Two distinct motifs for Zic-r.a drive specific gene expression in two cell lineages

Izumi Oda-Ishii, Deli Yu and Yutaka Satou
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Original submission

Decision letter

MS ID#: DEVELOP/2020/196725
MS TITLE: Non-canonical weak binding sites for Zic-r.a drive lineage specific gene expression
AUTHORS: Izumi Oda-Ishii, Deli Yu, and Yutaka Satou

I have now received all the referees' reports on the above manuscript, and have reached a decision. I am sorry to say that the outcome is not a positive one. The referees' comments are appended below, or you can access them online: please go to Development's submission site and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees raise some significant concerns about your paper, and are not strongly in favour of publication. Having looked at the manuscript myself, I agree with their views, and I must therefore, reject your paper.

I do realise this is disappointing news, but Development receives many more papers than we can publish, and we can only accept manuscripts that receive strong support from referees.

I do hope you find the comments of the referees helpful, and that this decision will not dissuade you from considering Development for publication of your future work. Many thanks for sending your manuscript to Development.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, the authors detail the contribution of non-canonical Zic-r.a binding sites to expression control of Tbx6-r.b in ascidian embryos. While the work is generally technically sound (with some suggestions for improvement below), unfortunately, I do not think that the authors have sufficiently explained the potential significance of this work to the field. As is briefly discussed in the paper, there is already evidence that non-canonical TF binding motifs can contribute to gene expression already, so it is not clear that this work represents a significant advance in that area. Is there something different about having primary/secondary motifs versus simply having strong/weak

binding sites that are better or worse matches to a sole binding motif? There may be something particularly of interest in the cis-regulatory element dissected in this paper, but if so, it is not explained in either the introduction or conclusion section.

Comments for the author

Specific comments to the authors (3 and 6 are major, the rest are minor):

1. I liked the way Figure 1 was laid out. It was easy to figure out which cells were which in the in situs.

2. (minor) I am used to seeing binding site deletions denoted by a "delta" symbol, not the "mu" symbol. You may want to consider changing the naming scheme of deletions in the paper.

3. (major) The introduction lacks a description of the key question of the study and the context for why this is a significant question to answer.

4. (minor) It's not clear how the details about Zic-r.b and Snail relate to the story at hand (lines 43-46)

5. (minor) I liked that the sample sizes and fractions of embryos showing an expression pattern are specified. To make this slightly more rigorous, one could add statistical tests to compare the percentages reported. This would be particularly important in Figure 3F.

6. (major) The paper should include a better justification for its use of both 189 bp and 3.4 kb reporter constructs for different experiments. What is known about the expression patterns/TF binding sites of the entire 3.4 kb region, the 189 bp and other subregions within the 3.4 kb region? Are there multiple enhancers within that 3.4 kb region? Does the 189 bp region recapitulate all Tbx6-r.b expression throughout a specific developmental time window?

7. (minor) What were the precise settings and PWMs used with Paster at line 112? What was the significance cutoff referred to at line 119?

8. (minor) In figure 2B, it would be nice to see the p-values of the Zic binding sites, since the scores are hard to interpret on their own.

9. (minor) In the work with the mouse primary and secondary Zic motifs, was there a quantitation of the difference in affinities of these binding sites? This might give some context into how much stronger Zic-r.a protein expression needs to be to bind the secondary sites (line 167).

10. (minor) I liked that Fig. S1 showed the sequences for the altered reporters. It would be nice to have in the Methods an explanation of how the mutations to eliminate binding sites were decided upon.

11. (minor) Add units to the bar graphs in Figure 2D.

12. (minor) What do you think is driving expression in 35% of embryos in the muZic-p/d construct in Figure 2E?

13. (minor) Why might the 5xZic + Beta-catenin MO result in a (significantly?) higher fraction of Gfp expressing embryos than without the MO as see in Fig. 3F?

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript, the authors detail the contribution of non-canonical Zic-r.a binding sites to expression control of Tbx6-r.b in ascidian embryos. While the work is generally technically sound (with some suggestions for improvement below), unfortunately, I do not think that the authors have sufficiently explained the potential significance of this work to the field. As is briefly discussed in the paper, there is already evidence that non-canonical TF binding motifs can contribute to gene expression already, so it is not clear that this work represents a significant advance in that area. Is there something different about having primary/secondary motifs versus simply having strong/weak binding sites that are better or worse matches to a sole binding motif? There may be something particularly of interest in the cis-regulatory element dissected in this paper, but if so, it is not explained in either the introduction or conclusion section.

[Response]
We have found that Zic recognizes two distinct motifs. We believe that having two distinct motifs is different from “having strong/weak binding sites that are better or worse matches to a sole binding motif”. In the former case, such motifs are recognized in different ways by a transcription factor. In the latter case, strong and weak motifs are recognized similarly by a transcription factor. In the present version of the manuscript, we added an experiment to show that the two Zic-binding motifs are indeed recognized by different sets of the zinc finger domains (Figure 6).
Our finding raises the possibility that transcription factors that have multiple recognition motifs utilize such motifs to induce specific expression. Secondary motifs are not easily identified by the ordinary Selex method. Therefore we may need to reconsider how gene regulatory network dynamics is regulated by taking into account of such motifs.

[Reviewer Comment]
Reviewer 1 Comments for the Author:
Specific comments to the authors (3 and 6 are major, the rest are minor):

1. I liked the way Figure 1 was laid out. It was easy to figure out which cells were which in the in situs.

[Response]
For every photograph showing in situ hybridization, we labelled cells by arrowheads with the same color code described in Figure 1. We now believe that readers can easily identify cells.

[Reviewer Comment]
2. (minor) I am used to seeing binding site deletions denoted by a "delta" symbol, not the "mu" symbol. You may want to consider changing the naming scheme of deletions in the paper.

[Response]
We changed the construct names in accordance with the reviewer’s comment (Fig. 3)

[Reviewer Comment]
3. (major) The introduction lacks a description of the key question of the study and the context for why this is a significant question to answer.

[Response]
We totally re-wrote Introduction. We believe that the present version of the manuscript is much more readable.

[Reviewer Comment]
4. (minor) It's not clear how the details about Zic-r.b and Snail relate to the story at hand (lines 43-46)

[Response]
We removed this part from Introduction.

[Reviewer Comment]
5. (minor) I liked that the sample sizes and fractions of embryos showing an expression pattern are specified. To make this slightly more rigorous, one could add statistical tests to compare the percentages reported. This would be particularly important in Figure 3F.

[Response]
We included a statistical test between two constructs in accordance with the reviewer's comment. The p-value is written in the main text (line 133).

[Reviewer Comment]
6. (major) The paper should include a better justification for its use of both 189 bp and 3.4 kb reporter constructs for different experiments. What is known about the expression patterns/TF binding sites of the entire 3.4 kb region, the 189 bp and other subregions within the 3.4 kb region? Are there multiple enhancers within that 3.4 kb region? Does the 189 bp region recapitulate all Tbx6-r.b expression throughout a specific developmental time window?

[Response]
In accordance with the reviewer's comment, we explained the details of these constructs (Line 151-159).

[Reviewer Comment]
7. (minor) What were the precise settings and PWMs used with Paster at line 112? What was the significance cutoff referred to at line 119?

[Response]
We included the parameters we used in Materials and Methods (line 288).

[Reviewer Comment]
8. (minor) In figure 2B, it would be nice to see the p-values of the Zic binding sites, since the scores are hard to interpret on their own.

[Response]
P-values are calculated by the program. However, the cutoff p-value is determined using sample size adjusted information content in this program. In our case, ln (p-value) for Zic-p was -10.99 and ln (p-value) for Zic-d was -8.70, while the cutoff p-value was -9.913. Although it is possible to include these p-values, we think that these values are misleading. Therefore, as we wrote in the main text (line 89-92), we say that Zic-p was significant but Zic-d was not significant. Because we now include the parameters we used in Materials and Methods, readers can know what significant means. Therefore, we think that showing scores is the best way to avoid misunderstanding.

[Reviewer Comment]
9. (minor) In the work with the mouse primary and secondary Zic motifs, was there a quantitation of the difference in affinities of these binding sites? This might give some context into how much stronger Zic-r.a protein expression needs to be to bind the secondary sites (line 167).

[Response]
To our knowledge, there is no quantification of the difference in affinities of two binding sites for mouse Zic.

[Reviewer Comment]
10. (minor) I liked that Fig. S1 showed the sequences for the altered reporters. It would be nice to have in the Methods an explanation of how the mutations to eliminate binding sites were decided upon.

[Response]
Figure S1 is now referred to in Materials and Methods.

[Reviewer Comment]
11. (minor) Add units to the bar graphs in Figure 2D.

[Response]
The Y-axis represents relative band intensity, and we included this in Figure 2D.

[Reviewer Comment]
12. (minor) What do you think is driving expression in 35% of embryos in the muZic-p/d construct in Figure 2E?

[Response]
It is possible that there are additional Zic-binding sites. It is also possible that Zic-r.a is not the only activator of Tbx6-r.b. However, because expression of the mutant construct was apparently reduced, it is obvious that these Zic binding sites are important for expression of Tbx6-r.b.

[Reviewer Comment]
13. (minor) Why might the 5xZic + Beta-catenin MO result in a (significantly?) higher fraction of Gfp expressing embryos than without the MO as see in Fig. 3F?

[Response]
In the current version of the manuscript, we removed the “the 5xZic + Beta-catenin MO” experiment.

Resubmission

First decision letter

MS ID#: DEVELOP/2021/199538

MS TITLE: Two distinct motifs for Zic-r.a drive specific gene expression in two cell lineages

AUTHORS: Izumi Oda-Ishii, Deli Yu, and Yutaka Satou

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 be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

Reviewer 1

Advance summary and potential significance to field

The manuscript by Oda-Ishii et al. explores regulatory sequences targeted by the ZIC transcription factor, Zic-r.a. The authors identify non-canonical sequences employed by Zic-r.a that bear little to no resemblance to the previously defined canonical sequences. They show that the non-canonical sequences bind Zic-r.a with much lower affinity than the canonical sequences and that these lower-affinity sequences are required for the proper expression pattern of a reporter gene derived from the regulatory sequence of a Zic-r.a target, Tb6-r.b. For example, the authors show that replacing the non-canonical sites with canonical sites results in ectopic expression of the reporter in tissues where both the gene and the reporter are normally silent. This result raises the question of whether the so-called canonical sites are biologically relevant. The authors show that the canonical high-affinity sites do appear to be biologically relevant in Zic-r.a target genes (identified in this study) that are activated at later stages in development, in neuronal cells at the tailbud stage. Lastly the authors find through a structure-function assay that Zic-r.a may differentially depend on different zinc-finger domains to activate reporters with canonical vs. non-canonical binding sites.

The results presented in this manuscript are significant for three reasons:
(1) they add to the growing appreciation that biology relies on “sub-optimal” binding sites rather than optimal high-affinity binding sites to express genes in precise patterns,
(2) they shed light on the role and targets of a key developmental regulator, Zic-r.a, and
(3) they open the door to further exploration of the use of dissimilar binding sites by individual transcription factors.

Comments for the author

The experiments are logical and the results are convincing. No further experiments appear necessary for this paper. The questions and comments below are for clarity only:

Questions/Comments:
1. To confirm the biological relevance of the non-canonical Zic binding sites the authors deleted two non-canonical Zic sites from a 189 bp reporter construct. This resulted in a reduction in the number of embryos that expressed the reporter at the 32-cell stage from 90% to 35% (Figure 2E). This raises questions that would be helpful for the authors to address in the text:
A. How do you explain the fact that 35% of the embryos still express the reporter? This appears to be a standard in the ascidian field for accepting the importance of a regulatory site; for non-ascidian experts, a short explanation of this read-out/interpretation would be helpful.
B. Also, it was not reported in this figure whether removal of the non-canonical sites affected expression of the reporter at the 16-cell stage. This would be interesting to know since the authors postulated that Zic-r.a may not need to bind directly to DNA when co-regulating expression with B-catenin/Tcf7.

2. To test if high levels of Zic-r.a can activate expression on its own, the authors injected Zic-r.a mRNA into the embryo and found that indeed it results in ectopic expression of Tb6-r.b, including in the animal hemisphere. From this result, they conclude that this “confirms” that Zic-r.a can express Zic-r.a independently of b-catenin. However, it is possible that ectopic Zic-r.a results in ectopic b-catenin expression. Therefore, this result is not so much confirmatory, as consistent with the previous data showing that Zic-r.a functions independently of b-catenin. In fact, the previous data (expression in wild type embryo in cells that lack b-catenin and direct DNA binding) is more confirmatory than the ectopic expression assay.

3. Changing non-canonical sites to canonical sites:
The authors observed that the canonical-site reporter (2xcano>Gfp) is ectopically expressed in cells that do not express Zic-r.a. As a result, the authors switched to a longer reporter that represses this ectopic expression. However, this result is interesting in itself because it suggests that transcription factors other than Zic-r.a may target the canonical site. If this is true, then it should be considered when speculating about the evolution of canonical and non-canonical sites. It would be interesting for the authors to speculate on this finding in the discussion.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, Oda-Ishii and colleagues explore how Zic-r.a, a maternal transcription factor, specifies posterior fate in ascidian embryos as its direct target, Tbx6-r.b, does not contain "typical" Zic-r.a binding sites in its regulatory region. The authors find that Zic-r.a binds to sites dissimilar from the canonical motif based on an in vitro selection assay and that it activates Tbx6-r.b in a sub-lineage of muscle cells. These sites with non-canonical motifs have low a affinity for Zic-r.a. Therefore, it activates Tbx6-r.b only in cells expressing Zic-r.a at high levels. Consistent with these results, the authors demonstrate that Zic-r.a expressed in late embryos activates neural genes through canonical binding sites. As such, they propose that the different zinc-finger domains of Zic-r.a are important for driving reporters with the canonical and non-canonical sites, respectively—that the non-canonical motif is not a divergent version of the canonical motif. In sum, the authors propose that Zic-r.a recognizes two distinct motifs to activate two sets of genes at two time points.

Comments for the author

Overall, the studies were well controlled. The inclusion of single-cell transcriptomics (even if it was a re-analysis), TALENs, biochemistry quantitative imaging, etc. was fantastic!

While it may be nice to have further quantitative images for each experiment (looking at the levels of transgene expression, for example in figure 1 with the), the critical experiments and quantification were all included, and sufficient for their conclusions.

One major issue with the manuscript is the lack of references or discussion about the range of work that overlaps with this work. For example, while the authors include Badis et al., and other genomics-based work exploring non-canonical motifs, there are several studies that should be referenced and mentioned. For example, Mango's work on pha4 (timing), Crocker et al., (low-affinity sites)., Farley et al., (low-affinity sites, ectopic), Fuqua et al., (timing, ectopic), etc. While the mechanisms are different from the varied zinc-finger domains, they should be mentioned none-the-less to place this work in a broader context. Adding further discussion on these previous studies may also help the work reach a broader audience—because as it stands the discussion and focus is narrow.

I would also encourage the authors to speculate on how the large diversity of zinc-fingers could be due to their modular nature. Here the authors provide compelling new data, and I would encourage further speculation.

Could the authors comment on possible splice variants of the zinc finger? Do any exist?

Fig. S2 was compelling, and I would encourage the authors to move it into the the main figure, as it helps aid interpretation.

Overall, it was great to review an elegant study that addressed open questions in the transcription field in a comprehensive way.

Reviewer 3

Advance summary and potential significance to field

This manuscript presents data demonstrating that the key tunicate maternal determinant, Macho-1 (referred to as Zic-r.a) has the capacity to differentially regulate transcription using two distinct DNA binding domains which bind to distinct binding sites. The authors thoroughly support this finding through extensive analysis of knockdown embryos and manipulations of reporter constructs.

Their results have important implications regarding the topology of gene regulatory networks (GRNs) and the limitations of using canonical binding sites in constructing models of GRN structure and function.

Comments for the author

We have a few concerns about this manuscript as listed below.

Major Concerns

1. The introduction and discussion are poorly composed and under-developed.
In the introduction, the authors provide a thorough summary of specific background information regarding muscle lineage specification in ascidians. However, they fail to provide an overview regarding broader implications of their work. In particular, they do not provide any overview of current ideas about divergent bindings sites for transcription factors and how emerging evidence for multiple distinct binding sites has impacted models of GRN structure and function in tunicates as well as other model organisms. This overview is also lacking in the discussion. They also mention enhancer tuning in their discussion but they fail to provide any background on this topic in their introduction and do not provide any discussion of what their findings contribute to this important topic. The authors should greatly expand the scope of their introduction and discussion to provide a more thorough and accurate summary of key underlying concepts and references to previous relevant studies in tunicates and other organisms. The lack of a well-developed broad perspective greatly reduces the potential impact of this manuscript.

2. The authors fail to provide a model to clarify their overall conclusions and help the reader understand the implications of their results. It is strongly suggested that the authors provide additional panels to each of their figures that illustrate the hypothesis that was supported by these results and then provide a final figure or panel at the end of the paper containing a model that clearly illustrates their conclusions (including lineage specific, differential regulation of Zic target genes, the use of different zinc fingers for differential binding and other key hypotheses).
Minor concerns –

Line 63: In “Hudson 2016”, this reviewer cannot find the MO experiment you reference. The authors should clarify.

Figure 1: The authors should add labels of genes being probed by ISH so the readers can avoid hunting through the legends. This should be performed throughout.

Line 82: The authors state that no Zic-r.a canonical binding sites are present in the upstream region of Tbx6-r.b, but in the paper you reference (Yagi 2004) the results show there are two Zic-r.a binding sites in the upstream region. Please clarify.

Figure 3: Control images are lacking; images of controls alongside experimental embryos would greatly aid the reader in more quickly understanding results. At minimum a note such as “please see Fig1C for WT expression embryo” should be added in all relevant situations.

The authors should move the summary from the discussion regarding the quiescence of the germ line to the appropriate section of the results (Fig 3, lack of Tbx6 expression in B6.3) and provide appropriate references.

Line 148: The authors should clarify the relevance of the following statement, “In other words, these cells are cousins of B5.1”

Figure 4: The authors should provide control images and more thorough labeling (see previous comment). Additionally, the same label is applied to two different constructs: 2Xcano>GFP in 4B and 4D are not the same, and the former is 189bp and the latter 3.4kb. This should be correctly indicated. Furthermore it would be valuable to see the results of the long constructs in 16-cell embryos.
Also, “neural cells” could be clarified here and throughout the manuscript by providing cell lineage designations.

Line 162: Here and throughout the manuscript it is often unclear whether the authors are referring to maternal or zygotic Zic-r.a. The authors should add the appropriate designation whenever Zic-r.a is mentioned to help clarify this point.

Line 168: The authors should explain why they chose these 20 genes.

Figure 5: 5C shows two different regions of Claudin regulatory DNA, however this is not mentioned or explained in the legend.

Line 190: This sentence is problematic. The authors should clarify what genes/regulatory elements that are referring to when they mention “two and one canonical motif sites”.

Line 233: The authors should summarize previous findings regarding Zic and snail regulation that are mentioned here and clearly delineate their idea that these findings along with their own findings suggest independent evolution of regulatory shifts under “common selective pressure.”

Line 238: The description of the structure/function of the upstream Zic-r enhancer is unclear and imprecisely worded. The authors should clarify this point.

Line 248: Is it possible that the lack of a neural expression they mentioned is due to a lack of required neural co-factors that bind in association with Zic-R to these neural enhancers? The authors should consider this model or explain why this is not a suitable explanation.

Line 266: the authors should include identifiers for all of the genes examined in this study, not just the three they list.

First revision

Author response to reviewers' comments

[Reviewer’s Comment]
Reviewer 1 Advance Summary and Potential Significance to Field: The manuscript by Oda-Ishii et al. explores regulatory sequences targeted by the ZIC transcription factor, Zic-r.a. The authors identify non-canonical sequences employed by Zic-r.a that bear little to no resemblance to the previously defined canonical sequences. They show that the non-canonical sequences bind Zic-r.a with much lower affinity than the canonical sequences and that these lower- affinity sequences are required for the proper expression pattern of a reporter gene derived from the regulatory sequence of a Zic-r.a target, Tb6-r.b. For example, the authors show that replacing the non-canonical sites with canonical sites results in ectopic expression of the reporter in tissues where both the gene and the reporter are normally silent. This result raises the question of whether the so-called canonical sites are biologically relevant. The authors show that the canonical high-affinity sites do appear to be biologically relevant in Zic-r.a target genes (identified in this study) that are activated at later stages in development, in neuronal cells at the tailbud stage. Lastly, the authors find through a structure-function assay that Zic-r.a may differentially depend on different zinc-finger domains to activate reporters with canonical vs. non- canonical binding sites.

The results presented in this manuscript are significant for three reasons: (1) they add to the growing appreciation that biology relies on “sub-optimal” binding sites rather than optimal high-affinity binding sites to express genes in precise patterns, (2) they shed light on the role and targets of a key developmental regulator, Zic-r.a, and (3) they open the door to further exploration of the use of dissimilar binding sites by individual transcription factors.

Reviewer 1 Comments for the Author:
The experiments are logical and the results are convincing. No further experiments appear necessary for this paper. The questions and comments below are for clarity only:

[Response]
We thank this reviewer's support for our manuscript.

[Reviewer's Comment] Questions/Comments:
To confirm the biological relevance of the non-canonical Zic binding sites, the authors deleted two non-canonical Zic sites from a 189 bp reporter construct. This resulted in a reduction in the number of embryos that expressed the reporter at the 32-cell stage from 90% to 35% (Figure 2E). This raises questions that would be helpful for the authors to address in the text: A. How do you explain the fact that 35% of the embryos still express the reporter? This appears to be a standard in the ascidian field for accepting the importance of a regulatory site; for non-ascidian experts, a short explanation of this read-out/interpretation would be helpful.

[Response]
In response to the comment, we changed the sentences as follows:
(original) These mutations reduced reporter expression in the B6.4 lineage of 32-cell embryos (Fig. 2E). Therefore, it is highly likely that Zic-r.a binds to the Zic-d and Zic-p binding sites to activate Tbx6-r.b in the B6.4 lineage at the 32-cell stage.
(revised) These mutations reduced reporter expression in the B6.4 lineage of 32-cell embryos (Fig. 2E), although 35% of embryos still expressed the reporter and therefore the reporter may contain additional unrecognizable Zic-r.a binding sites. Our observation indicates that Zic-r.a binds to the Zic-d and Zic-p binding sites to activate Tbx6-r.b in the B6.4 lineage at the 32-cell stage. **(Line 112-114)**

[Reviewer's Comment]
B. Also, it was not reported in this figure whether removal of the non-canonical sites affected expression of the reporter at the 16-cell stage. This would be interesting to know since the authors postulated that Zic-r.a may not need to bind directly to DNA when co-regulating expression with B-catenin/Tcf7.

[Response]
In the revised version of the manuscript, we included the data in Figure S2. The mutation construct reduced the number of embryos with reporter expression at the 16-cell stage. Because one Zic-r.a binding site abuts on an important Tcf7 binding site (Figure S1), and because we previously showed that Zic-r.a and Tcf7 physically interacts with each other, it is possible that Zic-r.a binds to this site with the help of ß-catenin/Tcf7. Therefore, we discussed this possibility.

(A sentence added to Results) In addition, these mutations also reduced reporter expression in B5.1 of 16-cell embryos (Fig. S2), and therefore these sites are necessary for expression in B5.1 (see Discussion). **(Line 115-117)**

(original) The B5.1 lineage contains less Zic-r.a than does the B6.4 lineage; therefore, it is likely that the Zic-d and Zic-p binding sites cannot respond sufficiently at the 16-cell stage. Thus, the weak non-canonical motif is utilized by reducing and tuning the activity of the enhancer, resulting in *Tbx6-r.b* expression in the B6.4 lineage, but not in the B5.1, or other lineages.
(revised) The B5.1 lineage contains less Zic-r.a than does the B6.4 lineage; therefore, it is likely that the Zic-d and Zic-p binding sites cannot respond sufficiently at the 16-cell stage. On the other hand, because the Zic-d site abuts the Tcf7 binding site (Fig. S1), and because Zic-r.a and Tcf7 can interact (Oda-Ishii et al., 2016), Zic-r.a may bind to these non-canonical sites with the help of Tcf7 in 16-cell embryos. Thus, the weak non-canonical motif is utilized by reducing and tuning the activity of the enhancer, and it induces *Tbx6-r.b* expression in the B6.4 lineage without the help of ß-catenin/Tcf7.

(Line 243-249)

[Reviewer's Comment]
2. To test if high levels of Zic-r.a can activate expression on its own, the authors injected Zic-r.a mRNA into the embryo and found that indeed it results in ectopic expression of Tb6-r.b, including in the animal hemisphere. From this result, they conclude that this "confirms" that Zic-r.a can express Zic-r.a independently of b-catenin. However, it is possible that ectopic Zic-r.a results in ectopic b-catenin expression. Therefore, this result is not so much confirmatory, as consistent with the previous data showing that Zic-r.a functions independently of b-catenin. In fact, the previous data (expression in wild type embryo in cells that lack b-catenin and direct DNA binding) is more confirmatory than

the ectopic expression assay.
[Response]
We think that this is unlikely with the following reasons. First, nuclear β-catenin is observed in the A-line cells, where Zic-r.a protein is rarely present, and in the B-line cells, where Zic-r.a protein is more abundantly present (Oda et al., 2018, Dev. Biol. 437:50-59). Second, knockdown of Zic-r.a does not affect expression of *Fgf9/16/20* and *Foxd*, which β-catenin, but not Zic-r.a, regulates (Oda et al., 2016, Plos Genetics 12:e1006045).
However, we agree that we cannot completely rule out the possibility that the reviewer pointed out. Therefore, in accordance with the reviewer's comment, we softened the tone of this sentence as follows:

(original) Overexpression of Zic-r.a evoked Tbx6-r.b ectopic expression at the 16-cell stage, even in the animal hemisphere, where no nuclear β-catenin is present (Fig. 3C, C'). This result confirms that a high concentration of Zic-r.a can evoke *Tbx6-r.b* expression independently of β-catenin.
Because it is expected that more binding sites recruit more Zic-r.a, we next tested modified *Tbx6-r.b* reporter constructs (Fig. S1BC).
(revised) Overexpression of *Zic-r.a* evoked *Tbx6-r.b* ectopic expression at the 16-cell stage, even in the animal hemisphere, where no nuclear β-catenin is expected (Fig. 3C, C'). Our result was consistent with the hypothesis that a high concentration of Zic-r.a can evoke *Tbx6-r.b* expression independently of β-catenin, although we cannot completely rule out the possibility that *Zic-r.a* overexpression induced nuclear translocation of β-catenin ectopically in the animal hemisphere. **(Line 135-139)**
To further test this hypothesis, we next tested modified *Tbx6-r.b* reporter constructs, because it is expected that more binding sites recruit more Zic-r.a (Fig. S1BC). **(Line 140-141)**

[Reviewer's Comment]
3. Changing non-canonical sites to canonical sites:

The authors observed that the canonical-site reporter (2xcano>Gfp) is ectopically expressed in cells that do not express Zic-r.a. As a result, the authors switched to a longer reporter that represses this ectopic expression. However, this result is interesting in itself because it suggests that transcription factors other than Zic-r.a may target the canonical site. If this is true, then it should be considered when speculating about the evolution of canonical and non-canonical sites. It would be interesting for the authors to speculate on this finding in the discussion.
[Response]
The 189-bp upstream region with 2xcano mutations evoked ectopic expression at the gastrula stage in cells in which *Zic-r.a* is not expressed. Because these are nerve cord cells in which a paralog, *Zic-r.b*, is expressed (Imai et al., 2002, Development 129, 2723), and Zic-r.b has a binding motif similar to the canonical motif for Zic-r.a (Yagi et al., 2004, Development 131, 1279). Therefore, it is possible that Zic-r.b induced ectopic expression and the longer upstream sequence (3.4k) contained an element that suppressed ectopic expression.

To mention this possibility, we inserted the following sentence:
(inserted sentence) It is possible that a paralog Zic-r.b, which is expressed in nerve cord cells (Imai et al., 2002) and binds to a sequence similar to the canonical site (Yagi et al., 2004b), activates the reporter. **(Line 172-173)**

[Reviewer's Comment]
Reviewer 2 Advance Summary and Potential Significance to Field:
In this manuscript, Oda-Ishii and colleagues explore how Zic-r.a, a maternal transcription factor, specifies posterior fate in ascidian embryos as its direct target, Tbx6-r.b, does not contain "typical" Zic-r.a binding sites in its regulatory region. The authors find that Zic-r.a binds to sites dissimilar from the canonical motif based on an in vitro selection assay and that it activates Tbx6-r.b in a sub-lineage of muscle cells. These sites with non-canonical motifs have low a affinity for Zic-r.a. Therefore, it activates Tbx6-r.b only in cells expressing Zic-r.a at high levels. Consistent with these results, the authors demonstrate that Zic-r.a expressed in late embryos activates neural genes through canonical binding sites. As such, they propose that the different zinc- finger domains of Zic-r.a are important for driving reporters with the canonical and non-canonical sites, respectively—that the non-canonical motif is not a divergent version of the canonical motif. In sum, the authors propose that Zic- r.a recognizes two distinct motifs to activate two sets of genes at two time points.

Reviewer 2 Comments for the Author:
Overall, the studies were well controlled. The inclusion of single-cell transcriptomics (even if it was a re-analysis), TALENs, biochemistry, quantitative imaging, etc. was fantastic! While it may be nice to have further quantitative images for each experiment (looking at the levels of transgene expression, for example in figure 1 with the), the critical experiments and quantification were all included, and sufficient for their conclusions.

One major issue with the manuscript is the lack of references or discussion about the range of work that overlaps with this work. For example, while the authors include Badis et al., and other genomics-based work exploring non- canonical motifs, there are several studies that should be referenced and mentioned. For example, Mango's work on pha4 (timing), Crocker et al., (low- affinity sites)., Farley et al., (low-affinity sites, ectopic), Fuqua et al., (timing, ectopic), etc. While the mechanisms are different from the varied zinc- finger domains, they should be mentioned none-the-less to place this work in a broader context. Adding further discussion on these previous studies may also help the work reach a broader audience—because as it stands the discussion and focus is narrow.
[Response]
We appreciate this constructive comment. According to the advice, we revised the manuscript as follows:

Introduction: we added the following sentences.
Many studies have shown that low-affinity binding sites for transcription factors are important for driving temporally or spatially controlled gene expression. These include a study of a temporal control by Pha-4 in nematodes (Gaudet and Mango, 2002), spatial control of *Otx* in ascidians (Farley et al., 2015), and temporal and spatial control of *Svb* in flies (Crocker et al., 2015; Fuqua et al., 2020). In these studies, each binding site that transcription factors recognize is represented by a single motif, and low-affinity sites are regarded as its divergent versions. **(Line 47-52)**

Discussion: We changed the following paragraph
(original) Transcription factors recognize specific DNA sequences. Several studies have suggested that some transcription factors can recognize two or more motifs……
(revised) On the other hand, the non-canonical-motif sites are low-affinity sites. Low-affinity sites are often used to control gene expression temporally and spatially (Crocker et al., 2015; Farley et al., 2015; Fuqua et al., 2020; Gaudet and Mango, 2002). While most low-affinity sites have been thought to be variants of high-affinity sites, the non-canonical motif recognized by Zic-r.a is not. Several studies have suggested that some transcription factors can recognize two or more motifs… **(Line 287-290)**

[Reviewer's Comment]

I would also encourage the authors to speculate on how the large diversity of zinc-fingers could be due to their modular nature. Here the authors provide compelling new data, and I would encourage further speculation.
[Response]
We thank this constructive advice. We added a sentence at **Line 295**: (added sentence) Zic-r.a is a transcription factor with five zinc finger domains. Our data indicate that this modular nature enables this transcription factor to recognize multiple motifs.

[Reviewer's Comment]
Could the authors comment on possible splice variants of the zinc finger? Do any exist?
[Response]
No, we do not think that isoforms are present, because extensive EST assays have not identified splicing variants. To explain this, we added the following sentences to Discussion.

(added sentences) *Zic-r.a* is composed of three exons and the five zinc finger domains are encoded by these three exons. While expressed sequence tags have been obtained from 34 cDNAs of seven cDNA libraries (Satou et al., 2005; Tassy et al., 2010), none of them indicates alternative splicing. Therefore, it is not likely that different isoforms recognize different motifs, but it is more likely that a single isoform recognizes the canonical and non-canonical motif sites. **(Line 297-301)**

[Reviewer's Comment]
Fig. S2 was compelling, and I would encourage the authors to move it into the the main figure, as it helps aid interpretation.
[Response]
Following the advice, we moved the result of middle tailbud embryos to Figure 5. We did not move the result of late tailbud embryos from Figure S4 (Figure S2 in the original version), because this information may somewhat be redundant.

[Reviewer's Comment]
Overall, it was great to review an elegant study that addressed open questions in the transcription field in a comprehensive way.
[Response]
We thank this reviewer for constructive advice and his/her support on our manuscript.

[Reviewer's Comment]
Reviewer 3 Advance Summary and Potential Significance to Field:
This manuscript presents data demonstrating that the key tunicate maternal determinant, Macho-1 (referred to as Zic-r.a) has the capacity to differentially regulate transcription using two distinct DNA binding domains which bind to distinct binding sites. The authors thoroughly support this finding through extensive analysis of knockdown embryos and manipulations of reporter constructs. Their results have important implications regarding the topology of gene regulatory networks (GRNs) and the limitations of using canonical binding sites in constructing models of GRN structure and function.
[Response]
We thank this reviewer for his/her positive assessment on our manuscript.

[Reviewer's Comment]
Reviewer 3 Comments for the Author:
We have a few concerns about this manuscript as listed below. Major Concerns

1. The introduction and discussion are poorly composed and under-developed. In the introduction, the authors provide a thorough summary of specific background information regarding muscle lineage specification in ascidians. However, they fail to provide an overview regarding broader implications of their work. In particular, they do not provide any overview of current ideas about divergent bindings sites for transcription factors and how emerging evidence for multiple, distinct binding sites has impacted models of GRN structure and function in tunicates as well as other model organisms. This overview is also lacking in the discussion. They also mention enhancer tuning in their discussion but they fail to provide any background on this topic in their introduction and do not provide any discussion of what their findings contribute to this important topic. The authors should greatly expand the scope of their introduction and discussion to provide a more thorough and accurate summary of key underlying concepts and references to previous relevant studies in tunicates and other organisms. The lack of a well- developed broad perspective greatly reduces the potential impact of this manuscript.
[Response]
We thank this reviewer for this helpful comment, and revised the manuscript as follows:

We added the following sentences to Introduction **(Line 47-52)**.
Many studies have shown that low-affinity binding sites for transcription factors are important for driving temporally or spatially controlled gene expression. These include a study of a temporal control by Pha-4 in nematodes (Gaudet and Mango, 2002), spatial control of *Otx* in ascidians (Farley et al., 2015), and temporal and spatial control of *Svb* in flies (Crocker et al., 2015; Fuqua et al., 2020). In these studies, each binding site that transcription factors recognize is represented by a single motif, and low-affinity sites are regarded as its divergent versions.

We changed the following paragraph in Discussion **(Line 287-290)**
(original) Transcription factors recognize specific DNA sequences. Several studies have suggested that some transcription factors can recognize two or more motifs……
(revised) On the other hand, the non-canonical-motif sites are low-affinity sites. Low-affinity sites are

often used to control gene expression temporally and spatially (Crocker et al., 2015; Farley et al., 2015; Fuqua et al., 2020; Gaudet and Mango, 2002). While most low-affinity sites have been thought to be variants of high-affinity sites, the non-canonical motif recognized by Zic-r.a is not. Several studies have suggested that some transcription factors can recognize two or more motifs……

We also added a paragraph to Discussion **(Line 302-307)**
(added paragraph) Gene regulatory networks for embryonic fate specification have been studied extensively. Knockout, knockdown, and overexpression assays to identify network connections have been complemented by reporter assays and chromatin immunoprecipitation assays to identify direct interactions. However, it is common that not all peaks identified by chromatin immunoprecipitation assays contain recognizable binding sites. Non-canonical sites may be present within such peak regions.

[Reviewer's Comment]
2. The authors fail to provide a model to clarify their overall conclusions and help the reader understand the implications of their results. It is strongly suggested that the authors provide additional panels to each of their figures that illustrate the hypothesis that was supported by these results and then provide a final figure or panel at the end of the paper containing a model that clearly illustrates their conclusions (including lineage specific, differential regulation of Zic target genes, the use of different zinc fingers for differential binding and other key hypotheses).
[Response]
In response to the comment, we added a panel to Figure 6 (Fig. 6DE), and created Figure 7.

[Reviewer's Comment] Minor concerns –
Line 63: In "Hudson 2016", this reviewer cannot find the MO experiment you reference. The authors should clarify.
[Response]
We apologize this mistake. We had misunderstood something. We deleted this sentence. **(Line 69)**

[Reviewer's Comment]
Figure 1: The authors should add labels of genes being probed by ISH so the readers can avoid hunting through the legends. This should be performed throughout.
[Response]
We added labels of genes to *in situ* hybridization photographs (Fig. 1, Fig. 3, and Fig. 4).

[Reviewer's Comment]
Line 82: The authors state that no Zic-r.a canonical binding sites are present in the upstream region of Tbx6-r.b, but in the paper you reference (Yagi 2004) the results show there are two Zic-r.a binding sites in the upstream region. Please clarify.
[Response]
To clarify this point, we changed the following sentence.

(original) Although our previous study showed that Zic-r.a preferentially binds to sequences similar to 5′-GCAGCGGGGGG-3′ (Yagi et al., 2004a) (Fig. 2A), no similar sequences are found in the *Tbx6-r.b* upstream region, using the computer program Patser (Hertz and Stormo, 1999) and the *Ciona* position weight matrix (Yagi et al., 2004a)
(revised) Although Zic-r.a preferentially binds to sequences similar to 5′-GCAGCGGGGGG-3′ (Fig. 2A) and potential binding sites are identified between -1095 and -1547 nucleotide positions from the transcription start site of *Tbx6-r.b* (Kugler et al., 2010; Yagi et al., 2004a), no similar sequences are found in the 189-bp upstream sequence of *Tbx6-r.b*, using the computer program Patser (Hertz and Stormo, 1999) and the *Ciona* position weight matrix (Yagi et al., 2004a). **(Line 87-91)**

[Reviewer's Comment]
Figure 3: Control images are lacking; images of controls alongside experimental embryos would greatly aid the reader in more quickly understanding results. At minimum a note such as "please see Fig1C for WT expression embryo" should be added in all relevant situations.
[Response]
We included photographs as a control for Figure 3C (Figure 3C'). We also add the following sentence

for Figure 3D-F.
(revised) see Figure 1E for expression of the wild-type construct **(Line 544-545)**

[Reviewer's Comment]
The authors should move the summary from the discussion regarding the quiescence of the germ line to the appropriate section of the results (Fig 3, lack of Tbx6 expression in B6.3) and provide appropriate references.

[Response]
We inserted the following sentence at the point where the reviewer suggested.

(inserted sentence) On the other hand, the absence of *Tbx6-r.b* mRNA in B6.3 is consistent with the earlier finding that the B6.3 cell pair is transcriptionally silent (Kumano et al., 2011; Shirae-Kurabayashi et al., 2011). **(Line 132-134)**

Meanwhile, we did not change the sentences in Discussion, because we feel that removal of these sentences from Discussion will made this part unreadable.

[Reviewer's Comment]
Line 148: The authors should clarify the relevance of the following statement, "In other words, these cells are cousins of B5.1"
[Response]
We removed this ambiguous sentence.

(original) Ectopic expression was found mainly in b-line cells, which are derived from cells where *Zic-r.a* mRNA is localized at the 4-cell stage. In other words, these cells are cousins of B5.1 (see Figure 1A).
(revised) Ectopic expression was found mainly in b-line cells, which are derived from cells where *Zic-r.a* mRNA is localized at the 4-cell stage (see Figure 1A). **(Line 148)**

[Reviewer's Comment]
Figure 4: The authors should provide control images and more thorough labeling (see previous comment). Additionally, the same label is applied to two different constructs: 2Xcano>GFP in 4B and 4D are not the same, and the former is 189bp and the latter 3.4kb. This should be correctly indicated. Furthermore it would be valuable to see the results of the long constructs in 16-cell embryos. Also, "neural cells" could be clarified here and throughout the manuscript by providing cell lineage designations.
[Response]
We add the following sentence for Figure 4B.
(added sentence) see Figure 1E for expression of the wild-type construct **(Line 552)**

We changed the labels for Figure 4D, and now labels are different between Figure 4B and Figure 4D. We included expression of the 3.4kbp constructs in 16-cell embryos in Figure S3, and explained the results in Line **175** and Line **180**.

We inserted the following sentence for clarification.
(inserted sentence) Judging from its mRNA expression pattern (Satou et al., 2002) and its protein distribution pattern (Oda-Ishii et al., 2016), it is likely that these neural cells are derived from the a-, b-, and A-lineages. **(Line 167-169)**

[Reviewer's Comment]
Line 162: Here and throughout the manuscript it is often unclear whether the authors are referring to maternal or zygotic Zic-r.a. The authors should add the appropriate designation whenever Zic-r.a is mentioned to help clarify this point.
[Response]
In response to the reviewer's comment, we revised the following sentences:

(original) Meanwhile, we found that Zic-r.a expressed in late embryos activates neural genes through canonical sites.
(revised) Meanwhile, we found that Zic-r.a expressed zygotically in late embryos activates neural genes through canonical sites. **(Line 19)**

(original) In addition, at the tailbud stage, this mutant construct was expressed ectopically in neural cells, in which *Zic-r.a* is expressed (Satou et al., 2002).
(revised) In addition to maternal expression, *Zic-r.a* is zygotically expressed in neural cells at the tailbud stage (Satou et al., 2002). **(Line 166-167)**

(original) To confirm whether Zic-r.a controls these genes, we mutated *Zic-r.a* by injecting a pair of TALEN mRNAs whose products were designed to target the third zinc-finger domain.
(revised) To confirm whether zygotically expressed *Zic-r.a* controls these genes, we mutated *Zic-r.a* by injecting a pair of TALEN mRNAs whose products were designed to target the third zinc-finger domain. **(Line 199)**

(original) This is probably because *KY.Chr7.686* is expressed not only in cells with *Zic-r.a* expression, but also in other cell populations (Fig. 5B; Fig. S4).
(revised) This is probably because *KY.Chr7.686* is expressed not only in cells with zygotic *Zic-r.a* expression, but also in other cell populations (Fig. 5B; Fig. S4). **(Line 211)**

(original) While Zic-r.a activates Tbx6-r.b cooperatively with β-catenin and Tcf7 in the B5.1 lineage of 16-cell embryos (Oda-Ishii et al., 2016), Zic-r.a alone activates Tbx6-r.b in the B6.4 lineage of 32-cell embryos.
(revised) While maternal Zic-r.a activates *Tbx6-r.b* cooperatively with β-catenin and Tcf7 in the B5.1 lineage of 16-cell embryos (Oda-Ishii et al., 2016), Zic-r.a alone activates *Tbx6-r.b* in the B6.4 lineage of 32-cell embryos. **(Line 239)**

(original) Third, in late embryos, Zic-r.a, which is expressed in the nervous system, activates neural genes through canonical-motif sites.
(revised) Third, in late embryos, Zic-r.a, which is zygotically expressed in the nervous system, activates neural genes through canonical-motif sites. **(Line 271)**

[Reviewer's Comment]
Line 168: The authors should explain why they chose these 20 genes. [Response]
We realized that the criteria were unclear in the original writing. We believe that our intent now becomes clear in the revised sentence.
(original) Among greatly downregulated genes (NOIseqsim P>0.99; reads-per-million values >50), we chose 20 genes, …
(revised) Among greatly downregulated genes, we chose 20 genes (NOIseqsim P>0.99; reads-per-million values >50), …**(Line 191)**

[Reviewer's Comment]
Figure 5: 5C shows two different regions of Claudin regulatory DNA, however this is not mentioned or explained in the legend.
[Response]
We added the following sentences in the legend.
(added sentence) Note that the upstream region of *Claudin* contains two sites. Nucleotide sequences and positions of these canonical-motif sites are shown in Figure S6. **(Line 566-568)**

[Reviewer's Comment]
Line 190: This sentence is problematic. The authors should clarify what genes/regulatory elements that are referring to when they mention "two and one canonical motif sites".
[Response]
In response to the reviewer's comment, we revised this sentence.

(original) In their 1-kb upstream regions, two and one canonical-motif sites were recognized by the Patser program (Hertz and Stormo, 1999) and the *Ciona* position weight matrix (Yagi et al., 2004a) (Fig. S5)

(revised) In the 1-kb upstream regions of *Claudin* and *KY.Chr7.686*, two and one canonical-motif sites were recognized by Patser (Hertz and Stormo, 1999) and the *Ciona* position weight matrix (Yagi et al., 2004a) (Fig. S6) **(Line 213-215)**

[Reviewer's Comment]
Line 233: The authors should summarize previous findings regarding Zic and snail regulation that are mentioned here and clearly delineate their idea that these findings along with their own findings suggest independent evolution of regulatory shifts under "common selective pressure."
[Response]
Following the advice, we revised the sentence as follows:

(original) Similarly, another Zic gene, *Zic-r.b*, and *Snail* are expressed in the B5.1 and B6.4 lineages at the 32-cell stage, and the regulatory mechanisms regulating these genes differ between these two lineages (Imai et al., 2016; Tokuoka et al., 2018).
(revised) Similarly, another Zic gene, *Zic-r.b*, and *Snail* are expressed in the B5.1 and B6.4 lineages at the 32-cell stage, and the regulatory mechanisms regulating these genes differ between these two lineages; *Zic-r.b* is activated in the daughter cells of B5.1 by maternal Gata.a, while the expression in B6.4 is not lost in Gata.a morphants (Imai et al., 2016); *Snail* is activated in the daughter cells of B5.1 by *Tbx6-r.b*, and in B6.4 by constitutively active form of Raf (Tokuoka et al., 2018). **(Line 261-263)**

[Reviewer's Comment]
Line 238: The description of the structure/function of the upstream Zic-r enhancer is unclear and imprecisely worded. The authors should clarify this point.
[Response]
We think that the reviewer may misunderstand the sentence. We do not say anything about "the upstream Zic-r enhancer", but discuss the upstream region of *Tbx6-r.b*. To make this point clearer, we revised the sentence as follows:

(original) Second, in B6.4 of the 32-cell embryo, a high level of expression allows Zic-r.a to bind to non-canonical-motif sites in the upstream regulatory region of *Tbx6-r.b*.
(revised) Second, in B6.4 of the 32-cell embryo, a high level of Zic-r.a expression allows it to bind to non-canonical-motif sites in the upstream regulatory region of *Tbx6-r.b*. **(Line 269-271)**

[Reviewer's Comment]

Line 248: Is it possible that the lack of a neural expression they mentioned is due to a lack of required neural co-factors that bind in association with Zic-R to these neural enhancers? The authors should consider this model or explain why this is not a suitable explanation.
[Response]
In response to the comment, we added the following sentence:

(added sentence) It is also possible that Zic-r.a activates neural genes combinatorially with additional factors specific to neural cells. **(Line 275-277)**

[Reviewer's Comment]
Line 266: the authors should include identifiers for all of the genes examined in this study, not just the three they list.
[Response]
We now included gene identifiers for *Foxa.a*, *Foxd*, *Neurog*, *Claudin*, *Pou2*, *Noto1*, and *Ef1a*. **(Line 318-320)**

Second decision letter

MS ID#: DEVELOP/2021/199538

MS TITLE: Two distinct motifs for Zic-r.a drive specific gene expression in two cell lineages

AUTHORS: Izumi Oda-Ishii, Deli Yu, and Yutaka Satou
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.