Brachyury controls *Ciona* notochord fate as part of a feed-forward network

Wendy M. Reeves, Kotaro Shimai, Konner M. Winkley and Michael T. Veeman
DOI: 10.1242/dev.195230

Editor: Stephen Wilson

Review timeline
Original submission: 20 July 2020
Editorial decision: 1 September 2020
First revision received: 9 November 2020
Editorial decision: 2 December 2020
Second revision received: 16 December 2020
Accepted: 18 December 2020

Original submission

First decision letter

MS ID#: DEVELOP/2020/195230

MS TITLE: Brachyury controls Ciona notochord fate as part of a feedforward network and not as a unitary master regulator

AUTHORS: Wendy Reeves, Kotaro Shimai, Konner Winkley, and Michael Veeman

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 substantial revisions to your manuscript. One issue picked up by two of the referees is that setting up the idea of Brachyury as a single master regulator is a bit of a straw man as there is little evidence to support this idea. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

We are aware that you may 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

In the current study, the authors conducted genome-wide transcriptome analyses following misexpression and CRISPR-mediated gene knockdown of Brachyury in Ciona. The analyses revealed that only subsets of genes among those enriched in the notochord-specific transcriptome were affected by these treatments, leading the authors to conclude that Bra is not a unitary master regulatory gene for notochord fates in Ciona. They also provided evidence indicating that Foxa.a is a part of the notochord regulatory gene network.

Comments for the author

The authors in this manuscript challenged again the concept of Brachyury being the notochord master regulatory gene in Ciona (Reeves et al, 2017, Development). However, I am not sure whether this gene has ever been defined as a master regulatory gene in Ciona. The three references are cited in the manuscript as the studies that originally described Bra as the master regulator of notochord fates (Hotta et al, 1999; Imai et al, 2006; Takahashi et al, 1999). However, I failed to find any description of "master" in these studies (I didn't read them through but simply searched them for "master"). Furthermore, in an earlier study (Yasuo and Satoh, 1998), it was discussed that 'it is unclear whether expression of Bra is sufficient for ‘‘full’’ notochord differentiation in ascidian notochord precursors'. I suggest the authors to tone down their emphasis on this matter (they have already made a point in their previous study!!).

I listed below several points that the authors might want to address to improve their study:

1) Figure 1D indicates that a great number of upregulated genes in Foxa.a>Bra embryos are not among the notochord-enriched genes previously identified by FACS-seq. Do they express in notochord in control embryos? The info might be in suppl tables but it is not easy for me to decode them.

2) It is not clear to me how "significant" and "not-significant" genes were defined in Figure 2C. Related to this, 3 out of 7 "not-significant" genes tested by in situ were shown to be Bra-dependent (Figure 3B). What does "not-significant" mean??

3) Among the upregulated genes in Etr1>Bra/Etr1>Foxa.a embryos (240 genes) and Foxa.a>Bra embryos (255 genes), how many of them are overlapped?

4) For Figure 6B, how many genes were analyzed? I am surprised to see that only a very few genes exhibit enriched Bra and FoxA motifs in their -1.5Kb regions. Could the authors comment on this in their result or discussion section?

Reviewer 2

Advance summary and potential significance to field

The manuscript “Brachyury controls Ciona notochord fate as part of a feedforward network and not as a unitary master regulator” is a well-designed study that highlights the complexities associated with GRNs in regulating cell and tissue types, such as the Ciona notochord. The authors show that Bra/Brachyury and Foxa.a function as part of a type 1 feed-forward GRN. The GRN involving Bra and Foxa.a acts downstream of FGF signaling to regulate the expression of notochord-enriched genes. This study enhances the field by showing that the previously characterized master transcriptional regulator, Bra, is part of a wider network of transcription factors that regulate gene expression. Overall, this is clearly presented work.

Comments for the author

1. The authors show that FGF signaling directly or indirectly regulates Foxa.a expression in the notochord. The authors claim that the "precise start or end points to this dependency" are not

mapped. Could the authors speculate a bit more on how they think FGF signaling is regulating Foxa.a, especially in regard to FGF9/16/20 and FGF8/17/18 (Yasou and Hudson, 2007)? Some more discussion about this would be very helpful. In addition, I would encourage the authors to consider a more direct approach toward inhibiting FGF signaling, by using an FGF receptor inhibitor, such as the FGF receptor 1 inhibitor, SU5402 (Mohammadi et al., 1997 Science). Inhibiting FGF more directly will make a stronger argument for FGF's specific role in acting upstream of Foxa.a.

2. In the introduction, pg 3 line 10, rearrange sentence to say "...but instead gives rise to a mass of cells....".

3. Supplemental tables 1 and 2 should include a column that contains the standard gene name, like that found in supplemental table 3. In addition, It would be helpful if the authors provided common gene names for the genes upregulated and downregulated in Figure 1C.

4. The X-axis in the plot in Figure 2B should be labeled.

5. In the Results section, under subsection "Bra misexpression only induces a subset of notochord-enriched genes" pg 5, line 14 "….921 twofold-enriched…", two and fold should be two words.

6. Under subsection "Some notochord-enriched genes are independent of Brachyury", pg 6, second paragraph, the text does not clearly depict the genes we should be looking at in figure 3. For example, where stated "As expected notochord expression was lost.....showing large decreases in the Bra CRISPR RNAseq.", it is not entirely clear as to which part of Figure 3 this is referring to? Perhaps placing a parenthesis indicating orange boxes would be more helpful?

7. In regard to Figure 4A and 4B, What is the fluorescent expression being shown? Is it a protein fusion or stain? The micrograph should properly label the fluorescent fusion protein or stain depicted in the micrographs.

8. Regarding Figure 5A, for consistency purposes, the authors should consider including the fluorescent reporter in the micrograph labels (i.e. Etr1>H2B-Venus).

9. Regarding Figure 5B, it would be helpful if the authors provided common gene names for the genes upregulated and downregulated as recommended for figure 1C.

10. For the reporter assay in Figure 6D, it would be nice to have representative images from this experiment, either in the figure or in a supplemental figure. I'm also concerned about the quantification here given the noted variable transfection efficiencies between experiments. It would strengthen the results to have a method for normalization of transgene dose. For instance this could be done by adding a ubiquitous promoter driving a spectrally distinct fluorescent protein to the experimental plasmids, which has been done in other systems.

11. In the discussion section, pg 14, first paragraph, line 7 "Another potential role is as...". This sentence is too complex and repeats noise and inadvertent. Please rephrase. Perhaps "Another potential role is to act as persistence detectors and noise filters to prevent stochastic fluctuations in transcription and/or signaling from inadvertently triggering unintentional and irreversible cell fate decisions"?

Reviewer 3

Advance summary and potential significance to field

In this manuscript the authors challenge the hypothesis that Brachyury may be a unitary "master regulator" of notochord fate in Ciona. They find that the ability of Brachyury to induce the notochord gene expression programme is context-specific, that it acts in conjunction with other factors to more comprehensively induce notochord gene expression, and that FGF acts upstream of key regulator Foxa.a. They support this with analyses indicating enrichment of T-box and forkhead binding sites in proximal cis-regulatory elements of notochord genes. They finally explore

regulation of Brachyury itself through sequence and deletion analysis of Brachyury upstream sequences, and present a resulting GRN for notochord specification.

The requirement for Brachyury in notochord is well known, however, the specifics of its function in this context are not completely understood. This manuscript does advance our understanding, however, I feel that the idea that it is not a unitary regulator is not wholly surprising. Typically in order to induce/repress cell fates changes in chromatin accessibility/nucleosome localisation are required. There are multiple models for how this might be achieved, e.g. pioneer transcription factors engaging nucleosome-rich chromatin to recruit remodellers, or cooperativity of a cohort of factors to competitively bind DNA thus evicting nucleosomes.
However, given there is no compelling evidence that T-box factors act as pioneers (unlike certain Fox and Sox factors) I am not aware of any convincing model wherein Brachyury individually was likely to be a unitary "master regulator". For T-box factors that are sufficient to induce cell fates e.g. Eomes in zebrafish, this appears to be through induction of a limited number of other potent cell fate regulators e.g. Nodal, rather than intrinsic properties of the T-box factor. This does not detract from the value of the novel experiments and regulatory relationships that are defined in the manuscript. However, given the prior evidence not in favour of Brachyury being a unitary "master regulator" including the literature the authors cite in the introduction, the emphasis seems slightly odd to someone outside the Ciona community. This may be a minor point and easily fixed.

Comments for the author

My experience largely relates to mammalian and zebrafish biology, so I am not an expert in Ciona development or experimentation. However, overall this seems to be a reasonable and well executed study. I do, however, have one major experimental concern and some additional minor comments:

Major:
The authors rely on tissue-specific enhancers to drive Brachyury expression in different regions of the embryo. What do we know of the levels of Brachyury induced by the different enhancers and the timing of expression relative to each other than the endogenous gene? How can we know whether differences in gene expression induction are due to cell type-specific differences in the ability to respond to Brachyury rather than differences in dose and temporal exposure to Brachyury? It may be possible to address this using existing data, but it is critical to determining the validity of results.

Further comments:
Page 6 - Salla, Col2a1, Col11A1/2 and CHDH, why are these genes mentioned? Where are they on the plot (Fig. 2C)?
Page 8 - "Of the remaining genes, Foxa.a was slightly but significantly decreased (p-value < 0.1) at the 112-cell stage". This seems like a fairly permissive interpretation of significance.
Pages 9 and 10 - Use of the terminology "only a few" in multiple places is not very specific nor seems to capture what is being explained Figure 1C and similar panels - I feel that these Excel conditional formatting-like figures would be more informative if the gene names were shown Figure 1D - this is confusing. Can the numbers be placed/labelled as per 1B so hey sum to 265 and we know what is common to different conditions?
Figure 4C - The table is not presented in a publication-ready manner Figure 6D - Why is the Zic plot kinked? It should ideally be fixed or explained in the legend.
Supplementary tables - these are not ideally accessible without gene names, which should be added. I also think a header lines should be added to explain each table. lfcSE should also be explained.

Importantly, it needs to be clear that the RNA-seq data have/will be submitted to suitable repository e.g. NCBI GEO for universal access. Ideally such a submission would have been available to reviewers since there is great need to broadly vet suitability.

First revision

Author response to reviewers' comments

My coauthors and I thank all three reviewers for their helpful comments. We are glad to see that all three reviewers are in agreement that we've made useful advances in understanding the gene regulatory networks for Ciona notochord fate. I think that we've been able to address all the reviewer concerns in this revised submission. Our responses to individual comments are in italics below.

Reviewer 1 Advance Summary and Potential Significance to Field:
In the current study, the authors conducted genome-wide transcriptome analyses following misexpression and CRISPR-mediated gene knockdown of Brachyury in Ciona. The analyses revealed that only subsets of genes among those enriched in the notochord-specific transcriptome were affected by these treatments, leading the authors to conclude that Bra is not a unitary master regulatory gene for notochord fates in Ciona. They also provided evidence indicating that Foxa.a is a part of the notochord regulatory gene network.

Reviewer 1 Comments for the Author:
The authors in this manuscript challenged again the concept of Brachyury being the notochord master regulatory gene in Ciona (Reeves et al, 2017, Development). However, I am not sure whether this gene has ever been defined as a master regulatory gene in Ciona. The three references are cited in the manuscript as the studies that originally described Bra as the master regulator of notochord fates (Hotta et al, 1999; Imai et al, 2006; Takahashi et al, 1999). However, I failed to find any description of "master" in these studies (I didn't read them through but simply searched them for "master"). Furthermore, in an earlier study (Yasuo and Satoh, 1998), it was discussed that 'it is unclear whether expression of Bra is sufficient for ‘‘full’’ notochord differentiation in ascidian notochord precursors'. I suggest the authors to tone down their emphasis on this matter (they have already made a point in their previous study!!).

Reviewer 1 is correct that these other papers do not use the phrase 'master regulator'. They do, however, all make the argument that misexpression of Bra transforms other cell types into notochord. The abstract of Takahashi '99, for example, states 'Here, we show that the misexpression of the Brachyury gene (Ci-Bra) of Ciona intestinalis is sufficient to transform endoderm into notochord.'. The reviewer is correct that Yasuo and Satoh '98 expressed some uncertainty about whether the transformation to notochord was complete, but all of these papers that argue that ectopic Brachyury expression is sufficient to transform other cell types to notochord make the implicit argument that Brachyury is a master regulator. I agree, however, that our original submission lacked nuance in how that was discussed, and we should not have implied that other authors used a phrase that they did not. We've updated the text, including changing the title, to 'tone down' the master regulator emphasis as requested and give a more balanced perspective.

Reviewer 1 is also correct that we started to chip away at the idea of Bra as a master regulator in our 2017 Development paper. I don't feel like that paper was definitive, however, in addressing this topic. We only misexpressed Bra with a single enhancer construct for that paper and there was no loss of function data. The data presented here is much more thorough and supports conclusions that can be far more firmly stated. That said, it was not our intention to sound unduly critical of Takahashi '99 or the other seminal ascidian Brachyury papers. These are all wonderful papers that made sound conclusions based on the data available at the time. It is only the availability of far better transcriptional profiling methods nowadays that allows for more nuanced interpretations. I probably wouldn't have switched to Ciona as a postdoc if I hadn't read and loved the Takahashi '99 paper as a grad student! I don't want to completely strip the manuscript of master regulator framing because I think that the view of Bra as a uniquely necessary and sufficient regulator of notochord fate has been widespread in the Ciona community and requires careful attention. I hope that our revised and toned down text will be seen as a good compromise between different perspectives.

I listed below several points that the authors might want to address to improve their study:

1) Figure 1D indicates that a great number of upregulated genes in Foxa.a>Bra embryos are not among the notochord-enriched genes previously identified by FACS-seq. Do they express in notochord in control embryos? The info might be in suppl tables but it is not easy for me to decode them.

Although this information is not available in the supplemental figures for the current paper, it was addressed in our previous paper. We searched publicly available in situ hybridization results on ANISEED and also tested 18 genes ourselves by in situ (Fig S2 of Reeves et al 2017). Of the genes with in situ information that were upregulated by Foxa.a>Bra expression but not identified as notochord enriched by FACSseq, essentially all are either broadly expressed in multiple tissues, enriched in other tissues besides notochord or not visibly expressed. We've added a sentence to the text to refer people to the earlier paper and clarify this important topic.

2) It is not clear to me how "significant" and "not-significant" genes were defined in Figure 2C. Related to this, 3 out of 7 "not-significant" genes tested by in situ were shown to be Bra-dependent (Figure 3B). What does "not-significant" mean??

This was based on statistical testing for differential expression using DESEQ2 and a p-value threshold (adjusted for multiple comparisons) of 0.05. This is discussed in the Methods but we have added text to the figure legend to make it more clear.

3) Among the upregulated genes in Etr1>Bra/Etr1>Foxa.a embryos (240 genes) and Foxa.a>Bra embryos (255 genes), how many of them are overlapped?

This is a really interesting question that we should have discussed in the original submission. Most of the genes are overlapping between the two sets. Of the 240 genes upregulated by Etr>Bra/Fox and the 255 genes upregulated by Foxa.a>Bra, 193 of them are upregulated in both (statistically significant padj <=0.05). If you look simply at the direction of the effect, the overlap is higher. Of the 255 genes significantly upregulated by Fox>Bra, 252 have increased expression in Etr>Bra/Fox. Of the 240 genes significantly upregulated by Etr>Bra/Fox, 238 have increased expression in Fox>Bra.This supports the idea that coexpression with FoxA (either endogenous FoxA with the FoxA>Bra construct or exogenous FoxA in the Etr>Bra/Etr>FoxA cocktail) is important for Bra's ability to induce a subset of notochord genes. We've added a discussion of this question to the text.

4) For Figure 6B, how many genes were analyzed? I am surprised to see that only a very few genes exhibit enriched Bra and FoxA motifs in their -1.5Kb regions. Could the authors comment on this in their result or discussion section?

At stage 16, we analyzed the 693 genes that were 2x notochord enriched by FACSseq and used a comparison set of 1000 genes that were the most depleted in notochord (relative to the non-notochord control). At stage 19, we analyzed the 658 genes that were 2x notochord enriched by FACSseq and 1000 genes that were the most depleted. In total between the two stages, there were 921 notochord enriched genes analyzed. We've added more of these details to the Methods. The graph in 6B is showing the Z-scores for the relative enrichment of the different binding motifs between the notochord enriched and notochord depleted gene sets. These Z-scores aren't exactly the same as a p-value, but you can think of them in a similar way in terms of being a statistical metric of support,for the enrichment of a particular motif in the set of target sequences versus the control sequences. The Z-scores aren't a direct measurement of the absolute number of sites in each set/enhancer. This was poorly phrased in our original submission and we have rephrased it to be more clear.

Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript "Brachyury controls Ciona notochord fate as part of a feedforward network and not as a unitary master regulator" is a well-designed study that highlights the complexities associated with GRNs in regulating cell and tissue types, such as the Ciona notochord. The authors show that Bra/Brachyury and Foxa.a function as part of a type 1 feed-forward GRN. The GRN involving Bra and Foxa.a acts downstream of FGF signaling to regulate the expression of notochord-enriched genes. This study enhances the field by showing that the previously

characterized master transcriptional regulator, Bra, is part of a wider network of transcription factors that regulate gene expression. Overall, this is clearly presented work.

Reviewer 2 Comments for the Author:
1.The authors show that FGF signaling directly or indirectly regulates Foxa.a expression in the notochord. The authors claim that the "precise start or end points to this dependency" are not mapped. Could the authors speculate a bit more on how they think FGF signaling is regulating Foxa.a, especially in regard to FGF9/16/20 and FGF8/17/18 (Yasou and Hudson, 2007)? Some more discussion about this would be very helpful. In addition, I would encourage the authors to consider a more direct approach toward inhibiting FGF signaling, by using an FGF receptor inhibitor, such as the FGF receptor 1 inhibitor, SU5402 (Mohammadi et al., 1997, Science). Inhibiting FGF more directly will make a stronger argument for FGF's specific role in acting upstream of Foxa.a.

We've added a sentence speculating that the temporal aspects of FoxA regulation by FGF may be similar to Brachyury's regulation by FGF where FGF9/16/20 and FGF8/17/18 have separable roles in induction versus maintenance and FGF acts over a considerable length of time and not as a momentary cue. I'm hesitant to speculate too much about this though because it could be addressed by conceptually straightforward experiments that we haven't gotten to yet that fall outside the immediate scope of this paper (in situs for FoxA on a time course of U0126 addition, FoxA in situs in FGF9/16/20 and FGF8/17/18 MO embryos etc).

Using an FGF receptor inhibitor is not a bad idea, but U0126 treatment is widely used in the Ciona community as a proxy for FGF inhibition. Vegetal FGF signaling in the early embryo is required to induce multiple cell fates beyond notochord and has received a great deal of experimental attention. There have been extensive in situ screens for signaling molecules in early Ciona development and no other ligands likely to induce MAPK pathway activity have been found to have relevant expression patterns. It's theoretically possible that some other ligand has been missed, but the fact that combined knockdown of FGF9/16/20 and FGF8/17/18 completely eliminates notochord makes me pretty confident that FGFs are the main players here. I think it makes more sense to save this topic for some future manuscript where we use CRISPR or MOs to actually knock out or knock down FGF9/16/20 and/or FGF8/17/18 and assess the effects on FoxA expression. This would be more compelling in the long run than another drug experiment here. We have made some edits to the manuscript, however, to make it more clear that U0126 inhibits MAPK pathway activity and not FGF per se.

2. In the introduction, pg 3 line 10, rearrange sentence to say "...but instead gives rise to a mass of cells....".

Agreed. We have made this change.

3. Supplemental tables 1 and 2 should include a column that contains the standard gene name, like that found in supplemental table 3. In addition, It would be helpful if the authors provided common gene names for the genes upregulated and downregulated in Figure 1C.

We have added gene names to Supplemental tables 1 and 2. Due to space constraints in Fig 1C (and the fact that some of the genes do not have clear homologs in other species to aid in assigning them standard names), we have provided the gene names for the tissue markers in supplemental table 2.

4. The X-axis in the plot in Figure 2B should be labeled.

Agreed. We have corrected this figure to include the x-axis label.

5. In the Results section, under subsection "Bra misexpression only induces a subset of notochord-enriched genes" pg 5, line 14 "….921 twofold-enriched…", two and fold should be two words.

Agreed. We have made this correction.

6. Under subsection "Some notochord-enriched genes are independent of Brachyury", pg 6, second

paragraph, the text does not clearly depict the genes we should be looking at in figure 3. For example, where stated "As expected, notochord expression was lost.....showing large decreases in the Bra CRISPR RNAseq.", it is not entirely clear as to which part of Figure 3 this is referring to? Perhaps placing a parenthesis indicating orange boxes would be more helpful?

We have edited the text to clarify which parts of the figure are being referenced.

7. In regard to Figure 4A and 4B, What is the fluorescent expression being shown? Is it a protein fusion or stain? The micrograph should properly label the fluorescent fusion protein or stain depicted in the micrographs.

The embryos were stained with Alexafluor-conjugated phalloidin. We ended up removing these images and turning this figure into a table as the key information was better suited to a table, but we've added this info to other figure legends where relevant

8. Regarding Figure 5A, for consistency purposes, the authors should consider including the fluorescent reporter in the micrograph labels (i.e. Etr1>H2B-Venus).

Good point. We have made this modification to the figure.

9. Regarding Figure 5B, it would be helpful if the authors provided common gene names for the genes upregulated and downregulated as recommended for figure 1C.

We have included the gene names in the associated supplemental table 2 (as described in response 3 above)

10.For the reporter assay in Figure 6D, it would be nice to have representative images from this experiment, either in the figure or in a supplemental figure. I'm also concerned about the quantification here given the noted variable transfection efficiencies between experiments. It would strengthen the results to have a method for normalization of transgene dose. For instance this could be done by adding a ubiquitous promoter driving a spectrally distinct fluorescent protein to the experimental plasmids, which has been done in other systems.

We've added a set of representative reporter images to this figure as requested. The effects seen with the different TFBS mutations are quite large (~90% decrease in median expression) so I'm not particularly concerned about the data being confounded by variability in electroporation efficiency. There is some variability from electroporation to electroporation, but it's not on that large a scale for electroporations with reasonable capacitances. We exclude electroporations with low reported capacitances characteristic of poor transfection and then quantify embryos from multiple electroporations for each construct. More sophisticated methods might be needed to be confident in really small changes in reporter activity but I find the current approach to be convincing for large effects like the ones reported here. The use of a coelectroporated control reporter with a different tag is a good idea, and we have actually done that in another paper when we were investigating a subtler aspect of regionalized gene expression within the notochord (Harder et al, Dev Bio, 2019), but it imposes some other challenges on the imaging process and we did not pursue it here. Such approaches will become increasingly important in the future, however, as cis-regulatory studies in Ciona become increasingly quantitative. Until recently the Ciona norm has just been to count the fraction of X-gal stained embryos expressing a lacZ transgene at all, so our quantitative imaging here is a step in the right direction.

11. In the discussion section, pg 14, first paragraph, line 7 "Another potential role is as...". This sentence is too complex and repeats noise and inadvertent. Please rephrase. Perhaps "Another potential role is to act as persistence detectors and noise filters to prevent stochastic fluctuations in transcription and/or signaling from inadvertently triggering unintentional and irreversible cell fate decisions"?

Agreed. We have made this change.

Reviewer 3 Advance Summary and Potential Significance to Field:
In this manuscript the authors challenge the hypothesis that Brachyury may be a unitary "master

regulator" of notochord fate in Ciona. They find that the ability of Brachyury to induce the notochord gene expression programme is context-specific, that it acts in conjunction with other factors to more comprehensively induce notochord gene expression, and that FGF acts upstream of key regulator Foxa.a. They support this with analyses indicating enrichment of T-box and forkhead binding sites in proximal cis-regulatory elements of notochord genes. They finally explore regulation of Brachyury itself through sequence and deletion analysis of Brachyury upstream sequences, and present a resulting GRN for notochord specification.

The requirement for Brachyury in notochord is well known, however, the specifics of its function in this context are not completely understood. This manuscript does advance our understanding, however, I feel that the idea that it is not a unitary regulator is not wholly surprising. Typically in order to induce/repress cell fates changes in chromatin accessibility/nucleosome localisation are required. There are multiple models for how this might be achieved, e.g. pioneer transcription factors engaging nucleosome-rich chromatin to recruit remodellers, or cooperativity of a cohort of factors to competitively bind DNA thus evicting nucleosomes.
However, given there is no compelling evidence that T-box factors act as pioneers (unlike certain Fox and Sox factors) I am not aware of any convincing model wherein Brachyury individually was likely to be a unitary "master regulator". For T-box factors that are sufficient to induce cell fates e.g. Eomes in zebrafish, this appears to be through induction of a limited number of other potent cell fate regulators e.g. Nodal, rather than intrinsic properties of the T-box factor. This does not detract from the value of the novel experiments and regulatory relationships that are defined in the manuscript. However, given the prior evidence not in favour of Brachyury being a unitary "master regulator" including the literature the authors cite in the introduction, the emphasis seems slightly odd to someone outside the Ciona community. This may be a minor point and easily fixed.

This is a really interesting point about Brachyury being unlikely to have pioneer factor activity and thus unlikely to be a true master regulator. This isn't something we gave much thought to in preparing the original submission but it's deeply relevant and we've worked a discussion of this topic into the revised manuscript. It's clear from prior work that misexpressing Bra outside the notochord in ascidian embryos can induce numerous notochord markers, so until recently it was very reasonable to think that it was a true master regulator regardless of the specific transcriptional regulatory mechanisms involved. Our work here and in our 2017 Development paper shows that this transformation to notochord is incomplete, but it certainly has a non-trivial ability to partially reprogram other cells into a more notochord-like transcriptional profile. There has unfortunately been very little cell type specific epigenetic profiling in the early Ciona embryo, so we don't actually have strong expectations for whether pioneer factor activity plays a pivotal role in notochord cell fate specification.

The recent whole-embryo ATACseq data of Madgwick et al show surprisingly few changes between stages, at least to my eye, and many genes seem to have open chromatin at enhancers well before they are actually expressed. This is whole-embryo data though, so we have no idea what is going on in specific lineages. Most of the early Ciona cell-fate decisions take place in the context of cell divisions where replication fork passage may transiently open chromatin for the access of non-pioneer TFs, and I have a gut feeling that such a rapidly developing organism must have poised polymerase complexes preloaded on many genes. It certainly seems reasonable that non-pioneer TFs would be less able to completely reprogram other cell types than pioneer TFs, but I think there are plausible ways that non-pioneer TFs like Bra could potentially act as true master regulators in Ciona. I agree though that aspects of how we framed the paper around the idea of Bra as a master regulator were overstated, and we have toned that down in response to Reviewers 1 and 3

Reviewer 3 Comments for the Author:
My experience largely relates to mammalian and zebrafish biology, so I am not an expert in Ciona development or experimentation. However, overall this seems to be a reasonable and well executed study. I do, however, have one major experimental concern and some additional minor comments:

Major:
The authors rely on tissue-specific enhancers to drive Brachyury expression in different regions of

the embryo. What do we know of the levels of Brachyury induced by the different enhancers and the timing of expression relative to each other than the endogenous gene? How can we know whether differences in gene expression induction are due to cell type-specific differences in the ability to respond to Brachyury rather than differences in dose and temporal exposure to Brachyury? It may be possible to address this using existing data, but it is critical to determining the validity of results.

We went back and reanalyzed our RNAseq data to directly quantify the expression of each of the three transgenes using reads for the Venus YFP fusion that all three Brachyury constructs were tagged with. We found that the three enhancers induced Venus expression at similar levels, with both Etr and Tbx driving expression levels around 60% of Foxa.a levels.

Foxa.a<Venus-Bra FPKM = 136
Etr>Venus-Bra FPKM = 77
Tbx>Venus-Bra FPKM = 83

It is important to keep in mind that each of these enhancers drives expression in different numbers of cells. As a fraction of the total embryo at the 112 cell stage (when most cells are of similar size and all of these enhancers are active),

Foxa.a 22/112 cells
Etr 20/112 cells
Tbx 12/112 cells

Normalized to the fraction of cells that are expressing, Tbx is very close to Foxa and Etr is still within a two-fold range. Although they are not identical, we are confident that the expression levels of Venus-Bra in the three misexpression experiments are quite close.

Endogenous Foxa and Tbx expression can be strongly detected by in situ hybridization at the 16-cell stage, and the Tbx reporter has been shown to be detectable as early as the 32-cell stage. Endogenous Etr is strongly expressed by the 110 cell stage. We haven't specifically tested when we can first detect these transgenes, but all are expressed long before we harvested embryos for RNAseq at the early tailbud stage. I would note that Etr>Bra was considerably more effective at reprogramming cells towards a notochord-like transcriptional profile compared to Tbx>Bra even though it is both somewhat later in onset and somewhat weaker in expression (after correcting for the number of expressing cells). That said, it is certainly true that there might be some other enhancer capable of driving Bra at a specific stage or in a specific cell type that might lead to a more extensive reprograming, and we cannot 100% exclude there being some role for the strength and/or temporal onset of expression in the differences seen between the different driver constructs. Note that our initial goal here was to test some other enhancers to see whether they might have a more profound ability to induce the expression of notochord-enriched genes than what we had seen with FoxA>Bra in our 2017 paper. The Etr1 and Tbx6 enhancers were instead found to be even weaker in this respect, so we focused our efforts on the combinatorial cocktail experiments, the CRISPR knockout and the GRN dissection. The fact that we observe similar notochord gene upregulation in the Foxa.a>Bra embryos and the Etr>Bra+Fox embryos also supports our hypothesis that a key difference between Foxa.a>Bra and Etr>Bra response is the lack of endogenous Foxa.a transcriptional activator expression in the majority of neural tissues expressing Etr

We've revised the text to include the new quantitation of transgene expression levels and a discussion of the temporal details. We've also added a more nuanced discussion of the assumptions involved.

Further comments:
Page 6 - Salla, Col2a1, Col11A1/2 and CHDH, why are these genes mentioned? Where are they on the plot (Fig. 2C)?

SallA is interesting because it is a transcription factor and these collagens are well-known early notochord genes. We agree though that this was a bit random as presented. We've removed the specific mention of these genes by name to streamline this section.

Page 8 - "Of the remaining genes, Foxa.a was slightly but significantly decreased (p-value < 0.1) at the 112-cell stage". This seems like a fairly permissive interpretation of significance.

This is indeed somewhat permissive but we were only using p-values here as a way to prioritize candidate genes for other studies. We validated FoxA by in situ hybridization and found that it

was indeed strongly affected by U0126 treatment but the decrease in notochord expression was extensively offset by an increase in part of the endoderm. Given this validation, it was useful to be able to use this less stringent 0.1 threshold to include FoxA on what is now called Table 1. Note that the standard p-value threshold of 0.05 can be a useful convention but it represents an arbitrary balance between type I and type II statistical errors. We're using p-values that have been corrected for multiple comparisons, so most of the differentially expressed genes in the 0.05 <Padj <0.1 range are expected to be true positives and not false positives. We don't want this to be distracting though, so we've kept these details in the table and streamlined the text.

Pages 9 and 10 - Use of the terminology "only a few" in multiple places is not very specific nor seems to capture what is being explained

We found three places in the manuscript where we used that phrasing. We've left one of them where it seemed apt but edited the other two to be more specific.

Figure 1C and similar panels - I feel that these Excel conditional formatting-like figures would be more
informative if the gene names were shown

As described in response to reviewer 1, we have added the gene names (where assigned) to the supplemental table 2

Figure 1D - this is confusing. Can the numbers be placed/labelled as per 1B so hey sum to 265 and we know
what is common to different conditions?

This is an excellent idea. The figure has been relabeled as suggested.

Figure 4C - The table is not presented in a publication-ready manner

It's not clear what this reviewer was specifically concerned about here, but we decided that the images weren't really needed so we reformatted this data into a table instead of a figure and have followed the Development table formatting requirements.

Figure 6D - Why is the Zic plot kinked? It should ideally be fixed or explained in the legend.

The central notches in the box and whisker plots show a confidence interval of the median, which was outside the quartile range for that construct. That is expected behavior for this plotting library and doesn't indicate anything distressing about the data. We did not explain this properly in the original submission so we've added some clarifying text to the figure legend.

Supplementary tables - these are not ideally accessible without gene names, which should be added. I also think a header lines should be added to explain each table. lfcSE should also be explained.

This is an excellent suggestion. We have added the gene names to all supplemental tables, added informative header lines with titles and changed the column header for lfcSE to the more informative Log2FoldChange StdError (Since it is the standard error for the log2foldchange.).

Importantly, it needs to be clear that the RNA-seq data have/will be submitted to suitable repository e.g. NCBI GEO for universal access. Ideally such a submission would have been available to reviewers since there is great need to broadly vet suitability.

The data is now publicly available in GEO and the accession numbers are in the revised manuscript.

Second decision letter

MS ID#: DEVELOP/2020/195230

MS TITLE: Brachyury controls Ciona notochord fate as part of a feedforward network

AUTHORS: Wendy M Reeves, Kotaro Shimai, Konner M Winkley, and Michael T Veeman

The referees are largely happy with your revisions and there are just a few points to address before we proceed to publication. 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.

Reviewer 1

Advance summary and potential significance to field

In the current study, the authors conducted genome-wide transcriptome analyses following misexpression and CRISPR/Cas9-mediated gene KO of Brachyury in Ciona. The analyses revealed that only subsets of genes among those enriched in the notochord-specific transcriptome were affected by these treatments, indicating that Brachuyry is not a unitary master regulator of notochord fate. Importantly, co-misexpression of Brachyury and Foxa.a lead to an increase in the number of upregulated notochord-specific genes. Consistently, a computational analysis of upstream regions of notochord-specific genes revealed an enrichment of both Bra and Foxa binding sites. Although Foxa was a well-known regulator of notochord fate both in vertebrates and ascidians, the current study provides a genome-wide view of its involvement in the process.

Comments for the author

Please find below three suggestions:
1) A statistical analysis would be required for Fig 5E while I agree with the interpretation of the authors.
2) It would be great if the authors could provide the list of genes whose upstream sequences exhibit enrichment of Bra and Foxa binding sites and relate them to those upregulated in Foxa.a>Bra and Etr>Bra/Etr>Foxa.a.
3) There are two studies, one in frog (Jim Smith's lab: O'Reilly et al, 1995, PMID: 7789266) and the other in ascidian (Nori Satoh's lab: Shimauchi et al, 2001, PMID: 11461000), both showing a synergistic activity of Bra and Foxa in promoting notochord fates. I suggest the authors to acknowledge these studies.

Finally, I have mixed feelings toward this study since the involvement of Foxa in notochord fate specification is well appreciated both in vertebrates and ascidians and the idea that Bra and Foxa act together to control gene expression in notochord lineage is not new. So, I am not entirely sure whether the current study represents a "sufficient" conceptual advance in the field. However, their genome-wide approach should be commended and it has generated important resources.

Reviewer 2

Advance summary and potential significance to field

The manuscript “Brachyury controls Ciona notochord fate as part of a feedforward network and not as a unitary master regulator” is a well-designed study that highlights the complexities associated with GRNs in regulating cell and tissue types, such as the Ciona notochord. The authors show that Bra/Brachyury and Foxa.a function as part of a type 1 feed-forward GRN. The GRN involving Bra and Foxa.a acts downstream of FGF signaling to regulate the expression of notochord-enriched genes. This study enhances the field by showing that the previously characterized master transcriptional regulator, Bra, is part of a wider network of transcription factors that regulate gene expression. Overall, this is clearly presented work.

Comments for the author

I'm satisfied with the changes made to the manuscript and explanations given for reasons where changes were not made as suggested. I support publication in Development.

Reviewer 3

Advance summary and potential significance to field

I feel that the revisions made to the manuscript have provided the required clarifications and a greater balance.

Comments for the author

I have no further concerns or suggestions.

Second revision

Author response to reviewers' comments

Please find below three suggestions:
1) A statistical analysis would be required for Fig 5E while I agree with the interpretation of the authors.

We've added the statistical analysis requested. All three mutant constructs are different from the control by the Wilcoxon rank sum test with p-values of less than 0.0005.

2) It would be great if the authors could provide the list of genes whose upstream sequences exhibit enrichment of Bra and Foxa binding sites and relate them to those upregulated in Foxa.a>Bra and Etr>Bra/Etr>Foxa.a.

In lieu of providing this list of genes, we've instead added a sentence about the strengths and weaknesses of TFBS enrichment analysis. Any such list will have many false negatives (because there are likely to be many important cis-regulatory regions outside the putative regions we defined, and also because current TFBS models may not identify all true binding sites) and many false positives (because most TF binding motifs are relatively short, common sequences and many occurrences won't be functionally important). These sources of error aren't critical for TFBS enrichment analysis because they are shared across the different gene sets being compared, but they are very problematic on the level of individual genes or binding sites in the absence of chipseq data or extensive reporter assays.

3) There are two studies, one in frog (Jim Smith's lab: O'Reilly et al, 1995, PMID: 7789266) and the other in ascidian (Nori Satoh's lab: Shimauchi et al, 2001, PMID: 11461000), both showing a synergistic activity of Bra and Foxa in promoting notochord fates. I suggest the authors to acknowledge these studies.

We've added these two references as requested. There is indeed extensive prior evidence from numerous model organisms that FoxA and Bra are both important in some way for notochord fate. Our new insights here are in the form of the specific GRN edges identified, the realization that Ciona notochord induction involves extensive feedforward circuitry, and the transcriptomic inferences about partial reprogramming.

Third decision letter

MS ID#: DEVELOP/2020/195230

MS TITLE: Brachyury controls Ciona notochord fate as part of a feedforward network

AUTHORS: Wendy M Reeves, Kotaro Shimai, Konner M Winkley, and Michael T Veeman
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.