



Newt Hoxa13 has an essential and predominant role in digit formation during development and regeneration

Takashi Takeuchi, Haruka Matsubara, Fumina Minamitani, Yukio Satoh, Sayo Tozawa, Tomoki Moriyama, Kohei Maruyama, Ken-ichi T. Suzuki, Shuji Shigenobu, Takeshi Inoue, Koji Tamura, Kiyokazu Agata and Toshinori Hayashi
DOI: 10.1242/dev.200282

Editor: Patrick Tam

Review timeline

Original submission:	1 October 2019
Editorial decision:	29 October 2019
First revision received:	27 October 2021
Editorial decision:	16 November 2021
Second revision received:	14 January 2022
Accepted:	21 January 2022

Original submission decision letter

MS ID#: DEVELOP/2019/185181

MS TITLE: The roles of hox 13 genes in newt limb development and regeneration

AUTHORS: Takashi Takeuchi, Fumina Minamitani, Kazuki Koriyama, Yukio Satoh, Ken-ichi Suzuki, Shuji Shigenobu, Takeshi Inoue, Kiyokazu Agata, and Toshinori Hayashi

Dear Dr. Takeuchi,

I have now received all the referees' reports on the above manuscript, and have reached a decision. I am sorry to say that the outcome is not a positive one. The referees' comments are appended below, or you can access them online: please go to [Development's submission site](#) and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees raise some significant concerns about your paper, and are not strongly in favour of publication. Having looked at the manuscript myself, I agree with their views, and I must therefore, reject your paper.

I do realise this is disappointing news, but Development receives many more papers than we can publish, and we can only accept manuscripts that receive strong support from referees.

I do hope you find the comments of the referees helpful, and that this decision will not dissuade you from considering Development for publication of your future work. Many thanks for sending your manuscript to Development.

Reviewer 1 Advance Summary and Potential Significance to Field:

The question of whether regeneration is a recapitulation of development is long-standing and important. Examining candidate genes in answering this question is a sensible approach because there is now extensive knowledge of the genes and pathways that control tetrapod limb development, and these can now be considered in animals that also regenerate limbs. Shared function between development and regeneration might be most likely among genes required for

patterning, i.e., how to form the shape of the limb once the progenitor cells have already been amassed. In this manuscript Takeuchi et al. characterize the role of *hox13* genes in newt development and regeneration. They explore whether *hox13* paralogs are essential for limb regeneration and whether they have same or distinct functions between development and regeneration in Iberian ribbed newts (*Pleurodeles waltl*). They show that double or triple mutants of *hox13* paralogs, *a13*, *c13* and *d13* via CRISPR/Cas9 system result in digit loss in developing newts which also persisted during regeneration. In general, this work provides information regarding the role of *hox13* paralogs during development which could serve an important role for further investigation of the roles of *hox13* genes in limb regeneration along with other developmental markers.

In regard to whether Hox genes have same or distinct function during development, the authors conclude that *hox13* paralogs have same function based on the similarities in the phenotypes observed between double (*hoxa13* and *hoxd13*) and triple mutants (*hoxa13*, *hoxc13* and *hoxd13*).

Reviewer 1 Comments for the Author:

This paper is likely to serve as a reference point for other researchers working with genetically-modified salamanders. Controls for claiming edits to a specific gene are causative for resulting phenotypes deserve scrutiny. Here, there are a couple of core issues with this work:

1. Specificity: Have authors tried rescuing the observed phenotype by adding back paralogs individually or in combination? This, in addition to testing whether *hox13* paralogs indeed perform similar functions, would also serve as an important control to show that the observed phenotype does not result from an off-target effect of CRISPR/Cas9. This is especially relevant in this case for two reasons. First, they are using F0 animals. This was done to save time (generation times in these animals are quite long, 1 year or more), and it was justified by the authors as acceptable because the editing rates at the loci they targeted were extremely high. Yet, without crossing the F0 animals, they are actually even more likely to be observing off-target effects (especially with this extremely high editing efficiency) due to unknown loci also being edited highly and there being no attempt to purify the alleles via crossing them out. Secondly, they are in the enviable situation where the phenotypes are essentially fully penetrant, and so it is completely conceivable that even a crude rescue experiment might cause a shift in the phenotype toward wild type. They ought to do a rescue experiment. This could be done in both development and regeneration. Partial rescue would be sufficient and obvious. For example, even without additional genetics, they could introduce the wild-type versions of these *hox13* genes alone or in combination and ask if there is any improvement in autopod formation. This could be done via electroporation into the early bud or into the early blastema. The authors also seem to rely on the phenotypes "making sense," i.e., they match their expectations based on the roles of these genes in the literature. The only effort they make to demonstrate specificity is to use more than one guide RNA. This could set a dangerous precedent in the literature; rescue should certainly be attempted.

2. Additionally, it is stated the wild-type siblings that are used as a control to CRISPR/Cas9 mutants are non-injected. It would be ideal to use a non-targeting CRISPR/Cas9 control for a better comparison of the phenotypes observed between mutants and control. Using uninjected controls is highly unusual.

3. Their core claim is that these genes are doing the same thing in development and regeneration. However, they have not cleanly separated these two processes because they only examined regeneration in the context of limbs that were already improperly developed, presumably because they lacked *hox13* gene activities. To better discern the role of these genes specifically during regeneration it might be better to perturb the functions of them on a wild-type background after amputation. This could perhaps be accomplished by editing directly in blastema cells (which has been shown to be effective in axolotls, and in this case since the phenotypes are so dramatic it might work even with modest editing rates). Another approach could be to use electroporation or a virus to express dominant-negative versions of *hoxa13*, *hoxc13*, and/or *hoxd13*. Alternatively, genes can be added back on a mutant background after amputation to test if they are sufficient to induce digit formation. It would also be intriguing to test whether adding back (rescuing) the mutants at different developmental (or regeneration) time points would have impact on the observed phenotype.

While these three core questions are critical for the authors to make the claims they make in the paper, there are some additional questions that would strengthen the work. Some of these questions might have already been tackled, and presenting information about them in the paper would be helpful. Have authors performed mutations of paralogs individually? It would be useful to compare the phenotype resulting from individual mutants to the combinatorial ones which would help to determine whether the phenotype is enhanced or not when genes are mutated in combination? One of the proposed questions in the introduction is whether the downstream signaling of hox genes is the same or different. Have authors looked at any downstream signaling events that are specific to different hox13 paralogs? Adding this information could also strengthen the argument that the observed phenotypes are due to the intended targets, though in a way this is also circular since it relies on the current scientific knowledge from other models about the effects of loss-of-function mutations in hox13 paralogs.

Minor edits

In Figure 1B there is a typo. "cording region" should be corrected as "coding region".

In Figure 1 age of animals that blastemas are collected to perform RT-PCT to check the expression of the paralogs is missing.

In Materials and Methods under the Preparation of RNPs and microinjection, "underlined" in the bracket is misspelled.

In the last paragraph of "Hox13 genes were mutated in the crisprants" section the referred mouse study is missing the reference. The same study is referred in the next section too and missing reference there as well.

"The remaining three animals in ad crisprants had only 1,2 and 4 digits in their four limbs, respectively, indicating that they lacked 14-17 digits." What does that "lacked 14-17" digit mean?

Reviewer 2 Advance Summary and Potential Significance to Field:

A creative novel combination of CRISPR/Cas9 manipulations to produce F0 compound mutant experimental animals. In terms of Hox gene function in limb development the conceptual result is confirmatory, the novelty is extension to non-mammalian tetrapods, and regeneration.

Reviewer 2 Comments for the Author:

Takashi Takeuchi and colleagues offer convincing demonstration that in simultaneous absence of Hox13 group A and D genes digits do not form during newt development. This is a first such publication in any amphibian model. Furthermore, they show that with respect to the combined function of Hoxa13 and Hoxd13 regeneration is analogous to development. This constitutes a good premiss to argue for the utility of this methodology in using gene loss-of-function approaches to study regeneration. Their "para-genetic" method of investigating gene function without genetic analysis in vivo is of considerable interest to laboratories working with species relying on external fertilisation, and probably beyond. The technical design and the molecular documentation are exemplary. I recommend publication in the Techniques and Resources section.

A revision of the title and abstract to show the primarily technical nature of the advance seems necessary.

Some shortcomings are evident, however.

1/Regrettably, the function of Hoxa13 and Hoxd13 were not investigated independently. The case of Hoxd13 in particular would be paradigmatic from an evolutionary point of view, since Hox gene clusters show very strict molecular organisation in mammals, which is dramatically relaxed in reptiles and amphibia. The Hoxd11-Hoxd13 region is altered in addition due to the absence of Hoxd12, associated with important DNA sequence expansion in *Notohophthalmus viridescens* and presumably also in other amphibia, and this may be related to perplexing differences evident in the patterns of digit complements between amphibia and mice. Given the apparent facility of

applying the tools developed by this research group it seems inexplicable why they did not run these simple experiments but explored a potential contribution of Hoxc13 instead.

2/ One would like to know if these compound homozygous individuals are capable to reproduce. Both Hoxa13 and Hoxd13 are required for the development of external genitals in mice and man, leading to sterility in mutants, which may be absent in the compound mutant newt crispants, further enhancing their experimental value.

3/The crispant model does not allow for investigating gene dose dependent effects. In the concrete example for instance, mouse breeding showed that depending on the number of wild type alleles of the Hoxa13 or Hoxd13 loci, complete absence of digit development was the final state when all four alleles were absent, yet in presence of one normal allele of the four, polydactyly occurred, and further increases of the Hox13 dose resulted in stabilization of the pentadactyl pattern. Such investigations would be useful to explore the evolutionary mechanisms, and their apparent impossibility represent limitations to the import of the crispant methodology "in understanding the molecular mechanisms that enabled the fin to limb transition and its biological significance". Notwithstanding these points, the summary conclusion of the manuscript is well supported, indicating that simultaneous absence Hoxa13 and Hoxd13 result in loss of digit development both during metamorphosis and regeneration in *Pleurodeles waltl*. This however does not exclude the possibility that significant differences may exist in the relative functions of Hoxa13 and Hoxd13 as compared to mammals.

Minor points

Title hox 13 Running title Hox13, should be the same notation

Running title should be modified Hox13 is not a substance or object, it is a concept ... which is not expected to have a function in a material sense

p 11 underlined

p12 Competing interests paragraph occurs twice

Reviewer 3 Advance Summary and Potential Significance to Field:

Using F0 crispants generated by Cas/CRISPR mutation, the authors report clear evidence supporting the functional conservation of Hox13 genes in autopod development in the axolotl, *P. waltl*. This is of high interest to the field.

Reviewer 3 Comments for the Author:

This manuscript reports clear evidence of a strong evolutionary conservation of function for Hox13 genes in autopod development and additional evidence of a similar requirement for these genes in regeneration in the *P. waltl*. While of strong interest to the field, the manuscript needs significant editing for clarity and grammar.

The title should more actively describe the interesting findings, not just state what question was addressed.

Scientifically:

Figure 1: More technical detail should be reported in Figure 1, including Suppl Data #2, but also more details incorporated in the diagram/cartoon in panel B. That hoxc13 is expressed so weakly and fails to have a clear phenotype in the results reported warrants further exploration, particularly given its possible expression divergence from mammals. Both additional qPCR primer sets and in situ hybridization experiments could confirm whether this gene is really expressed and, if so, in FL, HL or both.

Figure 2: The penetrance of the phenotype is not separated into FL and HL. Clarity on the total number of animals examined and what FLs and HLs from each were assayed is important. Are partial phenotypes in one or the other?

Figure 4: Was regeneration performed on FLs, HLs, both? Also, what happened if animals presenting partial phenotypes were amputated? Did phenotype reproduce what was observed upon initial deletion?

Figure 5: A reiteration of the developmental phenotype here is not warranted unless one is comparing a change. Showing both FL and HL and statistics would be valuable here.

Reviewer 4 Advance Summary and Potential Significance to Field:

The manuscript by Takeuchi et al addresses the fundamental question in developmental and regenerative biology of whether limb regeneration uses the same mechanisms as limb development. Mainly due to technical limitations in animals with the ability to regenerate appendages, functional testing of genes in regenerating appendages have been limited in the literature.

Although the conceptual basis of this manuscript is simple, these authors have pioneered the development of the Iberian ribbed newt animal system as a model for studying limb regeneration. The limited results that are presented here is a culmination of many other previous studies that has made the experiment here (knocking out three genes in one animal) seem easy. The efficiency that has been observed using the Iberian ribbed newt is higher than most other animals including axolotl salamanders.

Overall, the data are highly convincing and there is little argument that the *hox13* paralogs are necessary for distal limb development and regeneration. This may not surprise most people that study limb regeneration, but it was more of an assumption than an understanding of the function in distal *hox* genes. The study also addresses for the first time at the genetic level that the proximodistal patterning that determines limb region identity is conserved and re-used during limb regeneration. Therefore, the study adds novel and significant new understanding to limb regeneration.

Reviewer 4 Comments for the Author:

Technically, there is little to highlight as the study is a simple, yet clever approach to disabling three *Hox13* paralogs in a single animal using a single guide RNA.

I am unclear whether whole mount in situ hybridization is a robust and easy approach in the Iberian newt, but if it is straightforward it would strengthen the manuscript if ISH was used to show *Hox13* expression in the developing limb and even regenerating limb. This would lead to a more complete story, but considering the swath of information on *Hox13* genes during limb development it reasonable to assume that the gene expression pattern is conserved in salamander limb development and regeneration.

In the discussion portion of the manuscript, I suggest that the authors discuss and predict what they think may occur if the same experiment would be done in *hox11* paralogs. Is it likely that the entire zeugopod is missing? These results also suggest that *hox* genes don't work in a gradient manner in regenerating salamander limbs, but determines the identity of whole segments.

Response to reviewers' comments

This paper added a great deal of results to the data from the previous paper we submitted (DEVELOP/2019/185181). As a result, we found several new important results. For example, we showed that there is a big difference in *Hox13* paralog functions between mice and newts, which has novel implications for the mechanisms of vertebrate limb development and fin-to-limb transition. Therefore, the entire paper, including title and abstract, has been completely revised. In addition, we were able to respond appropriately to all comments from the previous reviewers. For example, we clearly excluded the possibility of off-target effects by establishing germline mutants. The summary of revision and new findings, and specific point-by-point responses to previous reviewers' comments are listed as follows:

The summary of the revision

1. We produced single, double, triple and quadruple *Hox13* paralog mutants and analyzed the limb development and regeneration of a total of 12 crispant groups (new Tables S1-6, new Figs. 2, 3, 6, S2, S3 and S4). In a previous paper, only two crispant groups were analyzed (corresponding to *ad-1* and *acd* in this paper).
2. We showed the expression patterns of *Hoxa13*, *c13*, and *d13* in developing forelimbs and blastemas (new Figs. 1 and 8A).
3. We also showed the expression patterns of *Shh*, *Hoxd11* and *Hoxd13* in *Hox13* crispants (new Fig. 4).
4. We revealed that newt *Hoxd13* has functions in digit formation by the induction of *Hoxd13* expression in limb buds of *a* crispants, in which almost no digit structure was formed (new Fig. 5). This result also showed that knockout of *Hoxa13* can be rescued by the paralogous gene, *Hoxd13*.
5. We confirmed that the phenotype in *Hoxa13* crispants (almost no digit structure was formed in limb development and regeneration), which is a main result in this paper, is the same as that in the germline mutants (new Fig. 7).
6. We suggest a crucial contribution of *Hoxa13* function in fin-to-limb transition (new Fig. 8B).

The summary of new findings

1. Among the newt *Hox13* paralogs, *Hoxa13* in particular plays a predominant role in digit formation. The evidence is that (1) *Hoxa13* crispants and *Hoxa13* germline mutants lacked all digit structures other than one phalange-like piece. (2) *Hoxc13* crispants, *Hoxc13* germline mutants, and *Hoxd13* crispants showed no phenotypes, and (3) *Hoxb13* expression was not detected in the developing limbs (new Figs. 2, 3, 7, S1 and S3).
2. Newt *Hoxd13* expression is spatio-temporally restricted in the limb buds, (new Figs. 1 and 8A) and the expression is strongly dependent on *Hoxa13* (new Fig. 4) unlike mice, although newt *Hoxd13* has functions in digit formation (new Fig. 5).

Specific point-by-point responses to previous reviewers' comments

We thank reviewers for constructive comments that helped us improved the quality of our paper. We have substantially revised the paper to improve its impact and general relevance as follows:

Reviewer 1

Major comments

1. *Have authors tried rescuing the observed phenotype by adding back paralogs*

We rescued the phenotype of *Hoxa13* crispants by *Hoxd13* (new Fig. 5). Ideally, *Hoxa13* should be used. However, we used *Hoxd13*, because induction of *Hoxa13* expression resulted in extremely high lethality and also it was necessary to investigate the function of *Hoxd13*. Furthermore, we confirmed that the phenotype in *Hoxa13* crispants (almost no digit structure was formed in limb development and regeneration), which is a main result in this paper, is the same as that in the germline mutants (new Fig. 7). These results clearly show that the phenotypes observed in at least *Hoxa13* crispants is *Hoxa13*-specific and not due to off-target effects. Because the phenotype in *Hoxa13* crispants is almost the same as other double, triple and quadruple *Hox13* paralog mutants (no substantial digits), we think that these experiments are sufficient in this paper.

2. *It would be ideal to use a non-targeting CRISPR/Cas9 control for a better comparison of the phenotypes observed between mutants and control.*

Although using a non-targeting CRISPR/Cas9 control is ideal, *Hoxc13* and *d13* crispants showed no phenotypes in limb development and regeneration (new Figs. 2, 3, 6, S3 and S4), showing that these experiments functioned as other ideal controls. In addition, we have knocked out many genes including tyrosinase and the phenotype showing loss of all digits has not been observed in other than *Hox13*.

3. *To better discern the role of these genes specifically during regeneration it might be better to perturb the functions of them on a wild-type background after amputation.*

The reviewer was concerned that we *examined regeneration in the context of limbs that were already improperly developed*. However, we thought it was feasible to examine the functions of *Hox13* in limb regeneration by amputation at the stylopod, since the stylopods of *Hox13* crispants were considered to be normal (new Figs. 2 and S3), and all distal regions were reconstructed from dedifferentiated stylopod cells by amputation at the stylopod. On the other hand, the autopod cells in *Hox13* crispants became abnormal during development. Therefore, regeneration by amputation at the autopod would be from cells that were already abnormal and would not be appropriate for studying the function of *Hox13* in limb regeneration. These comments are inserted in this paper (p11, line 297- 303).

4. *Have authors performed mutations of paralogs individually?*

Yes, we produced single *Hox13* paralog mutants including germline mutants other than mutants of *Hoxb13*, expression of which could not be detected in the developing limbs and blastemas (new Fig. S1), and obtained new important results (Figs. 2-7, S3 and S4). We compared the phenotypes to each other and with double, triple and quadruple *Hox13* paralog mutants and analyzed the genetic interaction.

5. *Have authors looked at any downstream signaling events that are specific to different hox13 paralogs?*

We do not know of any truly *Hox13* paralog-specific downstream signals. Instead, we investigated the expression of *Shh*, which is activated directly by *Hox13*, and found that the expression intensity decreased in *Hoxa13* single and *Hoxa13/d13* double crispants (new Fig. 4A), suggesting the regulation of *Shh* by *Hox13* in the newt limb.

Minor comments

1. *In Figure 1B there is a typo. "cording region" should corrected as "coding region".*
2. *In Figure 1 age of animals that blastemas are collected to perform RT-PCT to check the expression of the paralogs is missing.*

All were revised according to the reviewers' suggestions.

3. *In Materials and Methods under the Preparation of RNPs and microinjection, "underlined" in the bracket is misspelled.*
4. *In the last paragraph of "Hox13 genes were mutated in the crispants" section the referred mouse study is missing the reference. The same study is referred in the next section too and missing reference there as well.*
5. *The remaining three animals in ad crispants had only 1,2 and 4 digits in their four limbs, respectively, indicating that they lacked 14-17 digits." What does that "lacked 14-17" digit mean?*

All corresponding parts are deleted due to overall revision of this paper.

Reviewer 2

Major comments

1. *I recommend publication in the Techniques and Resources section.*

We believe that this major revision makes this article more suitable for a Research article.

However, if the editors decide that it would be better published in Techniques and Resources, we will be happy to accept it.

2. *Regrettably, the function of Hoxa13 and Hoxd13 were not investigated independently.*

We produced single *Hoxa13* and *d13* crispants as well as *Hoxa13* germline mutants, and showed *Hoxa13* predominance in newts (new Figs. 2-7, S3 and S4).

3. *One would like to know if these compound homozygous individuals are capable to reproduce.*

We tried to obtain F1 animals from many *Hoxa13*, *c13*, *d13* triple or *a13* and *d13* double crispants. However, sufficient fertility was not observed in both male and female crispants with strong phenotypes (class3 and 4). Therefore, we used crispants with weak phenotypes (class 2), and confirmed germline transmission of mutant alleles of these genes. Part of these results is described in Results (new Fig. 7) and Material and methods (p19, line 549-553). These results suggest that compound knockout of *Hoxa13* and *d13* affect fertility in male and female newts.

4. *The crispant model does not allow for investigating gene dose dependent effects. In the concrete example for instance, mouse breeding showed that depending on the number of wild type alleles of the Hoxa13 or Hoxd13 loci, complete absence of digit development was the final state when all four alleles were absent, yet in presence of one normal allele of the four, polydactyly occurred, and further increases of the Hox13 dose resulted in stabilization of the pentadactyl pattern. Such investigations would be useful to explore the evolutionary mechanisms, and their apparent impossibility represent limitations to the import of the crispant methodology “in understanding the molecular mechanisms that enabled the fin to limb transition and its biological significance”. Notwithstanding these points, the summary conclusion of the manuscript is well supported, indicating that simultaneous absence Hoxa13 and Hoxd13 result in loss of digit development both during metamorphosis and regeneration in *Pleurodeles waltl*. This however does not exclude the possibility that significant differences may exist in the relative functions of Hoxa13 and Hoxd13 as compared to mammals.*

We agree with this comment. Recently, we were finally able to obtain double heterozygous F1 newts (*Hoxa13*^{+/-}, *d13*^{+/-}) from crispants, and are currently waiting for their sexual maturation. If the results are available in time for the deadline, the data will be added to a revised version of this paper.

As described in this paper, we showed that the relative functions of *Hoxa13* and *Hoxd13* are largely different from those in mice. *Hoxa13* has a predominant function for digit formation and the predominance is probably due to the restricted expression pattern of *Hoxd13* in limb buds and the strong dependence of *Hoxd13* expression on *Hoxa13*. We think that these results have novel implications for the mechanisms of fin-to-limb transition because the predominance of *Hoxa13* function both in newt limbs and fish fins, but not in mouse limbs, suggests the crucial contribution of *Hoxa13* but not *d13* in fin-to-limb transition. These results and suggestions are described in this paper (Figs. 2-8, S3 and S4).

Minor comments

1. *Title hox 13 Running title Hox13, should be the same notation.*

2. *Running title should be modified Hox13 is not a substance or object, it is a concept ... which is not expected to have a function in a material sense*

3. *p 11 underlined*

4. *p12 Competing interests paragraph occurs twice*

All corresponding parts are corrected, deleted, or changed due to overall revision of this paper.

Reviewer 3

1. *the manuscript needs significant editing for clarity and grammar.*

In the overall revision, we did our best to improve the editing.

2. *The title should more actively describe the interesting findings, not just state what question was addressed.*

Due to new findings, we changed the title. We tried to describe our findings actively.

3. *Figure 1: More technical detail should be reported in Figure 1, including Suppl Data #2, but also more details incorporated in the diagram/cartoon in panel B.*

Previous Fig. 1B was revised to new Fig. S2, and we added the position of helices. Because there is much information about target regions of gRNAs in this schematic diagram, we used Table S1 and S7 for other information.

4. *That *hoxc13* is expressed so weakly and fails to have a clear phenotype in the results reported warrants further exploration, particularly given its possible expression divergence from mammals. Both additional qPCR primer sets and in situ hybridization experiments could confirm whether this gene is really expressed and, if so, in FL, HL or both.*

We retried RT-PCR and also performed in situ hybridization, and confirmed *hoxc13* expression at least in the developing limbs.

5. *Figure 2: The penetrance of the phenotype is not separated into FL and HL. Clarity on the total number of animals examined and what FLs and HLs from each were assayed is important. Are partial phenotypes in one or the other?*

6. *Figure 4: Was regeneration performed on FLs, HLs, both?*

In this paper, we focused only FLs for simplicity and detailed information about phenotypes are shown in new Figs. 4B and 6C.

7. *Also, what happened if animals presenting partial phenotypes were amputated? Did phenotype reproduce what was observed upon initial deletion?*

Yes, developmental phenotypes generally repeated in the limb regeneration. For simplicity, we describe the phenotypes of animals in which developmental phenotypes were strong (new Figs. 6 and S4).

8. *Figure 5: A reiteration of the developmental phenotype here is not warranted unless one is comparing a change. Showing both FL and HL and statistics would be valuable here.*

In this paper, we show developmental and regenerative phenotypes separately (development: Figs. 2, 3 and S3; regeneration: Figs. 6, S4 and S5). As mentioned above, we focused only FLs for simplicity. Detailed information is shown in Fig. 6B.

Reviewer 4

1. *it would strengthen the manuscript if ISH was used to show *Hox13* expression in the developing limb and even regenerating limb.*

We showed *Hox13* expression patterns in limb development and regeneration (new Fig. 1).

2. *In the discussion portion of the manuscript, I suggest that the authors discuss and predict what they think may occur if the same experiment would be done in *hox11* paralogs.*

We produced *Hox11* crispants and are currently analyzing the phenotypes. We hope to show the data in another future paper.

Original submissionFirst decision letter

MS ID#: DEVELOP/2021/200282

MS TITLE: Newt Hoxa13 has an essential and predominant role in digit formation during development and regeneration

AUTHORS: Takashi Takeuchi, Haruka Matsubara, Fumina Minamitani, Yukio Satoh, Sayo Tozawa, Tomoki Moriyama, Kohei Maruyama, Ken-ichi T Suzuki, Shuji Shigenobu, Takeshi Inoue, Koji Tamura, Kiyokazu Agata, and Toshinori Hayashi

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the comments of reviewer 1 and 3 can be satisfactorily addressed. Please attend to the comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1*Advance summary and potential significance to field*

This manuscript, titled Newt Hoxa13 has an essential and predominant role in digit formation during development and regeneration shows crispant and genetic mutants in the Hox13 paralogs and study of their defects in autopod formation and regeneration in newts is of high interest and represents an excellent contribution to our understanding of Hox genes in development, especially limb development. This is a highly interesting and significant contribution to the literature. This reviewer suggests stating Hox13 genes have an important role in newt limb, not just Hoxa13 as submitted (but consider this authors' choice).

Comments for the author

Below are comments generated during review which this reviewer recommends for clarity:

Two statements in the abstract lacked clarity:

Here, we produced single to quadruple Hox13 paralog mutants using the CRISPR/Cas9 system and also germline mutants in newts (*Pleurodeles waltl*), which have strong regenerative capacities -this sentence is unclear - what is 'also' also to?

The predominance of Hoxa13 function both in newt limbs and fish fins, but not in mouse limbs, suggests a crucial contribution of Hoxa13 function in fin-to-limb transition.

-the data that demonstrates differential contribution is solid and interesting, but subfunctionalization and changes in regulation don't clearly support the fin-to-limb transition aspect. There are many other possibilities that account for interesting changes in regulation.

Lines 105-108: The names of the four genes mutated should be specified here, presumably Hoxa13, Hoxb13, Hoxc13 and Hoxd13. Further, it seems the same compliment of Hox13 genes are present in newt, but explicitly stating this early in the manuscript would be useful.

Line 142-145: This is post-amputation blastemas presumably, but more detailed information on what age injury was performed, how many days post-injury for last blastula would be helpful for those not familiar.

Line 161 through 167. It seems a total of 9 gRNAs and 2-5 gRNAs per four paralogs does not correlate. Perhaps one of these two statements need modification?

Figure 2: Strongly recommend referring to the crisprants with more clear names; i.e. Hoxa13 #2sgRNA, not a-2 which has no meaning to the reader.

Line 203-204: c and d - author means Hoxc13, Hoxd13? This occurs throughout. Being more specific on genes and gRNA construct numbers would be very beneficial.

Do not refer to 'classes', name the genes disrupted.

Line 237-241: While it may be due to this reader's ability to accurately follow the nomenclature used, the data seem to show that Hoxa13/Hoxd13 only are required for limb development.

Including Hoxc13 crisprants did not appear to change phenotypes other than that shown at bottom right of Figure 2B. It seems that is a variation on the same phenotype as Hoxa13/Hoxd13 - not a role for Hoxc13. Hoxa13/Hoxc13 double crisprants should be generated to support this if important to authors.

Line 257-259: it seems loss of autopod region, as clearly shown by authors, accounts for expression shift. If the autopod region is mostly gone, there is the most straightforward (parsimonious) explanation that Hoxd11 is where it's supposed to be - in zeugopod which is shifted distally as autopod is not there.

Were there no germline Hoxd13 mutants generated? It would be interesting to know what germline single Hoxd13 mutants looked like.

The commentary on potential evolutionary differences in Hoxa13/Hoxd13 in fin-to-limb are interesting in discussion, but might be shortened. The abstract should make less definitive claims, though the discussion is interesting.

Reviewer 2

Advance summary and potential significance to field

The paper identifies a role for Hox13 genes in newt limb development and potentially also in limb regeneration. It also proposes an evolutionary implication of the epistasis observed between hoxa13 and hoxd13.

Comments for the author

The paper is much better and more interesting now. The rescue experiments are an important addition, and.

they've found something novel about hoxa13 being epistatic to hoxd13 newt limb development.

They have a new interpretation about how these findings may impact understanding of evolution of limb development.

They've also reported some germline phenotypes for the mutants. I still think it's problematic to not do the knockdown experiments in wild-type animals/limbs to assess a specific role for these genes in limb regeneration. However, they now do a better job explaining their position on this issue. I do think this is important, but I also recognize that to do the experiment well, it could require lengthy experimentation because they are working in newts.

Reviewer 3

Advance summary and potential significance to field

The authors have significantly modified the study based upon recommendations during a previous review. It is a stronger manuscript now clearly showing the predominant importance of Hoxa13 over other hox13 paralogs. This work provides important functional data on the role of hox genes during limb development and regeneration. Although the role of Hoxa13 predominance in the fin to limbs transition is difficult to functionally demonstrate, the discussion on this evolutionary story is important for the field of limb development.

Comments for the author

I do not encourage further revision to the manuscript considering the authors added a significant amount of new experiments to support their claims based upon reviewer comments. There was one small suggestion based upon the following statement. "One major reason why genetic analyses have not been performed in non-mammalian tetrapods is a lack of reverse genetic techniques other than in rodents. In particular, gene disruption had not been achieved in animals that can regenerate their limbs." This statement is somewhat misleading as Crispr/Cas9 was used in *X. laevis* and axolotls before 2018 who both regenerate their limbs.

First revisionAuthor response to reviewers' comments**Response to reviewers**

Note: this text is also uploaded as one of Supplementary material under the same title.

We thank reviewers for constructive comments that helped us improved the quality of our paper. We have revised the paper to improve its impact and general relevance as follows:

Reviewer 1

1. *Here, we produced single to quadruple Hox13 paralog mutants using the CRISPR/Cas9 system and also germline mutants in newts (Pleurodeles waltl), which have strong regenerative capacities*
-this sentence is unclear - what is 'also' also to?

In accordance with this comment, the corresponding parts were revised as follows (Lines 42-44).

"Here, we produced single to quadruple *Hox13* paralog mutants using the CRISPR/Cas9 system in newts (*Pleurodeles waltl*), which have strong regenerative capacities, and also produced germline mutants."

2. *The predominance of Hoxa13 function both in newt limbs and fish fins, but not in mouse limbs, suggests a crucial contribution of Hoxa13 function in fin-to-limb transition.*
-the data that demonstrates differential contribution is solid and interesting, but subfunctionalization and changes in regulation don't clearly support the fin-to-limb transition aspect. There are many other possibilities that account for interesting changes in regulation.

This comment has been addressed in the response to comment 10.

3. *Lines 105-108: The names of the four genes mutated should be specified here, presumably Hoxa13, Hoxb13, Hoxc13 and Hoxd13. Further, it seems the same compliment of Hox13 genes are present in newt, but explicitly stating this early in the manuscript would be useful.*

In accordance with this comment, the following text was added to Lines 112-113 in Introduction.

"Newts have four *Hox13* paralogs (*Hoxa13*, *b13*, *c13*, and *d13*)."

4. *Line 142-145: This is post-amputation blastemas presumably, but more detailed information on what age injury was performed, how many days post-injury for last blastula would be helpful for those not familiar.*

This information was added in Figure legends of Fig. 1.

5. *Line 161 through 167. It seems a total of 9 gRNAs and 2-5 gRNAs per four paralogs does not correlate. Perhaps one of these two statements need modification?*

The gRNAs, which target each paralog, are as follows.

Hoxa13: G22, G24, G43 and G27; 4 gRNAs

Hoxb13: G34 and G27; 2 gRNAs

Hoxc13: G22, G36 and G27; 3 gRNAs

Hoxd13: G25, G22, G26, G41 and G27; 5 gRNAs

We thought that this content would be easier to understand if Fig. S2 in the previous supplementary material could be referred to immediately. We have therefore moved this figure to the main text (new Fig. 2).

6. *Figure 2: Strongly recommend referring to the crispants with more clear names; i.e. Hoxa13 #2sgRNA, not a-2 which has no meaning to the reader.*
Line 203-204: c and d - author means Hoxc13, Hoxd13? This occurs throughout. Being more specific on genes and gRNA construct numbers would be very beneficial.
Do not refer to 'classes', name the genes disrupted.

If we use target gene names and gRNAs for crispant groups, these become very long and difficult to understand. For example, “*Hoxa13, b13, c13, d13*; G22 and G35 gRNA”. We referred to it as *abcd-1*. We thought that the reason why it was difficult to understand the correspondence between gRNA and target genes, and the contents of each crispant group was because the figure and table showing them were included in Supplementary material (Previous Fig. S2 and Table S1). Therefore, we have moved the figure and table to the main text (new Fig. 2 and Table 1). In addition, to aid understanding of crispant groups, the following text has been revised and added as examples (Lines 177-181).

“For example, only *Hoxa13* was targeted in *a* crispant groups (*a-1* and *a-2*). The gRNAs used were different between *a-1* and *a-2* (Table 1). In *ac* and *ad* crispants, *Hoxa13/Hoxc13* and *Hoxa13/Hoxd13* were doubly targeted, respectively. Similarly, in *acd* and *abcd* crispants, *Hoxa13/Hoxc13/Hoxd13* and all four paralogs were multiply targeted, respectively (Table 1).”

7. *Line 237-241: While it may be due to this reader's ability to accurately follow the nomenclature used, the data seem to show that Hoxa13/Hoxd13 only are required for limb development. Including Hoxc13 crispants did not appear to change phenotypes other than that shown at bottom right of Figure 2B. It seems that is a variation on the same phenotype as Hoxa13/Hoxd13 - not a role for Hoxc13. Hoxa13/Hoxc13 double crispants should be generated to support this if important to authors.*

The data for *Hoxa13/Hoxc13* double crispants were presented in the previous manuscript as *ac* crispants (previous Figs. 2, 3 and 6; New Figs. 3, 4 and 7). We think that it was difficult to understand the content of the crispant groups as mentioned in the response to comments 6 above. We hope that the response to comment 6 will resolve this issue.

8. *Line 257-259: it seems loss of autopod region, as clearly shown by authors, accounts for expression shift. If the autopod region is mostly gone, there is the most straightforward (parsimonious) explanation that Hoxd11 is where it's supposed to be - in zeugopod which is shifted distally as autopod is not there.*

After consideration of this comment, the following text has been added to Lines 257-258.

“If the former is the case, then the zeugopod was in the most distal region.”

9. *Were there no germline Hoxd13 mutants generated? It would be interesting to know what germline single Hoxd13 mutants looked like.*

We generated germline *Hoxd13* mutants and showed the data in the main text (Lines 343- 352), the new Figs. 8 and S6.

10. *The commentary on potential evolutionary differences in Hoxa13/Hoxd13 in fin-to- limb are interesting in discussion, but might be shortened. The abstract should make less definitive claims, though the discussion is interesting.*

In accordance with comment 2 and this comment, the corresponding parts of discussion and abstract were shortened and changed respectively (Lines 475-509 and Lines 52). In the discussion, total of 143 words have been deleted.

Reviewer 3

"One major reason why genetic analyses have not been performed in non-mammalian tetrapods is a lack of reverse genetic techniques other than in rodents. In particular, gene disruption had not been achieved in animals that can regenerate their limbs." This statement is somewhat misleading as Crispr/Cas9 was used in X. laevis and axolotls before 2018 who both regenerate their limbs.

In accordance with the comments, the corresponding parts were revised (Lines 93-100).

Second decision letter

MS ID#: DEVELOP/2021/200282

MS TITLE: Newt Hoxa13 has an essential and predominant role in digit formation during development and regeneration

AUTHORS: Takashi Takeuchi, Haruka Matsubara, Fumina Minamitani, Yukio Satoh, Sayo Tozawa, Tomoki Moriyama, Kohei Maruyama, Ken-ichi T Suzuki, Shuji Shigenobu, Takeshi Inoue, Koji Tamura, Kiyokazu Agata, and Toshinori Hayashi

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This is a very interesting contribution to our existing knowledge about limb development and the evolutionary conservation of Hox gene function in skeletal patterning.

Comments for the author

The authors have very thoughtfully addressed the minor concerns raised in the first review.