Hox13 genes are required for mesoderm formation and axis elongation during early zebrafish development

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MS TITLE: hox13 genes are required for mesoderm formation and axis elongation during early zebrafish development

AUTHORS: Zhi Ye and David Kimelman

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 referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. The reviewers appreciate the importance of the observation that Hox13 genes are required to maintain axis elongation. Nevertheless, Referee 1 and 2 raise the question of what effect Hox13 attenuation has in a normal wild type ntl background. I also think this is an important question to address. Similar to the referees, I was also confused about whether the appropriate genotypes are used in some of the comparisons, notably Fig 3 and Fig 7. For example, comparing the effects of Hox13b over expression in wild-type and ntlcs would be helpful. In addition, given recent evidence (from Stanier and colleagues) of unintended consequences of CRISPR mutations on the expression of related genes, it seems important to investigate whether there is a similar effect in the mutants you describe.

The referees also have several additional questions and comments that should help you strengthen and clarify your study.If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

This study by Ye and Kimelman examines the role of members of Hox13 paralogous group in embryonic axis elongation in the zebrafish. Based on data from various overexpression studies in amniote embryos, Hox13 genes have been proposed to play a key role in the termination of embryonic axis elongation by regulating the balance between axial progenitor proliferation/apoptosis and differentiation, though antagonism with pro-axis elongation transcriptional regulators and signals (e.g. Cdx2 and Wnt). Unexpectedly, the study shows that this is not the case in the fish. The authors demonstrate that the expression of some Hox13 members (Hoxa13b, Hoxd13a) starts earlier than expected, by the end of gastrulation/early somitogenesis. They then show that CRISPR-based inactivation (via removal of Homeobox binding site) of these two genes results in a range of posterior defects which are mainly revealed by placing the mutants in lower temperatures. The authors subsequently show that these effects are taking place in mutant fish also carrying a cold sensitive mutation of the Tbxta gene (ntlcs) suggesting that the Hox mutations enhance the impact of the ntlcs mutation at lower temperatures. Mechanistically the Hox deficient phenotype appears to be a consequence of attenuated paraxial mesoderm production from neuromesodermal progenitors (NMPs) due to reduced Wnt signalling and expanded RA signalling activity which result in a switch from mesoderm to neurectoderm (indicated by expanded Sox2 expression) as well as defective cell movements. The latter phenotype can be rescued by grafting of mutant cells in a wild type (wt) environment suggesting non-cell autonomous effects. Surprisingly, the authors also demonstrate that Hoxa13b overexpression also results in severe posterior defects and reduction of key axis elongation genes similar to the loss-of-function phenotype. Overall the manuscript is well-written and the experiments and resulting data support to a large extent the conclusions made by the authors. The study makes an important point about the interpretation of Hox overexpression experiments and potentially sheds light to a previously unappreciated role for Hox13 genes in axis elongation.

Comments for the author

I would like to comment on a few technical issues that require further clarification/attention:
1) As far as I am aware Hoxa13/Hoxd13 double mutant mice have not been reported to exhibit posterior truncations (e.g. Fromantail-Ramain et al Development 1996). On the contrary, the effect of the inactivation of the two equivalent Hox13 members in an ntlcs background in this study looks obvious suggesting potential differences between amniote and anamniote embryos. However, I am wondering whether the authors have tried the Hox13 attenuation experiments in a normal wild type ntl background. I am aware that they provide evidence of a posterior defect phenotype following Hox13 attenuation at a high temperature correlating to a “normal” ntl embryo phenotype but my worry is that there may be secondary effects of the ntlcs mutation that could have been missed making the interpretation of the Hox13 mutation effects challenging. Also it is unclear whether the effect observed on the expression of various tested markers (e.g. mesodermal/Wnt associated transcripts- see Fig.4 and 7d) is at least partly due to the ntlcs mutation. Showing expression data for these markers in ntlcs only controls would be informative. More importantly, repeating the Hox 13 attenuation experiments in a wt ntl background would shed light to the role of these genes in a more definitive manner.
2) If I am not mistaken the overexpression (OE) experiments in Fig 7 were done in a normal wt ntl background so I am wondering about the value of the comparison of the qPCR data in Fig 7d showing expression of affected genes under Hoxa13b OE in wt ntl background vs mutant Hoxa13b mutant in a ntlcs background. In my opinion, it would be more informative to compare Hoxa13b OE data in ntlcs fish versus their Hoxa13b mutant counterparts or alternatively present data from experiments in a wt ntl context (see point above).
3) The incorporation of some additional evidence about the specificity of the CRISPR-based gene inactivations would strengthen the study. Could the observed phenotypes be attributed to CRISPR-associated off-target effects? For example a rescue experiment involving overexpression of the

functional form of the affected Hox13 gene(s) in a mutant background could address the specificity issue.
4) Given the reported role of trunk Hox genes as positive regulators of axis elongation (Young et al Dev Cell 2009) and the potential (direct or indirect) interaction between different Hox members (e.g. Zhou et al PloS One 2017) it would be interesting to examine how the expression of trunk Hox members is affected in the Hox13 inactivation and OE experiments.

Reviewer 2

Advance summary and potential significance to field

In this study, the authors investigated the effect of the combined loss of hoxa13a and hoxd13b on the elongation of the zebrafish body axis. They also discover a novel genetic interaction with a temperature sensitive mutation in tbxta. Overall, this is an important contribution to the field since, despite their extensive study across a range of species, the requirement of Hox genes during posterior body axis elongation has not been elucidated. Most studies so far have focussed on using over-expression of hox genes at non-physiological levels. This has lead to the proposition that posterior Hox genes are important for terminating posterior body extension in vertebrates. However, the authors clearly point out that the expression of genes in the Hox13 class commences in the tailbud much earlier that would be expected for a role only in the termination of axis elongation. By examining the phenotypic consequences of hoxa13a and hoxd13b loss of function, the authors conclude that they are instead required for maintenance of neuromesodermal progenitors due to the increased expression of the neural marker sox2 at the expense of mesoderm. Transplant experiments are performed that suggest that this function is non-cell autonomous.

Comments for the author

Overall, I would support this work being published in Development, however there are several major points of concern that should be addressed prior to acceptance.

Major comments

1) The double mutant phenotype results in a dramatic tail defect in which almost a third of the body axis is missing. It is difficult to understand how this absence of tissue can be solely explained by an imbalance between neural and mesodermal specification. Could the authors provide more information on the timing in the onset of this phenotype, and ascertain whether an increases apoptosis of cells is occurring either prior to, or after the alterations in gene expression observed?

2) The analysis of Wnt pathway inhibitor expression is interpreted as an ’extension’ of these gene expression domains into the tailbud. However, the observed changes in expression patterns could equally be a consequence of a loss of cells within the tailbud, leading to a relative shift in these domains. Could the authors provide additional reference points with which to measure these expression domains against to determine whether they indeed are a posterior-directed expansion?

3) Transplant experiments are shown in which mutant cells are transplanted into wild-type embryos. In-line with the concerns over alterations in tailbud size/cell number raised above, it could be informative to determine the average size of each transplanted clones between wild type and mutant donors. Furthermore, were reverse transplants performed, in which wild type cells are transplanted into mutants. This would further strenghten the argument about cell autonomy.

4) In the generation of the CRISPR mutant lines, how did the authors control for off-target effects?

5) While a number different markers of paraxial mesoderm have been observed to be reduced in the mutants, only one marker of neural tissue (sox2) is observed to be expanded into the mesodermal territory. As the interpretation of the mutant phenotype rests heavily on the disruption of a neural vs. mesodermal cell fate decision, more markers of neural tissue should be examined to determine the degree to which cells have been re-specified as neural tissue or whether the transformation is restricted to only sox2.

Minor comments

1) 3B,C axis labels too small

2) Figure 4. Images have a very white background that makes it difficult to see the shape of the tailbuds in each of the images (Particularly for B-D). Is it possible to reduce the contrast slightly, or indicate the edge of the tailbud in each case?

Reviewer 3

Advance summary and potential significance to field

In this study, Ye and Kimelman, investigate the role of posterior Hox13 genes in the development of the posterior body. Using a loss of function approach (CRISPR/Cas9 system) in zebrafish embryos they suggest that Hox13 genes provide robustness to the Brachyury dependent NMp niche, which maintains Wnt signalling and prevents RA from accumulating in the tailbud. Furthermore, using overexpression studies they provide evidence that the overexpression of Hox13 genes has similar disruptive effects in the maintenance of the niche. Collectively, their data suggest that Hox13 genes are important for the maintenance of the niche and mesoderm formation in the fish. While these observations are interesting, further investigation is required to understand the mechanism by which Hox13 genes are involved in the process of axis elongation. An important concern is that the use of the ntlcs line makes the interpretation of the results difficult due to the large phenotypic variability caused by the different levels of Brachyury expression in these mutants. The generation of a knockout line of all Hox13 genes would be essential to clarify the role of these genes in axis elongation and the maintenance of NMp niche in zebrafish.

Comments for the author

Major comments

- The authors suggest that hoxa13b and hoxd13a are the most abundant of the hox13 genes in the tail. However, they don't show the QPCR data and from the in situs it is not clear if this is really the case. Additionally, even if this is true, it doesn't exclude the possibility that the other hox13 genes are equally important for tail development.

- What is the phenotype of the double mutant double homozygote hoxa13bΔ16/Δ16;hoxd13ains4/ins4? The authors claim one fish survived, but they do not present evidence that it has a more severe phenotype at early stages (Page 6);

- What is the phenotype of the Hoxd13a knockouts? Are there any posterior defects in the ntlcs ; Hoxd13a-/- ?

- In Figure 3C, the authors use as wild type control the no tail cold sensitive and the hox13 mutations are introduced in the same genetic background. Therefore, when they analyze the somites of the hox13 mutants at 21C and 18C they can't exclude that any effect they see is actually due to the no tail mutation.

- The authors observe mesodermal defects in ntlcs; Hoxa13-/- embryos maintained at 18.5 °C as compared to wt embryos. Are the same mesodermal defects observed in the ntlcs embryos at 18.5°C? The authors in Figure 4 should show in parallel in situs of the ntlcs embryos developed at 18.5 °C.

- In Figure 4, it's not clear what is the wildtype control that the authors use?

- In figure 4, although the authors show a reduction in the levels of tbxta in the NMp area, it would be necessary to complement this with an immunostaining for NMps to show conclusively that they are not being maintained;

- The phenotypes that the authors observe from the crosses between hoxa13 and hoxd13 mutants are very variable and largely depend on the ntlcs mutation at low temperatures. This makes it difficult to identify which of the observed effects are caused by the lower levels of brachyury or the specific Hox13 mutation. The authors should analyse the effect of eliminating all hox13 genes in a wild type background (and not in the ntlcs background). This will provide a more informative answer on the role of hox13 genes in the regulation of Brachyury and maintenance of the niche during axial elongation.

- In ntlcs; Hoxa13-/- embryos, have the authors observed any effect in the proliferation or cell death of tailbud cells?

Minor comments

- P4. Last paragraph "A major caveat….with the one exception of the mouse Hoxb13 mutant that causes a minor increase in the number of somites"
The Hoxb13 mutant mice, apart from the 2 additional somites, have overgrowth of neural structures (wider and longer spinal cord), ectopic dorsal root ganglia. Thus the effects are more pronounced than those that the authors describe in the manuscript.

- In all figures the authors should clearly indicate the genotype of the fish that they use as a wild type.

- In figure 3B column 6, the authors state that the phenotypes shown are the result of a cross between two homozygotes, but in the legend it's marked as a homozygous x heterozygous cross;

- Page 8, refers to Figure 4B when it should be 4A; "Interestingly, the expression of tbxta in the notochord (Figure 4B, black arrowhead)"

- In Figure 7D, the qPCR data are missing error bars; Is this only one experiment?

First revision

Author response to reviewers' comments

Response to Reviewer 1

We are grateful to the reviewer for the positive comments about the importance of the work and the quality of the writing, as well as the helpful comments. The reviewer may also be interested to know that at an international EMBO meeting in early September another group presented unpublished studies using a very different approach in mouse embryos that has come to the same general conclusion about the role of the *hox* genes in promoting axis extension as we propose here, demonstrating that this is a conserved feature in vertebrates.

1A) As far as I am aware Hoxa13/Hoxd13 double mutant mice have not been reported to exhibit posterior truncations (e.g. Fromantail-Ramain et al Development 1996). On the contrary, the effect of the inactivation of the two equivalent Hox13 members in an ntlcs background in this study looks obvious suggesting potential differences between amniote and anamniote embryos.
However, I am wondering whether the authors have tried the Hox13 attenuation experiments in a normal wild type ntl background. I am aware that they provide evidence of a posterior defect phenotype following Hox13 attenuation at a high temperature correlating to a "normal" ntl embryo phenotype but my worry is that there may be secondary effects of the ntlcs mutation that could have been missed making the interpretation of the Hox13 mutation effects challenging.

The reviewer is indeed correct that mouse *Hoxa13;Hoxd13* mutants do not show posterior defects, although a caveat is that we don't know if these are the most abundant *Hox13* genes in the mouse tailbud as is true in zebrafish. And as mentioned above, work will eventually be published showing

that the mouse is not different than fish with regards to the role of the *Hox13* genes. However, with regards to the reviewer's specific concern, we have backcrossed our *hoxa13;hoxd13* mutant into a wild-type background (since it took multiple crosses that is the reason it has taken so long to produce the revised manuscript). We now show that the *hoxa13;hoxd13* mutation in a *ntl* wt background produces embryos that are completely normal similar to that observed in mouse. Importantly, we show that embryos from a *hoxa13;hoxd13* mutation in a *ntl* wt background are hypersensitive to a *ntl* morpholino with respect to axis truncation, providing an independent confirmation that a loss of Hoxa13 and Hoxd13 interacts with the Ntl-Wnt autoregulatory loop, and that this is not due to a secondary effect of the *ntl cs* mutation. This important new data is provided in a new section of the paper on page 8 and Supplementary Figure S3. We also modified the text in the Abstract and Introduction to account for these results.

1B) Also it is unclear whether the effect observed on the expression of various tested markers (e.g. mesodermal/Wnt associated transcripts- see Fig.4 and 7d) is at least partly due to the ntlcs mutation. Showing expression data for these markers in ntlcs only controls would be informative.

We agree with the reviewer and have made a new Figure S4 that presents these results for comparison.

2) If I am not mistaken the overexpression (OE) experiments in Fig 7 were done in a normal wt ntl background so I am wondering about the value of the comparison of the qPCR data in Fig 7d showing expression of affected genes under Hoxa13b OE in wt ntl background vs mutant Hoxa13b mutant in a ntlcs background. In my opinion, it would be more informative to compare Hoxa13b OE data in ntlcs fish versus their Hoxa13b mutant counterparts or alternatively present data from experiments in a wt ntl context (see point above).

Based on the reviewer's comment we have now examined the Hoxa13b OE in the backgrounds of ntl wt, ntl cs/+ and ntl cs/cs, and we find the same phenotype in all cases. That makes sense with what we also know about ntl cs, since after the heat shock (at 40 C), we raised the embryos at the permissive temperature (29 C), where we would not expect a phenotype from the ntl cs mutation. We have added text to the Figure 7 legend to address this point.

3) The incorporation of some additional evidence about the specificity of the CRISPR-based gene inactivations would strengthen the study. Could the observed phenotypes be attributed to CRISPR-associated off-target effects? For example, a rescue experiment involving overexpression of the functional form of the affected Hox13 gene(s) in a mutant background could address the specificity issue.

We have looked into this further based on the reviewer's comment. The *hoxd13a* gRNA has no targets with 1 or 2 mismatches and only a single target with 3 mismatches, but that target is located in the middle of an intron in *deltaD*, and there is no reason to think based on the published literature that DeltaD would affect the Wnt/Brachyury loop. The *hoxa13b* gRNA has no targets with 1 mismatch, and only one target with 2 mismatches, but that is in a long non- coding RNA. It also three targets with 3 mismatches, with two of those in introns or UTRs. It does have one target that could possibly be significant based on position in the transcript, which is the intron-exon junction of hydroxysteroid (17-beta) dehydrogenase 2, a protein not studied in embryos. We sequenced this just to be sure and found no sequence changes in our fish. But most importantly, none of the genes that the *hoxa13b* or *hoxd13a* gRNAs might target in an off-target scenario are expressed in the tailbud based on RNA-seq data we have from wildtype tailbud explants. Finally, we note that we isolated 3 independent *hoxd13a* mutants (in the *hoxa13b* mutant background) and all give the same phenotype and all enhance the *hoxa13b* phenotype. Thus, based both on possible off-targets, their lack of expression in the tailbud and the phenotypes of independent isolates, we do not believe that there is any reason to consider off targets as a cause for the observed phenotypes, but it is good that we went through and checked all of this.

4) Given the reported role of trunk Hox genes as positive regulators of axis elongation (Young et al Dev Cell 2009) and the potential (direct or indirect) interaction between different Hox members (e.g. Zhou et al PloS One 2017) it would be interesting to examine how the expression of trunk Hox members is affected in the Hox13 inactivation and OE experiments.

Based on the reviewer's comments we checked several trunk *hox* genes by qPCR in both gain and loss of function experiments. Indeed, we found that overexpression of Hoxa13b did suppress the expression of all the trunk *hox* genes we examined, but in the loss of function experiments we saw very little or no change in expression. We can't, of course, rule out that other three Hox13 factors (a13a, c13a and c13b) are providing redundancy in the loss of function experiments, but we would say that the phenotype we observe in our mutants is not due to changes in trunk hox gene expression.

Response to Reviewer 2

We are grateful to the reviewer for supporting publication. The reviewer may also be interested to know that at an international EMBO meeting in early September another group presented unpublished studies using a very different approach in mouse embryos that has come to the same general conclusion about the role of the *hox* genes in promoting axis extension as we propose here, demonstrating that this is a conserved feature in vertebrates.

1) The double mutant phenotype results in a dramatic tail defect in which almost a third of the body axis is missing. It is difficult to understand how this absence of tissue can be solely explained by an imbalance between neural and mesodermal specification. Could the authors provide more information on the timing in the onset of this phenotype, and ascertain whether an increases apoptosis of cells is occurring either prior to, or after the alterations in gene expression observed?

We agree with the reviewer that this is a dramatic change in phenotype, and except for the fact that the notochord is normal in our mutants, this is the same phenotype as is observed in the *ntl* null mutants. The phenotype (loss of tail) seems to be due in part to the fact that the extra neural tissue cannot extend the body as can the mesoderm, which is lost in all of these mutants. In addition, there is indeed increased apoptosis in the neural tissue of *ntl* null mutants at late-somitogeneis stages, but not at the mid-somitogenesis stages when the mesoderm-to-neural transformation phenotype is clearly observed (Marlow, 2004). We checked for apoptosis during the mid-somitogenesis stages in our strongly affected mutants (the stages when we examined gene expression) and saw little apoptosis, just as is seen in wildtype embryos, but did observe increased apoptosis at late somitogenesis stages, just as with the *ntl* null mutants, again supporting the view that the strongly affected *hoxa13;d13* mutants are the same as *ntl* null mutants except for the notochord.. As for changes in gene expression, we were able to see changes in *ntl* expression as early as the 8-12 somite stage, but we found that 15 somite stage embryos were the easiest to visualize since one can get good images from the lateral side.

2) The analysis of Wnt pathway inhibitor expression is interpreted as an 'extension' of these gene expression domains into the tailbud. However, the observed changes in expression patterns could equally be a consequence of a loss of cells within the tailbud, leading to a relative shift in these domains. Could the authors provide additional reference points with which to measure these expression domains against to determine whether they indeed are a posterior- directed expansion?

We checked for apoptosis in the tailbud and there is no loss of cells causing the genes to shift. However, we think the wording was not good and we have changed the text to say that "the expression of both genes was found to be closer to the posterior end of the embryo" (page 9), which is clear from the in situs. The only point we had intended to make is that if the Wnt inhibitors are closer to the Wnt source at the back end of the embryo, however that occurs, that is likely to diminish the overall levels of active Wnt ligand at the posterior end. We appreciate the clarification.

3) Transplant experiments are shown in which mutant cells are transplanted into wild-type embryos. In-line with the concerns over alterations in tailbud size/cell number raised above, it could be informative to determine the average size of each transplanted clones between wild type and mutant donors. Furthermore, were reverse transplants performed, in which wild type cells are transplanted into mutants. This would further strengthen the argument about cell autonomy.

We transplanted 30-50 cells and we have added that to the text. We did try the reverse transplant as the reviewer suggests but as there is no muscle cells at the back of the embryo and only a lump

of neural tissue, we did not feel that we could get any really informative results.

4) In the generation of the CRISPR mutant lines, how did the authors control for off-target effects?

We have looked into this further based on the reviewer's comment. The *hoxd13a* gRNA has no targets with 1 or 2 mismatches and only a single target with 3 mismatches, but that target is located in the middle of an intron in *deltaD*, and there is no reason to think based on the published literature that DeltaD would affect the Wnt/Brachyury loop. The *hoxa13b* gRNA has no targets with 1 mismatch, and only one target with 2 mismatches, but that is in a long non- coding RNA. It also three targets with 3 mismatches, with two of those in introns or UTRs. It does have one target that could possibly be significant based on position in the transcript, which is the intron-exon junction of hydroxysteroid (17-beta) dehydrogenase 2, a protein not studied in embryos. We sequenced this just to be sure and found no sequence changes in our fish. But most importantly, none of the genes that the *hoxa13b* or *hoxd13a* gRNAs might target in an off-target scenario are expressed in the tailbud based on RNA-seq data we have from wildtype tailbud explants. Finally, we note that we isolated 3 independent *hoxd13a* mutants (in the *hoxa13b* mutant background) and all give the same phenotype and all enhance the *hoxa13b* phenotype. Thus, based both on possible off-targets, their lack of expression in the tailbud and the phenotypes of independent isolates, we do not believe that there is any reason to consider off targets as a cause for the observed phenotypes, but it is good that we went through and checked all of this.

5) While a number different markers of paraxial mesoderm have been observed to be reduced in the mutants, only one marker of neural tissue (sox2) is observed to be expanded into the mesodermal territory. As the interpretation of the mutant phenotype rests heavily on the disruption of a neural vs. mesodermal cell fate decision, more markers of neural tissue should be examined to determine the degree to which cells have been re-specified as neural tissue, or whether the transformation is restricted to only sox2.

This is a very good point and we examined two more markers, *sox19a* and *pou5f3/oct4*. We also found both of these markers to be expanded into the prospective mesodermal territory like *sox2*, and we have included this new data as Figure S7.

1) 3B,C axis labels too small

We have fixed this.

2) Figure 4. Images have a very white background that makes it difficult to see the shape of the tailbuds in each of the images (Particularly for B-D). Is it possible to reduce the contrast slightly, or indicate the edge of the tailbud in each case?

Good suggestion, thank you. We have added a dashed line to indicate the posterior end of the tailbud.

Response to Reviewer 3

We are grateful to the reviewer for the helpful comments on the manuscript. The reviewer may also be interested to know that at an international EMBO meeting in early September another group presented unpublished studies using a very different approach in mouse embryos that has come to the same general conclusion about the role of the *hox* genes in promoting axis extension as we propose here, demonstrating that this is a conserved feature in vertebrates.

The authors suggest that hoxa13b and hoxd13a are the most abundant of the hox13 genes in the tail. However, they don't show the QPCR data and from the in situs it is not clear if this is really the case. Additionally, even if this is true, it doesn't exclude the possibility that the other hox13 genes are equally important for tail development.

We have observed the same data about the relative levels not only from qPCR but also from multiple RNA-seq experiments, all of which show that *hoxa13b* and *hoxd13a* are the most abundant. If the reviewer and editor think it is important to present the qPCR data we can add that. We also

note that for the *hox13* in situs shown in Figure 1A we developed them for different lengths of time to optimally show the tailbud expression (please see the legend to Figure 1), and so this doesn't represent relative levels of expression. We agree with the reviewer that the less abundant *hox13* genes expressed in the tail (*hoxc13a*, *hoxc13b* and *hoxa13a*) could play a role, and indeed we think the reason that lack of these doesn't cause a phenotype is due to redundancy. From some more recent work that we will soon submit for publication, we actually think that the redundancy could include all of the posterior *hox* genes (*hox9-13*) so that even if we knocked out all of the *hox13* genes we would still be dealing with redundancy.

What is the phenotype of the double mutant double homozygote hoxa13bΔ16/Δ16;hoxd13ains4/ins4? The authors claim one fish survived, but they do not present evidence that it has a more severe phenotype at early stages (Page 6).

In the double mutants raised at 29 C most of the larvae are normal but a certain percentage show minor posterior defects. Most of those die somewhere during the larval stages and the fish we showed was a rare case of one surviving to adulthood (because we put larvae as a group in a nursery tank at 5 days post- fertilization there is no way for us to tell when they die).

What is the phenotype of the Hoxd13a knockouts? Are there any posterior defects in the ntlcs ; Hoxd13a-/-?

We never raised *hoxd13* mutants as single mutants and instead raised them in the background of *hoxa13* mutants since we were looking to see if *hoxd13* mutations enhanced the *hoxa13* mutant phenotype.

In Figure 3C, the authors use as wild type control the no tail cold sensitive and the hox13 mutations are introduced in the same genetic background. Therefore, when they analyze the somites of the hox13 mutants at 21C and 18C they can't exclude that any effect they see is actually due to the no tail mutation.

Because we see a strong enhancement of the *ntl cs* phenotype when *hoxa13* is mutated, and especially when *hoxa13* and *hoxd13* are mutated (Figure 3), our conclusion is that the *hox13* mutations are enhancing the *ntl cs* defect. For example, at 21 C, in *ntl cs* alone there are no class 1 (null phenotype) embryos and only 2% class 2, whereas in *ntl cs* with *hoxa13* and *hoxd13* mutated there are 15% class 1 and 50% class 2.

The authors observe mesodermal defects in ntlcs; Hoxa13-/- embryos maintained at 18.5 °C as compared to wt embryos. Are the same mesodermal defects observed in the ntlcs embryos at 18.5°C? The authors in Figure 4 should show in parallel in situs of the ntlcs embryos developed at 18.5 °C.

This is an excellent point and we have added a new Figure S4 to show exactly this.

In Figure 4, it's not clear what is the wildtype control that the authors use?

This is a WIK/AB hybrid line we use as our lab wildtype (see first sentence in the Methods). From previous studies (Kimelman, 2016 and unpublished data) we know that *ntl cs* kept at 29 C shows the same expression of markers as the WIK/AB wildtype.

In figure 4, although the authors show a reduction in the levels of tbxta in the NMp area, it would be necessary to complement this with an immunostaining for NMps to show conclusively that they are not being maintained.

The NMPs are defined as the overlap of *tbxta/brachyury* and *sox2*. Once *tbxta* is lost then there are no NMPs. There are no good antibodies available for Tbxta or Sox2 in zebrafish.

The phenotypes that the authors observe from the crosses between hoxa13 and hoxd13 mutants are very variable and largely depend on the ntlcs mutation at low temperatures. This makes it difficult to identify which of the observed effects are caused by the lower levels of brachyury or the specific Hox13 mutation. The authors should analyse the effect of eliminating all hox13 genes in a wild type

background (and not in the ntlcs background). This will provide a more informative answer on the role of hox13 genes in the regulation of Brachyury and maintenance of the niche during axial elongation.

We would again make the point that what we are seeing is a synergism between a loss of Hox13 activity and a reduction in Tbxta activity, which is a regulator of *wnt* expression. In work we are in the process of completing, we have identified two enhancer elements in the *tbxta* promoter that have binding sites for all the posterior Hox factors and for Tcf, the mediator of Wnt signaling. We mention this just to make the point that these factors are working together and it not as separate molecular entities. While a quintuple *hox13* mutant would be interesting, even if we could raise fish with this many *hox13* genes mutated, we feel that this is beyond what is necessary for the present work.

In ntlcs; Hoxa13-/- embryos, have the authors observed any effect in the proliferation or cell death of tailbud cells?

Based on the reviewer's suggestion we examined apoptosis. During the mid- somitogenesis stages when we start seeing the mesodermal defects there is no increase in apoptosis in the embryos we show in this work. At later stages there is apoptosis in the neural tissue as was also shown for *ntl* null mutants (Marlow, 2004), most likely because the neural tissue is not receiving signals from the missing mesoderm. As for proliferation, we previously showed that there is very little proliferation in the tailbud of zebrafish embryos (Bouldin, 2014).

P4. Last paragraph "A major caveat….with the one exception of the mouse Hoxb13 mutant that causes a minor increase in the number of somites"
The Hoxb13 mutant mice, apart from the 2 additional somites, have overgrowth of neural structures (wider and longer spinal cord), ectopic dorsal root ganglia. Thus the effects are more pronounced than those that the authors describe in the manuscript.

Thank you for the clarification. We have changed the text.

In all figures the authors should clearly indicate the genotype of the fish that they use as a wild type.

Since we are already running up against the word limit, to save space we have described the nature of the wildtype fish at the start of the Method section as we use the same wildtype for all experiments.

In figure 3B column 6, the authors state that the phenotypes shown are the result of a cross between two homozygotes, but in the legend it's marked as a homozygous x heterozygous cross.

The legend states "The specific *hoxd13a* mutation is shown; all fish were homozygous for both *hoxa13b*$^{\Delta 16}$ and *ntl*^{cs}." The labeling on the columns is just the hoxd13 mutation. If this is not clear, we would be open to an alternative suggestion for writing this sentence in the legend.

Page 8, refers to Figure 4B when it should be 4A; "Interestingly, the expression of tbxta in the notochord (Figure 4B, black arrowhead)"

Thank you for the correction, it is now fixed..

In Figure 7D, the qPCR data are missing error bars; Is this only one experiment?

Thank you for catching that. The data was from three independent biological replicates and we had omitted to include the error bars. This is now fixed.

Second decision letter

MS ID#: DEVELOP/2019/185298

MS TITLE: hox13 genes are required for mesoderm formation and axis elongation during early zebrafish development

AUTHORS: Zhi Ye and David Kimelman
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 nice study shedding light into the role of Hox13 genes in the biology of NMPs and axis elongation. The authors also demonstrate elegantly that the findings of overexpression experiments should be approached with caution.

Comments for the author

I am happy with the revised version of the manuscript and I support its publication.

Reviewer 2

Advance summary and potential significance to field

In this study, the authors investigated the effect of the combined loss of hoxa13a and hoxd13b on the elongation of the zebrafish body axis. They also discover a novel genetic interaction with a temperature sensitive mutation in tbxta. Overall this is an important contribution to the field since, despite their extensive study across a range of species, the requirement of Hox genes during posterior body axis elongation has not been elucidated. Most studies so far have focussed on using over-expression of hox genes at non-physiological levels. This has lead to the proposition that posterior Hox genes are important for terminating posterior body extension in vertebrates. However, the authors clearly point out that the expression of genes in the Hox13 class commences in the tailbud much earlier that would be expected for a role only in the termination of axis elongation. By examining the phenotypic consequences of hoxa13a and hoxd13b loss of function, the authors conclude that they are instead required for maintenance of neuromesodermal progenitors due to the increased expression of the neural marker sox2 at the expense of mesoderm. Transplant experiments are performed that suggest that this function is non-cell autonomous.

Comments for the author

The authors have satisfactorily addressed all my concerns

Reviewer 3

Advance summary and potential significance to field

The manuscript has substantially improved after revisions and should be accepted for publication in Development.

Comments for the author

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