nature portfolio

Peer Review File

Retinoic acid breakdown is required for proximodistal positional identity during axolotl limb regeneration

Corresponding Author: Dr Timothy Duerr

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)
This is a very interesting paper on proximodistal patterning during limb regeneration in axolotls which establishes the mechanism by which RA signals in the limb and some genes which may be involved in proximodistal regionalization. Most importantly it establishes that it is the RA breakdown enzyme which creates different positional concentrations of RA rather than differential synthesis which is what most of us would have expected. It will have an important part to play in understanding the genetic basis of how positional identity is established and regenerated in the limb as well as in identifying the gene pathways involved.

The authors begin by examining the distribution of known patterning genes – Meis1, Meis2, Hoxa9, Hoxa11, Hoxa13 in regeneration blastemas from different level amputations and over time. HCR-FISH, PCR and single cell RNA seq data are used for the analysis which shows the nested expression of the Hox genes as seen in other developing limbs and they also analyze axolotl limb buds as well. Since RA is well known to respecify the proximodistal axis of the regenerating limb they then ask whether there are any differential distributions of the genes in the RA pathway (receptors, synthetic enzymes, breakdown enzymes) which could explain the concentration dependent action of RA. For example there could be a higher production of RA by one of the enzymes which could generate the differential proximodistal effects on blastema respecification. Very interestingly they show that it is Cyp26b1, one of the breakdown enzymes that is responsible for the differential concentration of RA, rather than its synthesis. If this enzyme is then inhibited with a compound called TAL the limb axes are then respecified in exactly the same manner as RA administration.
A RNA sequencing experiment is then performed to generate genes potentially responsible for this respecification and a relatively small number are identified (138) which fall into the appropriate category (up in distal TAL, up in proximal) including Shox and Shox2 which then become the subject of further study. It is interesting to note that 2 genes previously proposed to play a role in the PD axis, Prod1 and Tig1 are not identified here in this sequencing analysis. Any comments why?
Shox is expressed proximally both in development and regeneration and likely interacts negatively with Hoxa13 expressed distally. They then make a CRISPR mutant of Shox which develop with shorter stylopods and zeugopods, failing to undergo endochondral ossification. These mutants can regenerate their limbs and respond to the duplicating effects of TAL, but do not undergo ossification. The discussion elaborates on a model which can explain the results and proposes a combination of segments and position within segments as a way of establishing positional identity rather than just a straight forward gradient from proximal to distal limb regions. This goes some way to explaining older observations of half-segment duplications after RA treatment. I also liked the fact that gene expression of both limb development and regeneration is described which is very unusual and allows for interesting comparisons between the two processes.

I have a couple of comments on the ms which cause me some confusion.
One comment relates to expression levels of the genes that are studied which is difficult to follow in the text e.g.
1. Data on Meis 1 and Meis 2, lines 310-322, first 2 paragraphs. I find these two paragraphs difficult to follow because that data seems contradictory e.g.
Line 311 – “Meis1 and Meis2 expression decreases in progressively distal amputations”
Line 313 – “We observed Meis1 expression in mesenchymal and epithelial cells while Meis2 was undetected”
Line 316 – “We then visualized Meis1 and Meis2 in DBs at 7, 10, and 14 DPA and found Meis1 was expressed only in the epithelium while Meis2 was nonexistent”
Line 328 – “Epithelial expression of Meis1 and Meis2 did not differ between PBs and DBs at any time point or amputation

location (Fig. S2D)"
There are 2 contradictions here – is Meis 2 present distally or not (Fig. 1C says yes) and is Meis 1 present in both epithelium and mesenchyme or just epithelium?
2. Hox expression: Line 340 – "Additionally, we found that Hoxa9, Hoxa11, and Hoxa13 were predominately expressed in mesenchymal cells". So why is there a relatively high level of epithelium expression in Fig. S2J?
3. Raldh expression: Line 365 – "Raldh1 and Raldh3 were primarily expressed in the epithelium..". But Fig S6E shows nice mesenchymal expression of Raldh3 as so does the in situ in S6G.
4. Line 509 "This indicates that high levels of epithelial expression in blastemas obscured detection by RNA-seq. High epithelial expression also prevented Cyp26b1 detection by RNA-seq (Fig. 2C-D)". There are also differences in levels between the HCR-FISH and qRT-PCR results. This all adds to my confusion. Is it well established that high levels of expression in one tissue can obscure RNA-seq results? I would think not otherwise single cell RNA-seq would not be possible.
I think the figures using HCR-FISH PD intensity plots (Fig. 1F, 1L, 2E) provide the best visualization of gene expression levels that the text struggles to make clear (worth a thousand words).

The second comment refers to the interpretation of the Shox CRISPR mutants.
Since the CRISPR mutants do have proximal elements and can regenerate them I think it is a bit of a leap to say that Shox acts downstream of Meis1 to establish proximal limb positional identity (line 618). There are several other places where you say 'Shox has a role in establishing stylopod and zeugopod, not autopod, positional identity during both limb development and regeneration'. I think most people would interpret this to mean that in the absence of Shox, the mutants should have missing proximal elements which they clearly do not. Are you therefore saying that Meis1 establishes positional identity and Shox is responsible for endochondral ossificiation at that positional identity determined by Meis1? If so this should be made clearer in the text.

Reviewer #2

(Remarks to the Author)
Retinoic acid has long known to be major upstream factor instructing the establishment of proximal-distal patterning during salamander limb regeneration, but the underlying mechanisms are not yet fully elucidated. This paper provides new insights into how retinoic acid levels are regulated in the blastema and along the PD axis, through a string of experiments involving inhibition of the distal factor CYP26B1, which recapitulates classical RA-treatment induced phenotypes and reprograms cells towards a proximal-like molecular profile. It further uncovers the role of the RA-downstream factor SHOX in promoting endochondral ossification in a PD position-dependent manner, through the generation of SHOX knockout crispants.

Notably, along the paper, both some of the proximal 'classical' factors (such as Meis1, Meis2, 5'HoxA genes) and the molecules in focus (Cyp261b and Cyp261a, Shox and Shox2), are spatially characterized by HCR-FISH and quantified at key stages of limb regeneration. This quantification along the distal blastema P-D axis is in itself new and allows detailed visualisation of spatial distributions that hint at potential causal relationships between factors that were previously not known. Temporal and spatial dynamics are taken into account to provide a model for how retinoic acid impacts on PD events during limb regeneration, i.e. through the activation of SHOX which results in a position-dependent endochondral ossification.

The work is insightful and the experiments overall support the conclusions. Nevertheless, a few points can be further clarified, including data used for scRNAseq analysis, the link between Meis1 activation and Shox expression, and the experiments on Shox F0 crispants. Further, we provide a few suggestions-corrections for data presentation that we feel would help the readers.

Specific comments:

• The paper presents an important work of spatial characterization of MEIS 1 and 2, Cyp26b1, SHOXs and the 5' Hox genes expression, that would strongly benefit from displaying the individual channels along the merged images on Fig.1D,J, Fig.2C, Fig.3E, Fig.4F, Fig.5A, F and I, Fig.7B and D, perhaps adding as supplementary figures in some cases. In general, the size of the insets should also be slightly larger for better visualisation.

• Line 312 - The dataset from Li et al 2021 was reanalyzed and used throughout the work, where it is claimed to be a distal blastema (DB) dataset. However, in the original article it is not clear to me whether this dataset is DB or PB (proximal blastema) – the schematic figure seems to indicate it is a distal blastema, but elsewhere (legends, main text and supplementary file) it is mentioned that an 'upper forearm amputation' was performed, leading to the idea that the amputation was done at the stylopod level. Could you please clarify why you assume it is a DB dataset?

• Regarding the same dataset, it is stated in the supplementary file of Li et al 2021 that the 22d timepoint corresponds to the palette stage. Since there is no corresponding stage in the current work and that the genes studied have a time-sensitive expression pattern, could you comment on the decision to include the 22d datapoint for the RNA-seq data analysis?

• Line 317 – Please mention Supplementary figures 3 and 4 in the text, when the respective data is first mentioned.

• Still regarding the HCR-FISH analysis and expression intensity charts, is often not easy to judge how significant the differences between gene expression values are on the intensity plots like e.g. Fig.1F and L, where the lines represent mean expression values. Would it be possible to represent the standard deviation as e.g. a shadowed area accompanying the lines of (all) these charts?

• Line 384 – Please correct the legend title of Figure 2 for better accuracy; Also, please consider adjusting the claims that 'Cyp26b1 expression at 7, 10 and 14 dpa closely mirrors (…)' for clarity and precision.

• Line 378 – Could you please rephrase 'both assays indicate that Cyp26a1 is 'lowly expressed'? In Fig2A the average relative expression values for Cyp26a1for the stylopod and autopod blastema at 10dpa are respectively close to 6.5-3 and to 1, which is in general similar or higher than that of the Cyp26b1 in the autopod blastema, so not relatively 'lowly expressed' according to the RT-PCR.

• Line 366 – Could you please provide further clarification and comments on why you excluded Raldh3 as a modulator of RA signaling? The RT-PCR data does support a differential expression along blastemas originated from different levels of the P-D axis, in a manner consistent with RA decreasing concentration. Further, it is shown in the next section that RA synthesis is necessary for limb duplications to occur, so the Raldh3 data could be relevant here.

• Line 387 – Please consider substituting 'associated' with e.g. 'correlated'

• Line 449 – What kind and what is the extent of 'skeletal abnormalities' are observable upon DIS or RAA-only treatments? Which segments does it affect?

• Line 452 – Visualization of results in Figure S9 could be improved by adding arrows pointing to the morphological alterations observed. Please add scalebar as well.

• Line 507 – Could you please also provide the HCR-FISH for the DMSO 14DPA distal blastema in Fig.4F?

• Line 508 – Would be desirable to provide the RT-PCR validation of the RNA-seq for key genes involved in patterning, including Meis1, Meis2 and Cyp26b1 and others, and perhaps for genes that could be interesting to substantiate the hypothesis present in the discussion regarding Cyp26b1 expression, namely Fgf8 or Shh.

• Line 509 - Please rephrase this and following sentences for a more accurate versions, mainly by confining to conclusions that directly follow from the respective experiments and bearing in mind the fact that TAL treatment only partially shifts cells to a more proximal-like profile and, as acknowledged, also impacts in gene expression beyond DB vs PB paradigm.

• Line 542 – Have you verified whether SHOX also increases in PBs upon TAL-treatment?

• Line 576 - While the F0 Shox knockout crispants showed a consistent phenotype coupled with a generally high mutation rate, it would be great if this phenotype 'smaller limbs than controls, with significantly smaller stylopods and zeugopods' could also be verified in F1s, if available. If not available, then it would be important to provide information on the correlation between genotype (as percentage of mutated reads leading to loss of function) and the observed phenotypes.

• Supplementary figure 12 - Among the genotypes, please identify which ones are predicted to lead to e.g. truncations, vs. to silent mutations? This is important to determine whether mosaic mosaicisms affects the observed phenotypes. A similar concern applies for the limb regeneration experiment.

• Line 609 - It is also not clear from the text whether the shorter limbs obtained after full regeneration are due to an overall size reduction or affect specifically the stylopod and zeugopod. A quantification of limbs segments, similar to what was done in Fig.1C would allow to appreciate this aspect.

• Due to the above-mentioned concerns, the limitations associated to the use of F0s should be acknowledged somewhere in the text.

• Line 565 - Would it be possible to simultaneously visualize and quantify Meis1 and Shox by HCR-FISH in 14DPA proximal blastemas to determine the extent of overlay? In addition, an analysis of the potential Meis1 binding sites in the SHOX promoter could strengthen the hypothesis.

• Line 615 – Could you provide quantification analysis of the HoxA13 expression on 10DPA SHOX crispants vs. non transgenic animals? Perhaps it's because of the individual channels are missing in Fig.1J, but the Hoxa13 expression domain is not clearly distinguished in non-transgenic PBs at 10dpa, while it is clear in the Shox cripants in Fig.7D.

• Line 617 – This sentence might be overstating the point, and will benefit from a more balanced phrasing.

• Line 692 – TIG1 is RA-responsive in stylopod-derived blastemas and has a distinct temporal expression profile than that of Shox – expression peaks at 3-7 dpa and quickly decreasing in zeugopod blastemas from 10dpa onwards (Oliveira et al 2022). It is likely that TIG1 was not found in the RNAseq dataset due to different expression timing and either acts upstream

or in parallel during the establishment of limb P-D pattern.

• A label of ‘P’ ‘D’ could be considered to be added either on the charts or legend of Fig.1 F,L, Fig.2E, Fig.5H, for prompter interpretation.

• Please increase the size of numerical labeling of charts like the ones of Fig.1B,C,G,H, I throughout the manuscript, for better readability.

• Amputation plane and inset delineation color of the HCR-FISH images is often not clear when overlaid onto such colorful images – could it be changed to a white/grey line?

• Scalebar is missing in several panels, including Fig.3B, Fig.5I, Fig.6D, Fig.7A, B and E.

Dr. Max H Yun & Dr. Catarina Oliveira (co-reviewed)

Reviewer #3

(Remarks to the Author)
Duerr and colleagues have investigated how proximodistal positional identity is established during axolotl limb regeneration. Specifically, studies from the 1980s identified that retinoic acid specifies proximal limb identity but how retinoic acid signaling levels are established remained unknown. The authors describe two significant observations; (a) CYP26B1, an enzyme responsible for retinoic acid breakdown, is responsible for regulating retinoic acid levels in the blastema; and (b) Shox, a retinoic acid responsive gene, regulates proximal distal skeletal element formation during limb regeneration. Both of these findings would be of broad interest to developmental biologists and the regenerative medicine communities. The first observation builds upon existing work that retinoic acid signaling exists along the urodele proximal distal axis and that its disruption results in abnormal skeletal morphologies (Maden, 1998, Nguyen et al., 2017, Scadding and Maden, 1986). The findings in this study demonstrate that it’s actually the breakdown of retinoic acid that patterns the blastema. The second, begins to delve into the downstream mechanisms behind retinoic acid positional identity and their functional significance. This is a field of research that has long been idle and only recently started to gain traction. This study, is very timely, and offers fresh new insights. Overall, I am very supportive of the data and concepts presented in this manuscript and with some minor clarifications (as detailed below), would recommend it for publication.

1. The authors analyse the expression of a variety of genes using HCR-FISH. In general, most of these images are at low magnification to demonstrate the expression pattern throughout the limb. It would be helpful to show high magnification images in addition so that the individual ‘dots’ can be visualised. For example, Figure 1D, expression for Meis2 cannot be seen in any of the images despite relative counts in the associated graph. In addition the highlighted box for distal blastema at 10DPA seems to be more proximal than expected (compared to the proximal image).

2. Since Meis1 was only detected at very low levels using scRNA-seq however appears to have increased expression using HCR-FISH, can the authors also show the scrambled probe control for this tissue to ensure the signal is specific? Epithelium is generally very ‘sticky’ and can give false results.

3. Why does 5uM TAL inhibit limb regeneration in its entirety? Does this have off target effects and cause cell death? Is this compound cytotoxic to cells?

4. Images corresponding to Supplementary Table 5 in certain cases would be appreciated (i.e. showing the defects).

5. In line 508, the authors state that ‘high levels of epithelial expression in blastemas obscured detection by RNA-seq’. Epithelial cells don’t contribute to the blastema and therefore must come from the overlying tissue (e.g. AEC) obscuring the results. Can this sentence be amended to reflect this distinction.

6. Since Shox crispants successfully regenerated limbs and progressed through typical stages of regeneration, it would be interested to determine if there was any compensation by Shox2 during this process to ensure proximal distal identity remained intact.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The paper has been considerably clarified by attention to the previous review comments and now represents a very significant contribution to the understanding of limb pattern formation in the proximodistal axis during regeneration. The results are also related to limb development in axolotls, chick and mice and therefore has an evolutionary aspect as well. I recommend acceptance.

Reviewer #2

(Remarks to the Author)
The authors have addressed all concerns raised from our side, and we consider the manuscript ready for publication. We congratulate Dr Duerr and all for this interesting and significant study.

Dr Max H Yun & Dr Catarina Oliveira

Reviewer #3

(Remarks to the Author)
I appreciate the efforts the authors have made in providing additional images, additional information or explanation to satisfy all of my concerns. I am very happy with all of their corrections and believe the manuscript clearly represents an advancement to our current understanding of how positional identity is established during proximal-distal patterning during axolotl limb regeneration. I am wholly supportive of its publication in Nature Communications.

Reviewer #4

(Remarks to the Author)
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REVIEWER COMMENTS

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A RNA sequencing experiment is then performed to generate genes potentially responsible for this respecification and a relatively small number are identified (138) which fall into the appropriate category (up in distal TAL, up in proximal) including Shox and Shox2 which then become the subject of further study. It is interesting to note that 2 genes previously proposed to play a role in the PD axis, Prod1 and Tig1 are not identified here in this sequencing analysis. Any comments why?
Shox is expressed proximally both in development and regeneration and likely interacts negatively with Hoxa13 expressed distally. They then make a CRISPR mutant of Shox which develop with shorter stylopods and zeugopods, failing to undergo endochondral ossification. These mutants can regenerate their limbs and respond to the duplicating effects of TAL, but do not undergo ossification. The discussion elaborates on a model which can explain the results and proposes a combination of segments and position within segments as a way of establishing positional identity rather than just a straight forward gradient from proximal to distal limb regions. This goes some way to explaining older observations of half-segment duplications after RA treatment. I also liked the fact that gene expression of both limb development and regeneration is described which is very unusual and allows for interesting comparisons between the two processes.

I have a couple of comments on the ms which cause me some confusion.

One comment relates to expression levels of the genes that are studied which is difficult to follow in the text e.g.

1. Data on Meis 1 and Meis 2, lines 310-322, first 2 paragraphs. I find these two paragraphs difficult to follow because that data seems contradictory e.g.
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Line 328 – "Epithelial expression of Meis1 and Meis2 did not differ between PBs and DBs at any time point or amputation location (Fig. S2D)"
There are 2 contradictions here – is Meis 2 present distally or not (Fig. 1C says yes) and is Meis 1 present in both epithelium and mesenchyme or just epithelium?

Our HCR-FISH results detected very little *Meis2* expression in PBs and DBs. Further, we observed significantly higher *Meis1* expression in the mesenchyme of PBs compared to DBs. However, *Meis1* was not differentially expressed in the epithelium of PBs and DBs. Considering mesenchymal cells are the cell type that convey positional identity, our findings would suggest that *Meis1* expression in the mesenchyme is what drives proximal positional identity in PBs. We have rewritten portions of these two paragraphs to more clearly convey low *Meis2* expression in DBs and both epithelial and mesenchymal *Meis1* expression. The paragraphs can now be found on lines 114-148, and read "To explore how patterning genes regulate RA signaling in PBs and DBs, we examined the expression of known RA-responsive homeobox genes, including Meis1 and Meis2 15, 31. We assessed the expression of these genes in limbs amputated at the upper stylopod (US), lower stylopod (LS), upper zeugopod (UZ), and autopod levels at 10 DPA using qRT-PCR and found that Meis1 and Meis2 expression decreases in progressively distal amputations (Fig. 1A-C). We next reanalyzed blastema scRNA-seq data from proximally amputated limbs (Fig. S1) to identify cell types expressing Meis1 and Meis2 32, and found Meis1 expression in mesenchymal and epithelial cells, while Meis2 was undetected (Fig. S2A-B).

We then visualized Meis1 and Meis2 in DBs at 7, 10, and 14 DPA and found Meis1 was primarily expressed in the epithelium while Meis2 was lowly expressed in the mesenchyme and epithelium (Fig. 1D-E, Fig. S2C-D, Fig. S3). Similarly, PBs at 7 DPA exhibited low mesenchymal Meis1 and Meis2 expression (Fig. S2C). At 10 DPA, Meis1 was significantly higher in the mesenchyme of PBs, and Meis2 was elevated in PBs but was generally lowly expressed (Fig. 1D-E, Fig. S4). By 14 DPA, Meis1 was significantly higher in PBs and localized to the proximal-most mesenchyme cells, creating a distal zone devoid of Meis1 expression (Fig. 1D-F, Fig. S5). A similar Meis1 expression pattern was observed during axolotl (Fig. S3E, Fig. S6A), mouse, and chick limb development 33, 34, suggesting an evolutionarily conserved role reutilized for limb regeneration. Meis2 was significantly higher in PBs at 14 DPA but was generally lower than Meis1 (Fig. 1D-E). Neither Meis1 nor Meis2 expression differed in the epithelium of

PBs and DBs at any time point (Fig. S2D), underscoring the importance of mesenchymal cells in conveying PD positional identity within the blastema."

2. Hox expression: Line 340 – "Additionally, we found that Hoxa9, Hoxa11, and Hoxa13 were predominately expressed in mesenchymal cells". So why is there a relatively high level of epithelium expression in Fig. S2J?

Indeed, these genes show some expression in the epithelium. We have updated the text to reflect this epithelial expression. The changes can be found on lines 157-158 and now reads "Additionally, we found that Hoxa9, Hoxa11, and Hoxa13 were expressed in mesenchymal and epithelial cells (Fig. S2F-H)."

3. Raldh expression: Line 365 – "Raldh1 and Raldh3 were primarily expressed in the epithelium..". But Fig S6E shows nice mesenchymal expression of Raldh3 as so does the in situ in S6G.

We have revised these lines to more accurately reflect our results. It is located on lines and reads "Raldh1 and Raldh2 expression did not differ between PBs and DBs, and Raldh3 did not exhibit a spatiotemporal expression pattern comparable to that of Meis, Hoxa11, and Hoxa13 (Fig. S10, Fig. S11)." We have also added a paragraph in the discussion on the possibility of differences in RA synthesis levels in PBs and DBs. The paragraph can be found on lines 681-705 and reads "While our model specifically focuses on RA degradation in PBs and DBs, it does not address RA synthesis along the regenerating PD axis. Our results present two possibilities: 1) heightened levels of RA synthesis at more proximal amputations and 2) identical levels of RA synthesis at any amputation level that is fine-tuned at each amputation location by RA degradation. Regarding the former, our qRT-PCR analysis revealed that Raldh3 expression was significantly higher in proximal amputations compared to distal amputations, suggesting that elevated RA levels in PBs are generated primarily through Raldh3 rather than Raldh1 or Raldh2. However, this finding was inconsistent with our FISH and RNAseq results, which showed no differences in expression of Raldh1, Raldh2, and Raldh3 between PBs and DBs. Furthermore, inhibiting RA synthesis in PBs and DBs caused skeletal abnormalities in the final regenerate at each amputation location. These results are more consistent with the possibility that RA synthesis is uniform along the PD axis. Nevertheless, our results do not exclude either scenario, and future studies are needed to ascertain RA synthesis levels along the PD axis. Regardless of either possibility, we observed expression of that RA synthesis genes in the epithelium of DBs. An outstanding question related to this is why RA would be synthesized in the regenerating limb only to be degraded depending on amputation location. While our work does not address this complexity directly, we speculate that RA has several roles during limb regeneration outside of providing proximal limb positional identity. RA is important for directing nerves to their targets, including epithelial and neuromuscular junctions 47. Furthermore, RA plays an essential role in replenishing epithelial cells during physiological growth 48, which may contribute to the scarless wound healing following injuries in salamanders 49."

4. Line 509 "This indicates that high levels of epithelial expression in blastemas obscured detection by RNA-seq. High epithelial expression also prevented Cyp26b1 detection by RNA-seq (Fig. 2C-D)". There are also differences in levels between the HCR-FISH and qRT-PCR results. This all adds to my confusion. Is it well established that high levels of expression in one tissue can obscure RNA-seq results? I would think not otherwise single cell RNA-seq would not be possible.
I think the figures using HCR-FISH PD intensity plots (Fig. 1F, 1L, 2E) provide the best visualization of gene expression levels that the text struggles to make clear (worth a thousand words).

Considering how highly expressed *Cyp26b1* and *Meis1* are in the epithelium of both PBs and DBs, we reasoned that the difference in mesenchymal expression would be too small to observe in bulk RNAseq. We have revised the phrasing of this paragraph for clarity. It can be found on line 396-424 and reads "Comparisons between DMSO treated DBs and either DMSO treated PBs or 1 μM TAL treated DBs enabled identification of RA-responsive genes associated with proximal or distal limb identity. We found that 138 DEGs (FDR <0.1) were shared between both comparisons (Fig. 4D, Table S6). Among these genes, we identified several that have known roles in patterning the developing or regenerating limb, including Pax9 40, Evx1 41, and Alx4 42 (Fig. 4E). Notably absent were Meis1, Meis2, and Cyp26b1 despite our previous results (Fig. 1-2) and their known roles as RA-responsive genes involved in PD patterning 18, 22, 39, 43. We suspect these genes were absent from our RNAseq analysis due to equally high levels in the wound epithelium of PBs and DBs (Fig. S2D, J, Fig. S12D). However, we observed significantly different expression levels of mesenchymal Cyp26b1, Meis1, and Meis2 in DBs treated with 1 μM TAL compared to DMSO treated DBs at 14 DPA (Fig. 3E-F, Fig. 4F-G, Fig. S16O), indicating that these genes are both involved in patterning and responsive to TAL treatment. Together, our results show that TAL treatment induces DBs to adopt a more proximal positional identity, likely mediated by RA-responsive patterning genes differentially expressed between PBs and DBs."

The second comment refers to the interpretation of the Shox CRISPR mutants.
Since the CRISPR mutants do have proximal elements and can regenerate them I think it is a bit of a leap to say that Shox acts downstream of Meis1 to establish proximal limb positional identity (line 618). There are several other places where you say 'Shox has a role in establishing stylopod and zeugopod, not autopod, positional identity during both limb development and regeneration'. I think most people would interpret this to mean that in the absence of Shox, the mutants should have missing proximal elements which they clearly do not. Are you therefore saying that Meis1 establishes positional identity and Shox is responsible for endochondral ossificiation at that positional identity determined by Meis1? If so this should be made clearer in the text.

We believe that *Meis1* establishes proximal positional identity and that *Shox* is a downstream target of *Meis1* responsible for endochondral ossification of proximal elements, not de-novo formation of proximal skeletal elements. We have revised our text accordingly. It can be found on lines 622-625 and now reads "The observations that

Hoxa13 and Meis1 expression are unaffected by Shox knockout (Fig. 7D-F), along with Meis1 and Shox colocalization (Fig. 5H-I), suggest that Shox acts downstream of Meis1 to regulate endochondral ossification of proximal skeletal elements."

Reviewer #2 (Remarks to the Author):

Retinoic acid has long known to be major upstream factor instructing the establishment of proximal-distal patterning during salamander limb regeneration, but the underlying mechanisms are not yet fully elucidated. This paper provides new insights into how retinoic acid levels are regulated in the blastema and along the PD axis, through a string of experiments involving inhibition of the distal factor CYP26B1, which recapitulates classical RA-treatment induced phenotypes and reprograms cells towards a proximal-like molecular profile. It further uncovers the role of the RA-downstream factor SHOX in promoting endochondral ossification in a PD position-dependent manner, through the generation of SHOX knockout crispants.

Notably, along the paper, both some of the proximal 'classical' factors (such as Meis1, Meis2, 5'HoxA genes) and the molecules in focus (Cyp261b and Cyp261a, Shox and Shox2), are spatially characterized by HCR-FISH and quantified at key stages of limb regeneration. This quantification along the distal blastema P-D axis is in itself new and allows detailed visualisation of spatial distributions that hint at potential causal relationships between factors that were previously not known. Temporal and spatial dynamics are taken into account to provide a model for how retinoic acid impacts on PD events during limb regeneration, i.e. through the activation of SHOX which results in a position-dependent endochondral ossification.

The work is insightful and the experiments overall support the conclusions. Nevertheless, a few points can be further clarified, including data used for scRNAseq analysis, the link between Meis1 activation and Shox expression, and the experiments on Shox F0 crispants. Further, we provide a few suggestions-corrections for data presentation that we feel would help the readers.

Specific comments:

• *The paper presents an important work of spatial characterization of MEIS 1 and 2, Cyp26b1, SHOXs and the 5' Hox genes expression, that would strongly benefit from displaying the individual channels along the merged images on Fig.1D,J, Fig.2C, Fig.3E, Fig.4F, Fig.5A, F and I, Fig.7B and D, perhaps adding as supplementary figures in some cases. In general, the size of the insets should also be slightly larger for better visualisation.*

We have added supplementary figures with individual channels and larger insets for each *in-situ* hybridization in the manuscript. These can be found in Fig. S3, Fig. S6, Fig. S7, Fig. S9, Fig. S11, Fig. S13, Fig. S14, Fig. S16, Fig. S17, Fig. S18, Fig. S19, Fig. S20, and Fig. S22.

• *Line 312 - The dataset from Li et al 2021 was reanalyzed and used throughout the work, where it is claimed to be a distal blastema (DB) dataset. However, in the original article it is not clear to me whether this dataset is DB or PB (proximal blastema) – the schematic figure seems to indicate it is a distal blastema, but elsewhere (legends, main text and supplementary file) it is mentioned that an 'upper forearm amputation' was performed, leading to the idea that the amputation was done at the stylopod level. Could you please clarify why you assume it is a DB dataset?*

We have reached out to the authors of the Li paper and confirmed that the blastemas used were in fact PBs. We have revised our text accordingly reflecting the proximal nature of these blastemas.

• *Regarding the same dataset, it is stated in the supplementary file of Li et al 2021 that the 22d timepoint corresponds to the palette stage. Since there is no corresponding stage in the current work and that the genes studied have a time-sensitive expression pattern, could you comment on the decision to include the 22d datapoint for the RNA-seq data analysis?*

The scRNAseq analysis was included to corroborate claims of tissue specific expression of genes visualized via FISH rather than temporal expression. We wished to include samples from every blastema stage within this dataset to maximize the number of cells within our analysis. We carefully avoid making claims on temporal expression using the scRNAseq dataset. Furthermore, removal of the 22d timepoint does not impact our interpretations.

• *Line 317 – Please mention Supplementary figures 3 and 4 in the text, when the respective data is first mentioned.*

We have now included references to Fig. S4 and Fig. S5 in lines 129 and 131, respectively.

• *Still regarding the HCR-FISH analysis and expression intensity charts, is often not easy to judge how significant the differences between gene expression values are on the intensity plots like e.g. Fig.1F and L, where the lines represent mean expression values. Would it be possible to represent the standard deviation as e.g. a shadowed area accompanying the lines of (all) these charts?*

We have reported the 99% confidence interval for each curve. This can be seen in Fig. 1, Fig. 2, Fig. 5, Fig. S5, Fig. S8, Fig. S10, and Fig. S12.

• *Line 384 – Please correct the legend title of Figure 2 for better accuracy; Also, please consider adjusting the claims that 'Cyp26b1 expression at 7, 10 and 14 dpa closely mirrors (…)' for clarity and precision.*

We have revised the text and figure caption. The revisions can be found on lines 208-210 and now reads "Finally, the spatiotemporal expression of Cyp26b1 within the

mesenchyme resembled that of Hoxa11 and Hoxa13 while appearing negatively correlated with Meis1 (Fig. 2E).” The figure caption is located on lines 1167-1168 and now reads “Figure 2: Cyp26b1 is differentially expressed in PBs and DBs and is related to Meis1, Hoxa11, and Hoxa13 expression”

• *Line 378 – Could you please rephrase ‘both assays indicate that Cyp26a1 is ‘lowly expressed’? In Fig2A the average relative expression values for Cyp26a1for the stylopod and autopod blastema at 10dpa are respectively close to 6.5-3 and to 1, which is in general similar or higher than that of the Cyp26b1 in the autopod blastema, so not relatively ‘lowly expressed’ according to the RT-PCR.*

We have revised this sentence. It can be found on lines 201-203 and now reads “This difference in Cyp26a1 detection between our HCR-FISH and qRT-PCR results is likely due to the higher sensitivity of qRT-PCR.”

• *Line 366 – Could you please provide further clarification and comments on why you excluded Raldh3 as a modulator of RA signaling? The RT-PCR data does support a differential expression along blastemas originated from different levels of the P-D axis, in a manner consistent with RA decreasing concentration. Further, it is shown in the next section that RA synthesis is necessary for limb duplications to occur, so the Raldh3 data could be relevant here.*

We excluded *Raldh3* because it does not show a similar spatiotemporal expression pattern as *Meis1*, *Hoxa11*, or *Hoxa13*. In addition, *Raldh3* was not RA responsive or differentially expressed between PBs and DBs in our RNAseq, and we observed no defects in skeletal patterning from RALDH inhibition. However, our results cannot exclude that RA synthesis differs in PBs and DBs or if RA synthesis is consistent along the PD axis. We have added a paragraph in the discussion on lines 681-705 clarifying this point and have rephased our language to provide flexibility regarding the importance of RA synthesis in this process.

Specifically, we discuss that while CYP26B1-mediated degradation is the dominant mechanism regulating RA levels, RA synthesis (Raldh1-3) may still contribute to establishing basal RA levels.

• *Line 387 – Please consider substituting ‘associated’ with e.g. ‘correlated’*

We have revised our phrasing accordingly. The new revisions are located on lines 208-210 and read “Finally, the spatiotemporal expression of Cyp26b1 within the mesenchyme resembled that of Hoxa11 and Hoxa13 while appearing negatively correlated with Meis1 (Fig. 2E).”

• *Line 449 – What kind and what is the extent of ‘skeletal abnormalities’ are observable upon DIS or RAA-only treatments? Which segments does it affect?*

We have revised our text and included examples of abnormalities in Fig. S15. In text revisions can be found on lines 321-323. It reads “Animals treated with DIS or RAA alone showed either no phenotype or minor skeletal abnormalities from both proximally and distally amputated limbs, but nothing that appeared to affect PD patterning (Fig. S15A-B, Table S4, Table S5).”

• *Line 452 – Visualization of results in Figure S9 could be improved by adding arrows pointing to the morphological alterations observed. Please add scalebar as well.*

We have added arrows to skeletal abnormalities and added a scale bar to the top left image. Abnormalities include digit number reductions and fused skeletal elements.

• *Line 507 – Could you please also provide the HCR-FISH for the DMSO 14DPA distal blastema in Fig.4F?*

We have included an image of the DMSO treated limb for this figure (Fig. 4F) and it showed little *Meis2* expression and epithelial, but not mesenchymal, *Meis1* expression

• *Line 508 – Would be desirable to provide the RT-PCR validation of the RNA-seq for key genes involved in patterning, including Meis1, Meis2 and Cyp26b1 and others, and perhaps for genes that could be interesting to substantiate the hypothesis present in the discussion regarding Cyp26b1 expression, namely Fgf8 or Shh.*

We decided to quantify 11 genes using HCR-FISH instead of qPCR to provide quantitative and spatial gene expression measurements which showed trends similar to those observed in our RNA-seq data. These data can be found in Fig. 16D-N. Regarding the hypothesis in the discussion, several other studies have observed similar trends for *Shh* and *Fgf8* in PBs, DBs and RA treated DBs (Voss et al. 2018, Nguyen et al. 2017). Identifying upstream regulators of *Cyp26b1* is a goal our lab. Our ongoing work aims to experimentally test potential upstream regulators of Cyp26b1 using additional functional assays and will be a focus of future studies.

• Line 509 - Please rephrase this and following sentences for a more accurate versions, mainly by confining to conclusions that directly follow from the respective experiments and bearing in mind the fact that TAL treatment only partially shifts cells to a more proximal-like profile and, as acknowledged, also impacts in gene expression beyond DB vs PB paradigm.

We have modified the text for clarity. The relevant changes can be found on lines 352-357 and reads “DBs treated with 0.1 or 1 μM TAL were more transcriptionally similar to each other compared to either DMSO treated PBs or DBs (Fig. 4A, Fig. S14A). TAL-treated DBs exhibited transcriptomes more akin to DMSO treated PBs, suggesting a shift towards a more PB-like positional identity (Fig. 4A, Fig. S16A-N). However, the transition from DB to PB is likely incomplete, impacting gene expression beyond just positional information.”

• *Line 542 – Have you verified whether SHOX also increases in PBs upon TAL-treatment?*

We have performed this experiment and included it in the manuscript. It can be found in Fig. S17B-C. Our analysis of these results can be found on lines 457-461 and reads "We also observed that while Shox2 expression in PBs treated with 1 μM TAL was identical to DMSO treated PBs, Shox expression was significantly lower in PBs treated with 1 μM TAL compared to control PBs (Fig. S17B-C). These results suggest that Shox is more sensitive to heightened RA levels observed in PBs treated with 1 μM TAL than Shox2."

• *Line 576 - While the F0 Shox knockout crispants showed a consistent phenotype coupled with a generally high mutation rate, it would be great if this phenotype 'smaller limbs than controls, with significantly smaller stylopods and zeugopods' could also be verified in F1s, if available. If not available, then it would be important to provide information on the correlation between genotype (as percentage of mutated reads leading to loss of function) and the observed phenotypes.*

We have validated this shortened stylopod and zeugopod phenotypes in F1 homozygous *Shox*$^{-/-}$ animals. These results can be found in Fig. S20. We have also annotated the mutation type for each mutated read in Fig. S20. Modifications to the text can be found on lines 546-551 and reads "To confirm this finding, we generated F1 homozygous Shox knockout axolotls (Shox-/- animals) by crossing two Shox crispants. Like the F0 crispants, Shox-/- animals exhibited shortened stylopodial and zeugopodial elements, while their autopods appeared relatively unaffected (Fig. S20E). This indicates that despite mosaicism, F0 crispants display the same limb phenotype as F1 Shox-/- animals."

• *Supplementary figure 12 - Among the genotypes, please identify which ones are predicted to lead to e.g. truncations, vs. to silent mutations? This is important to determine whether mosaic mosaicisms affects the observed phenotypes. A similar concern applies for the limb regeneration experiment.*

We have annotated the mutation type for each mutated read in Fig. S20.

• *Line 609 - It is also not clear from the text whether the shorter limbs obtained after full regeneration are due to an overall size reduction or affect specifically the stylopod and zeugopod. A quantification of limbs segments, similar to what was done in Fig.1C would allow to appreciate this aspect.*

We have quantified the length of each segment in *Shox*$^{-/-}$ animals following amputation and included this analysis in Fig. S21. We describe the result with the following text on lines 579-584 and reads "Shox crispant limbs remained shortened after fully regenerating, suggesting that Shox is dispensable for limb regeneration but crucial for patterning the regenerating stylopodial and zeugopodial segments (Fig. 7A). This

finding was also confirmed in Shox-/- animals, which regenerated similarly sized autopods as controls but significantly smaller stylopods and zeugopods (Fig. S21)."

• *Due to the above-mentioned concerns, the limitations associated to the use of F0s should be acknowledged somewhere in the text.*

We have acknowledged the use of mosaic F0 cripsants in the text and confirmed that the phenotypes we see in these animals are identical to *Shox*$^{-/-}$ animals. This can be found on lines 546-551 and reads "To confirm this finding, we generated F1 homozygous Shox knockout axolotls (Shox-/- animals) by crossing two Shox crispants. Like the F0 crispants, Shox-/- animals exhibited shortened stylopodial and zeugopodial elements, while their autopods appeared relatively unaffected (Fig. S20E). This indicates that despite mosaicism, F0 crispants display the same limb phenotype as F1 Shox-/- animals"

• *Line 565 - Would it be possible to simultaneously visualize and quantify Meis1 and Shox by HCR-FISH in 14DPA proximal blastemas to determine the extent of overlay? In addition, an analysis of the potential Meis1 binding sites in the SHOX promoter could strengthen the hypothesis.*

We have now included an HCR-FISH visualizing *Meis1* and *Shox* expression in PBs (Fig. 5I). The new data are described on lines 473-475, which reads "At 14 DPA, Shox remained significantly more highly expressed in PBs but was restricted to the Meis1+ proximal mesenchyme, leaving a distal subset of Hoxa13+ cells devoid of Shox expression (Fig. 5F-J, Fig. S17G-H)."

We have also performed an analysis of *Meis1* binding sites in the *Shox* promoter and included it in the manuscript (Table S7). The text modification can be found on lines 482-483 and reads "Indeed, the Shox promoter contains several putative Meis1 binding sites (Table S7)."

• *Line 615 – Could you provide quantification analysis of the HoxA13 expression on 10DPA SHOX crispants vs. non transgenic animals? Perhaps it's because of the individual channels are missing in Fig.1J, but the Hoxa13 expression domain is not clearly distinguished in non-transgenic PBs at 10dpa, while it is clear in the Shox cripants in Fig.7D.*

We have performed HCR-FISH and qRT-PCR for *Meis1* and *Hoxa13* in *Shox*$^{-/-}$ blastemas. The results can be found in Fig. 7, and the analysis on lines 592-597. The text reads "We next assessed Meis1 and Hoxa13 expression in Shox-/- PBs and DBs and found their spatial expression patterns unchanged compared to controls (Fig. 7D, Fig. S22), consistent with findings from Shox2 KO mice 46. Correspondingly, the relative expression of these genes in Shox-/- PBs and DBs was unchanged from controls (Fig. 7E-F). These results suggest that PD positional identity is unaffected by the absence of Shox."

We have also provided supplemental figures for each FISH image in the manuscript, including individual channels without pseudodots and high magnification insets to visualize the FISH dots. These can be found in Fig. S3, Fig. S6, Fig. S7, Fig. S9, Fig. S11, Fig. S13, Fig. S14, Fig. S16, Fig. S17, Fig. S18, Fig. S19, Fig. S20, and Fig. S22.

• *Line 617 – This sentence might be overstating the point, and will benefit from a more balanced phrasing.*

We have rephased this sentence for clarity. It be found on lines 622-625 and now reads "The observations that Hoxa13 and Meis1 expression are unaffected by Shox knockout (Fig. 7D-F), along with Meis1 and Shox colocalization (Fig. 5H-I), suggest that Shox acts downstream of Meis1 to regulate endochondral ossification of proximal skeletal elements."

• *Line 692 – TIG1 is RA-responsive in stylopod-derived blastemas and has a distinct temporal expression profile than that of Shox – expression peaks at 3-7 dpa and quickly decreasing in zeugopod blastemas from 10dpa onwards (Oliveira et al 2022). It is likely that TIG1 was not found in the RNAseq dataset due to different expression timing and either acts upstream or in parallel during the establishment of limb P-D pattern.*

We have modified our text to clarify this point. The modifications can be found in the discussion on lines 729-732 and reads "However, our RNAseq represents only a small portion of gene expression during the entire duration of limb regeneration. Indeed, Tig1 expression appears strongest at earlier timepoints, which may explain its absence from our dataset."

• *A label of 'P' 'D' could be considered to be added either on the charts or legend of Fig.1 F,L, Fig.2E, Fig.5H, for prompter interpretation.*

We have added labels to the intensity charts indicating proximal and distal along the x axes.

• *Please increase the size of numerical labeling of charts like the ones of Fig.1B,C,G,H, I throughout the manuscript, for better readability.*

Font size on all charts was increased.

• *Amputation plane and inset delineation color of the HCR-FISH images is often not clear when overlaid onto such colorful images – could it be changed to a white/grey line?*

We have changed the color of the amputation plane lines to white.

• *Scalebar is missing in several panels, including Fig.3B, Fig.5I, Fig.6D, Fig.7A, B and E.*

A scale bar has been added to each figure.

Dr. Max H Yun & Dr. Catarina Oliveira (co-reviewed)

Reviewer #3 (Remarks to the Author):

Duerr and colleagues have investigated how proximodistal positional identity is established during axolotl limb regeneration. Specifically, studies from the 1980s identified that retinoic acid specifies proximal limb identity but how retinoic acid signaling levels are established remained unknown. The authors describe two significant observations; (a) CYP26B1, an enzyme responsible for retinoic acid breakdown, is responsible for regulating retinoic acid levels in the blastema; and (b) Shox, a retinoic acid responsive gene, regulates proximal distal skeletal element formation during limb regeneration. Both of these findings would be of broad interest to developmental biologists and the regenerative medicine communities. The first observation builds upon existing work that retinoic acid signaling exists along the urodele proximal distal axis and that its disruption results in abnormal skeletal morphologies (Maden, 1998, Nguyen et al., 2017, Scadding and Maden, 1986). The findings in this study demonstrate that it's actually the breakdown of retinoic acid that patterns the blastema. The second, begins to delve into the downstream mechanisms behind retinoic acid positional identity and their functional significance. This is a field of research that has long been idle and only recently started to gain traction. This study, is very timely, and offers fresh new insights. Overall, I am very supportive of the data and concepts presented in this manuscript and with some minor clarifications (as detailed below), would recommend it for publication.

1. The authors analyse the expression of a variety of genes using HCR-FISH. In general, most of these images are at low magnification to demonstrate the expression pattern throughout the limb. It would be helpful to show high magnification images in addition so that the individual 'dots' can be visualised. For example, Figure 1D, expression for Meis2 cannot be seen in any of the images despite relative counts in the associated graph. In addition the highlighted box for distal blastema at 10DPA seems to be more proximal than expected (compared to the proximal image).

We have provided supplemental figures for each FISH image in the manuscript, including individual channels without pseudodots and high magnification insets to visualize the FISH dots. We have additionally corrected the inset boxes and ensured they are positioned correctly relative to the larger image. These can be found in Fig. S3, Fig. S6, Fig. S7, Fig. S9, Fig. S11, Fig. S13, Fig. S14, Fig. S16, Fig. S17, Fig. S18, Fig. S19, Fig. S20, and Fig. S22.

2. Since Meis1 was only detected at very low levels using scRNA-seq however appears to have increased expression using HCR-FISH, can the authors also show the

scrambled probe control for this tissue to ensure the signal is specific? Epithelium is generally very 'sticky' and can give false results.

scRNAseq, according to 10x Genomics (https://kb.10xgenomics.com/hc/en-us/articles/360001539051-What-fraction-of-mRNA-transcripts-are-captured-per-cell) at best, captures roughly 1/3 of the total transcripts within a cell. We have found HCR-FISH to be more sensitive than scRNA-seq leading to stronger signal sometimes, but this is on a case-by-case basis. Indeed, epithelium often gives false results especially when performing immunohistochemistry. However, we do not observe this noise with HCR FISH in the many other probe sets we have used, as evidenced by the distinct absence of signal in the epithelium with probes for other genes such as *Shox*, *Shox2*, and *Hoxa13* in Fig. 5. Essentially, the specificity of many other probe sets (in this manuscript and in others from our lab, including Lovely et al. 2022) act as controls for Meis1 and we have observed similar results for negative probe controls run on limb blastemas. To further clarify specificity, we have added individual channel images and zoomed-in views in the supplemental figures. These can be found in Fig. S3, Fig. S6, Fig. S7, Fig. S9, Fig. S11, Fig. S13, Fig. S14, Fig. S16, Fig. S17, Fig. S18, Fig. S19, Fig. S20, and Fig. S22.

3. Why does 5uM TAL inhibit limb regeneration in its entirety? Does this have off target effects and cause cell death? Is this compound cytotoxic to cells?

Excessive exposure to retinoids has been shown to inhibit regeneration (Maden 1983- The effect of vitamin A on the regenerating axolotl limb), similar to what we observed from our results. Furthermore, retinoids are known to inhibit cell cycling in a variety of cell types (Lotan 1980- Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells). Indeed, slower rates of DNA synthesis and fewer mitotic events are observed in blastema cells upon RA treatment (Maden 1983). For this reason, we believe that high TAL concentrations raise the level of RA to a level deleterious to cycling cells, either slowing their cell cycle or preventing them from dividing entirely. Yet, we cannot rule out the possibility that high levels of TAL are cytotoxic or reprogramming cells into a non-limb cell identity.

4. Images corresponding to Supplementary Table 5 in certain cases would be appreciated (i.e. showing the defects).

We have revised our text and included examples of abnormalities in Fig. S15. In text revisions can be found on lines 321-323. It reads "Animals treated with DIS or RAA alone showed either no phenotype or minor skeletal abnormalities from both proximally and distally amputated limbs, but nothing that appeared to affect PD patterning (Fig. S15A-B, Table S4, Table S5)."

5. In line 508, the authors state that 'high levels of epithelial expression in blastemas obscured detection by RNA-seq'. Epithelial cells don't contribute to the blastema and therefore must come from the overlying tissue (e.g. AEC) obscuring the results. Can this

sentence be amended to reflect this distinction.

We have revised our text for clarity. Revisions can be found 396-424 and reads "Comparisons between DMSO treated DBs and either DMSO treated PBs or 1 μM TAL treated DBs enabled identification of RA-responsive genes associated with proximal or distal limb identity. We found that 138 DEGs (FDR <0.1) were shared between both comparisons (Fig. 4D, Table S6). Among these genes, we identified several that have known roles in patterning the developing or regenerating limb, including Pax9 40, Evx1 41, and Alx4 42 (Fig. 4E). Notably absent were Meis1, Meis2, and Cyp26b1 despite our previous results (Fig. 1-2) and their known roles as RA-responsive genes involved in PD patterning 18, 22, 39, 43. We suspect these genes were absent from our RNAseq analysis due to equally high levels in the wound epithelium of PBs and DBs (Fig. S2D, J, Fig. S12D). However, we observed significantly different expression levels of mesenchymal Cyp26b1, Meis1, and Meis2 in DBs treated with 1 μM TAL compared to DMSO treated DBs at 14 DPA (Fig. 3E-F, Fig. 4F-G, Fig. S16O), indicating that these genes are both involved in patterning and responsive to TAL treatment. Together, our results show that TAL treatment induces DBs to adopt a more proximal positional identity, likely mediated by RA-responsive patterning genes differentially expressed between PBs and DBs."

6. Since Shox crispants successfully regenerated limbs and progressed through typical stages of regeneration, it would be interested to determine if there was any compensation by Shox2 during this process to ensure proximal distal identity remained intact.

We have now made significant changes and additions to the section titled: "Shox is not required for limb regeneration but is essential for proximal limb patterning" addressing this point. Among the changes, we have performed HCR-FISH and qRT-PCR for *Shox2* in *Shox*$^{-/-}$ blastemas. The results can be found in Fig. 7D and Fig. 7G, and the analysis on lines 597-620 and reads "Furthermore, while Shox2 was expressed in the posterior mesenchyme of both control and Shox-/- DBs, it was generally more lowly expressed in Shox-/- DBs (Fig. 7D, G). In contrast, Shox2 expression expanded into the anterior mesenchyme of Shox-/- PBs, though its levels remained unchanged compared to controls (Fig. 7D, G). This suggests that Shox2 compensates for the loss of Shox in Shox-/- PBs."

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.