nature portfolio

Peer Review File

Nerve dependent regulation of H3K27me3 during the induction of patterning competency in regenerating Axolotl limb cells.

Corresponding Author: Professor Catherine McCusker

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Raymond et al. developed an extension of the ALM model in which the authors replaced tissue transplantation with RA injection. Using this system, the authors analyzed changes in H3K27me3 and the redundant roles of nerves and FGF/BMP signaling pathways. The finding that RA injection induces Shh and leads to accessory limb is not surprising and I am not sure if it really deserves a new name (CALM) as it almost reads like suggesting a completely new methodology. However, using this system, the authors went on to describe potential chromatin changes associated with genes involved in regeneration and how they associate with FGF/BMP pathways. Epigenetic studies are increasing in the field of regeneration, so the study is timely from that perspective. This is a small and well-reported paper with sufficient information on experimental design and replication, and the conclusions are supported by the data. I have only minor comments:
1. Throughout the figures, the authors show many heat maps, but as a reader, we do not have an easy way to read the significance of these genes. I know the authors note the gene sets they used at the top of the heatmap, but I still found it very hard to look at. Could the authors at least make some key known and relevant genes bold in these figures? This should at least make them easier to understand and associate.
2. Line 40 states that axolotls regenerate complete limbs throughout their life cycle, it is not completely correct as this ability decreases after metamorphosis (PMID: 27499857). Please correct this statement.
3. Line 85 states "will only express patterning genes and cues consistent with the axial position of the wound". This may be true, but there is no comprehensive study evaluating this to conclude "only" patterning genes.
4. Line 397. The authors note that Wnt activation can induce regeneration in other tetrapods. More relevant to this work, Fgf activation has been suggested to induce regeneration (PMID: PMID: 11319858) or Bmp inhibition has been suggested to also impair regeneration competence in other tetrapods and as a downstream element of FGF (PMID: PMID: 16938438 and PMID: 34105722). Relevant work should be cited.

Reviewer #2

(Remarks to the Author)
Review of: Development of a model system to study the regulation of patterning 1 competency in regenerating axolotl limbs.

Raymond et al., use ectopic blastemas to dissect the signaling machinery of limb patterning in axolotl regeneration. The authors lay out a comparative transplantation that modifies the well-studied accessory limb model (ALM) to allow pharmacological modification of patterning signals. They named this paradigm, the CALM that is comprised by an anterior surgery, CALM-A and a posterior surgery, CALM-P. Through gene expression analysis and epigenetic modifications, they identified FGF and BMP signals as the initiators of what they name patterning competency, probably exerted through epigenetic modification as these molecules are upstream.

The work is interesting because it can help dissect the acquisition of competency to make new structures in cells that otherwise are situated in a determined position and function.

There are some points that should be addressed or clarified:

1) Some concerns arise from the format of the manuscript. The abstract portraits a methodological abstract, given less importance to the conclusions of the experiments, which is the main focus of the discussion. It would help the reader to define the focus of this manuscript. Additionally, it is not clear to me what the novelty in the methodology is since these experiments have been done before by the authors, specifically the CALM-A. It would seem that it is rather the validation of a known surgical paradigm to uncover patterning competency

2) Line 183, 184: “We observed a detectable increase in mitotic cells in the blastema mesenchyme between 5 and 6 days postsurgery (Fig 2d), indicating that the acquisition of patterning competency correlates with the activation of the cell cycle.”

This statement is vague, since there is green florescence at day 3, a correlation of competency and cell cycle should be further validated.

3) The differential response of mature tissue and blastema to retinoic acid, is a quite interesting, however, it is not further explored here. To test the direct competency of RA, manipulation of the retinoic acid pathway, such an antagonist, would provide a stronger support to the results presented here.

4) Line 219-223: “Together, the expression data from the developing blastemas (Fig 2f) and denervated CALM-P blastemas (Fig 2g) and the accessory limb data from the denervated CALM-A blastemas (Fig 2h and Supplemental Table 1.1) indicate that redifferentiating blastema cells lose patterning competency.”

This statement should be supported with maybe a staining of cells that have expression of a differentiation marker and the loss of the competency marker.
If I understand correctly, denervation close to the blastema was done to ensure that outgrowth would happen while a shift in expression of competency markers can be done short term (after 4 days). If this is the case, the authors should expand on this important distinction that makes the experiment different form other setups. And it would be important that they show when this reinnervation happens, if after 4 days, when the tissue is harvested, there is some regrowth of the nerves or not.

5) Figure 2l-m shows the opposite from what was expected, increase of a repressive mark in the limb patterning genes, although by qRTPCR, these genes were shown to increase, the argument made by the authors is that the presence of the repressive mark does not prevent the expression. The size of the animals in the ChipSeq experiment is quite big, 13-16 cm, while the animals in the qRTPCR experiment are 5-7 cm and the quantification of H3K27me3 was done in 9-12.5 cm, yet they use 4 days equivalently. This could be a reason for the discrepancy as it is well known that regeneration can differ as animal grow and age.

6) The authors mention a couple of times MEIS2 and TIG1 as an example of proximo distal marker that changes with RA even in uninjured tissue, however in contrast to MEIS2, there is no change in TIG1 in any of their data.
How is this explained? Did they not find any significant change? Please comment on this discrepancy.

7) Line 273-275: “Here, we assayed expressional changes and accessory limb formation in CALM-A (performed on A-lateral wounds without nerves) to test whether all of these factors (B2FF) are required to induce competency or whether a combination of only two factors (F2F8, B2F8) is sufficient (Fig 3a-c).”

The correlation of cell cycle and patterning competency is rather weak based on the evidence presented, given that during the blastema formation many processes are happening also including cell death, and that wouldn’t correlate cell death with pattern competency.

8) There is a mention in the text of a Supplemental figure 5, this was not found

Reviewer #3

(Remarks to the Author)
In this manuscript, Raymond et al. present an advancement on the well-established accessory limb model, which they call the competency accessory limb model (CALM), as a new system to study the molecular processes of inducing patterning competency in a systematic reductionist manner. Overall, it is an interesting model and gives some new insights into the epigenetic control of limb regeneration. It is slightly unclear how the role of H3K27me3 plays in the CALM model relates to its role in natural limb regeneration.
However, the way in which the data is currently presented makes the manuscript extremely difficult to read and extract what the main findings of the manuscript actually are.

Specific points:
1. Figure 1: all panels are too small. In all panels, the nerve needs to be bigger, and a different color is used to illustrate the nerve.
2. Where is RA injected, what dose is used.
3. Is the downregulation of shh after RA injection dose-dependent? How long does it take to see the downregulation of shh,

hours, days?
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5. Fig2D, you should not use red and green; color blind people cannot distinguish them,. Please change the colors.
6. Figure2F legends too small, what do the dark versus light bars represent?
7. Figure 2F meis is upregulated, is Tig1 also upregulated in your models?
8. Figure 2: do deinnervated CALM-A or CALM-P blastema cells simply stop dividing in addition to losing patterning competency?
9. ChiP data should be moved to a separate figure.
10. Some of the data showing that there are changes in H3K27me3 levels in the induced blastema should be included in the manuscript, as this is an important premise that future experiments rely upon. Do these changes occur only in cells in the blastema or do they also occur in mature tissue adjacent to the injury site? If the enzymatic activity of EzH2 is inhibited does this inhibit patterning of the limb in the CALM models and in normal regeneration.
11. Figure 4 A, illustration too small, the circles are. So small the colors cannot be seen, hence the data is very difficult to interpret.
12. Figure 4 : need to remove “lorem Ipsum” panel A, bottom section below WNT3A
13. The Tanka lab has a manuscript on Bioarchivs, Otsuki et al., which describes the molecular basis for positional memory and shows that posterior cells in the mature limb express Hand2, maintaining them in a competent state to respond to injury by turning on shh. Hand2 cells are stabilized in their posterior identity and protected from being reprogrammed via a Hand2-shh feedback mechanism. Whereas anterior Alx4 expressing cells can more easily be reprogrammed. This work and how it is related to the findings in this. manuscript should be discussed in the Discussion section of this manuscript.
14. a model figure at the end clearly illustrating the new insights this body of work brings to the field of limb regeneration would be really useful.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I appreciate the responses of the authors and the amount of work to take in consideration the previous comments.

Reviewer #3

(Remarks to the Author)
The authors have done a lot of work to overall improve the manuscript. however there are still two points I think need to be adressed:

1. Two reviewers have asked about Tig1 in this model, the authors need to include qRT-PCR data for Tig1 and Meis in their model in the manuscript, this could for example be included Figure 2. Tig1 and Meis are two genes that are well-charatcterized in limb regeneration to be RA responsive genes, so it is essential to characterize them in this model.

2. The authors have now included a model figure to summarize their data, however the illustration really does not summarize the work of this paper and should be revised to reflect the model used and the findings in that model or removed.

Responses to Reviewers' concerns:

Summary: We first would like to thank the reviewers for their careful review of our manuscript and thoughtful comments. While the reviews were largely enthusiastic and supportive of the publication, it was clear from the comments that a considerable amount of rewriting and figure modification was needed for us to convey a clearer story. Because of this, the revised manuscript was almost completely rewritten from the original form. We reframed the paper's focus from developing and validating the competency assay (CALM) to investigating the biology of patterning competency. We also better-defined patterning competency, which helped us articulate the main thesis of this work. In short, we feel that the reviewer's insightful comments have led to a significantly improved manuscript, but more importantly, they have helped us further develop our own thinking about patterning competency.

Some of the reviewers also suggested additional experimentation to strengthen or flesh out the story. We made considerable effort to perform most of the requested experiments, and the significant change in the story frame made some of these experiments more crucial than others. Below, we describe in more detail how we addressed the individual concerns of each reviewer. Our responses are italicized and prefaced by "Author response".

Reviewer #1:

Raymond et al. developed an extension of the ALM model in which the authors replaced tissue transplantation with RA injection. Using this system, the authors analyzed changes in H3K27me3 and the redundant roles of nerves and FGF/BMP signaling pathways. The finding that RA injection induces Shh and leads to accessory limb is not surprising and I am not sure if it really deserves a new name (CALM) as it almost reads like suggesting a completely new methodology. However, using this system, the authors went on to describe potential chromatin changes associated with genes involved in regeneration and how they associate with FGF/BMP pathways. Epigenetic studies are increasing in the field of regeneration, so the study is timely from that perspective. This is a small and well-reported paper with sufficient information on experimental design and replication, and the conclusions are supported by the data. I have only minor comments:

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Author response: We have reduced the number of heatmaps in the main paper to focus on the ones that addressed the key questions that we were asking about patterning competency. The other heatmaps have largely been moved to supplemental materials for readers who are interested in this data. As Reviewer 1 suggested, we highlighted the

most significant genes in each map. We have now also performed Panther analyses on a number of the heat maps to identify biological processes that were overrepresented in gene clusters. This helped us further digest these heatmaps and present them in a way that is easier for and more meaningful to readers.

2. Line 40 states that axolotls regenerate complete limbs throughout their life cycle, it is not completely correct as this ability decreases after metamorphosis (PMID: 27499857). Please correct this statement.

Author response: This sentence in question was removed during the rewriting of the introduction.

3. Line 85 states "will only express patterning genes and cues consistent with the axial position of the wound". This may be true, but there is no comprehensive study evaluating this to conclude "only" patterning genes.

Author response: We have rewritten this sentence to be less definitive.

4. Line 397. The authors note that Wnt activation can induce regeneration in other tetrapods. More relevant to this work, Fgf activation has been suggested to induce regeneration (PMID: PMID: 11319858) or Bmp inhibition has been suggested to also impair regeneration competence in other tetrapods and as a downstream element of FGF (PMID: PMID: 16938438 and PMID: 34105722). Relevant work should be cited.

Author response: The discussion point in question was removed during the rewriting of the manuscript.

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Raymond et al., use ectopic blastemas to dissect the signaling machinery of limb patterning in axolotl regeneration. The authors lay out a comparative transplantation that modifies the well-studied accessory limb model (ALM) to allow pharmacological modification of patterning signals. They named this paradigm, the CALM that is comprised by an anterior surgery, CALM-A and a posterior surgery, CALM-P. Through gene expression analysis and epigenetic modifications, they identified FGF and BMP signals as the initiators of what they name patterning competency, probably exerted through epigenetic modification as these molecules are upstream.

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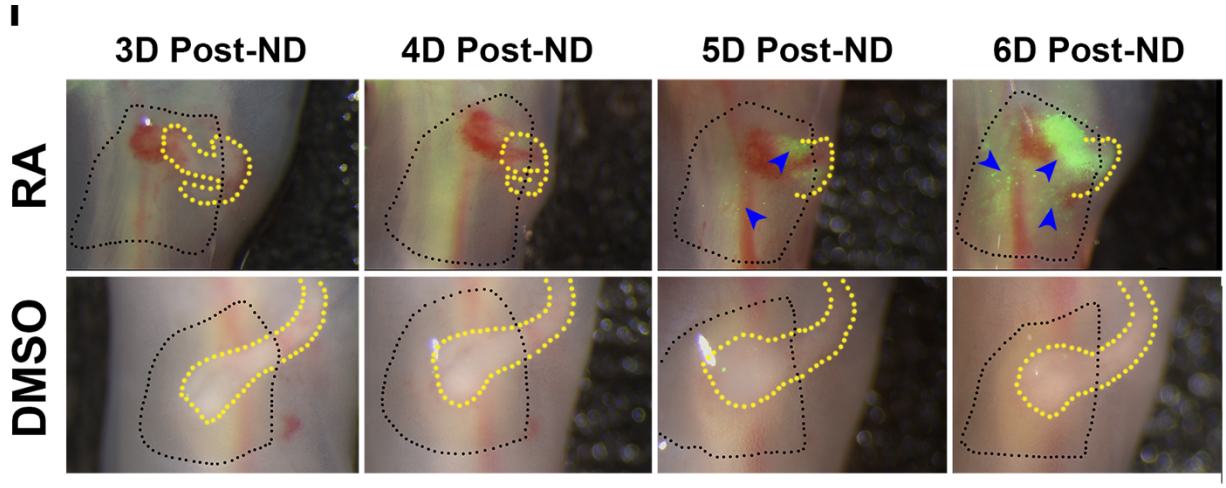
Author response: The biggest change to the manuscript was to distil the main storyline of the manuscript better. We have reframed the manuscript such that the main focus is on our experimental findings from the CALM assay and other in vivo manipulations. These studies have unveiled the specific timing of patterning competency induction (which takes a surprisingly long time!), the requirements to maintain this property in the blastema, identified the molecular signals required and sufficient to induce patterning competency, and epigenetic changes that are specific to these signals (from all the other ones downstream of the nerve) in patterning competent cells. We have also characterized pathway-specific modifications on limb patterning genes and identified the ErBB pathway as the most significant target of the epigenetic changes downstream of the pattern-competency-inducing signals. Together, these findings provide foundational insights into the property of patterning competency as a springboard for future investigations.

Last, while the CALM assay is largely based on a known surgical paradigm, this previous paradigm can be used in many different ways to answer different questions about regeneration. The validation experiments we performed in the current manuscript not only show that this system can be employed to specifically assay patterning competency but also establish clear parameters for implementing this assay in future experiments. This constitutes the first regenerative assay to test for competency in specific regenerative signals. We have explained these points in more detail in the text.

2) Line 183, 184: “We observed a detectable increase in mitotic cells in the blastema mesenchyme between 5 and 6 days postsurgery (Fig 2d), indicating that the acquisition of patterning competency correlates with the activation of the cell cycle.” This statement is vague, since there is green florescence at day 3, a correlation of competency and cell cycle should be further validated.

Author response: The fluorescence at day 3 is located at the end of the severed nerve and not within the wound/blastema cells, although further validation is required to strengthen this correlation. Because of this, we have removed this data from the figure and replaced it with new time course data from RARE—GFP transgenic axolotl (Figure panel below). We think that the new data, which shows the timing of when the transgene is activated by RA treatment in ectopic blastemas, is more meaningful in terms of characterizing the onset of competency.



3) The differential response of mature tissue and blastema to retinoic acid, is a quite interesting, however, it is not further explored here. To test the direct competency of RA, manipulation of the retinoic acid pathway, such an antagonist, would provide a stronger support to the results presented here.

Author response: We agree that the response of mature tissue to RA is very interesting, especially given that it is known that mature limb tissue is resistant to the pattern-reprogramming effects of exogenous RA. Notably, the intensity of fluorescent transgenic signal activated in RARE-GFP (new image data added in Figure 2i) is much brighter in the blastema compared to the mature tissue following RA treatment -future highlighting this differential response

The current study uses exogenous RA as a tool to determine when the wounded limb cells broadly become responsive to patterning signals (not just RA). It is likely that the phenotypes that we have documented here encompassed both direct and indirect effects of these treatments. For example, the inhibition of Shh expression in the CALM-P occurs within hours of RA (new data in Figure 2e), while it takes almost a week to detect a significant increase in the expression of Shh in the CALM-A. These differences in response time could reflect how "far" downstream of ectopic RA signaling these responses are, although more experiments would be required to know for sure.

While endogenous RA is essential for blastema induction, whether it plays a direct role in regenerative patterning has not been definitively established. One problem identified by the Monaghan lab at Northeastern University is that RA signaling antagonists often have many of the same effects on gene expression as exogenous RA itself (Nguyen et al., 2017). While it is not completely understood why this occurs, the current hypothesis is that the RA receptors largely act as transcriptional repressors (when not bound to RA). Thus, the antagonists are likely inhibiting this inhibitory activity.

However, since the current study is focused on understanding when and how broad patterning competency is achieved, we feel that exploring the effect of exogenous RA

on mature tissue, which is not patterning competent, is beyond the scope of the current study. We have added further explanations in the text to highlight some of these points. We hope that the data presented here will help inform future studies from those interested in dissecting the role of endogenous RA on gene regulation in limb cells in different competency states.

4) Line 219-223: “Together, the expression data from the developing blastemas (Fig 2f) and denervated CALM-P blastemas (Fig 2g) and the accessory limb data from the denervated CALM-A blastemas (Fig 2h and Supplemental Table 1.1) indicate that redifferentiating blastema cells lose patterning competency.”

This statement should be supported with maybe a staining of cells that have expression of a differentiation marker and the loss of the competency marker.
If I understand correctly, denervation close to the blastema was done to ensure that outgrowth would happen while a shift in expression of competency markers can be done short term (after 4 days). If this is the case, the authors should expand on this important distinction that makes the experiment different form other setups. And it would be important that they show when this reinnervation happens if after 4 days, when the tissue is harvested, there is some regrowth of the nerves or not.

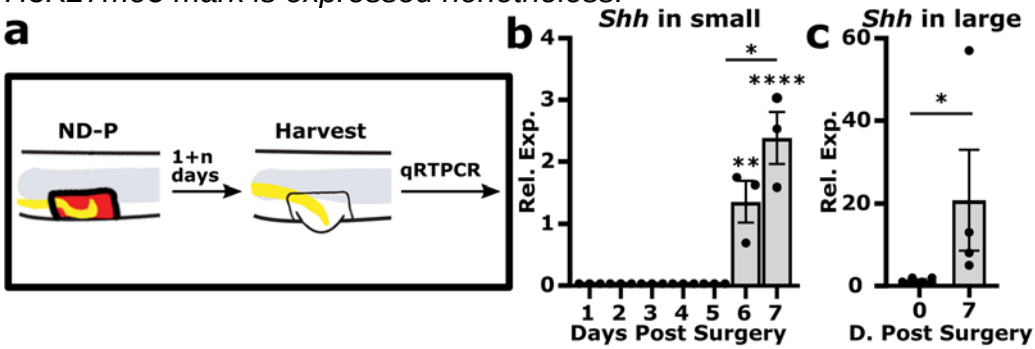
Author response: Unfortunately, a bona fide marker for patterning competency has not yet been established. While markers for redifferentiating or differentiated limb cells do exist, to our knowledge, all of these markers take a relatively long time to be expressed in blastema cells -and this experiment requires a marker that would be detectable within 4 days. While the role of nerves in the induction and maintenance of dedifferentiation in limb cells is well established, to our knowledge, we do not know of any studies that have definitively shown that denervated blastemas have increased differentiation at this time point. However, our past research has shown that denervated blastemas do prematurely specify their patterning information (McCusker et al., 2013) -which indicates that the cells are taking a step toward redifferentiating. Therefore, we have rewritten the text that refers to this experiment to more thoroughly and accurately represent these uncertainties.

As requested, we have improved our distinction between the near-blastema denervation and the other (more proximal denervations) in two ways. First, we have expanded the description of our reasoning behind this difference in the text. We have also made changes in the figures to make the distinction more prominent and avoid confusion.

5) Figure 2l-m shows the opposite from what was expected, increase of a repressive mark in the limb patterning genes, although by qRTPCR, these genes were shown to increase, the argument made by the authors is that the presence of the repressive mark does not prevent the expression. The size of the animals in the ChipSeq experiment is quite big, 13-16 cm, while the animals in the qRTPCR experiment are 5-7 cm and the quantification of H3K27me3 was done in 9-12.5 cm, yet they use 4 days equivalently. This could be a reason for the discrepancy as it is well known that regeneration can

differ as animal grow and age.

Author response: This is a good point. Our anecdotal observations are that blastema development occurs at relatively the same rate in these different-sized animals, however, we have not performed a thorough comparison of gene expression. We have included new data in Supplemental Figure 1c that shows Shh expression significantly increased 7-days after ND-surgery in the large animal range (14-20cm) at the same time point that the samples were collected for both sequencing analyses. This data shows that at least one of the genes with a significant increase in the repressive H3K27me3 mark is expressed nonetheless.



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How is this explained? Did they not find any significant change? Please comment on this discrepancy.

Author response: We apologize for the confusion. We did not perform expressional analyses for Tig1 in this study. The Tig1 data that we referred to in the text was published by the Yun lab (Oliveira et al., 2022). We have modified the text, only discussing Tig1 in the discussion section, to improve the clarity on this point.

7) Line 273-275: “Here, we assayed expressional changes and accessory limb formation in CALM-A (performed on A-lateral wounds without nerves) to test whether all of these factors (B2FF) are required to induce competency or whether a combination of only two factors (F2F8, B2F8) is sufficient (Fig 3a-c).”

The correlation of cell cycle and patterning competency is rather weak based on the evidence presented, given that during the blastema formation many processes are happening also including cell death, and that wouldn’t correlate cell death with pattern competency.

Author response: We agree that many other processes could also correlate with the induction of patterning competency. We have removed this point from the text -and have also removed the fluorescent FUCCI transgenic time course data from Figure 2.

8) There is a mention in the text of a Supplemental figure 5, this was not found

Author response: Thank you for identifying this error. In the current manuscript, we have included the missing supplemental figure.

Reviewer #3 (Remarks to the Author):

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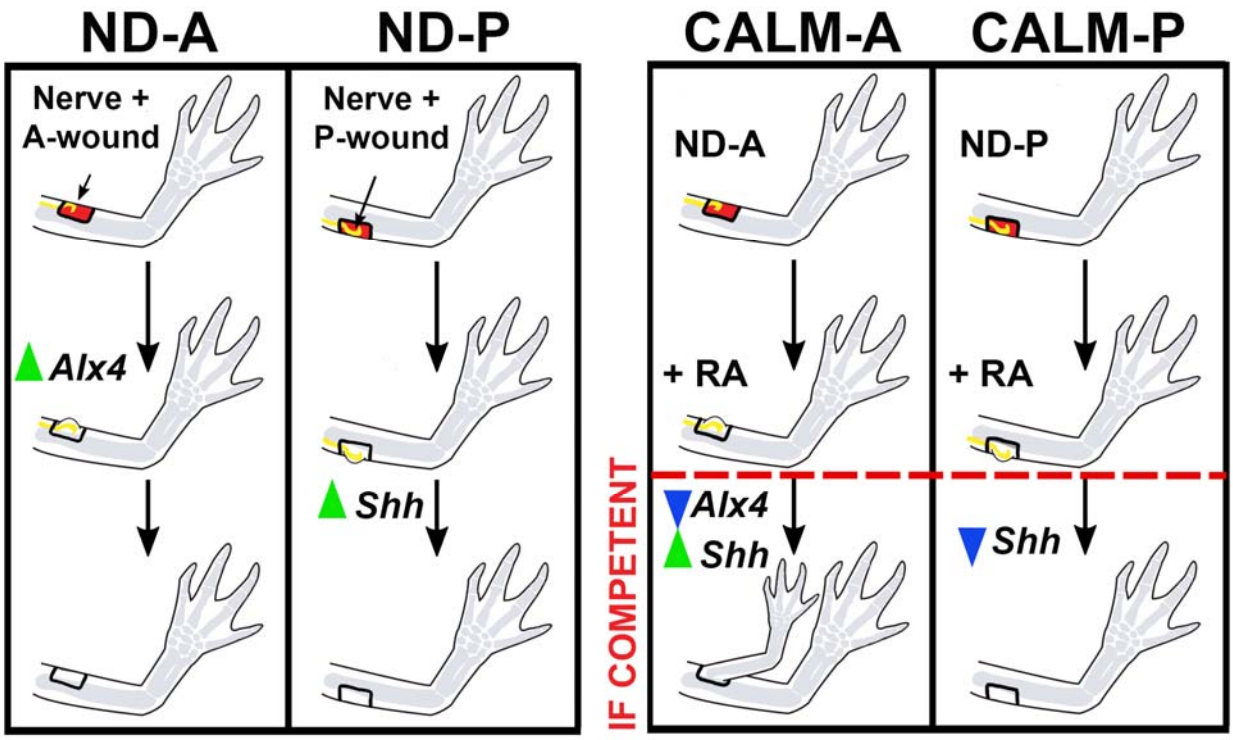
However, the way in which the data is currently presented makes the manuscript extremely difficult to read and extract what the main findings of the manuscript actually are.

Specific points:
1. Figure 1: all panels are too small. In all panels, the nerve needs to be bigger, and a different color is used to illustrate the nerve.

Author response: Thank you for letting us know. We have simplified Fig 1 and increased the size of the panels in Figs 1 and 2 and the text in the remaining figures a larger font to make them easier to see. We have also significantly modified the text and how the data is presented so that the main findings of the manuscript are more evident.



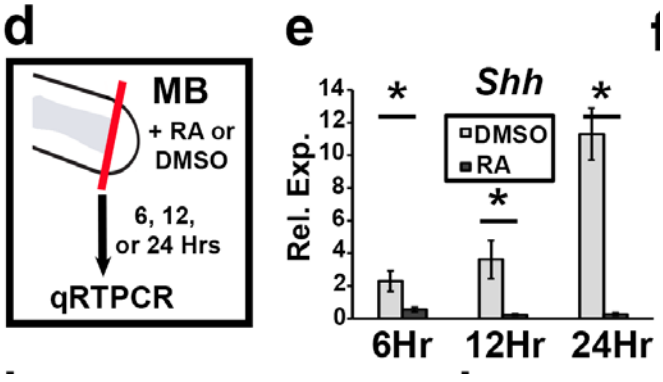
2. Where is RA injected, what dose is used.

Author response: This information is included in the materials and methods section. It was a single dose of RA (150 mg/Kg) injected into the dermal space in the trunk of the animal.

3. Is the downregulation of shh after RA injection dose-dependent? How long does it take to see the downregulation of shh, hours, days?

To address part of this, in figure 2e we now include data that shows that Shh expression downregulation occurs within 6 hours following RA treatment in midbud blastemas.

It would be interesting to determine whether this effect on Shh is dose-dependent since the proximalization phenotype of RA is well known to be dose-dependent. However, since the objective of this study was to understand patterning competency, we felt that investigating the dose dependency of RA is out of the scope.



4. Figure 2, again, all panels are too small.

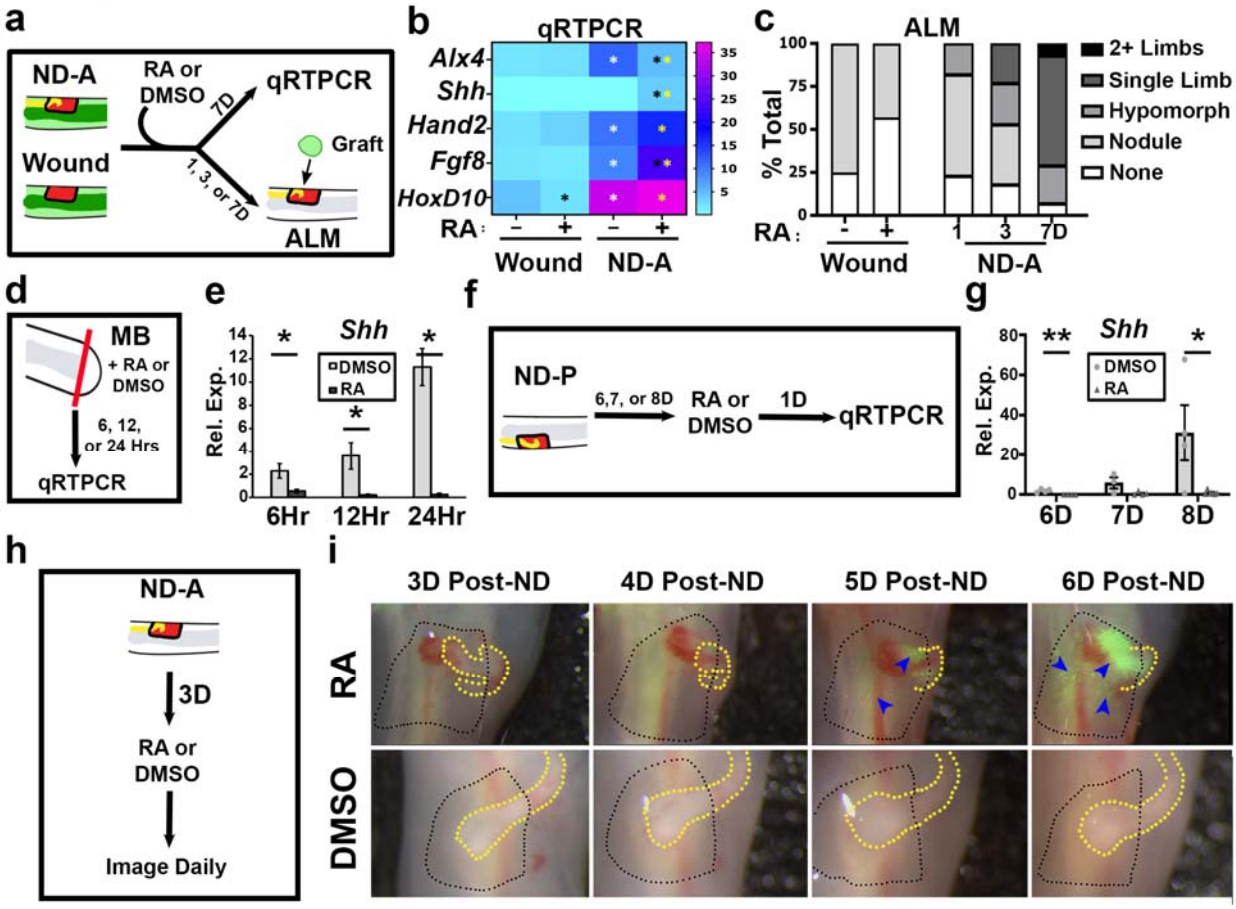
Author response: We apologize; we have made the panels and font size in Figure 2 larger. We have made two figures form the data in the original figure 2 so that it is larger and easier to visualize.

5. Fig2D, you should not use red and green; color blind people cannot distinguish them,. Please change the colors.

Author response: Due to comments from Reviewer 2, we have decided to remove this panel from the manuscript. We have additionally ensured that red and green are not present within other panels.

6. Figure2F legends too small, what do the dark versus light bars represent?

Author response: We have increased the size of the panels and the key for this panel. The dark bars refer to samples from RA treated animals, the light grey bars are from animals that received the DMSO (control) treatment.



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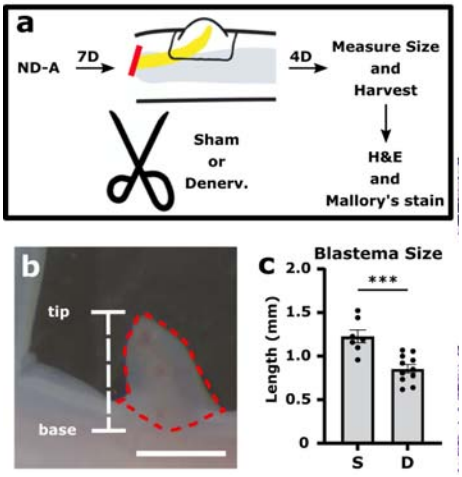
Author response: We apologize for the confusion. We did not perform expressional analyses for Tig1 in this study. The Tig1 data that we referred to in the text was published by the Yun lab (Oliveira et al., 2022). We have modified the text, only discussing Tig1 in the discussion section, to improve the clarity on this point.

8. Figure 2: do deinnervated CALM-A or CALM-P blastema cells simply stop dividing in addition to losing patterning competency?

Author response: We have included new data that indirectly addresses this question - measurements of the lengths of the ND-blastemas 4-days post-denervation compared to mock denervations. The data shows a significant reduction in the denervated blastema length and is now included in a new Supplemental Figure 2. This is likely the result of decreased proliferation and increased apoptosis, which has been previously documented to occur in denervated blastemas (Goldhammer and Tassava 1987, Lehrberg and Gardiner 2015)

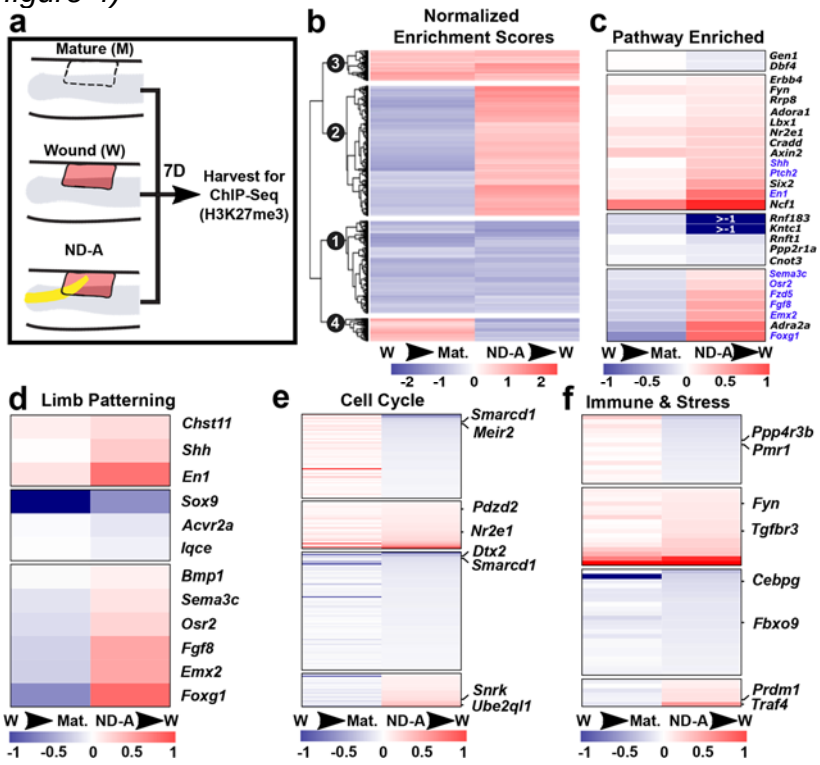
Based on the comments from Reviewer 2, we have removed our discussion about the correlation between cell cycle activation and patterning competency. Given these

changes, we felt that a deeper investigation of cell proliferation/cell cycle is outside of the scope of the current study.



9. ChiP data should be moved to a separate figure.

Author response: We have now moved the ChiP-seq data into a separate Figure (new figure 4)



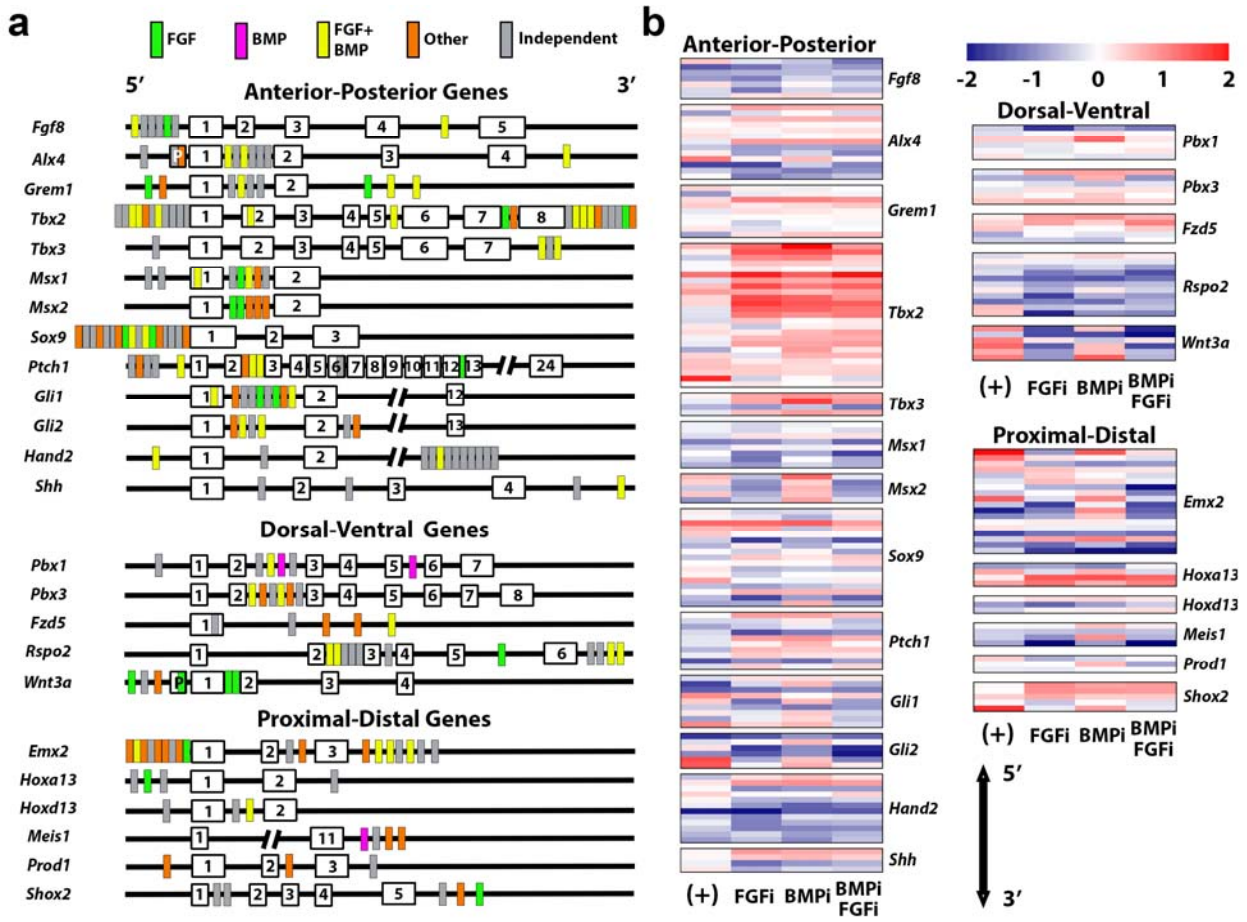
10. Some of the data showing that there are changes in H3K27me3 levels in the induced blastema should be included in the manuscript, as this is an important premise that future experiments rely upon. Do these changes occur only in cells in the blastema or do they also occur in mature tissue adjacent to the injury site? If the enzymatic activity of EzH2 is inhibited does this inhibit patterning of the limb in the CALM models and in normal regeneration.

Author Response: Mature limb tissue samples were used to compare H3K27me3 in all of the induced blastema samples. This data, which shows that there are changes in H3K27me3, is provided in figures 3, 4, and 5, and supplemental figures 2, 4, and 5. While it would be very interesting to see how far away the blastema the effect on H3K27me3 levels is seen in the mature tissue, we felt that this was outside of the scope of the current study, which focused on patterning competency.

Our data indicates that the induction of patterning competency is more complicated than the regulation of Ezh2 activity. First, ChIP-Seq and CUT&RUN analyses show that there are location-specific differences in H3K27me3 (some increased, some decreased) on the DNA of patterning competent cells. Since some gene regions show a decrease, this indicates that H3K27me3 may be actively removed, which would depend on different epigenetic regulators. Further research is needed to determine whether the suppression or activation (or both) of specific gene targets is essential for patterning competency. Second, our data shows that increased H3K27me3 on Shh does not inhibit its expression -further indicating alternate mechanisms of regulation. Therefore, while the H3K27me3 data we provide in the current manuscript identifies genomic regions that are actively regulated by patterning competency-inducing signals, these regions will need to be further tested for a role in this cellular state. Given all this, it is unclear how we would interpret the outcome of globally suppressing Ezh2 on regeneration.

11. Figure 4 A, illustration too small, the circles are. So small the colors cannot be seen, hence the data is very difficult to interpret.

Author response: We apologize for the scale issue. We have remade the illustration so that it is larger and the colors are easier to see.



12. Figure 4 : need to remove “lorem Ipsum” panel A, bottom section below WNT3A

Author response: Thank you for identifying this oversite. We have removed it from the figure.

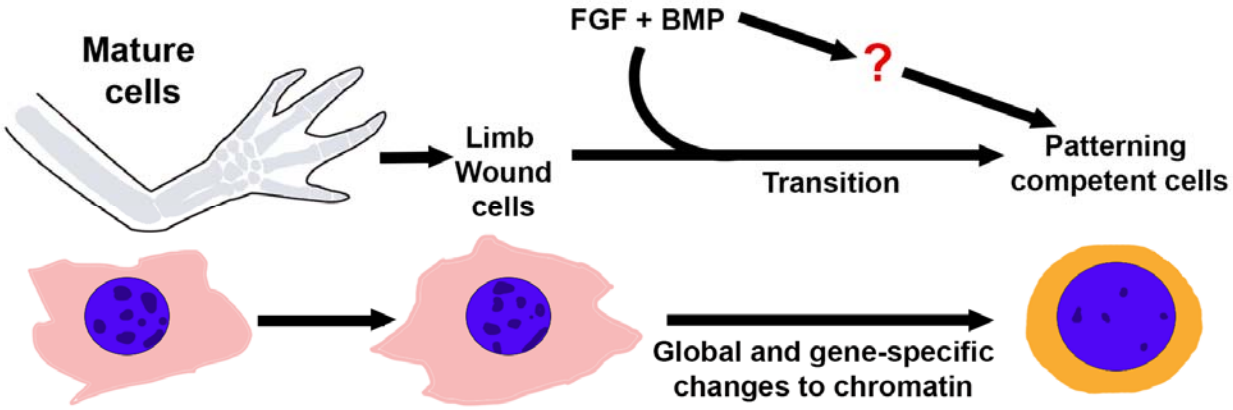
13. The Tanka lab has a manuscript on Bioarchivs, Otsuki et al., which describes the molecular basis for positional memory and shows that posterior cells in the mature limb express Hand2, maintaining them in a competent state to respond to injury by turning on shh. Hand2 cells are stabilized in their posterior identity and protected from being reprogrammed via a Hand2-shh feedback mechanism. Whereas anterior Alx4 expressing cells can more easily be reprogrammed. This work and how it is related to the findings in this. manuscript should be discussed in the Discussion section of this manuscript.

Autor response: The pre-print from Dr. Tanakas’ team provides very interesting new insights regarding cellular memory and the patterning mechanisms of the regenerating limb tissue. Since these attributes are downstream of the property that we are focusing on here (patterning competency), we have referenced the Otsuki et al. study in the introduction, where we describe the patterning signals that A and P blastema cells need

to become competent to respond to. This allowed us to stay on the theme of patterning competency and how it might be induced, in the discussion.

14. a model figure at the end clearly illustrating the new insights this body of work brings to the field of limb regeneration would be really useful.

Author response: We have now included a summary figure at the end that illustrates the new insights that this work brings to the field.



Responses to reviewer criticisms:

We appreciate the re-review of this manuscript and the favorable decision of “minor revisions” to this work. Below, we address (in italicized text) the additional concerns from Reviewer # 3.

Reviewer 3:

The authors have done a lot of work to overall improve the manuscript. however there are still two points I think need to be addressed:

1. Two reviewers have asked about Tig1 in this model, the authors need to include qRT-PCR data for Tig1 and Meis in their model in the manuscript, this could for example be included Figure 2. Tig1 and Meis are two genes that are well-charatcterized in limb regeneration to be RA responsive genes, so it is essential to characterize them in this model.

We greatly appreciate your further discussion of this concern with the editor and reconsideration of this point.

We agree that characterizing the expression of these and other Pr/Di genes known to be responsive to RA will add valuable context to the CALM model. Our main problem is that it will take us upward of 6 months to grow a sufficient number of animals at the correct size to perform this analysis. Additionally, regardless of the outcome of this analysis, the data is unlikely to change the interpretation of the current findings in the manuscript. We have added to the discussion section the need to validate these Pr/Di genes in the CALM assay in the future.

2. The authors have now included a model figure to summarize their data, however the illustration really does not summarize the work of this paper and should be revised to reflect the model used and the findings in that model or removed.

We ultimately decided to remove Figure 7 from the manuscript.