Identification of in vivo Hox13-binding sites reveals an essential locus controlling zebrafish brachyury expression

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MS TITLE: Identification of in vivo Hox13 binding sites reveals an essential locus controlling zebrafish brachyury expression

AUTHORS: Zhi Ye, Christopher R Braden, Andrea Wills, 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. Each of the referees makes helpful suggestions as to how to strengthen and clarify aspects of your study. Some of these will require additional analyses or substituting experimental data. Referee 1 would like you to investigate whether Hox13 is pioneering or activating regulatory elements and suggests how to explore this. This seems a constructive suggestion that would greatly enhance the 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.

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

This work aims to understand how posterior Hox genes control axial elongation and cell-type specification. This paper builds on the authors' recent observations placing Hoxb13 in the pathway maintaining the NM pool to allow for axial elongation. The proposed model suggests Hox13 maintains the mesoderm NM potential, leaving an outstanding question: how does Hoxb13 maintain NM's mesoderm potential? Therefore, this works tackles this question head-on in a natural progression.

Cut&Run experiments with a Hoxb13 overexpression and a tagged endogenous Hoxb13 (kudos to the authors) revealed a collection of Hoxb13 genomic binding sites. Histone modifications mapping places Hoxb13 binding on putative regulatory elements even at the key tbxta gene, discovering an evolutionarily conserved regulatory mode.

I find this paper interesting in many ways: in vivo Hox binding at endogenous levels, tbxta enhancer dissection and characterization, and a regulatory model that links wnt/Hox/tbxta controlling NM progenitors. As a whole, going from binding to gene circuit controlling NM potential and axis elongation is by itself a significant contribution that deserves to be published. I think, however, the authors could add value by expanding the Hoxb13 binding analysis to keep clarifying the Hox13 regulatory role.

Comments for the author

The authors discuss potential Hoxb13 activity modes on the genome. I believe they might have the tools to push the model forward with 4 relatively easy new experiments. To test if Hox13 are pioneering or activating regulatory elements: What is the histone and accessibility landscape at Hoxb13 sites in a Hox13 compound mutant? Alternatively, histone and accessibility landscape at new Hoxb13 sites in the Hoxb13 overexpression?

A simple analysis might illuminate hypotheses about possible transcription factors working with Hoxb13 to activate or repress regulatory elements. Is there a different motif or footprint signature at Hoxb13 sites with 1) no K3me or K27ac, 2) K3me only and 3) 1) K3me + K27ac? Does the T vs C site difference correlate with enhancer activity? I also believe the analysis to the previous coment could be divided into these three categories.

Would the authors venture to discuss the "Guarantor" and "pioneer" relationship for Hox13? To me, they are non-opposing terms that can be conceptualized within this model, where the pioneer activity is what makes them guarantors.

Minor:

1. Maybe I am missing something, but I could not find the percentage of NM progenitors in the tailbuds for Cut&Run experiments. The percentage of these cells should be kept in the reader's mind when presenting cell-type specific models.

2. H3k4me1, 27ac and Hoxb13 binding tracks for the tbxa gene in the main figure would complement well the paper description and thought process in selecting enhancers.

3. Would it be possible to quantify transgene expression in Fig 5 to make it easier to read for fish embryo amateurs like me?

4. Adding “muscle” or “somite” and “neural tube” would help to read panels 5H and 5I information easily.

5. Would it be possible to compare the tbxta enhancer deletion with the mutant to compare this enhancer's strict requirement?

6. This might be a complicated experiment and perhaps unnecessary to finally nail this model, but tbxta overexpression should rescue the Hox element 1 mutant, right?

7. The authors seem to be missing some important references of recent papers which show Hox13 shaping chromatin landscapes (Desanlis, 2020 and Amandio, 2020).

8. “In order to monitor expression, GFP was placed after the 2A peptide, which allows GFP to be co-expressed with Hoxa13b as a separate protein”

9. Have the authors verified that the 2A peptide is properly cleaved and that Hoxa13b and GFP are expressed as two separate proteins, using a Western blot?

10. “Intriguingly, at a very specific position in the motif (Figure 3B) approximately half the fragments have a T, which is the base bound by all Hox proteins (Hox1-13), whereas in the other half a C is detected, which is bound specifically by the posterior Hox proteins (Hox9-13, Jolma et al., 2013). Thus, Hoxa13b binds both motifs at a similar frequency.” The authors may want to change the wording of their conclusion. To state that both motifs are bound at a similar frequency would require a motif scanning approach. MEME and DREME harbor their own biases. This may be a simple change in the tone to: “Thus, Hoxa13b binds the predicted motifs (TTTAT and TTTAC) in our system.”

Reviewer 2

Advance summary and potential significance to field

In this paper by Ye et al., the authors describe the use of a novel method applied for the first time in zebrafish, to identify transcription factor binding sites involved in the regulation and maintenance of tbxta expression in the tailbud Neuromesodermal Progenitor (NMp) population. They identify two Hox binding domains, which they term Hox Element 1 and Hox Element 2 and demonstrate that Hox Element 1 has a functional role in promoting the expression of tbxta in the posterior NMps. This is of particular interest to the field, as specific NMp drivers of tbxta expression have, so far have been elusive. Through their transcription factor binding studies, they demonstrate that Hox13, alongside TCF/Lef1 drive the expression of tbxta, which is something they themselves previously suggest1. The regulation of tbxta by Wnt is also something well documented by previous work2 and nice to see reproduced using this novel technique. Through mutation studies, by the removal of Hox Element 1 in vivo, they demonstrate that the embryo has a truncated posterior body, and appears to have defects in the terminal somites. The malformation of the terminal somites indeed supports the conclusion that this Hox Element 1 does indeed regulate NMp tbxta expression verses all tbxta expression and provides good experimental validation that the authors are indeed modulating NMp dynamics. Finally the authors identify Rbpj as another binding factor which uses Hox Element 1 to promote tbxta expression and demonstrate that its dominant negative form reduces tbxta expression, also causing posterior somite deformation.
This paper is of clear interest to a community interested in NMps and provides much needed clarity on the drivers of tbxta specifically in the NMp population. It also provides direct evidence of how Wnt drives tbxta expression at the nucleotide level, through Hox Element 1 via Rbpj. I support the publication of this work with the following amendments.

Comments for the author

Major Corrections:
1. The introduction should be expanded to introduce in more detail the contribution of NMPs in zebrafish embryos, as this would help the reader better interpret mutant phenotypes presented. Through the authors’ own, and the work of others both cited and uncited, it has been

demonstrated that the NMPs in zebrafish are unusual compared to mouse NMps in that they do not contribute to the full length of the body axis. Rather, cell proliferation ceases shortly after the tailbud stage and the tailbud NMp population contributes only to the most posterior aspect of the axis3,4. This should be clarified in the introduction, as the proper contextualisation of the author's observed phenotype upon deletion of Hox element 1 will demonstrate that they fit very well with the cellular contribution of tailbud NMPs to the posterior tip of the embryo.

2. There are currently measures of somite and neural tube volumes presented however there are no methods detailing how these measurements were made. It would be beneficial to the reader to understand the methods used to calculate volume and to see an image of this as part of a figure or supplementary figure. It is unclear to the reader currently how the volume measurements can be taken from the images presented and one assumes that these are confocal slices, however this is not stated. This is also the case for body length measurements.

3. In some figures, the conclusions from the text are not obvious to the reader. In particular, Figure 5B is purported to show expression, or lack thereof of GFP within the NMps however at the magnification of the image presented this is not immediately clear. It would significantly benefit the reader to show closer zoom ins of these image, or better still a fixed embryo stained with a GFP antibody and DAPI, imaged at high magnification. If possible, this could be coupled with a tbxta/sox2 FISH to really demonstrate GFP within the coexpressing NMp domain. To demonstrate the absence or presence of GFP within the somites, a slice view of one of these high magnification stain would be a real benefit.

4. Finally, the paper methodology lacks significant levels of detail including the primer sequences and methods used in the cloning of the cDNA sequences for a number of the constructs used. In addition, the methods lack any detail about how the immunofluorescence stains were created or imaged and currently many experiments would be difficult to replicate.

Minor Corrections:
There is variation in the formatting of gene names and in the use of non-standard English terms such as in vitro and in silico. These should be provided in italics with mRNA in italics, protein names in Roman.

The shortening of the term "transcription factor" to TF is seemingly unhelpful and may be better written in full for the benefit of the reader

Figure 1 – this shows a low contrast brightfield image, where the embryo is not clearly distinguishable from the background. It would benefit the reader if this contrast could be improved. Please also clarify if this is a slice view, or wholemount image? An alternative to a bright field image may be through use of a nuclear counterstain, if this was captured alongside the green signal in Video 1.
Figure 6 – the axis labels, points and line sizes on G, H and I are too small to be clearly viewed by the reader. There also appears to be a formatting error in I where the axis appears to break between +/+ and +/-
Figure S6A – it is not obviously clear to the reader where the somite boundaries are in these images due to overexposure. It would benefit the reader to reduce the brightness of these images, use a colour other than green, or more clearly mark the somite boundaries digitally or through an appropriate counterstain. Figure S6B, it would be beneficial if this could be shown with either DAPI or transmitted light to help orientate the reader. It would also benefit the reader if higher magnification images of the tailbud domain specifically could be shown to demonstrate the change in NMp contribution. There are a number of figures lacking correct/clear scale bars (Figure 6B-F & S6A-B). This is of particular importance when asking the reader to make comparisons between images in terms of size.

References
1. Ye, Z. & Kimelman, D. Hox13 genes are required for mesoderm formation and axis elongation during early zebrafish development. Development 147, (2020).
2. Martin, B. L. & Kimelman, D. Regulation of Canonical Wnt Signaling by Brachyury Is Essential for Posterior Mesoderm Formation. Dev. Cell 15, 121–133 (2008).

3. Bouldin, C. M., Snelson, C. D., Farr, G. H. & Kimelman, D. Restricted expression of cdc25a in the tailbud is essential for formation of the zebrafish posterior body. Genes {&} Dev. 28, 384–395 (2014).
4. Attardi, A. et al. Neuromesodermal progenitors are a conserved source of spinal cord with divergent growth dynamics. Development dev.166728 (2018) doi:10.1242/dev.166728.

Reviewer 3

Advance summary and potential significance to field

The work by Ye, Kimelman and colleagues follows very beautiful work published by this group recently identifying a highly unexpected role for Hox13 genes in supporting tail formation in fish. Here, the group take an unbiased approach to identify direct Hox13 targets, and follow up a potential direct regulation of Tbxt as a mechanism driving this novel function of Hox13 genes. This new manuscript at present may not have quite the level of impact for the field as the original identification of Hox13 and axis elongation, but the approach to identify novel Hox targets is well planned and important given the major lack of understanding in this aspect of Hox function. A deeper analysis of these targets as a whole, to complement the Tbtx work, could help in this regard.

Comments for the author

Specific comments:

For the Hoxa13b-FLAG KI line, I assume this was sequence verified? Please confirm the localisation presented in Fig1C using dual labelling for GFP and for endogenous Hoxa13b.

Regarding the in vivo enhancer analysis – driving GFP using various elements. As a mouse person, I find it difficult to appreciate the difference indicated. Are these stable transgenic lines, has copy number been taken into consideration? I assume if GFP is extinguished from NMPs and the tailbud is no longer expressing GFP in the “proximal” line at the stage of development depicted in Fig5, then when these fish are aged for another 10 somites, you will have a very clear GFP/non-GFP demarcation along the A-P axis, that will be absent in the other lines. Can the authors please perform this experiment.

The Hox element 1 mutant fish data is very interesting, however specific deletion of the Hox binding site alone is required for formal conclusions, particularly as there is a Wnt-responsive site that I presume is also deleted.

Regarding genotyping of Hox element 1 mutant fish: Again, this is coming from a mouse person, but I am very surprised the authors do not genotype every short fish of the 92 post-analysis, to make sure the phenotype 100% parallels genotype. Similarly, for the Tbtx expression analysis, was this 17% of 29 fish, or were the 29 fish 17% if a total. If the former, and you genotyped 5 fish, I suggest repeating with a larger cohort, and genotyping all fish after in situ, to define the % of mutant fish exhibiting reduced Tbtx expression.

Deeper characterisation of the Hox element 1 mutant fish would be helpful – are the fish shorter because there are reduced somite number, smaller somites etc. Please include Sox2-FISH images that were used for quantification.

Regarding the conclusion that “Thus, when Hox element 1 is deleted from the tbxta gene the NMPs switch from producing mesoderm to neural tissue in the most posterior end of the embryo.” I understand increased neural tube volume may imply decreased mesoderm production, but of course it could also be expansion post-allocation. Please show decreased mesoderm progenitor population specifically in the tailbud in support of the stated conclusion.

Similar comment for Rbpj experiments – please specifically delete the binding site alone to allow formal conclusion that it is directly regulating Tbtx expression.

Minor typo:
located 24.6 upstream of – add Kb

First revision

Author response to reviewers' comments

Reviewer 1

We greatly appreciate the reviewer's opinion that the work is interesting in many ways and that it is a significant contribution that deserves to be published. We address the reviewer's concerns below.

The authors discuss potential Hoxb13 activity modes on the genome. I believe they might have the tools to push the model forward with 4 relatively easy new experiments. To test if Hox13 are pioneering or activating regulatory elements: What is the histone and accessibility landscape at Hoxb13 sites in a Hox13 compound mutant? Alternatively, histone and accessibility landscape at new Hoxb13 sites in the Hoxb13 overexpression?

The reviewer raises some interesting questions, but unfortunately Zhi has had to return to China since his 3 year US visa ended, and he is in the process of searching for a job. He was the one who did the whole CUT&RUN procedure, which is very complicated and technically very challenging, and there is no one else left in the lab who can do this procedure. We discussed this issue with the editor, who agreed that it was not necessary for us to perform these experiments. We apologize to the reviewer for not being able to do this.

A simple analysis might illuminate hypotheses about possible transcription factors working with Hoxb13 to activate or repress regulatory elements. Is there a different motif or footprint signature at Hoxb13 sites with 1) no K3me or K27ac, 2) K3me only and 3) 1) K3me + K27ac?
Does the T vs C site difference correlate with enhancer activity? I also believe the analysis to the previous comment could be divided into these three categories.

We have performed the analyses the reviewer suggested. In the case of the methyl marks, the only clear thing we found is an association of an arid3a motif with K4me only in approximately one-third of the cases where we found a Hox site, and nothing obvious with either no K4me or K27ac or with K4me+K27ac. Based on a RNAseq dataset we created from tailbuds we do find arid3b and arid3c expressed in the tailbud, but there is no available in situ data on any of the arid3 genes and so we don't know if they are expressed ubiquituously in the embryo, or expressed specifically in the tailbud, unfortunately. Since we found this association in just one- third of the cases, and since it is K4me only, we weren't quite sure what to make of this result and so we didn't add anything to the text. However, if the reviewer feels we should mention this, we will add a sentence to the text. With regards to the T vs C, we found no significant differences. In other words, the presence of the T or C site did not correlate with enhancer activity, suggesting that other factors are the key determinants of enhancer activity. Since this analysis did not produce a clear finding, and since we are already over the word limit with the revisions suggested by the reviewers, we chose not to discuss this point in the text, but could add a sentence if the reviewer feels we should. Regardless of the outcome, we appreciate the reviewer suggesting that we perform these analyses since they were certainly very good things to investigate.

Would the authors venture to discuss the "Guarantor" and "pioneer" relationship for Hox13? To me, they are non-opposing terms that can be conceptualized within this model, where the pioneer activity is what makes them guarantors.

This is a very interesting idea and one we had not considered, so we greatly appreciate the reviewer suggesting it. We have added out thoughts about this to the Discussion on p. 17, along with the citations suggested by the reviewer (in minor point #7).

Minor:

1. Maybe I am missing something, but I could not find the percentage of NM progenitors in the tailbuds for Cut&Run experiments. The percentage of these cells should be kept in the reader's mind when presenting cell-type specific models.

It is hard to get an exact estimate of the number of NMPs in the tailbud, but we should point out that the Hoxa13b is expressed not just in the NMPs but also in the mesodermal region. We estimate that approximately 50% of the cells in the dissected tailbud express Hox13b, and we have added a sentence explaining this on page 7.

2. H3k4me1, 27ac and Hoxb13 binding tracks for the tbxa gene in the main figure would complement well the paper description and thought process in selecting enhancers.

Thank you for the suggestion. We have now moved this data from what was Fig. S3 to Fig. 4.

3. Would it be possible to quantify transgene expression in Fig 5 to make it easier to read for fish embryo amateurs like me?

We have found from making many Tol2 transgenic lines in zebrafish that there is variability in overall transgene expression from line to line, most likely due to the site of insertion. For this reason we don't think overall expression is a very good indicator, and thus we always focus on relative expression (ie the proximal promoter without the Hox enhancers has low tailbud expression relative to the notochord whereas inclusion of the Hox enhancers produces strong tailbud and notochord expression).

4. Adding "muscle" or "somite" and "neural tube" would help to read panels 5H and 5I information easily.

Thank you for this suggestion. We have added the words as suggested to the Figure (and just to be clear this is 6H and 6I).

5. Would it be possible to compare the tbxta enhancer deletion with the mutant to compare this enhancer's strict requirement?

It is a very interesting question why the enhancer deletion is less severe than the null tbxta (no tail) mutant, which has a complete absence of tail somites. We have now added a lengthy discussion on p. 18-19 to deal with this question. The simplest possibility is that the second Hox enhancer is redundant and mostly compensates. However, as discussed on p. 18-19, a not- mutually exclusive idea is that in zebrafish, which forms its body very rapidly, tbxta transcription post-gastrula plays a relatively minor role since in zebrafish there are a relatively small number of cells that remain bipotential after the gastrula stage. We therefore speculate that in other teleosts, with much longer bodies than zebrafish (eels, for example) that deletion of the Hox element might have a much stronger effect.

6. This might be a complicated experiment and perhaps unnecessary to finally nail this model, but tbxta overexpression should rescue the Hox element 1 mutant, right?

In theory the reviewer is right, that is exactly what we would predict. Unfortunately, the only tool we have is a heat shock line expressing Tbxta, but because the HS line causes uniform overexpression of Tbxta, including in the neural tissue and newly forming mesoderm (the presomitic mesoderm) it causes widespread changes in gene expression and will not produce a phenotypic rescue of the Hox element 1 mutation.

7. The authors seem to be missing some important references of recent papers which show Hox13 shaping chromatin landscapes (Desanlis, 2020 and Amandio, 2020).

Thank you very much for pointing this out. We have now included these citations in the Discussion on p. 17, where we discuss the pioneer factor idea mentioned above.

8."In order to monitor expression, GFP was placed after the 2A peptide, which allows GFP to be co-

expressed with Hoxa13b as a separate protein" 9.Have the authors verified that the 2A peptide is properly cleaved and that Hoxa13b and GFP are expressed as two separate proteins, using a Western blot?

Yes, we did a Western blot when we were first determining how stable the Hoxa13b-FLAG protein is and indeed found that none of the Hoxa13b-FLAG is produced as a GFP fusion protein. This fits exactly with what was shown in the original paper on the use of the 2A peptide in zebrafish (Provost, 2007). Since we were just using this to monitor expression and were not expecting to show it, it is very much not a publication quality blot but the data was completely clear.

10."Intriguingly, at a very specific position in the motif (Figure 3B) approximately half the fragments have a T, which is the base bound by all Hox proteins (Hox1-13), whereas in the other half a C is detected, which is bound specifically by the posterior Hox proteins (Hox9-13, Jolma et al., 2013). Thus, Hoxa13b binds both motifs at a similar frequency." The authors may want to change the wording of their conclusion. To state that both motifs are bound at a similar frequency would require a motif scanning approach. MEME and DREME harbor their own biases. This may be a simple change in the tone to: "Thus, Hoxa13b binds the predicted motifs (TTTAT and TTTAC) in our system."

Thank you, this is a good point and we have changed the text as suggested.

Reviewer 2

We are very appreciative of the reviewer's interest in this paper, and for supporting publication. We certainly agree that it helps us understand the direct regulation of tbxta during the somitogenesis stages.

Major Corrections:

1. The introduction should be expanded to introduce in more detail the contribution of NMPs in zebrafish embryos, as this would help the reader better interpret mutant phenotypes presented. Through the authors' own, and the work of others both cited and uncited, it has been demonstrated that the NMPs in zebrafish are unusual compared to mouse NMps in that they do not contribute to the full length of the body axis. Rather, cell proliferation ceases shortly after the tailbud stage and the tailbud NMp population contributes only to the most posterior aspect of the axis3,4. This should be clarified in the introduction, as the proper contextualisation of the author's observed phenotype upon deletion of Hox element 1 will demonstrate that they fit very well with the cellular contribution of tailbud NMPs to the posterior tip of the embryo.

Thank you for the suggestion to discuss more about the NMps in zebrafish. We tried to include this in the Introduction as suggested, but for the optimal flow of the paper it worked out better to bring this up in the Discussion (p. 18), after we had shown the Hox element 1 deletion mutant, since the logic of discussing the differences in zebrafish made more sense after this data is presented. This worked out very well since it allowed us to discuss some interesting issues related to the role of Tbxta in the post-gastrula embryo, which relates well to the work of Steventon and colleagues showing that in zebrafish the bipotential cells contribute only to the most posterior part of the body, and it makes us wonder if in other species with longer bodies (such as the eel, for example) if the Hox elements (and post-gastrula Tbxta) might play a bigger role. Hopefully one day those systems will be more amenable to experimentation.

2. There are currently measures of somite and neural tube volumes presented however there are no methods detailing how these measurements were made. It would be beneficial to the reader to understand the methods used to calculate volume and to see an image of this as part of a figure or supplementary figure. It is unclear to the reader currently how the volume measurements can be taken from the images presented and one assumes that these are confocal slices, however this is not stated. This is also the case for body length measurements.

Apologies for the omission. We have added a section to the Methods to explain how the imaging and calculations were done.

3. In some figures, the conclusions from the text are not obvious to the reader. In particular, Figure 5B is purported to show expression, or lack thereof, of GFP within the NMps however at the magnification of the image presented this is not immediately clear. It would significantly benefit the reader to show closer zoom ins of these image, or better still a fixed embryo stained with a GFP antibody and DAPI, imaged at high magnification. If possible, this could be coupled with a tbxta/sox2 FISH to really demonstrate GFP within the coexpressing NMp domain. To demonstrate the absence or presence of GFP within the somites, a slice view of one of these high magnification stain would be a real benefit.

Thank you for the suggestions. We have reworked Figure 5 to present the results better, and we have added a new supplemental figure (Figure S5) to show the GFP expression in the somites in a confocal slice, and to compare the GFP expression to tbxta/sox2 FISH to demarcate the NMps as the reviewer suggests.

4. Finally, the paper methodology lacks significant levels of detail including the primer sequences and methods used in the cloning of the cDNA sequences for a number of the constructs used. In addition, the methods lack any detail about how the immunofluorescence stains were created or imaged and currently many experiments would be difficult to replicate.

Thank you for noticing this. We have added much more detail to the Methods.

Minor Corrections:
There is variation in the formatting of gene names and in the use of non-standard English terms such as in vitro and in silico. These should be provided in italics with mRNA in italics, protein names in Roman.

Thank you for pointing out errors. We have gone through and corrected a few places where we felt we made an error, and if the reviewer happens to notice any other instances we missed we would appreciate knowing this. We would like to mention that we discovered during the proofs stage of our previous paper that Development has an unusual house style such that when referring to a single hox gene such as hoxa13b it is lowercase and italics, but when referring in general to Hox genes or Hox13 genes it is uppercase and Roman. In this manuscript we endeavored to match the Development house style (to make it easier on the copy editor), and perhaps many of the cases where there appeared to be variation in our formatting was just because of this unusual style of the journal.

The shortening of the term “transcription factor” to TF is seemingly unhelpful, and may be better written in full for the benefit of the reader.

Thank you for pointing this out. We were struggling with the word limit, and it is good to know that this was not a useful way to deal with it.

Figure 1 – this shows a low contrast brightfield image, where the embryo is not clearly distinguishable from the background. It would benefit the reader if this contrast could be improved. Please also clarify if this is a slice view, or wholemount image? An alternative to a bright field image may be through use of a nuclear counterstain, if this was captured alongside the green signal in Video 1.

We agree with the reviewer that the data could have been presented better and so we have reworked Figure 1 to make the images from the single confocal slice clearer, and we have added a snapshot from the 3D reconstruction.

Figure 6 – the axis labels, points and line sizes on G, H and I are too small to be clearly viewed by the reader. There also appears to be a formatting error in I, where the axis appears to break between +/+ and +/-

Thank you for the comments. We have reworked Figure 6 to present the data better. The error in I seems to have just been an issue with the conversion of the image to a PDF format since it looks fine in the original.

Figure S6A – it is not obviously clear to the reader where the somite boundaries are in these images due to overexposure. It would benefit the reader to reduce the brightness of these images, use a colour other than green, or more clearly mark the somite boundaries digitally or through an appropriate counterstain. Figure S6B, it would be beneficial if this could be shown with either DAPI or transmitted light to help orientate the reader. It would also benefit the reader if higher magnification images of the tailbud domain specifically could be shown to demonstrate the change in NMp contribution.

Thank you for the comments. We have reworked Figure S6A to show the data better.

There are a number of figures lacking correct/clear scale bars (Figure 6B-F & S6A- B). This is of particular importance when asking the reader to make comparisons between images in terms of size.

Thanks for pointing this out. Scale bars have been added.

Reviewer 3

We thank the reviewer for the very nice comments on our previous paper, and appreciate that the reviewer feels that the identification of in vivo Hox target genes is important.

Please confirm the localisation presented in Fig1C using dual labelling for GFP and for endogenous Hoxa13b.

Unfortunately, hoxa13b is expressed at a very low level and FISH does not work very well, even more so when combined with immunofluorescence (for the hoxa13b-FLAG) since the hybridization has to be done at a lower temperature. However, we have added to Figure 1 a panel showing ISH for hox13b at the same stage as we did the immunofluorescence and in the same orientation, so this should allow a comparison of the hoxa13b transcripts to the Hox13b- FLAG protein. This shows that the Hoxa13b-FLAG protein is in the NMps and posterior mesoderm as are the hoxa13b transcripts, but as the protein is stable it is also present in the more anterior PSM.

Regarding the in vivo enhancer analysis – driving GFP using various elements. As a mouse person, I find it difficult to appreciate the difference indicated. Are these stable transgenic lines, has copy number been taken into consideration? I assume if GFP is extinguished from NMPs and the tailbud is no longer expressing GFP in the "proximal" line at the stage of development depicted in Fig5, then when these fish are aged for another 10 somites, you will have a very clear GFP/non-GFP demarcation along the A-P axis, that will be absent in the other lines. Can the authors please perform this experiment.

It is possible that some of the lines have more than one copy and so we are not making any claims about overall expression levels. The point is that when we have just the proximal promoter there is strong expression in the gastrula stage but a lack of post-gastrula expression in the tailbud, whereas when we add the Hox element there is now post-gastrula expression in the tailbud (and in the posterior somites – see our new data shown in Figure S5A,B). As for looking for a demarcation in expression in the proximal line, we have looked at this and there is no clear border of expression. This is because the GFP expressed at the start of gastrulation turns over as time increases and the number of somites increase, and because (as shown by others) the cells from the tailbud intermix with the mesodermal cells from the gastrula stage.

The Hox element 1 mutant fish data is very interesting, however specific deletion of the Hox binding site alone is required for formal conclusions, particularly as there is a Wnt-responsive site that I presume is also deleted.

To eliminate the Hox binding site in Hox element 1 would require creating a knockin line that would replace the endogenous Hox element 1 with a mutant version. Knockin technology in zebrafish is still extremely difficult (it took us well over a year to get the knockin fish reported in this paper). We discussed this with the editor who agreed that such a mutant is beyond the scope of the present paper. We also were very careful not to overstate the case, but we think the idea that the Hox

binding site in Hox element 1 is important for *tbxta* regulation is the most parsimonious explanation given that 1) we identified this important enhancer using an unbiased screen for Hoxa13 binding sites; 2) this site has been perfectly conserved for hundreds of millions of years of evolution; 3) in our previous paper we showed that the Hox13 proteins contribute to *tbxta* expression.

Regarding genotyping of Hox element 1 mutant fish: Again, this is coming from a mouse person, but I am very surprised the authors do not genotype every short fish of the 92 post-analysis, to make sure the phenotype 100% parallels genotype. Similarly, for the Tbtx expression analysis, was this 17% of 29 fish, or were the 29 fish 17% if a total. If the former, and you genotyped 5 fish, I suggest repeating with a larger cohort, and genotyping all fish after in situ, to define the % of mutant fish exhibiting reduced Tbtx expression.

We have been genotyping the Hox element 1 mutants using PCR and high percentage agarose gels to look for the deletion, which is relatively low throughput (we had inadvertently left the screening conditions out of the Methods and this has now been added). In retrospect, it would have been good to switch to a high throughput method but as we had seen the same result repeatedly in preliminary analyses that the shorter embryos always genotyped as homozygous mutant, we felt that genotyping a subset in the of the embryos from the one experiment reported here is sufficient to make the point. We also point out that in addition to these embryos, we have seen the same result with the embryos we characterized for decrease muscle and those with increased neural tube.

We apologize for the confusion about the tbxta results. The reviewer is correct that we genotyped 5 embryos that had reduced tbxta expression and 100% were mutant. We have repeated the in situs and genotyped a larger number of embryos and that data is now included.

Deeper characterisation of the Hox element 1 mutant fish would be helpful – are the fish shorter because there are reduced somite number, smaller somites etc. Please include Sox2- FISH images that were used for quantification.

These mutant fish have the same number of somites but the most posterior somites are smaller as we documented. We had meant to state that the number of somites are unchanged and had left that out, so thank you for bringing this up; it has been added to page 12. The sox2 data is in Figure S6B.

Regarding the conclusion that “Thus, when Hox element 1 is deleted from the tbxta gene the NMPs switch from producing mesoderm to neural tissue in the most posterior end of the embryo.” I understand increased neural tube volume may imply decreased mesoderm production, but of course it could also be expansion post-allocation. Please show decreased mesoderm progenitor population specifically in the tailbud in support of the stated conclusion.

Thank you for the suggestion. We have now added in situs for tbx16 (the fish equivalent of Tbx6) to Figure 6, since tbx16 is the canonical marker of early mesoderm. The results show a clear reduction of tbx16 in homozygous mutant fish. The text is on p. 13.

Similar comment for Rbpj experiments – please specifically delete the binding site alone to allow formal conclusion that it is directly regulating Tbtx expression.

With regards to making a mutant in the Rbpj binding site in Hox element 1, the same difficulties as discussed above with regards to the Hox site apply, and here also the editor has agreed that making such a mutant is beyond the scope of the paper. We think that the idea that Rbpj is contributing to the regulation of *tbxta* expression is the most parsimonious explanation given that 1) Rbpja binds this site in vivo; 2) the Rbpja site has been perfectly conserved for hundreds of millions of years of evolution; 3) a dominant-negative Rbpja causes reduction of *tbxta* expression.

Minor typo:
located 24.6 upstream of – add Kb

Fixed. Thank you for noticing this.

Second decision letter

MS ID#: DEVELOP/2021/199408

MS TITLE: Identification of in vivo Hox13 binding sites reveals an essential locus controlling zebrafish brachyury expression

AUTHORS: Zhi Ye, Christopher R Braden, Andrea Wills, 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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. As you will see, Referee 3 remains concerned about how some of the conclusions are worded and would like the strength of these conclusions tempered or the alternatives more clearly stated. I agree with these points. 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

The new manuscript version maintains the submission essence and interest with significant improvements. I support the publication of this work as it is.

Comments for the author

I understand the technical limitations that impede adding new CUT&RUN experiments. Thus, it is not an unsatisfactory response to my comment.
This comment reflects my personal preference and not an effort to force the authors to include anything they do not want in their manuscript. However, a specific sequence signature, or absence of it, would be interesting to groups studying Hox binding. If the authors find the space, a short sentence stating they did not find any discriminating signature would be appreciated by many.

Reviewer 2

Advance summary and potential significance to field

The authors have satisfactorily addresses all concerns.

Comments for the author

I now recommend this article for publication

Reviewer 3

Advance summary and potential significance to field

Most points raised have been appropriately addressed
- Revised Fig1 shows the regional overlap (albeit not cellular) well.
- In vivo enhancer analysis images are clearer
- Increased genotyping post-Tbxta in situ with 100% correlation with genotype
- Clear analysis/reduction of Tbx16 in enhancer deletion line

I still maintain that Hox binding site deletion is required to support the way the manuscript is written. I fully appreciate that the data conceptually fits together very well and is an elegant model. However, without deletion of the specific Hox-binding site, one cannot make any assessment of the relative importance of Hox13 acting via direct positive regulation of Tbxt in axial elongation. Playing devil's advocate – what if functionally, everything came down to the Wnt-responsive site(s) within enhancer(s) - I agree may be unlikely for the evolutionary conservation reason listed, but it needs to be proven. At a minimum, I suggest the following sentences aren't currently supported:

Our study provides a direct connection between Hox13 and regulation of the Wnt/Brachyury loop.

These enhancer elements also each contain a conserved perfect consensus binding site for Tcf7/Lef1, which are the Wnt regulated transcription factors in cells, revealing how Hox factors and Wnt signaling intersect to promote tbxta expression.

Our results provide a molecular basis for understanding how Hox13 proteins act to promote axis formation

I also suggest calling the two enhancer elements something other than Hox element 1, 2 to reflect that they harbour multiple TF-binding sites and that the Hox sites within each have not been individually tested.

Comments for the author

No further comments

Second revision

Author response to reviewers' comments

Reviewer 1

I understand the technical limitations that impede adding new CUT&RUN experiments. Thus, it is not an unsatisfactory response to my comment. This comment reflects my personal preference and not an effort to force the authors to include anything they do not want in their manuscript. However, a specific sequence signature, or absence of it, would be interesting to groups studying Hox binding. If the authors find the space, a short sentence stating they did not find any discriminating signature would be appreciated by many.

We appreciate the reviewer's suggestion and we added to the text on page 9 to address these ideas.

Reviewer 3

I still maintain that Hox binding site deletion is required to support the way the manuscript is written. I fully appreciate that the data conceptually fits together very well and is an elegant

model. However, without deletion of the specific Hox-binding site, one cannot make any assessment of the relative importance of Hox13 acting via direct positive regulation of Tbxt in axial elongation. Playing devil's advocate – what if, functionally, everything came down to the Wnt-responsive site(s) within enhancer(s) - I agree may be unlikely for the evolutionary conservation reason listed, but it needs to be proven. At a minimum, I suggest the following sentences aren't currently supported:

Our study provides a direct connection between Hox13 and regulation of the Wnt/Brachyury loop.

We have changed the text to:
Our study provides a potential direct connection between Hox13 and regulation of the Wnt/Brachyury loop.

These enhancer elements also each contain a conserved perfect consensus binding site for Tcf7/Lef1, which are the Wnt regulated transcription factors in cells, revealing how Hox factors and Wnt signaling intersect to promote tbxta expression.

We have changed the text to:
These enhancer elements also each contain a conserved perfect consensus binding site for Tcf7/Lef1, which are the Wnt regulated transcription factors in cells, revealing how Hox factors and Wnt signaling could intersect to promote tbxta expression.

Our results provide a molecular basis for understanding how Hox13 proteins act to promote axis formation

We have changed the text to:
Our results provide a potential molecular basis for understanding how Hox13 proteins act to promote axis formation

I also suggest calling the two enhancer elements something other than Hox element 1, 2 to reflect that they harbour multiple TF-binding sites and that the Hox sites within each have not been individually tested.

We disagree about changing the name away from Hox element 1 and 2, although we certainly will make this change if the editor feels it is necessary. Since they were identified based on in vivo Hox binding, we feel it is reasonable to call them Hox elements. To just call them Upstream element 1 and 2 is too generic, and to call them Wnt elements is not very helpful since we know there are other Tcf sites (that don't have Hox binding) elsewhere in the promoter.

Third decision letter

MS ID#: DEVELOP/2021/199408

MS TITLE: Identification of in vivo Hox13 binding sites reveals an essential locus controlling zebrafish brachyury expression

AUTHORS: Zhi Ye, Christopher R Braden, Andrea Wills, 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.