An atlas of seven zebrafish hox cluster mutants provides insights into sub/neofunctionalization of vertebrate Hox clusters

Kazuya Yamada, Akiteru Maeno, Soh Araki, Morimichi Kikuchi, Masato Suzuki, Mizuki Ishizaka, Koumi Satoh, Kagari Akama, Yuki Kawabe, Kenya Suzuki, Daiki Kobayashi, Nanami Hamano and Akinori Kawamura
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Original submission

First decision letter

MS ID#: DEVELOP/2020/198325

MS TITLE: An atlas of zebrafish seven *hox* cluster mutants provides insights into sub/neofunctionalization of vertebrate *Hox* clusters

AUTHORS: Kazuya Yamada, Akiteru Maeno, Soh Araki, Morimichi Kikuchi, Masato Suzuki, Mizuki Ishizaka, Koumi Satoh, Kagari Akama, Yuki Kawabe, Kenya Suzuki, Daiki Kobayashi, and Akinori Kawamura

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.

As you will see, the reviews are mixed with two positive and one more critical review. All reviewers have criticisms and/or suggestions to improve your manuscript; reviewer 3 in particular would like more in depth analysis of some phenotypes to ensure that they are robust and that your conclusions are correct. After reading each other's reviews, this reviewer added a few more comments as below:

I realize that it may be difficult to get large n's with microCT, but n=3D1 or 2 with variable phenotypes is insufficient. With respect to pleural vertebrae the wild type situation is known to be quite variable: the number of ribs is in the range of 9-11, which means that vertebra 14 often carries a rib even in wild type (see PMID:14579374). Also the hemal arches that extend from the 15th vertebra are quite variable (see PMID: 32191876). The strong Weberian ossicle phenotype the authors describe in the hoxca cluster mutant is welloutside of normal wild type variability but the mutant phenotype may also be quite variable.

If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. 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.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Yamada et al., DEVELOP/2020/198325 This is a well planned and executed set of experiments. An original set of complete loss of function allelic series of zebrafish hox cluster mutants were derived and reported here in relevant detail, both in adults and in critical developmental stages. Phenotype documentations were acquired by state of the art techniques, all observations are limpid and well interpreted.

Comments for the author

A few small changes in textual presentation may help to improve the manuscript, some are suggested below:
1/page 4 line 6 internal instated of intemal
2/page 4 line 7- page 5 line 1 Building the rationale based on gene-by-gene comparisons is very laborious.
the complete cluster deletions are not easily exploitable to learn about correspondence at single gene level. Some other, simpler way to justify the project would seem preferable
3/page 5 line 7-9 it seems justified to list the original sources where individual murine Hox cluster deficiencies were reported for the first time, and not to complicate the introduction with compound mutants at this stage of the presentation.
4/page 5 line 12 deficiencies instead of mutants
5/page 5 line 19 common ancestors instead of primitive vertebrates
6/page 5 line 20 possessed instead of possess
7/page 5 line 21 second round instead of two rounds
8/page 6 line 21 each hox cluster loss of function alleles instead of seven hox cluster-deleted mutants
9/page 7 line 1-2, line 4 and elsewhere seven individual hox cluster deficiencies instead of individual seven hox cluster mutants.
10/page 9 line 5 The absence of hemizygous phenotypes of all mutant alleles should be stated. If applicable, it would be helpful to document any hemizygous adult deviation(s) from normal phenotypes in Supplemental Files.
11/page 16 line 13 evolutionary instead of evolutionally
12/page 16 line 17 specific instead of several
13/page 18 line 12 absence instead of deletion
14/page 27 line 18 reference should be completed
15/Care should be taken to compare similar deficiencies, if possible, e.g., in mice the deletion of Hoxb1-9 is not a complete HoxB cluster deficiency…

Reviewer 2

Advance summary and potential significance to field

Yamada and colleagues have explored the functions of the 7 Hox gene clusters of the zebrafish by deletion of each cluster from the genome. CRISPR/Cas has enabled this approach, but its achievement is still an impressive technical feat. The one cluster that the authors were unable to delete (Hoxcb) is the smallest, enabling deletion of the constituent genes. Thus, this is a comprehensive study of the phenotypic impact of removing each entire Hox cluster in turn, allowing direct comparison to the phenotypes associated with removing each of the four Hox clusters from mice.

The analysis involves assays of developing structures as well as high quality microCT scans of adult fish for those mutants that are viable. The phenotypes are considered primarily in the comparative context and reveal evidence of differential sub-functionalization in different lineages. For example, the Hoxd cluster is shown to be dispensable for fin development in zebrafish (with the two Hoxa clusters playing important roles), but not for limb development in mouse. The work is very nicely done, and while perhaps not a Â‘typicalÂ’ Development paper, makes an important contribution to our understanding of comparative developmental biology.

Comments for the author

There are a couple of obvious questions about axial patterning of the mutants that should be addressed to complete the study.

Major Points:
1. The authors make no comment on the precise AP position of the pelvic fins in the 5 viable mutants. While these look normal at first glance, this point should be considered, and if the position is unaffected this should be confirmed in a quantitative manner.

2. Another neglected question of interest is potential impact of loss of Hox clusters on the spinal cord/hindbrain boundary. Cdx4 mutants show a change in position of this boundary (Skromne et al 2007), and this is thought to be caused in part through changes in Hox gene expression, downstream of Cdx4. Analysis of motor neuron disposition with a suitable antibody - such as acetylated tubulin - would be one way to address this question.

3. In Fig. 3, the AP extent of the endochondral disk appears to be reduced in WT relative to the mutants, and in fact the whole disk looks small, a more representative example should be selected or if there is variation in the AP axis then it should be properly quantitated. The phenotypes should also be discussed in the light of published Hox expression data for the zebrafish fin – in particular the detailed analysis of Ahn and Ho in 2008.

Minor points:
Axial patterning of the vertebral column shows minor, but clear, homeotic phenotypes in a subset of the mutants. These are documented in the pleural ribs and there is also significant reduction of an element of the Weberian apparatus in the Hoxca cluster mutant – a specialized Ostariophysan structure, which I have personally hypothesized to be Hox influenced, so I was pleased to see that prediction borne out. However, this mutant also shows an intriguing increase in number of somites, as well as ultimate vertebra number, suggesting a role for this Hox cluster in posterior outgrowth – an issue that should be touched upon in the discussion.

The title would be better formatted as:
An atlas of seven zebrafish hox cluster mutants provides insights into sub/neofunctionalization of vertebrate Hox clusters

Reviewer 3

Advance summary and potential significance to field

In this manuscript, Yamada and colleagues generate homozygous deletions of each of seven Hox clusters in the zebrafish and complete an initial characterization of these mutants. The authors conclude that while zebrafish hox genes are required for similar developmental processes as mouse Hox genes, the allocation of functions to specific clusters has diverged during evolution. This was demonstrated previously in the case of hoxb1b which in fish carries the function of the mouse hoxa1 gene, however the current work extends this finding to other hox clusters. Furthermore, by looking at fish-specific anatomy of the Weberian apparatus, the authors identify a novel function for a gene(s) in the hoxca cluster in the development of the 3rd and 4th vertebrae.

While the work represents an impressive effort in generating seven independent hox cluster knockouts, the phenotypic characterization is broad and shallow, and limited largely to known or expected phenotypes (the loss of Mauthner neurons previously described in hoxb1a and hoxb1b mutants; jaw cartilage defects described in hoxb2a; hoxb2b double knock-downs, neuromast deposition defects described in hoxb8a mutants and vertebral defects predicted by mouse Hox mutants). Consequently, there are rather few major new insights to be gleaned, either about specific hox gene functions or about hox cluster evolution. I regret that I cannot recommend publication in Development.

Comments for the author

Specific criticisms:

The most novel aspect of the study is the micro-CT demonstration of vertebral transformations in viable zebrafish hox cluster deletions (hoxaa, hoxca, hoxda clusters), which though expected has never been described for single hox mutants in the zebrafish. Additionally, the defects in the Weberian apparatus, in hoxca mutants have not been described in individual hox mutants to my knowledge. However, it appears that the n's for these experiments are really very low: n=2 for the vertebral phenotypes and n=1 for the Weberian ossicle phenotype. Without more n's it is impossible to know how consistent these phenotypes are.

The characterization of the cranial cartilage phenotype is very superficial. The hoxab-/- mutant does not look normal in lateral view, and the nature of the cartilage defect in the hoxba-/- mutant is very unclear. Are there real changes in cartilage morphologies, or just in their orientation? The intact alcian preparations do not allow a full description of the defects. The authors should dissect the cartilages and measure the shapes and sizes of individual elements, the presence or absence of small elements like the interhyal, and note any fusions between elements.

Minor points:

The reduced tripus on the 3rd vertebra in the hoxca cluster deletion mutant is interpreted as an anterior homeotic transformation because it resembles the lateral process on the 2nd vertebra. However in losing its characteristic fan shape it equally (or more strongly) resembles the tp4 process on the 4th vertebrae. The authors should be more circumspect about their interpretation of this phenotype.

In the discussion, the authors hypothesize that hoxc6a and c6b may be responsible for defects in the formation of the Weberian apparatus, since their expression boundaries are at the 5th somite. Which vertebrae are derived from the 5th somite? If the 3rd vertebra is derived from a somite anterior to the anterior-most hox-6 expressing somite, are the authors proposing a non-autonomous effect of mutating it?
In the mouse, mutations of the hoxd3 gene cause transformations of the 1st and 2nd vertebrae, so it is not clear why the authors propose that a much more posterior hox

First revision

Author response to reviewers' comments

First of all, we would like to express our appreciation to the reviewers for their insightful comments, which have helped us to significantly improve our manuscript. We would like to answer the reviewers' comments as follows.

Reviewer 1 Comments for the Author:
Reviewer#1-1: page 4 line 6 internal instated of intimal

Response: We corrected the word (page 4, line 6).

Reviewer#1-2: page 4 line 7- page 5 line 1
Building the rationale based on gene-by-gene comparisons is very laborious.
the complete cluster deletions are not easily exploitable to learn about correspondence at single gene level. Some other, simpler way to justify the project would seem preferable

Response: Thank for this suggestion. We made this part in the Introduction more concise (page 4, lines 14-22).

Reviewer#1-3: page 5 line 7-9 it seems justified to list the original sources where individual murine Hox cluster deficiencies were reported for the first time, and not to complicate the introduction with compound mutants at this stage of the presentation.

Response: We appreciate the suggestion by the reviewer. In this sentence, we cited original papers describing the isolation of individual Hox cluster mutant mice for the first time (page 5, lines 1-2).

Reviewer#1-4: page 5 line 12 deficiencies instead of mutants

Response: Thank you for the suggestion. We replaced the word (page 5, line 5).

Reviewer#1-5: page 5 line 19 common ancestors instead of primitive vertebrates

Response: According to the reviewer's suggestion, we replaced the word (page 5, line 12).

Reviewer#1-6: page 5 line 20 possessed instead of possess

Response: Thank you for the comment. We corrected the word (page 5, line 12).

Reviewer#1-7: page 5 line 21 second round instead of two rounds

Response: According to the reviewer's suggestion, we replaced the word (page 5, line 14).

Reviewer#1-8: page 6 line 21 each hox cluster loss of function alleles instead of seven hox cluster-deleted mutants

Response: Thank you for the comment. We corrected the word (page 6, line 3).

Reviewer#1-9: page 7 line 1-2, line 4 and elsewhere seven individual hox cluster deficiencies instead of individual seven hox cluster mutants.

Response: We appreciate this comment by the reviewer. We tried to use seven individual *hox* cluster deficiencies instead of individual seven *hox* cluster mutants (page 7, lines 1-2, 4 etc).

Reviewer#1-10: page 9 line 5
The absence of hemizygous phenotypes of all mutant alleles should be stated. If applicable, it would be helpful to document any hemizygous adult deviation(s) from normal phenotypes in Supplemental Files.

Response: We appreciate this comment by the reviewer. Regarding the embryonic analysis, we already confirmed that the phenotypes of the hemizygous mutants are indistinguishable from those of sibling wild-type zebrafish. Therefore, we included sentences to mention this point in Figure legends (page 33, lines 6-7, 15-16, 20-21; page 35, lines 10-11). Micro-CT scan analysis was not carried out for hemizygous fish. However, we noticed that hemizygous fish for each hox cluster appears externally indistinguishable from wild-type fish. We added sentences to describe this point in the revised manuscript (page 20, lines 7-8).

Reviewer#1-11: page 16 line 13 evolutionary instead of evolutionally

Response: We are sorry for our mistake. We corrected the word (page 18, line 2).

Reviewer#1-12: page 16 line 17 specific instead of several

Response: Thank you for the suggestion. We replaced the word (page 18, line 6).

Reviewer#1-13: page 18 line 12 absence instead of deletion

Response: Thank you for the suggestion. We replaced the word (page 20, line 13).

Reviewer#1-14: page 27 line 18 reference should be completed

Response: We corrected the reference (page 30, lines 30-31).

Reviewer#1-15: Care should be taken to compare similar deficiencies, if possible, e.g., in mice the deletion of Hoxb1-9 is not a complete HoxB cluster deficiency…

Response: Thank you for the suggestion. So as not to confuse the readers, we would like to accurately use murine *Hoxb1-9*-deleted mutant instead of murine *HoxB* cluster mutant in the manuscript (page 2, line 12; page 14, lines 9, 13).

Reviewer 2 Comments for the Author: Major Points:
Reviewer#2-1: The authors make no comment on the precise AP position of the pelvic fins in the 5 viable mutants. While these look normal at first glance, this point should be considered, and if the position is unaffected this should be confirmed in a quantitative manner.

Response: We appreciate the comments by the reviewer. Regarding the five viable *hox* cluster mutants, we measured the relative position of the pelvic fin along the AP axis in a quantitative manner and included new data in Fig. S9.

Reviewer#2-2:. Another neglected question of interest is potential impact of loss of Hox clusters on the spinal cord/hindbrain boundary. Cdx4 mutants show a change in position of this boundary (Skromne et al 2007), and this is thought to be caused in part through changes in Hox gene expression, downstream of Cdx4. Analysis of motor neuron disposition with a suitable antibody - such as acetylated tubulin - would be one way to address this question.

Response: We agree with the reviewer that *Hox* genes may regulate the position of the spinal cord/hindbrain boundary. Since this is a very interesting and important issue in terms of the function of vertebrate *Hox* genes, we would like to examine the phenotype more carefully by using double or triple *hox* cluster homozygous mutants and report the results in the near future.

Reviewer#2-3: In Fig. 3, the AP extent of the endochondral disk appears to be reduced in WT relative to the mutants, and in fact the whole disk looks small, a more representative example should be selected or if there is variation in the AP axis then it should be properly quantitated. The phenotypes should also be discussed in the light of published Hox expression data for the zebrafish fin – in particular the detailed analysis of Ahn and Ho in 2008.

Response: Thanks you for pointing that out. We replaced the picture of wild-type with a more

representative one (Fig 3A'). In addition, the AP length of each endochondral disc was quantitatively examined and shown in Fig. 3Q. Furthermore, we discussed the phenotypes of pectoral fins by citing Ahn and Ho's paper (page 16, lines 10-24).

Minor points:
Reviewer#2-4: Axial patterning of the vertebral column shows minor, but clear, homeotic phenotypes in a subset of the mutants. These are documented in the pleural ribs, and there is also significant reduction of an element of the Weberian apparatus in the Hoxca cluster mutant - a specialized Ostariophysan structure, which I have personally hypothesized to be Hox influenced, so I was pleased to see that prediction borne out. However, this mutant also shows an intriguing increase in number of somites, as well as ultimate vertebra number, suggesting a role for this Hox cluster in posterior outgrowth - an issue that should be touched upon in the discussion.

Response: Thank you for the comments. In the discussion, we mentioned the possibility that the hox genes in the hoxca cluster may preferentially regulate posterior outgrowth (page 15, lines 21-24).

Review 2-5: The title would be better formatted as:
An atlas of seven zebrafish hox cluster mutants provides insights into sub/neofunctionalization of vertebrate Hox clusters

Response: We appreciate this suggestion by reviewer. According to the suggestion, we replaced the title.

Reviewer 3 Comments for the Author:

Specific criticisms:

Review 3-1: The most novel aspect of the study is the micro-CT demonstration of vertebral transformations in viable zebrafish hox cluster deletions (hoxaa, hoxca, hoxda clusters), which though expected has never been described for single hox mutants in the zebrafish. Additionally, the defects in the Weberian apparatus, in hoxca mutants have not been described in individual hox mutants to my knowledge. However, it appears that the n's for these experiments are really very low: n=2 for the vertebral phenotypes and n=1 for the Weberian ossicle phenotype.
Without more n's it is impossible to know how consistent these phenotypes are.

Response: Upon this critical comment, we additionally performed micro-CT analysis for viable *hox* mutants (n=2 for wild-type, n=2 for *hoxaa*-/-, n=1 for *hoxca*-/-, and n=2 for *hoxcb*-/-). For *hoxbb*-/- and *hoxda*-/- mutants, we could not obtain viable homozygous fish because the survival rate of *hox* cluster mutants is low, especially for *hoxbb* cluster mutants. In the revised manuscript, the total numbers of micro-CT analyses for the vertebral phenotypes and the Weberian apparatus of each mutant are as follows.

wild-type	n=7
hoxaa-/-	n=4
hoxbb-/-	n=2
hoxca-/-	n=3
hoxcb-/-	n=4
hoxda-/-	n=2

We analyzed these data and confirmed that the additional micro-CT data support our conclusions in the original manuscript.

This reviewer pointed out that the identity of the vertebral column in zebrafish is quite variable.

Although we admit that the vertebral identity of wild-type zebrafish is not always consistent, variation of the vertebral column is not so frequently observed, at least in our zebrafish (See Table S1). To show the reproducibility, we present 3D-movies of one wild-type and two mutant fish showing the phenotype of the Weberian apparatus and vertebral phenotypes (Movies 1-10). Following thie reviewer's suggestion, we touch upon the fact that vertebral identity is variable in zebrafish by citing a reference in the revised manuscript (page 12, lines 4-5). We also included a summary of vertebral phenotypes in Table S1.

Review 3-2: The characterization of the cranial cartilage phenotype is very superficial. The hoxab-/- mutant does not look normal in lateral view, and the nature of the cartilage defect in the hoxba-/- mutant is very unclear. Are there real changes in cartilage morphologies, or just in their orientation? The intact alcian preparations do not allow a full description of the defects. The authors should dissect the cartilages and measure the shapes and sizes of individual elements, the presence or absence of small elements like the interhyal,and note any fusions between elements.

Response: Thank you for pointing that out. We repeated Alcian blue staining and confirmed that craniofacial cartilage in *hoxab* mutants is indistinguishable from that in wild-type. As the picture may not be good, we replaced the picture with new ones (Fig. 2K and 2K').
To show the defects of jaw cartilages in *hoxba* cluster mutants more clearly, we took pictures of flat-mounted jaws and have shown them in Fig. S7.

Minor points:
Review 3-3: The reduced tripus on the 3rd vertebra in the hoxca cluster deletion mutant is interpreted as an anterior homeotic transformation because it resembles the lateral process on the 2nd vertebra. However in losing its characteristic fan shape it equally (or more strongly) resembles the tp4 process on the 4th vertebrae. The authors should be more circumspect about their interpretation of this phenotype.

Response: Thank you for the comments. We should have mentioned the phenotype of the Weberian apparatus in *hoxca* mutants in more detail. Morphologies of the 2nd and 4th vertebrae are quite different in zebrafish. The lateral process on the 2nd vertebra is non-bifurcated bone. On the other hand, the os suspenscrium (os) and the transverse process of vertebra 4 (tp4) are bifurcated from the bones on the 4th vertebra, and tp4 is further bifurcated (see Fig. 4 C). Our micro-CT scan analysis revealed that the fan-shaped tripus in *hoxca* cluster mutants is severely disrupted and the morphology of affected bones exhibits laterally-extending 'non-bifurcated' bones, although the shape of bones appears irregular. In addition, the lateral process is attached to the anterior portion of the centrum. The affected bones on the 3rd vertebra are similarly attached to the anterior portion of the centrum. These observations suggest that the altered bones on the 3rd vertebra of *hoxca* cluster mutants resemble the lateral process on the 2nd vertebra, which can be interpreted as anterior homeotic transformation. To explain the phenotype of *hoxca* cluster mutants in more detail, we added sentences in the manuscript (page 11, lines 7- 13).

Reviewer 3-4: In the discussion, the authors hypothesize that hoxc6a and c6b may be responsible for defects in the formation of the Weberian apparatus, since their expression boundaries are at the 5th somite. Which vertebrae are derived from the 5th somite? If the 3rd vertebra is derived from a somite anterior to the anterior-most hox-6 expressing somite, are the authors proposing a non-autonomous effect of mutating it? In the mouse, mutations of the hoxd3 gene cause transformations of the 1st and 2nd vertebrae, so it is not clear why the authors propose that a much more posterior hox.

Response: In zebrafish, the segmental relationship between somites and the vertebral column has been examined (Morin-Kensicki et al., Development, 2002). According to the results obtained by Eisen and colleagues, the first two pairs of somites do not contribute to the vertebral column in zebrafish. Therefore, we presume that the 3rd vertebra is mainly derived from the 5th somite, which corresponds to the anterior expression boundary of *hoxc6a* and *hoxc6b* genes (see Fig.6 in Morin-Kensicki et al). **[Information about unpublished data has been removed from the peer review report on the authors' request and in line with editorial policy.]**

We also recognize that the mutation of mouse *Hoxd3* results in homoeotic transformation of the cervical vertebrae in mice (Condie and Capecchi, Development, 1993). Although zebrafish possesses

hoxd3a, which is an ortholog of mouse *Hoxd3*, our *hoxda* cluster-deleted homozygous fish do not exhibit abnormal morphologies in the anteriormost vertebrae. We presume that there are functional differences of *Hox* genes between mice and zebrafish in formation of the vertebral column. We are now extensively investigating this point and hope that we can report the results in the near future.

Second decision letter

MS ID#: DEVELOP/2020/198325

MS TITLE: An atlas of seven zebrafish hox cluster mutants provides insights into sub/neofunctionalization of vertebrate Hox clusters

AUTHORS: Kazuya Yamada, Akiteru Maeno, Soh Araki, Morimichi Kikuchi, Masato Suzuki, Mizuki Ishizaka, Koumi Satoh, Kagari Akama, Yuki Kawabe, Kenya Suzuki, Daiki Kobayashi, Nanami Hamano, and Akinori Kawamura

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 referees' remaining concerns and comments can be satisfactorily addressed. Please attend to all of the reviewers' 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

A unique novel allelic series that should be instrumental in developmental genetic analysis of the Hox patterning system in zebrafish, a prominent model organism.

Comments for the author

I am pleased that the authors got and took the opportunity to revise this manuscript. Besides some minor points (see below) I recommend considering the following suggestion to further help the reader to grasp the potential uses of the presented hox cluster deficiencies by a more explicit exposition.

With respect to the evaluation of differential contribution of Hox genes in patterning the appendicular skeleton and the vertebral column in fish and mice, addition of a few phrases may be justified to complement the Discussion (at p18 line 18 perhaps).

Contribution of the hoxaa cluster was seen here in the fin fold, but not in the endoskeleton of early zebrafish, while the function of the hoxba cluster was unexpectedly revealed in the endoskeletal disk of pectoral fins. Several aspects of hoxa and hoxd involvement in zebrafish fin development

have been documented in the literature, but a function of the hoxb clusters seems a novel contribution of this manuscript.

The case of the hoxba cluster is further remarkable in that of all zebrafish clusters, hoxba is the only cluster in this species that contains a gene annotated as PG7, hoxb7a. Therefore, in homozygous hoxba deficient individuals no PG7 product should be present. From these premisses a hypothesis may be formulated, that the absence of hoxba cluster actually reflects to a large degree the absence of the hoxb7a gene product, which cannot be compensated for by any of the other clusters. Incidentally, the function of hoxb7a seems largely under-investigated, the loss of function allele has not yet been reported. In possession of this hoxba deficiency, and a presumably easily producible hoxb7a single mutant, the above hypothesis may be tested experimentally. A series of crosses may reveal precious information about genetic interaction between hoxb7a and other hoxba genes presumably avoiding lethality observed with the hoxba homozygous genotype. The results of such a genetic analysis could be profitably compared to an analogous condition which has been observed in mice in the case of Hoxa7 Hoxb7 double homozygous (doi.org/10.1016/S0925-4773(98)00126-9), which also represents a complete absence of PG7 gene product in mice. That genetic constitution revealed that in mouse both Hoxa7 and Hoxb7 redundantly contribute to upper thoracic and sternal patterning, but neither are required in forelimbs. Besides this apparent differential PG7 subfunctionalization in appendicular skeleton development, important novel information may ensue from the same crosses, concerning the genetic control of cervico-thoracic transition along the vertebral column, which is markedly different between fish and mammals. In the skeletal analyses of the five viable alleles, no obvious defects were observed in the anterior vertebral column, besides the Weberian apparatus, which was heavily dependent on hoxca. The single gene/cluster balanced mutants may hold the key to such advance, using the hoxba allele, but similar crosses may be planned exploiting hoxab, the other lethal allele as well.

Minor points:

p2 line 2
… Hox genes that specify positional identity along the body axis.
replace by
… Hox genes that control morphology and developmental timing along multiple body axes.

p2 line 8-14
… revealed functional discrepancies of… truncation of the limbs.
replace by
… revealed several species specific functional contributions of homologous Hox clusters along the appendicular axis, while important shared general principles were also confirmed, as exemplified by serial anterior vertebral transformations along the main body axis, observed in fish for the first time.

p2 line 16
…the ancient Hox cluster.
replace by
…the ancestral Hox cluster. This set of seven complete Hox cluster loss of function alleles provide a formidable resource for future developmental genetic analysis of the Hox patterning system in zebrafish.

p11 line 16 and other places
…suspenscrium… occurs systematically at several places,
…suspensorium… seem to be the appropriate choice ?

p15 line 18
…combinatorial…
change to
…combined…

p16 line 2
…results in anterior transformation……
change to

…results in malformations and fusions of anterior vertebrae…

p16 line 11
…to pattern the forelimb……
change to
…to pattern the distal limbs…

Reviewer 2

Advance summary and potential significance to field

Yamada and colleagues have explored the functions of the 7 Hox gene clusters of the zebrafish by deletion of each cluster from the genome. CRISPR/Cas has enabled this approach, but its achievement is still an impressive technical feat. The one cluster that the authors were unable to delete (Hoxcb) is the smallest, enabling deletion of the constituent genes. Thus, this is a comprehensive study of the phenotypic impact of removing each entire Hox cluster in turn, allowing direct comparison to the phenotypes associated with removing each of the four Hox clusters from mice.

The analysis involves assays of developing structures as well as high quality microCT scans of adult fish for those mutants that are viable. The phenotypes are considered primarily in the comparative context and reveal evidence of differential sub-functionalization in different lineages. For example, the Hoxd cluster is shown to be dispensable for fin development in zebrafish (with the two Hoxa clusters playing important roles), but not for limb development in mouse. The work is very nicely done, and while perhaps not a Â‘typicalÂ’ Development paper, makes an important contribution to our understanding of comparative developmental biology.

Comments for the author

The authors have done a thorough job of responding to reviewer comments, and the manuscript has been much improved as a consequence. In my opinion it is appropriate to publish once some minor issues with wording have been resolved.

Specifically:
p4. Line 18: "at the levels of whole Hox GENES remains to be determined" - I think genes ought to read clusters here.
p. 4 Line 25 "(For the murine..: - The F should not be capitalized.

p5 Line 8 "For viable five hox cluster homozygous adult fish,.. " would read better as "For adult fish of the five viable Hox cluster homozygous mutants,.."

p7 Line 4" Characterization of zebrafish seven individual hox cluster deficiencies during embryogenesis" would read better as " Characterization of seven individual zebrafish hox cluster deficiencies during embryogenesis"

In addition, I recommend careful proof reading by a native English speaker.

Reviewer 3

Advance summary and potential significance to field

The paper describes the phenotypes of whole Hox-cluster mutants generated by CRISPR/Cas9 in the zebrafish.

Comments for the author

The authors have addressed my main criticisms by adding more individuals to the phenotypic analysis. Overall, while the phenotypic characterization remains superficial, this criticism is offset

by the impressive effort involved in making all of these whole-cluster mutants. I am satisfied with the changes and can recommend moving forward with publication in Development. I hope that the authors will make these valuable mutants available to the community via an international stock center.

Second revision

Author response to reviewers' comments

First of all, we would like to express our appreciation to the reviewers for their insightful comments, which have helped us to significantly improve our manuscript. We would like to answer the reviewers' comments as follows.

Reviewer 1 Comments for the Author:

Reviewer#1-1: With respect to the evaluation of differential contribution of Hox genes in patterning the appendicular skeleton and the vertebral column in fish and mice, addition of a few phrases may be justified to complement the Discussion (at p18 line 18 perhaps).

Response: Thank you for the suggestion. We added the words at the end of Discussion (page 18, lines 20-23).

Reviewer#1-2: Contribution of the hoxaa cluster was seen here in the fin fold, but not in the endoskeleton of early zebrafish, while the function of the hoxba cluster was unexpectedly revealed in the endoskeletal disk of pectoral fins. Several aspects of hoxa and hoxd involvement in zebrafish fin development have been documented in the literature, but a function of the hoxb clusters seems a novel contribution of this manuscript.
The case of the hoxba cluster is further remarkable in that of all zebrafish clusters, hoxba is the only cluster in this species that contains a gene annotated as PG7, hoxb7a. Therefore, in homozygous hoxba deficient individuals no PG7 product should be present. From these premisses a hypothesis may be formulated, that the absence of hoxba cluster actually reflects to a large degree the absence of the hoxb7a gene product, which cannot be compensated for by any of the other clusters. Incidentally, the function of hoxb7a seems largely under-investigated, the loss of function allele has not yet been reported. In possession of this hoxba deficiency, and a presumably easily producible hoxb7a single mutant, the above hypothesis may be tested experimentally. A series of crosses may reveal precious information about genetic interaction between hoxb7a and other hoxba genes presumably avoiding lethality observed with the hoxba homozygous genotype. The results of such a genetic analysis could be profitably compared to an analogous condition which has been observed in mice in the case of Hoxa7 Hoxb7 double homozygous (doi.org/10.1016/S0925-4773(98)00126-9), which also represents a complete absence of PG7 gene product in mice. That genetic constitution revealed that in mouse both Hoxa7 and Hoxb7 redundantly contribute to upper thoracic and sternal patterning, but neither are required in forelimbs. Besides this apparent differential PG7 subfunctionalization in appendicular skeleton development, important novel information may ensue from the same crosses, concerning the genetic control of cervico-thoracic transition along the vertebral column, which is markedly different between fish and mammals. In the skeletal analyses of the five viable alleles, no obvious defects were observed in the anterior vertebral column, besides the Weberian apparatus, which was heavily dependent on hoxca. The single gene/cluster balanced mutants may hold the key to such advance, using the hoxba allele, but similar crosses may be planned exploiting hoxab, the other lethal allele as well.

Response: We deeply appreciate the reviewer for these valuable comments. Following the suggestions, we added several sentences to discuss the phenotype of zebrafish *hoxba* cluster mutants (page 14, lines 9-13). As the reviewer pointed out, we've been interested in the only PG7, *hoxb7a* in zebrafish. As we isolated *hoxb7a* mutants, we would like to describe our results in near future.

Reviewer#1-3:p2 line 2
… Hox genes that specify positional identity along the body axis.
replace by
… Hox genes that control morphology and developmental timing along multiple body axes.

Reviewer#1-4:p2 line 8-14
… revealed functional discrepancies of… truncation of the limbs. replace by
… revealed several species specific functional contributions of homologous Hox clusters along the appendicular axis, while important shared general principles were also confirmed, as exemplified by serial anterior vertebral transformations along the main body axis, observed in fish for the first time.

Reviewer#1-5:p2 line 16
…the ancient Hox cluster. replace by
…the ancestral Hox cluster. This set of seven complete Hox cluster loss of function alleles provide a formidable resource for future developmental genetic analysis of the Hox patterning system in zebrafish.

Response: We really appreciate these suggestions by this reviewer. We corrected the sentences in Abstract (page 2, lines 1-2, lines 8-12, lines 14-16).

Reviewer#1-6:p11 line 16 and other places

…suspenscrium… occurs systematically at several places,
…suspensorium… seem to be the appropriate choice ?

Response: We are sorry for our mistake. We corrected the word (page 11, line 16; page 34, line 13).

Reviewer#1-7:p15 line 18
…combinatorial…
change to
…combined…

Response: Thank you for pointing it out. We corrected the word (page 15, line 18).

Reviewer#1-8:p16 line 2
…results in anterior transformation…… change to
…results in malformations and fusions of anterior vertebrae…

Response: Thank you for the suggestion. We replaced the word (page 16, lines 7-8).

Reviewer#1-9:p16 line 11
…to pattern the forelimb…… change to
…to pattern the distal limbs…

Response: Thank you for the suggestion. We added the word (page 16, line 16).

Reviewer 2 Comments for the Author:
Reviewer#2-1: p4. Line 18: "at the levels of whole Hox GENES remains to be determined" - I think genes ought to read clusters here.

Response: Thank you for the comment. We replaced the word (page 4, line 18).

Reviewer#2-2: p. 4 Line 25 "(For the murine..: - The F should not be capitalized.

Response: We are sorry for our mistake. We corrected the word (page 4, line 24).

Reviewer#2-3: p5 Line 8 "For viable five hox cluster homozygous adult fish,.. " would read

better as "For adult fish of the five viable Hox cluster homozygous mutants,.."

Response: According to the reviewer's suggestion, we replaced the word (page 5, line 8).

Reviewer#2-4: p7 Line 4" Characterization of zebrafish seven individual hox cluster deficiencies during embryogenesis" would read better as " Characterization of seven individual zebrafish hox cluster deficiencies during embryogenesis"

Response: Thank you for the suggestion. We replaced the word (page 7, lines 4-5).

Reviewer 3 Comments for the Author:

Reviewer#3-1: I hope that the authors will make these valuable mutants available to the community via an international stock center.

Response: Thank you for the comment. The National BioResouce Project (Japan), in which the frozen sperms of our mutant fish have been deposited, internationally distributes mutant fish. In the case of more requests, we would like to deposit our mutants in other stock centers such as ZIRC.

Third decision letter

MS ID#: DEVELOP/2020/198325

MS TITLE: An atlas of seven zebrafish hox cluster mutants provides insights into sub/neofunctionalization of vertebrate Hox clusters

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ARTICLE TYPE: Techniques and Resources Article

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