Axial skeleton anterior-posterior patterning is regulated through feedback regulation between Meis transcription factors and retinoic acid

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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. 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 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.

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

The paper examines the role of the Meis genes in the establishment of the anterior-posterior axial skeleton patterning. They have found a cooperation between Meis1 and Meis2, demonstrated an involvement of the Hox genes functions and of the Raldh2 pathway. They also establish a hierarchy between Meis2 and Meis1 and demonstrate an effect on the myogenic pathways, showing phenotypes similar to those due to deletion of myogenic genes.

Comments for the author

Lopez-Delgado et al. have studied the role of Meis (1 and 2), in the establishment of the RA pathway, the expression of Hox genes and function and the establishment of the antero-posterior axial skeleton patterning. They have used several elegant conditional genetic models to answer this important developmental genetic question. These questions are complex and difficult to answer for the existence of multiple Meis genes, particularly Meis1 and Meis2 and for the involvement of other genetic, cell identity determining, functions like Hox and the Raldh2 pathway.
The AA analyze a vast and well detailed homeotic phenotype upon deletion of Meis2 which becomes more severe in the Meis1-Meis2 dKO, estanlishing a primary but not exclusive role of Meis2. The phenotypes exclude a role of Meis2 in the early posterior epiblast in axial patterning, and are independent of the time of deletion in the development of these tissues (three CRE lines used). In the axial skeleton patterning, Meis expression coincides in time and space with Hox genes expression in the posterior epiblast and explains the homeotic phenotypes observed. However, the deletion of Meis does not affect Hox genes expression, leaving an open question on how the homeotic phenotype is induced.
In addition to their effect in segmental identity, Meis KOs show a profound alteration of both patterning and myogenic pathways, with phenotypes very similar to Myf5, MRF4 and Myogenin-deficient mice, that explain the altered ribs patterning.
Moreover, other experiments also demonstrate a tight connection with the RA synthetic and degradative pathways, in particular the important role of Meis in the maintenance of Raldh2 expression and the demonstration of a positive regulatory loop in RA synthesis, which may well be the cause of axial mispatterning.
The experiments presented are extremely well done and presented, are extensive and complete and give very clear and insightsful answers. Overall, I believe this paper is an important genetic contribution to developmental biology which deserves a broad audience for the importance of the results, the deepness of the analysis and the clarity of the presentation.
My personal policy is not to propose additional experiments if not absolutely essential. To me the present results are clear, even if the authors do not give a molecular mechanism that explains how Meis affects Hox function without affecting Hox genes expression. However, I believe that the paper would benefit from a paragraph in the Discussion in which some possible explanations are suggested

Reviewer 2

Advance summary and potential significance to field

The present study describes a large amount of detailed work that could be of high interest to the developmental biology community and to the field of HOX and TALE transcription factor biology. While there is a rich literature available on roles of HOX proteins and TALE cofactors in organismal development, the phenotypic outcomes of compound tissue-specific loss of Meis genes in mouse models have been unknown to date and have been long awaited by the research community. Therefore, the present study is certainly timely.

However, there is serious unease regarding some of the data described and especially regarding some of the conclusions that are being put forth, which are not supported by adequate experimental evidence and are largely overplayed.

Comments for the author

The paper by López-Delgado et al. presents a detailed analysis of axial skeletal patterning in mice with loss-of-function (LOF) of *Meis1*, *Meis2* and *Raldh2* genes. The results presented are based on the characterization of tissue-specific LOF of *Meis1* and *Meis2* alleles using different *Cre* deleter lines and evaluation of the resulting skeletal phenotypes.

1) The Authors claim that compound loss of *Meis1/2* genes produces axial skeleton defects without affecting *Hox* gene transcription, including vertebral homeotic transformations and rib mis-patterning.

2) The Authors describe that while RALDH2 and MEIS positively regulate each other, *Raldh2* elimination recapitulates the defects associated with MEIS-deficiency and they state that *Meis* overexpression rescues the axial skeletal defects in *Raldh2* mutants.

3) The Authors propose a MEIS-RA positive feedback loop that is essential to establish anterior-posterior identities and pattern of the vertebrate axial skeleton.

In summary, the study describes a large amount of detailed work that could be of high interest to the developmental biology community and to the field of HOX and TALE transcription factor biology. While there is a rich literature available on roles of HOX proteins and TALE cofactors in organismal development, the phenotypic outcomes of compound tissue-specific loss of *Meis* genes in mouse models have been unknown to date. Therefore, the present study is warranted and welcomed. However, there is unease regarding some of the data described and especially regarding some of the conclusions that are being put forth, which are not supported by adequate experimental evidence.

Specific Comments:

a) The Authors purport the presence of homeotic transformations in compound *Meis* mutants, which -they state- are similar to those found in anterior-*Hox* and *Raldh2* mutants. This conclusion is based solely on morphological evaluations of skeletal preparations, a highly descriptive (and subjective) experimental approach. Only alcian blue/alizarin red-stained skeletal preparations are shown in Figure 2. This type of analysis for skeletal structures is not adequate and certainly cannot unequivocally demonstrate the presence of homeotic transformations. In light of the recent technological advances based on methods that enable imaging and measurements of skeletal elements highlighting all the feautures of the structures under analysis, alcian blue/alizarin red-stained skeletal preparations appear somewhat outdated. Lacking any type of analysis based on current techniques, such as OPT or microCT, the authors should at least provide larger insets that better illustrate the fine details of the skeletal elements that are affected in the mutants. At the present magnification it is impossible to adequately appreciate any of the subtle features of the skeletal elements under analysis (for example, see Figure 2). Evaluation of the skeletons that is made possible by the alcian blue/alizarin red- stained stained preparations photographed at the present (very small) magnification suggests that the skeletal abnormalities displayed by compound *Meis* mutants might be due to segmentation defects, a scenario that the Authors mention only tangentially, more than to real homeotic transformations.

b) The defects related to the occipital bone and C1 might share some similarities with phenotypes described in *Hoxd3* and *Hoxa3* mutants when *Meis2* is lost in single mutants. However, when *Meis1* is also inactivated on a *Meis2*-deficient background in compound mutants, the abnormalities of the occipital and cervical segments are very different and also much stronger than those reported so far in any *Hox* mutant (single or compound). Notably, these skeletal defects could simply be due to abnormalities in segmentation (see for example the skeleton in Figure 3O, or the rostral somites of mutant embryos analyzed by *in situ* hybridization in Figure 6B' and 6D)'. And again, one would need higher magnifications, or at least large insets,

to better judge the nature of the morphological defects, in the absence of OPT or similar high-resolution preparations.

c) Differences are also present between *Hox* and *Meis* phenotypes in the ribs. While for example loss of *Hox6* paralogs causes rib phenotypes, their characteristics are strikingly different from those observed in *Meis* mutants.

In light of all the considerations discussed in **b)** and **c)**, the Authors should be more careful in attributing the phenotypes observed in *Meis* compound mutants to homeosis. Statements like: *"Meis elimination produces axial skeleton defects without affecting Hox gene transcription, including vertebral homeotic transformations"* (in the Abstract) and "*… the anterior transformations observed in the cervical region may also affect the thoracic region…*" and again *"the type of defects were similar, with anterior transformations of C1-C3 (Figure 3G-I and 3M"* should be avoided. There is no experimental evidence that supports clear homeosis.

d) The Authors state that they have not observed alterations in *Hox* gene expression patterns or transcript abundance in *Meis* mutants. Therefore, they suggest that despite the profuse binding of MEIS proteins to the HOX complexes (Penkov et al., 2013), MEIS is not involved in *Hox* gene transcriptional regulation during axial skeleton patterning.

First, this Reviewer notes that the expression of some *Hox* genes is markedly perturbed in *Meis* mutants, in contrast to the above statement. This is clear when observing some of the photographs from *in situ* hybridization experiments that illustrate mRNA expression in developing embryos. For example, unless the Authors chose an embryos that is an outlier, *Hoxa3* expression in the *Meis* compound mutant embryo is markedly different, with reduced domains of expression and decreased levels of expression, as compared to the wildtype (Figure 4B). Also, expression of *Hoxb3* in the mutant embryo is significantly different when compared to the wildtype (Suppl Figure 3). The *in situ* hybridization results illustrating *Hoxb3* expression are somewhat buried inside the Suppl Materials, but nonetheless the differences are clear and marked. In the opinion of this reviewer, these differences cannot be completely disregarded and ignored.

In summary, while the authors conclude that "*transcriptional regulation of Hox genes is not involved in Meis regulation of axial skeleton patterning*", this reviewer does not agree with this unyielding conclusion, which is not adequately supported by the experimental findings.

Second, the Authors report the absence of *Hox* gene expression differences in wildtype versus mutant embryos assessed by RNA-seq analysis, in contrast to some of the marked differences they have found by *in situ* hybridization. The negative results obtained by RNA-seq could be easily due to dissection procedures, resulting in the analysis of different embryonic domains in the dissected tissues analyzed by RNA-seq. For example, dissected tissues could comprise more anterior or more posterior somites, which would dilute the overall differences in *Hox* gene expression. In addition, as it appears, the authors conducted their RNA-seq analysis at E9.0, while *in situ* hybridization experiments were performed mostly at E10.5 or at E8/8.5. No rationale is provided for the different timepoints analyzed using the 2 different methods. The different time-points analyzed by *in situ* hybridization and RNA-seq could easily explain the conflicting results obtained regarding *Hox* gene expression. Also, there is no indication of how many biological replicates were conducted for RNA-seq experiments. This point is critical to support the quality and reliability of of the RNA-seq experiments.

e) The Authors suggest the presence of direct *Raldh2* regulation by MEIS transcription factors. However, Panels I and K of Figure 6 clearly show that there appears to be only very little overlap between cells expressing *Raldh2* and *Meis* genes, respectively. The lack of robust co-expression is rather concerning and should be addressed.

f) The authors show a genetic rescue experiment to demonstrate that MEIS activity in the paraxial mesoderm is sufficient to rescue the *Raldh2* mutant phenotype. Hardly any experimental details are provided regarding these rescue experiments, which appear convincing at the pure morphological observation (Figure 7). However, the Authors do not consider the possibility that under the conditions of the rescue experiment *Raldh2* inactivation might become

suboptimal at best. If that were the case (which is not addressed) then the lack of a phenotype would not be the result of rescued RA deficiency by MEIS activity, but could be explained more likely by the presence of residual RALDH2 expression, which would enable the activation of functional levels of retinoic acid signalling in the embryo. To support a bona fide genetic rescue the Authors need to show that in these embryos *Raldh2* is fully inactivated in the domains where *Meis* is upregulated.

Absolute statements like: *"The complete rescue of Raldh2 mutants by Meis overexpression suggests that Meis is the main functional output of the positive regulatory loop between Meis and RA in the paraxial mesoderm"* (page 16) should be avoided, as they are not adequately supported by sufficiently robust and careful experimental evidence.

Minor Comments:
a) The nomenclature of genes and proteins is inaccurate: in the mouse, genes must be listed in Italics (*Meis*) and proteins in Plain Text, all letters upper case (MEIS). All nomenclature should be revisited throughout the text.

b) The Authors should add one last figure with a cartoon that summarizes the overall take-home message of the study, which is currently rather confusing.

Reviewer 3

Advance summary and potential significance to field

The authors sought to elucidate the function of Meis in the anterior-posterior patterning by generating compound mutant mice. They found that Meis mutations exhibited the anterior homeotic transformation of the vertebrae and ribs. Interestingly, Meis and Raldh2 seem to form a positive regulatory loop, and Rsldh2 mutation decreased Meis expression, leading to the anterior homeotic transformation of the vertebrae and ribs. Finally, the authors showed that overexpression of Meis2 rescued the skeletal defects of Rsldh2 mutation, suggesting that a proper level of Meis expression, which is dependent on RA signaling, regulates the AP patterning of the axial skeleton. This is an interesting work showing the role of Meis-RA positive regulatory loop in the AP patterning of the axial skeleton.

Comments for the author

The mechanistic analyses are still preliminary and need more clarification. Specific comments are indicated below.

1. In Figure 4A and B, the authors stated that Hox expression was not altered in Meis mutants, but it is not clear from the data. They should show which somite corresponded to the anterior boundary of each Hox expression with a higher magnification.

2. Meis mutants did not relocate Hox gene expression to more posterior somite at later stages, suggesting that Meis is involved in Hox gene expression. However, the authors concluded that the transcriptional regulation of Hox genes is not directly controlled by Meis. This conclusion is not clear, because they previously showed that Meis can directly control HoxA expression. The authors should clarify this issue.

3. Figure 6 showed that while Raldh2 expression was decreased in anterior somites of Meis mutants, the RA-responsive gene RARb was not changed. This result suggested that Meis is not important for RA signaling in somites, and I am not sure whether Meis-RA positive feedback loop exists here. The authors should clarify this issue. They should also examine the expression of another related enzyme, Raldh3, which could compensate for Raldh2 down-regulation.

4. The authors examined Raldh2, Cyp26b1, and RARb expression by in situ hybridization, but changes in the expression levels were rather small or not clear. Because in situ hybridization is not quantitative, the authors should perform qPCR to quantify the expression levels more precisely.

5. Meis1;Meis2 double KO mice showed segmentation defects in the cervical region (Figure 3O), suggesting that the segmentation clock does not work properly at early stages. The authors should examine the expression of the clock genes such as Hes7 and Lfng in the PSM of these mutants at E8–8.5.

6. The last line of page 12 is misleading. At E8.75, more than 10 somites are formed, and this sentence should be corrected.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
The paper examines the role of the Meis genes in the establishment of the anterior-posterior axial skeleton patterning. They have found a cooperation between Meis1 and Meis2, demonstrated an involvement of the Hox genes functions and of the Raldh2 pathway. They also establish a hierarchy between Meis2 and Meis1 and demonstrate an effect on the myogenic pathways, showing phenotypes similar to those due to deletion of myogenic genes.

Reviewer 1 Comments for the Author:
Lopez-Delgado et al. have studied the role of Meis (1 and 2), in the establishment of the RA pathway, the expression of Hox genes and function and the establishment of the antero-posterior axial skeleton patterning. They have used several elegant conditional genetic models to answer this important developmental genetic question. These questions are complex and difficult to answer for the existence of multiple Meis genes, particularly Meis1 and Meis2 and for the involvement of other genetic, cell identity determining, functions like Hox and the Raldh2 pathway.

The AA analyze a vast and well detailed homeotic phenotype upon deletion of Meis2 which becomes more severe in the Meis1-Meis2 dKO, estanlishing a primary but not exclusive role of Meis2. The phenotypes exclude a role of Meis2 in the early posterior epiblast in axial patterning, and are independent of the time of deletion in the development of these tissues (three CRE lines used). In the axial skeleton patterning, Meis expression coincides in time and space with Hox genes expression in the posterior epiblast and explains the homeotic phenotypes observed. However, the deletion of Meis does not affect Hox genes expression, leaving an open question on how the homeotic phenotype is induced.

In addition to their effect in segmental identity, Meis KOs show a profound alteration of both patterning and myogenic pathways, with phenotypes very similar to Myf5, MRF4 and Myogenin-deficient mice, that explain the altered ribs patterning.

Moreover, other experiments also demonstrate a tight connection with the RA synthetic and degradative pathways, in particular the important role of Meis in the maintenance of Raldh2 expression and the demonstration of a positive regulatory loop in RA synthesis, which may well be the cause of axial mispatterning.

The experiments presented are extremely well done and presented, are extensive and complete and give very clear and insightsful answers. Overall, I believe this paper is an important genetic contribution to developmental biology which deserves a broad audience for the importance of the results, the deepness of the analysis and the clarity of the presentation.

My personal policy is not to propose additional experiments if not absolutely essential. To me the present results are clear, even if the authors do not give a molecular mechanism that explains how Meis affects Hox function without affecting Hox genes expression. However, I believe that the paper would benefit from a paragraph in the Discussion in which some possible explanations are suggested.

R.-We thank the reviewer for the appreciation of our work. We think Meis affects Hox function in axial patterning though its ability to form complexes with Hox proteins, thereby affecting their affinity for and selectivity of targets. As suggested, we have included this view in the discussion.

Reviewer 2 Comments for the Author:

The paper by López-Delgado et al. presents a detailed analysis of axial skeletal patterning in mice with loss-of-function (LOF) of Meis1, Meis2 and Raldh2 genes. The results presented are based on the characterization of tissue-specific LOF of Meis1 and Meis2 alleles using different Cre deleter lines and evaluation of the resulting skeletal phenotypes.
1) The Authors claim that compound loss of Meis1/2 genes produces axial skeleton defects without affecting Hox gene transcription, including vertebral homeotic transformations and rib mis-patterning.
2) The Authors describe that while RALDH2 and MEIS positively regulate each other, Raldh2 elimination recapitulates the defects associated with MEIS-deficiency and they state that Meis overexpression rescues the axial skeletal defects in Raldh2 mutants.
3) The Authors propose a MEIS-RA positive feedback loop that is essential to establish anterior-posterior identities and pattern of the vertebrate axial skeleton.

In summary, the study describes a large amount of detailed work that could be of high interest to the developmental biology community and to the field of HOX and TALE transcription factor biology. While there is a rich literature available on roles of HOX proteins and TALE cofactors in organismal development, the phenotypic outcomes of compound tissue-specific loss of Meis genes in mouse models have been unknown to date. Therefore, the present study is warranted and welcomed. However, there is unease regarding some of the data described and especially regarding some of the conclusions that are being put forth, which are not supported by adequate experimental evidence.

R.-We thank the reviewer for the comments and address below the concerns raised.

Specific Comments:
a) The Authors purport the presence of homeotic transformations in compound Meis mutants, which -they state- are similar to those found in anterior-Hox and Raldh2 mutants. This conclusion is based solely on morphological evaluations of skeletal preparations, a highly descriptive (and subjective) experimental approach. Only alcian blue/alizarin red-stained skeletal preparations are shown in Figure 2. This type of analysis for skeletal structures is not adequate and certainly cannot unequivocally demonstrate the presence of homeotic transformations. In light of the recent technological advances based on methods that enable imaging and measurements of skeletal elements highlighting all the feautures of the structures under analysis, alcian blue/alizarin red-stained skeletal preparations appear somewhat outdated. Lacking any type of analysis based on current techniques, such as OPT or microCT, the authors should at least provide larger insets that better illustrate the fine details of the skeletal elements that are affected in the mutants. At the present magnification it is impossible to adequately appreciate any of the subtle features of the skeletal elements under analysis (for example, see Figure 2). Evaluation of the skeletons that is made possible by the alcian blue/alizarin red- stained stained preparations photographed at the present (very small) magnification suggests that the skeletal abnormalities displayed by compound Meis mutants might be due to segmentation defects, a scenario that the Authors mention only tangentially, more than to real homeotic transformations.

R.-We understand the limitations of classical staining methods compared to 3D image reconstruction approaches, especially regarding the quantitative analysis of shapes, however we would not be able to repeat the whole analysis by microCT or OPT at this point. Nonetheless, it is also true that the definition of homeosis “transformation of a body part into the likeness of something else” involves the qualitative appreciation of the observer and that the field has built solidly for decades on the basis of classical skeletal preparations. Some of the phenotypes observed, like the loss of ribs, cannot be taken as a proof of homeosis, because they could just mean an impairment in rib development and not a switch of identity. In the manuscript, we have not identified these cases as homeotic transformations, although they cannot be excluded. In some other cases, the position of specific structures like the anterior arch of the atlas or the tuberculi anterior appears shifted by one segment. These features have been long recognized in the field as

bona-fide homeotic transformations. While many of the vertebrae of the cervical region look very similar, the specific shape of vertebrae at the occipital-cervical junction allow to evaluate homeosis easily. It is true that there are defects in segmentation, but these only show overtly in strongly affected individuals with complete elimination of Meis function, while milder conditions mainly show left-right misalignment of the structures. The fact that the first vertebra tends to fuse with the exoccipital is an indication of its recruitment to the developmental program of the occipital region, which involves the fusion of derivatives from the first 5 somites. We think that the fact that the detected transformations are consistently anterior transformations at several levels of the A-P axis and the similarity of the observed phenotypes to those reported for Hox mutations that affect the same area, further strengthen our interpretations. We would like to point out that for the conclusions of our work, the relevant point is that the phenotypes are similar to those in Hox mutants and Raldh2 mutants; not whether the phenotypes represent "homeosis" or they do not, which of course, is arguable. Nonetheless, in consistency to previous reports on Hox and Retinoic acid metabolism mutants that describe similar defects as homeotic, we think we should keep this description. Following the advice of the reviewer, we have now included supplementary figures with magnification of the key phenotypes that we assigned to homeotic transformations.

b) The defects related to the occipital bone and C1 might share some similarities with phenotypes described in Hoxd3 and Hoxa3 mutants when Meis2 is lost in single mutants. However, when Meis1 is also inactivated on a Meis2-deficient background in compound mutants, the abnormalities of the occipital and cervical segments are very different and also much stronger than those reported so far in any Hox mutant (single or compound). Notably, these skeletal defects could simply be due to abnormalities in segmentation (see for example the skeleton in Figure 3O, or the rostral somites of mutant embryos analyzed by in situ hybridization in Figure 6B' and 6D)'. And again, one would need higher magnifications, or at least large insets, to better judge the nature of the morphological defects, in the absence of OPT or similar high-resolution preparations.

R.-This is a very interesting point and we concur with the reviewer that Meis functions go beyond those exerted by Hox transcription factors. We have now reflected this in our conclusions and in the scheme of the new figure 8, which summarizes them. Indeed, as shown in Figure 3O, absence or strong disruption of skeletal elements including imperfect segmentation is appreciable. Given that somites form and that the segmentation clock does not seem to be affected (see below in response to reviewer 3), we think Meis affects re-segmentation and that, most likely, this involves the alteration of cell adhesion/repulsion phenomena involved in re-segmentation. A different issue is whether these Meis functions go beyond Hox-related functions. The answer to this question is unknown, given that we do not know how does a completely Hox-less mouse looks like or even how the elimination of all Hox genes expressed in the occipital/cervical region looks like. As requested by the reviewer, we have included higher magnifications of the skeletal preparation of the most affected embryos. We also included this issue in the Discussion of the manuscript, and pointed out the differences with Hox mutant phenotypes in the results section.

c) Differences are also present between Hox and Meis phenotypes in the ribs. While for example loss of Hox6 paralogs causes rib phenotypes, their characteristics are strikingly different from those observed in Meis mutants.

In light of all the considerations discussed in b) and c), the Authors should be more careful in attributing the phenotypes observed in Meis compound mutants to homeosis. Statements like: "Meis elimination produces axial skeleton defects without affecting Hox gene transcription, including vertebral homeotic transformations" (in the Abstract) and "... the anterior transformations observed in the cervical region may also affect the thoracic region..." and again "the type of defects were similar, with anterior transformations of C1-C3 (Figure 3G-I and 3M" should be avoided. There is no experimental evidence that supports clear homeosis.

R.-I think the question of the homeotic transformations in the cervical/occipital region has already been addressed above. Regarding the ribs, we do not speak in the manuscript of homeotic transformations when describing the rib phenotypes. The reviewer is right in that phenotypes are clearly different from those shown by Hox mutants, but we do not claim this in the manuscript. With the marginal note, again, that we do not know how a hox-less rib cage looks like, it is clear that our manuscript does not provide any morphological evidence supporting the idea of very coincident functions here. This is mentioned on page 8, last paragraph. Nonetheless, at the

molecular level, Meis mutants affect pathways that have been shown to depend on Hox function during rib specification and therefore, we cannot rule out a Meis Hox cooperation in some aspects of rib specification and patterning. It is only in this context, that we mention in the discussion the possibility of Hox-Meis interactions in rib patterning and we think it is a valid speculation.

d) The Authors state that they have not observed alterations in Hox gene expression patterns or transcript abundance in Meis mutants. Therefore, they suggest that despite the profuse binding of MEIS proteins to the HOX complexes (Penkov et al., 2013), MEIS is not involved in Hox gene transcriptional regulation during axial skeleton patterning.

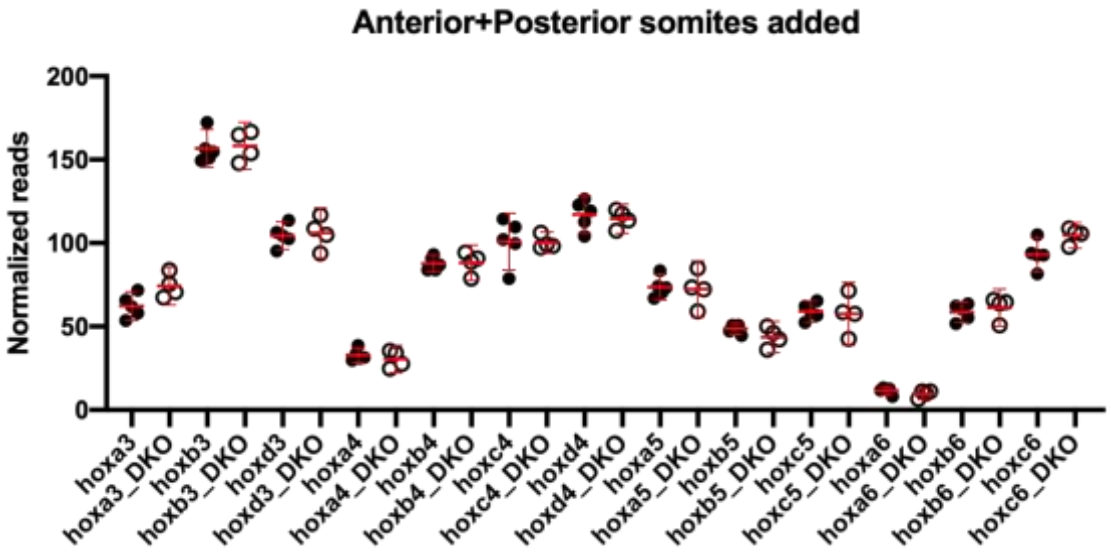
First, this Reviewer notes that the expression of some Hox genes is markedly perturbed in Meis mutants, in contrast to the above statement. This is clear when observing some of the photographs from in situ hybridization experiments that illustrate mRNA expression in developing embryos. For example, unless the Authors chose an embryos that is an outlier, Hoxa3 expression in the Meis compound mutant embryo is markedly different, with reduced domains of expression and decreased levels of expression, as compared to the wildtype (Figure 4B). Also, expression of Hoxb3 in the mutant embryo is significantly different when compared to the wildtype (Suppl Figure 3). The in situ hybridization results illustrating Hoxb3 expression are somewhat buried inside the Suppl Materials, but nonetheless the differences are clear and marked. In the opinion of this reviewer, these differences cannot be completely disregarded and ignored.
In summary, while the authors conclude that “transcriptional regulation of Hox genes is not involved in Meis regulation of axial skeleton patterning”, this reviewer does not agree with this unyielding conclusion, which is not adequately supported by the experimental findings.

R.-We agree with the reviewer in that *Hoxb3* expression changes in the mutants, but this is only so in the neural tube, not in the paraxial mesoderm, which is the focus of this manuscript. This is also true for *Hoxd3* and *Hoxd4* and it is indicated in the results section. In Figure 4B, the difference observed for a3 corresponds to the neural tube, which is not affected by Cre recombination in this embryo (recombined by *Dll1Cre*), so it is not related to the paraxial mesoderm expression of the gene but to experimental variability in the expression pattern of a3, which is very weak in the neural tube at this stage. In the b3, d3 and d4 RNA in situs on embryos completely devoid of Meis function (Figure 4), the alteration of expression is very clear in the neural tube (and we indicated it with an arrowhead). Besides keeping the mention to these changes of expression in the results section, we now mention it in the discussion, together with the published evidence on the ability of Meis to regulate Hox transcription in limb buds and neural tube. The reason to put *Hoxb3* in the supplementary figures was for better fitting of the panels in Figure 4 and because we already showed another group-3 representative in the main figure. We have now moved the *Hoxb3* panel to Figure 4.

Second, the Authors report the absence of Hox gene expression differences in wildtype versus mutant embryos assessed by RNA-seq analysis, in contrast to some of the marked differences they have found by in situ hybridization. The negative results obtained by RNA-seq could be easily due to dissection procedures, resulting in the analysis of different embryonic domains in the dissected tissues analyzed by RNA-seq. For example, dissected tissues could comprise more anterior or more posterior somites, which would dilute the overall differences in Hox gene expression.
In addition, as it appears, the authors conducted their RNA-seq analysis at E9.0, while in situ hybridization experiments were performed mostly at E10.5 or at E8/8.5. No rationale is provided for the different timepoints analyzed using the 2 different methods. The different time-points analyzed by in situ hybridization and RNA-seq could easily explain the conflicting results obtained regarding Hox gene expression. Also, there is no indication of how many biological replicates were conducted for RNA-seq experiments. This point is critical to support the quality and reliability of of the RNA-seq experiments.

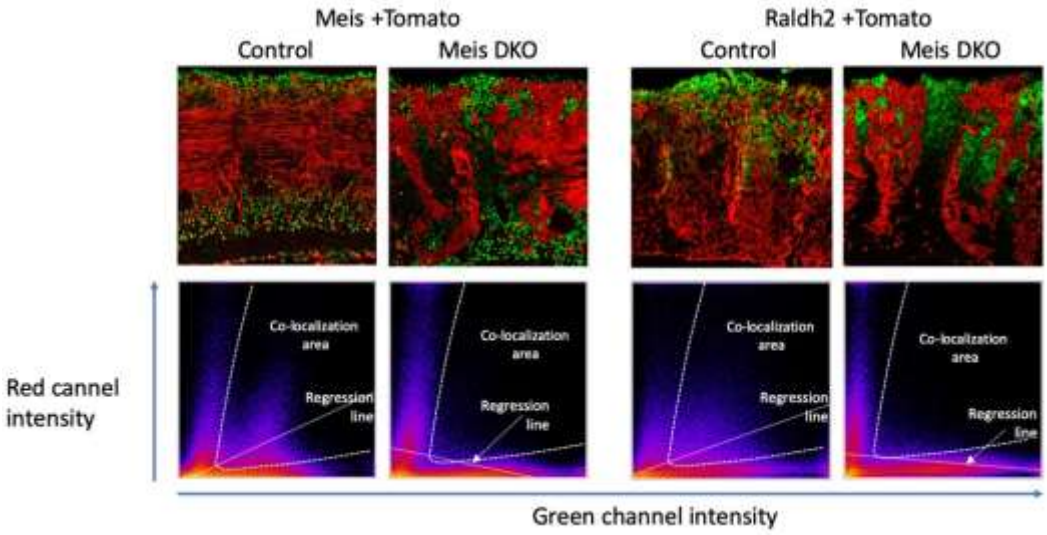
R.-The number of biological replicates (5 controls and 4 mutants) for the RNAseq experiments is indicated in the Methodology section. Given the progressive nature of AP axis development in the mouse, we do not think that a difference in the specific stage analyzed is very relevant here. We do not claim that the RNAseq results are relevant to determine the A-P border of expression of Hox genes; we understand that changes of 1-2 somites in the A-P border of expression would very likely not be reflected in RNAseq differences. We interpreted the RNAseq data, only after showing by RNA in situ that the *Hox* genes did not change their expression borders. Under these conditions,

given that RNAseq is a highly quantitative approach, we think it is a good measure of the amount of transcription of the Hox loci and allows us to conclude that the transcript levels of Hox genes is not modified by the lack of Meis activity (irrespective of their anterior borders of expression). Indeed, the reviewer is correct in that the dissection procedure can jeopardize the conclusions. Luckily, we kept track of the embryo identity through the RNAseq process for the anterior and posterior parts of the embryo. In this way we can add up the reads detected in the anterior and posterior parts of each embryo, thereby cancelling the possible effects of the variability in the dissection. The results of this analysis for paralog groups 3-6 is shown below for the reviewer and indicates a very coarse distribution of the reads for each gene (average and 95% confidence interval are shown). This analysis indicates that the dissection procedure did not compromise the conclusion that no quantitative differences were found in mRNA abundance between control and mutant embryos.



e) The Authors suggest the presence of direct Raldh2 regulation by MEIS transcription factors. However, Panels I and K of Figure 6 clearly show that there appears to be only very little overlap between cells expressing Raldh2 and Meis genes, respectively. The lack of robust co- expression is rather concerning and should be addressed.

R.-Panels 6I and K do not show co-localization of Raldh2 and Meis, because antibodies to detect these proteins are both made in rabbit, so we worked on adjacent sections. RNA in situ shows that both Meis and Raldh2 are expressed in all paraxial and lateral plate mesoderm (Figure 1E, L; 6C, Q). In the case of the analysis shown in 6I and 6K, we chose for comparison the line indicated in yellow, which is at an equivalent position for both sections. As indicated in the quantifications in I'' and K'', both Raldh2 and Meis are expressed along these lines. For comparison; in the mutant mosaic, shown in J'', Meis expression is kept in the non-tomato cells and lost in tomato cells. This shows that the signal detected in I'' is bona-fide Meis expression and that it is ubiquitous in the region. The same result is obtained for Raldh2 in K'' and L''. There is, in our opinion, no room for doubts regarding these experiments. Nonetheless, we realize that the intense dapi or Tomato staining masks the Meis and Raldh2 signal. We now provide green channels in isolation, where it can be seen that both Meis and Raldh2 are extensively expressed in somite cells. The strongest expression is in the upper part of the image, where the epithelial part of the somite is sectioned, while more central regions in which somite cells differentiate show lower -but detectable- levels. While we placed the line for quantification in the upper part, where a more solid expression is detected, the mosaic elimination can be seen elsewhere in the images and has been quantified below for the reviewer.



f) The authors show a genetic rescue experiment to demonstrate that MEIS activity in the paraxial mesoderm is sufficient to rescue the Raldh2 mutant phenotype. Hardly any experimental details are provided regarding these rescue experiments, which appear convincing at the pure morphological observation (Figure 7). However, the Authors do not consider the possibility that under the conditions of the rescue experiment Raldh2 inactivation might become suboptimal at best. If that were the case (which is not addressed) then the lack of a phenotype would not be the result of rescued RA deficiency by MEIS activity, but could be explained more likely by the presence of residual RALDH2 expression, which would enable the activation of functional levels of retinoic acid signalling in the embryo. To support a bona fide genetic rescue the Authors need to show that in these embryos Raldh2 is fully inactivated in the domains where Meis is upregulated.

Absolute statements like: “The complete rescue of Raldh2 mutants by Meis overexpression suggests that Meis is the main functional output of the positive regulatory loop between Meis and RA in the paraxial mesoderm” (page 16) should be avoided, as they are not adequately supported by sufficiently robust and careful experimental evidence.

R.-The *Meis2* overexpression allele contains EYFP linked to *Meis2* activation in the same mRNA through an IRES (as described in Roselló-Díez et al. Development 2014). We therefore checked *Raldh2* expression and *Meis2* activation simultaneously in somites of *Raldh2* floxed embryos in which the *Meis2* overexpression had been activated by *Dll1Cre*. As can be seen in the new supplementary figure 11, *Meis2* activation is extensive in these embryos and there is a perfect match between *Meis2* activation and elimination of *Raldh2* expression. The results reported in this experiment are therefore solid and clearly assignable to a rescue of the defects provoked by Raldh2 deficiency by Meis2 overexpression.

Minor Comments:
a) The nomenclature of genes and proteins is inaccurate: in the mouse, genes must be listed in Italics (Meis) and proteins in Plain Text, all letters upper case (MEIS). All nomenclature should be revisited throughout the text.
R.-Done

b) The Authors should add one last figure with a cartoon that summarizes the overall take- home message of the study, which is currently rather confusing.
R.-Done

Reviewer 3 Advance Summary and Potential Significance to Field:
The authors sought to elucidate the function of Meis in the anterior-posterior patterning by generating compound mutant mice. They found that Meis mutations exhibited the anterior homeotic transformation of the vertebrae and ribs. Interestingly, Meis and Raldh2 seem to form a

positive regulatory loop, and Rsldh2 mutation decreased Meis expression, leading to the anterior homeotic transformation of the vertebrae and ribs. Finally, the authors showed that overexpression of Meis2 rescued the skeletal defects of Rsldh2 mutation, suggesting that a proper level of Meis expression, which is dependent on RA signaling, regulates the AP patterning of the axial skeleton. This is an interesting work showing the role of Meis-RA positive regulatory loop in the AP patterning of the axial skeleton.

R.-We thank the reviewer for the appreciation of our work

Reviewer 3 Comments for the Author:
The mechanistic analyses are still preliminary and need more clarification. Specific comments are indicated below.

1. In Figure 4A and B, the authors stated that Hox expression was not altered in Meis mutants, but it is not clear from the data. They should show which somite corresponded to the anterior boundary of each Hox expression with a higher magnification.
R.-We have included a supplementary figure with magnified images and indicating the number of the rostral-most somite with Hox expression

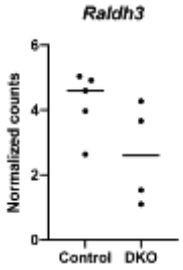
2. Meis mutants did not relocate Hox gene expression to more posterior somite at later stages, suggesting that Meis is involved in Hox gene expression. However, the authors concluded that the transcriptional regulation of Hox genes is not directly controlled by Meis. This conclusion is not clear, because they previously showed that Meis can directly control HoxA expression. The authors should clarify this issue.

R.-As we comment in the results and discussion section, Meis controls Hox transcription in the limb and in the neural tube, however, at the time when somites can be clearly counted in mutant embryos, we do not see any displacement of the boundaries in the complete elimination of Meis. At later stages, it is very difficult to count somites in the embryos in which Hox expression apparently does not relocate to more posterior positions, but only in some embryos. At these stages, these embryos appear “frozen” in development; they basically look like an E8.5 embryo with an elongated trunk and therefore, it is not possible to discern a direct action of Meis from a generalized blockade in development. Irrespective of these uncertainties, there are two important aspects that lead to the conclusion that Meis control of Hox transcription is not involved in the phenotypes observed: 1) We see normal boundaries of Hox expression in the *Delta1Cre*-recombined mutants, in which the homeotic phenotypes were found. 2) If transcriptional regulation of Hox genes was the cause of the homeotic phenotypes observed, the expected result would be a posteriorization of Hox expression boundaries (leading to anterior transformations) and not an anteriorization. Therefore, even if the anteriorization of Hox transcripts at late stages were true, it cannot be the basis of the observed phenotypes and we would still need to invoke post-transcriptional actions of Meis.

3. Figure 6 showed that while Raldh2 expression was decreased in anterior somites of Meis mutants, the RA-responsive gene RARb was not changed. This result suggested that Meis is not important for RA signaling in somites, and I am not sure whether Meis-RA positive feedback loop exists here. The authors should clarify this issue. They should also examine the expression of another related enzyme, Raldh3, which could compensate for Raldh2 down-regulation.

R.-We think the regulatory loop is solidly demonstrated by expression and genetic evidence, since Meis expression is reduced in Raldh2 mutants and vice-versa, the phenotypes of mutating either Raldh2 or Meis are largely coincident and Meis rescues the phenotypic defects in *Raldh2* mutants. A different question is whether this involves RA. Raldh enzymes are highly specialized in producing RA and no other function has been described for them. Several previous works showed that Raldh2 mutation leads to the complete elimination RA in paraxial mesoderm and nearly complete elimination in the rest of the embryo. Since we show that Raldh2 is completely lost in Meis mutant cells, it would be difficult to think that they synthesize normal levels of RA. As the reviewer points out, Raldh3 is responsible for the residual RA synthesis activity in Raldh2 mutant embryos, however there has been no previous report indicating that Raldh3 increases in Raldh2 mutants as a compensatory mechanism. Raldh3 is expressed at very low levels at the stages analyzed and in the RNAseq analyses we found a non-significant tendency to reduction in the mutants (see graph below). To confirm this result, we performed quantitative RT-PCR of *Dll1Cre*-recombined and

maternally/paternally recombined Meis1/2 floxed embryos and found that *Raldh3* was unchanged in 3/6 embryos and clearly reduced in 3/6 embryos of the *Dll1Cre* model (new figure S8), while 5/5 embryos showed clearly reduced levels in the maternally/paternally recombined embryos (new figure S8). The variability in the Dll1Cre-recombined embryos likely correlates with the variable mosaicism in this model and was not observed in the embryos with total elimination of *Meis1*/2. These results show not only that *Raldh3* does not compensate the reduction in *Raldh2* but that it also requires Meis for expression. We do not have an explanation for why Rarß does not change in Meis mutants, despite the obvious reduction in RA-synthesizing enzymes. In contrast, Cyp26b1, a well known direct RAR target, shows consistent reduction. Since not all *Raldh2* is lost from Meis mutant embryos, these embryos are not equivalent to a complete elimination of *Raldh2* and therefore are expected to show reduced but not absent RA levels. In fact, RA-less embryos do not develop properly beyond E8.5. One possibility is that Rarß requires further lower levels to switch off. In a recent study of *Raldh2* mutant embryos (Berenguer et al Plos Biol 2020), the reductions in Rarß were found not significant by RNAseq, which would support this view. Alternatively, the situation could be more complex and the transcriptional responses of Rarß be specifically be affected by Meis loss of function.
The similarity of the phenotypes observed between Meis mutants and Raldh2 or RARs mutants, and the opposite transformations in Cyp26b1 mutants or when RA is pharmacologically increased, are also strong functional support to the conclusion that Meis mutants show reduced RA levels and that this is related to the phenotypes characterized. We therefore think that the inconsistency observed for one target of the pathway does not compromise the conclusions. We have included these considerations in the discussion.



4. The authors examined Raldh2, Cyp26b1, and RARb expression by in situ hybridization, but changes in the expression levels were rather small or not clear. Because in situ hybridization is not quantitative, the authors should perform qPCR to quantify the expression levels more precisely.

R.-We have determined the reduction of *Raldh2*, *Raldh3* and *Cyp26b1* transcripts by qRT-PCR in the *Dll1Cre* model and in the Complete *Meis1/2* elimination model (Figure S8). We also did qPCR for *Rarb* in these two models, but did not find any differences with controls (Figure S8).
We think that the complete elimination of RALDH2 protein in Meis mutant cells is a very solid result, indicating the absolute requirement of Meis function for Raldh2 expression in somites. The variable results with the ISHs are due to mosaicism of the *Dll1Cre* recombination. Given that Meis directly and cell- autonomously represses *Cyp26b1* (Roselló-Díez et al 2014), the most likely scenario for this observation is an indirect effect of Meis deletion through reduction in RA levels.

5. Meis1;Meis2 double KO mice showed segmentation defects in the cervical region (Figure 3O), suggesting that the segmentation clock does not work properly at early stages. The authors should examine the expression of the clock genes such as Hes7 and Lfng in the PSM of these mutants at E8-8.5.

R.-We have studied the expression of Hes5 and Lnfg in mutant embryos and found no differences with control embryos. We show these results in the new Figure S7.

6. The last line of page 12 is misleading. At E8.75, more than 10 somites are formed, and this sentence should be corrected.

R.-Corrected

Second decision letter

MS ID#: DEVELOP/2020/193813

MS TITLE: Axial skeleton anterior-posterior patterning is regulated through feedback regulation between Meis transcription factors and retinoic acid

AUTHORS: Alejandra C. Lopez-Delgado, Irene Delgado, Vanessa Cadenas, Fatima Sanchez-Cabo, and Miguel Torres

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. 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 rsults show a mechanism for the role of Meis in the axial patterning that is Hox-independent and relies on the cooperation with Retinoic Acid.

Comments for the author

I stick to my previous evaluation.

Reviewer 3

Advance summary and potential significance to field

The authors have properly addressed most of my concerns, and I have only minor comments.

Comments for the author

Figures 4C and S5 showed that the anterior border of Hoxd4 is S6 in the control and S4 in the Meis mutants (with Zp3-Cre x Stra8-Cre) at S10-16 stages, suggesting that Hoxd4 expression is anteriorized. This anteriorization may not be involved in vertebral homeotic transformations, but “Meis elimination produces axial skeleton defects without affecting Hox gene transcription” in Abstract is somewhat misleading. Meis elimination does affect some Hox gene expression, and therefore this statement should be corrected.

The authors should explain what arrowheads stand for in Figure 4B.

Second revision

Author response to reviewers' comments

We have modified the text of Abstract and Figure 4 legend accordingly to the requests of reviewer 3

Third decision letter

MS ID#: DEVELOP/2020/193813

MS TITLE: Axial skeleton anterior-posterior patterning is regulated through feedback regulation between Meis transcription factors and retinoic acid

AUTHORS: Alejandra C. Lopez-Delgado, Irene Delgado, Vanessa Cadenas, Fatima Sanchez-Cabo, and Miguel Torres

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.