there are 4 alternative transcripts. The authors say that they identified only 2 splice variants, but they do not say how these were identified?

**We agree with the reviewer. While the predicted transcripts and nomenclature used change between Ensembl genome assemblies leading to the complexity of that figure, we believe it is best to follow already established nomenclature as much as possible. That being said, we recognize our naming of the transcripts was not clear within the text. We have expanded on this in the discussion to improve the clarity of this section.**

#3 - The authors should explain why did they target exon 7 to generate the Padi2 mutant.

**We now discuss this in the revised manuscript. We wanted to target the region before the catalytic subunit and key calcium binding regions.**

#4 - "These observations provide the first evidence that citrullination is not necessary for broadly normal development of a vertebrate." The author should re-phrase this sentence because citrullination in mammals were shown to have an impact on development. This paragraph should be re written and the precise Padi genes should be mentioned, because actually none of the papers cited in this paragraph are about Padi2.

**Changes have been made to the text to specify mammalian PAD1 and PAD6's contribution to early development. Conclusions about development have been changed to indicate zebrafish development.**

#5 - The authors refer that "The mammalian PAD2 is the predominant isozyme in skeletal muscle and nervous system" and they went on to show that in zebrafish Padi2 mutants the skeletal fibers have a normal morphology with more neuromuscular junctions. What about the number of myelinated axons, as these were shown to be less in mice mutant for Padi2?

**While we believe the observation of increased neuromuscular junctions in the *padi2* mutant larvae is interesting further investigation is outside the scope of this manuscript. With the publication of this paper, we will make this zebrafish mutant line available to the scientific community for further evaluation of neuronal and other phenotypes.**

#6 - The regenerate fin fold length in Padi2 mutants is smaller when compared with control wt larvae (Fig. 2B). These measures were done at 3 days post excision. As it takes 2-4 days for complete regrowth, the authors should quantify fin fold regenerate size at later stages. Only then it will be possible to claim that regeneration is impaired and not simply delayed.

**As suggested by the editor, we refer to the repair process as wound healing and the editor indicated that we should not pursue further studies related to regeneration in this brief report.**

#7 - In Fig.2B, it is possible to see that fin fold in Padi2 mutants are slightly bigger when compared with control wt larvae. These measures were done at 5 days post fertilization. Are the differences in size seen also at earlier stages, namely at the stages when the measurements of the regenerate size were done (i.e. 3 dpf)? Are these size differences seen only in the fin fold or are the larvae bigger as a whole?

**These control measurements were taken at a developmental timepoint corresponding to the regenerated fin. Fins were amputated at 2 dpf and allowed**

**to regenerate to 3 days post wound (3dpw) or 2 dpf unwounded larvae were measured 3 days later at 5 dpf. We have clarified these points in the revised text.**

#8 - In Fig. 2F, the authors claim that Padi2-deficient larvae have "... impaired neutrophil resolution from the wound at 48 hpw. I would argue that Padi2 mutants have more leucocytes in the wound to start with, and so the degree of decrease is in the same order as in the wt. In addition an increase in leucocytes is not necessarily negative, as it was shown that this early inflammation is important for successful regeneration in adult zebrafish. These issues should be discussed.

**We agree that this is an essential interpretation of the results and have reworded our conclusion within the discussion. Citrullination is an excellent candidate for the study of how a shift in the balance of a necessary signal either in excess or depletion can each have unique consequences. The role of regulated citrullination levels on neutrophil responses should be examined in future publications but is beyond the depth of discussion achieved in this manuscript.**

#9 - What is the cellular nature of the subpopulation with citrullinated histones? Are these notochord cells or are these some kind of blastema cells? Do these cells share notochord or blastema markers? Are these cells leucocytes? The authors should be careful with the term pluripotency because in the fins of zebrafish regeneration is not really done using bona fide stem cells but by mechanisms a de-differentiation. And as far as I know the level of pluripotency exhibited by these de-differentiated cells is no really pluripotency, actually they have a very narrow differentiation potential.

**We agree with the reviewer that this is an interesting question. To address this question we have used a reporter of the blastema (lepb:eGFP) (Kang, J. et al. 2016), to determine if these cells correspond to our identified cell population with citrullinated histones. Indeed, our findings suggest that nuclei with citrullinated histones associate with lepb:eGFP expression. We have added this to figure S3 H and I. This improves the paper substantially so thank you for the recommendation! We did not see overlap with leukocytes (data not shown).**

Reviewer #3 (Comments to the Authors (Required)):

Golenberg et al., here characterized the effects of knockout of a Ca<sup>2+</sup> dependent histone modifier on injury-induced leukocyte recruitment and larval regeneration.

For what it tries to show, this is a well-executed/controlled zebrafish gene KO study for an interesting enzyme that could transduce some of the physiological effects of wound-induced calcium signals. Notably, the authors have confirmed their gene KO approach, with orthogonal morpholino-mediated knockdown (in the supplement). I also really like how the authors characterize padi2's enzymatic activity (or lack thereof in the mutant). All the necessary controls for the presented experiments seem to be there. I have little technical concerns with the presented data.

It would be interesting to know whether the observed (moderate?) tail fin regeneration defects in larvae translate to notable adult tail fin regeneration defects. I think such an experiment would not be too difficult to do and could consolidate the idea that padi2 is important during regeneration, which most people still define as adult regeneration. Mechanistically, there remain of course many unanswered questions, some of them pointed out by the authors in their discussion. What are these padi2 positive cells in the notochord bead, and what do they do? How does padi2 mediated histone modification influence regeneration/inflammation? Etc. Whether there remain too many open mechanistic questions for this particular journal is an editorial call.

**We thank the reviewer for their enthusiasm. As discussed above, while we also believe effects on adult regeneration is interesting question, addition of this study as suggested by the editor is beyond the scope of the current manuscript.**

Regardless, the study touches on interesting biological aspects, i.e., how rapid wound signals are stored to inform long-term responses such as regeneration by activation of a less known histone modifier. So, I enjoyed reading the paper, which is also well-written. Maybe I would add to the discussion that there are many ways by which  $\text{Ca}^{2+}$  can influence wound healing/regeneration given the very many wound relevant,  $\text{Ca}^{2+}$  dependent pathways. Padi2 is one interesting possibility, but by far not the only one.

**We have modified the discussion to address this interesting question.**

January 24, 2020

RE: JCB Manuscript #201908164R

Dr. Anna Huttenlocher  
University of Wisconsin Madison  
Dept of Pediatrics & Medical Microbiol & Immunol University of Wisconsin 4225 Microbial Sciences Building  
1550 Linden Drive  
Madison, WI 53706

Dear Anna,

Thank you for submitting your revised manuscript entitled "Citrullination regulates wound responses and tissue regeneration in zebrafish". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

#### A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, <http://jcb.rupress.org/submission-guidelines#revised>. \*\*Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.\*\*

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2) Figures limits: Reports may have up to 5 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

- 5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.
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- 10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.
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- 14) A separate author contribution section following the Acknowledgments. All authors should be

mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

## B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, <http://jcb.rupress.org/fig-vid-guidelines>.

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

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

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

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

The authors have responded to all of my queries, all key issues have been resolved by addition of data and some by modification of text. It is a high-quality study with exciting novelties and the presentation has much improved. I am satisfied with most of the changes, where some minor issues remain, are listed below:

I would have liked to see that the authors give more respect the efforts of colleagues in the zebrafish user community by applying the standardised stage names. This is to the benefit of those readers also, who are not coming from the zebrafish regeneration community.

In response to my minor formatting requests, these have mostly not been implemented. It would help the reviewer if the figures are numbered in the assembled pdf. Bit of a pain of having to go through these checks myself on the prints and pdf to make sure I am looking at the right figure. Additionally, not adding legend under the figure panels, what I asked for the first-time round, was a choice by the authors, presumably to offer larger images to print. However, this is not necessary, I can always zoom in on the pdf to see details. I wish to note that adding them does help in reading the manuscript, rather than having to jump between main text, legends and figures. Please help each other in making the review process easier for all of us by offering the manuscripts edited in a user-friendly way.

Regarding the response to point 6:

I made a mistake in my review referring to Fig. 3B instead of Figure 3C (which shows the same larvae as in B and they should could be under the same panel numbering). I apologise for causing confusion. My point is that the tool used to detect wound response with the H4cit3 antibody needs controlling when claiming that the citrullination only occurs in wound healing as nicely shown by comparing Fig. S3G to Fig3C. This comparison would benefit from having them on the same figure.