THE EMBO JOURNAL

Manuscript EMBO-2015-92188

Postmitotic diversification of olfactory neuron types is mediated by differential activities of the HMG-box transcription factor SOX-2

Amel Alqadah, Yi-Wen Hsieh, Berta Vidal, Chieh Chang, Oliver Hobert and Chiou-Fen Chuang

Corresponding author: Chiou-Fen Chuang, University of Illinois at Chicago

Review timeline:

Submission date:	01 June 2015
Editorial Decision:	09 July 2015
Revision received:	12 July 2015
Editorial Decision:	05 August 2015
Revision received:	05 August 2015
Accepted:	06 August 2015

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 09 July 2015

Thank you for submitting your manuscript to The EMBO Journal. Two referees have now seen your study and their comments are provided below.

As you can see both referees appreciate your analysis reporting that Sox2 interacts with different transcription factors to promote cell type diversity. They also find the analysis well done. They raise a number of different points that shouldn't involve too much work to address. Referee #1 importantly raises the issue that relevant mammalian studies need to be cited as well.

Should you be able to address the concerns raised in full, then I would like to invite you to submit a revised version for our consideration. I should add that it is EMBO Journal policy to allow only a single round of revision, and that it is therefore important to address the raised concern at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

In the paper by Alqadah et al. the authors examine the generation of two types of olfactory neurons (AWB and AWC) in C. elegans. They show that the transcription factor Sox2 acts together with Lim4 to promote an AWB state and with Ceh36 to promote an AWC state. It is a rather extensive study that nicely demonstrates that Sox2 acts with different partner factors in regulating two sets of differentiation genes in AWB and AWC neurons.

1) In the vertebrate nervous system it is well established that Sox2 acts with different partner factors to regulate many different genes. For instance, Sox2 has been shown to act together with transcription factors such as Brn2, Pax6, Gli and Tcf, both in gene activation and repression. Studies like this should be referred to and discussed.

2) In the abstract/introduction the authors state that the paper "broadens the perspective of Sox2" as it shows a role for Sox2 in the regulation of genes in mature neurons and not only in stem cells. It should be noted that in many other systems (such as vertebrates) Sox2 expression is absent from mature neurons and mostly confined to stem and progenitor cells. Nevertheless, Sox2 has already been implicated in neuronal maturation e.g. GABAergic maturation in the cortex and the olfactory bulb of the mouse (Cavallaro et al. 2008, Development).

3) On page 13 the authors state that Sox2ot640 null mutant phenocopied the defects of loss of Ceh36. But in Sox2ot640 null mutants both AWB/AWC cells are lost and only AWC cells in Ceh36 mutants.

4) In Figure 6 the authors examine the cooperativity of Sox2, Ceh36 and Lim4 on the odr1 promoter.
A) the authors should provide better statistics for the luciferase experiments.
B) on page 14 it is stated "Apart from demonstrating the cooperativity of SOX-2, LIM-4 and CEH-36, the transfection results also demonstrate that these factors operate directly on terminal effectors, rather than acting via intermediary factors." Have any intermediary factors been examined in these experiments?
C) On page 14 it is further stated "This notion is consistent with the previous analysis that identified cis-regulatory modules required for expression of AWB expressed genes (Nokes et al, 2009)." What is meant with this statement?

5) In a table in Figure 7b the authors show that the loss of Lim4 and Sox2 motifs from the odr1 promoter results in a selective loss of reporter activity in AWB neurons. The authors should complement this table with images of the cells. Moreover, is the loss of Ceh36 motifs altering the activity of the transgene in AWB neurons?

6) On page 18 the authors outline a model in which Lim4 sequesters Sox2 from Ceh36. For this competition model to be relevant Ceh36 should be expressed in AWB neurons. Is that the case?

Referee #2:

In this manuscript Alqadah et al. identify a new function for the transcription factor SOX-2, famous for its important role in regulating pluripotency. The authors convincingly show that it functions in regulating cell fate in postmitotic neurons. This function of SOX-2 was identified in C. elegans in a forward genetic screen for mutants that affect AWC cell fate. This screen identified mutant ky707 and characterization of expression of many genes in this mutant background shows that in ky707 animals AWB fate has been changed into AWC-on fate. Using mapping, whole genome sequencing and transgene rescue the authors show that the ky707 phenotype is caused by G73E mutation of sox-2. Subsequently, they show the conservation of sox-2 by rescuing the ky707 phenotype by expressing the mouse Sox2 gene under control of the C. elegans sox-2 promoter. Finally the authors extensively characterize how mutation of sox-2 affects the cell fate of AWB.
I think it is a very good paper, with extensive analyses that convincingly support the claims of the

authors. It is well written and clear. The only bigger remark I have is that I would like to see a description of the expression pattern of sox-2. Now we only know it's expression in AWB and C, but for other researchers, examining sox-2 function it is essential to know its expression pattern. It does not have to be in great detail, a couple of pictures in the sup info and an overall description (e.g. x neurons in head, body wall muscle etc) would do.

Minor remarks
Page 8, line 9. I would rephrase "mutations of each" to "mutations in each".
Page 9, line 2. Please change "overlapped" to "overlapping".
Figure 3, Please correct "idnduction"
Figure 4C. The Y-axis goes from -0.1 to 0, to 0.4. Please change -0.1 to -1. I'm actually surprised that the avoidance index yields negative values. I would expect that an avoidance index of 1, would mean 100% avoidance; here 100% avoidance results in an index of -1. I would rather call this chemotaxis index, resulting in negative chemotaxis and thus avoidance.
Text and pictures in Figure 5 are too small.
Page 11, please add a space between 2.8 and kb.
Page 12, when the authors refer to fig 6C they mean 6D, and when they refer to 6D they mean 6E.
Page 14, line 8. Please remove "rather".
Figure 8B. The authors show that SOX-2 G73E still binds the sox-2 binding site, but in the model they show the protein as unbound. It is not clear to me why.
Page 17, line 8. Please remove "auto" from "autoregulates". It's already enough to say that it regulates its own expression.
Page 20, first line. Please rephrase to "str-2 has been found to be exquisitely sensitive to various sensory signals" or something similar.

1st Revision - authors' response 12 July 2015

Referee #1:

In the paper by Alqadah et al. the authors examine the generation of two types of olfactory neurons (AWB and AWC) in C. elegans. They show that the transcription factor Sox2 acts together with Lim4 to promote an AWB state and with Ceh36 to promote an AWC state. It is a rather extensive study that nicely demonstrates that Sox2 acts with different partner factors in regulating two sets of differentiation genes in AWB and AWC neurons.

1) In the vertebrate nervous system it is well established that Sox2 acts with different partner factors to regulate many different genes. For instance, Sox2 has been shown to act together with transcription factors such as Brn2, Pax6, Gli and Tcf, both in gene activation and repression. Studies like this should be referred to and discussed.

We had already mentioned some partner relationships in the Discussion (Kondoh review paper), including a specific partnership with Pax6 for lens development and Brn2 for progenitor development. But we had not mentioned the Gli and Tcf examples that the reviewer is referring to – we now do this in the discussion section on page 21.

2) In the abstract/introduction the authors state that the paper "broadens the perspective of Sox2" as it shows a role for Sox2 in the regulation of genes in mature neurons and not only in stem cells. It should be noted that in many other systems (such as vertebrates) Sox2 expression is absent from mature neurons and mostly confined to stem and progenitor cells. Nevertheless, Sox2 has already been implicated in neuronal maturation e.g. GABAergic maturation in the cortex and the olfactory bulb of the mouse (Cavallaro et al. 2008, Development).

We thank the referee for the comment. We had already referenced the Cavallaro 2008 paper for the following description in the discussion: Diminished generation of GABAergic neurons from cultured neural stem cells and reduced generation of retinal ganglion cells (RGCs) from progenitor cells have been observed in the absence of Sox2 (Cavallaro et al, 2008; Taranova et al, 2006), but whether Sox2 directly controls terminal differentiation of GABA neurons or RGCs is not known.
As stated in the abstract/introduction:

"Our findings provide novel insights into combinatorial codes that drive terminal differentiation programs in the nervous system and reveal a novel biological function of the deeply conserved Sox2 protein that goes beyond its well-known role in stem cell biology."
"Our paper significantly broadens the perspective of Sox2 by showing – for the first time – that Sox2 can also have a role in a terminal neuronal differentiation, through direct regulation of target genes that define the differentiated state of a neuron."

3) On page 13 the authors state that Sox2ot640 null mutant phenocopied the defects of loss of Ceh36. But in Sox2ot640 null mutants both AWB/AWC cells are lost and only AWC cells in Ceh36 mutants.

We thank the reviewer for catching this. We had meant to say that *sox-2* phenocopies *ceh-36* in the AWC neuron, not in both neurons. We have now clarified this and rephrased the sentence to state that *sox-2(ot640)* null mutants phenocopied loss of *ceh-36* in terminally differentiating AWC identity.

4) In Figure 6 the authors examine the cooperativity of Sox2, Ceh36 and Lim4 on the odr1 promoter.
A) the authors should provide better statistics for the luciferase experiments.
We have included additional statistic analysis for the luciferase experiments in Figure 7A. We have also changed luciferase activity from arbitrary unit to fold activation at the y-axis of the bar chart.

B) on page 14 it is stated "Apart from demonstrating the cooperativity of SOX-2, LIM-4 and CEH-36, the transfection results also demonstrate that these factors operate directly on terminal effectors, rather than acting via intermediary factors." Have any intermediary factors been examined in these experiments?
There may be a misunderstanding of what is meant by "intermediary factors". We simply meant to say that *sox-2*/*lim-4*/*ceh-36* act directly on terminal effector genes, and not by regulating some unknown factors that then directly activate effector genes. To avoid this confusion, we have removed the latter part of the sentence "rather than acting via intermediary factors."

C) On page 14 it is further stated "This notion is consistent with the previous analysis that identified cis-regulatory modules required for expression of AWB expressed genes (Nokes et al, 2009)." What is meant with this statement?
We apologize for the lack of clarity regarding this statement. In a study performed by Nokes et al, it was found that several genes expressed in AWB neurons are coregulated by a common cis-regulatory module. This cis-regulatory motif is required for expression of the genes in AWB neurons, which is consistent with the concept of a selector transcription factor-driven differentiation program in which terminal selector transcription factors act directly on terminal selector motifs in the regulatory sequences of terminal differentiation genes to govern neuronal identities (Hobert, 2008). As SOX-2, CEH-36, and LIM-4 are capable of operating directly on a terminal effector of AWB and AWC neurons, our results are consistent with previous analysis. However, as the reviewer points out, this statement is confusing to raise here in the Result section; we have simply deleted this sentence.

5) In a table in Figure 7b the authors show that the loss of Lim4 and Sox2 motifs from the odr1 promoter results in a selective loss of reporter activity in AWB neurons. The authors should complement this table with images of the cells. Moreover, is the loss of Ceh36 motifs altering the activity of the transgene in AWB neurons?

We have made a new Figure S5 to include images of transgenic worms expressing *odr-1* promoter GFP reporter constructs. Mutating *ceh-36* motif slightly decreased *odr-1p::GFP* expression in AWB neurons. We have included the data in the new Figure S5.

6) On page 18 the authors outline a model in which Lim4 sequesters Sox2 from Ceh36. For this competition model to be relevant Ceh36 should be expressed in AWB neurons. Is that the case?
The reviewer makes a good point. Although we are unable to observe *ceh-36* expression in AWB neurons in wild type animals, it may be transiently expressed in embryonic AWB

neurons and suppressed fairy quickly by *lim-4*. We have added this possibility to the manuscript.

Referee #2:

In this manuscript Alqadah et al. identify a new function for the transcription factor SOX-2, famous for its important role in regulating pluripotency. The authors convincingly show that it functions in regulating cell fate in postmitotic neurons. This function of SOX-2 was identified in C. elegans in a forward genetic screen for mutants that affect AWC cell fate. This screen identified mutant ky707 and characterization of expression of many genes in this mutant background shows that in ky707 animals AWB fate has been changed into AWC-on fate. Using mapping, whole genome sequencing and transgene rescue the authors show that the ky707 phenotype is caused by G73E mutation of sox-2. Subsequently, they show the conservation of sox-2 by rescuing the ky707 phenotype by expressing the mouse Sox2 gene under control of the C. elegans sox-2 promoter. Finally the authors extensively characterize how mutation of sox-2 affects the cell fate of AWB.
I think it is a very good paper, with extensive analyses that convincingly support the claims of the authors. It is well written and clear. The only bigger remark I have is that I would like to see a description of the expression pattern of sox-2. Now we only know it's expression in AWB and C, but for other researchers, examining sox-2 function it is essential to know its expression pattern. It does not have to be in great detail, a couple of pictures in the sup info and an overall description (e.g. x neurons in head, body wall muscle etc) would do.

We thank the referee for this feedback. We agree that the expression pattern of *sox-2* is important to understanding its function. The expression pattern of *sox-2* was described in a very recent study (Vidal et al, Development, in press). In addition to AWB and AWC neurons, *sox-2* is also expressed in other sensory neurons (IL1, IL2, URA, URB, OLL), interneurons (AIM, AIN, AVK, RIH) and motor neurons (RME), as well as in other tissues like head hypodermis, arcade cells and rectal epithelial cells (Vidal et al, in press). We have added a description of the expression pattern of *sox-2* in *C. elegans* on page 11.

Minor remarks

Page 8, line 9. I would rephrase "mutations of each" to "mutations in each".
The requested change has been made.

Page 9, line 2. Please change "overlapped" to "overlapping".
We have made the suggested change.

Figure 3, Please correct "idnduction"
We have corrected the typo in Figure 3.

Figure 4C. The Y-axis goes from -0.1 to 0, to 0.4. Please change -0.1 to -1. I'm actually surprised that the avoidance index yields negative values. I would expect that an avoidance index of 1, would mean 100% avoidance; here 100% avoidance results in an index of -1. I would rather call this chemotaxis index, resulting in negative chemotaxis and thus avoidance.

We agree that Figure 4C should be referred to as a chemotaxis index. We have edited the axis title of Figure 4C "Chemotaxis index" instead of "Avoidance index". We have also changed -0.1 to -1.

Text and pictures in Figure 5 are too small.
We have edited Figure 5 to make the text and pictures bigger.

Page 11, please add a space between 2.8 and kb.
We have made the suggested edit.

Page 12, when the authors refer to fig 6C they mean 6D, and when they refer to 6D they mean 6E.

We have fixed these errors in the manuscript.

Page 14, line 8. Please remove "rather".
We have removed the word "rather".

Figure 8B. The authors show that SOX-2 G73E still binds the sox-2 binding site, but in the model they show the protein as unbound. It is not clear to me why.
We thank the referee for pointing this out. Our EMSA data shows that SOX-2 G73E is not as efficient at binding its consensus site when adjacent to a LIM-4 target site (Figure 7C, D). Therefore, we initially chose to reflect this in the model. However, we agree with the referee's point that SOX-2G73E can still bind to the site. We have edited the model to show this in Figure 8B.

Page 17, line 8. Please remove "auto" from "autoregulates". It's already enough to say that it regulates its own expression.
The suggested change has been made.

Page 20, first line. Please rephrase to "str-2 has been found to be exquisitely sensitive to various sensory signals" or something similar.
We have made the change to the sentence.

2nd Editorial Decision 05 August 2015

Thank you for submitting your revised manuscript to The EMBO Journal.

As we have discussed, I noted your related and recently published Development paper and I was surprised that this manuscript was not provided during initial submission as it is relevant for the EMBO Journal submission. As you know it is our policy that "any related work under consideration, review, revision or accepted for publication elsewhere must accompany the submission if they are relevant to its scientific assessment."

I decided to run the revised version plus the related Development paper by referee #2 and an advisor and I have now heard back from both of them. As you can see both referees indicate that the Development paper should have been provided during initial review and are surprised that it was not done so. However, they also both find that the EMBO Journal submission goes beyond the Development paper and they do appreciate that we gain mechanistic insight into how Sox-2 regulates terminal differentiation. I have discussed the referee reports with my colleagues and we have decided that we will proceed with the publication of your manuscript. I have provided the link below so that you can make appropriate text modifications to better reflect the Development paper. Please also note that we will publish the review process file for this paper.

Also we have made some changes to our supplemental information please take a look http://emboj.embopress.org/authorguide#expandedview and modify accordingly.

Once I get the revised version back I will proceed with the acceptance of the paper for publication here.

REFEREE REPORTS

Referee #2:

The authors have very adequately addressed all issues raised in my original review.

In the meantime another paper from the same authors has appeared in Development (Vidal ea). In this paper, the authors describe the expression patterns of all sox genes in C. elegans and analyze a.o. the effect of a null mutation in sox-2. This analysis reveals that sox-2 is required for terminal differentiation of specific neurons.

In the manuscript currently under review by EMBO J, the authors claim the same (which is true), but state that this is the first publication that does so, which is not true. For example in the abstract they state that they uncover a "novel" function of sox-2, at the end of the introduction they state "by showing - for the first time - that Sox2 can also have a role in a terminal neuronal differentiation" and in the discussion they state "This is the first time that sox-2 is implicated in specific terminal neuron differentiation programs rather than earlier roles in neurogenesis.". These claims should be amended and refer to the Vidal et al paper.

The authors do refer to the Vidal et al. Development paper, when they mention the deletion allele, the expression pattern and in a paragraph in the results, where they briefly discuss that the ky707 allele has a very cell-specific effect, which differs from the effect of the null mutant. I'm surprised this manuscript was not included as a related manuscript in the original submission.

I must stress though that the current EMBO J manuscript goes way beyond the analysis provided in the Development paper and significantly increases our molecular and mechanistic understanding of how sox-2 regulates terminal differentiation in a cell specific manner.

Advisor:

I find it surprising that the authors made no mention of their previous Development paper while this one was at consideration at The EMBO Journal. I also note that nowhere in this paper do they mention that they previously already implicated SOX-2 and SOX-3 as terminal selector factors (for other neuron types) in their previous paper. They refer to it for alleles and expression patterns but that's about it. Moreover, they include this statement as the last sentence of the Intro: "Our paper significantly broadens the perspective of Sox2 by showing for the first time that Sox2 can also have a role in a terminal neuronal differentiation, through direct regulation of target genes that define the differentiated state of a neuron."

While it is true that their previous paper did not get into the mechanisms of SOX2 regulation of terminal differentiation genes, given the previous paper it is definitely not true that this is the first demonstration of a SOX factor acting as a terminal selector.

Having said that, this current paper does take the mechanism quite far.Leaving aside the above issue, the paper is very detailed and comprehensive and is quite well done.

While this turns out not to be the first report of a SOX factor's role in terminal differentiation, it is still a very good paper. However, they need to tone down statements like the above, and cite and discuss the previous manuscript extensively in the context of this work. But beyond that, I think this will really be an Editorial decision.

2nd Revision - authors' response 05 August 2015

Referee #2:

The authors have very adequately addressed all issues raised in my original review.

In the meantime another paper from the same authors has appeared in Development (Vidal ea). In this paper, the authors describe the expression patterns of all sox genes in C. elegans and analyze a.o. the effect of a null mutation in sox-2. This analysis reveals that sox-2 is required for terminal differentiation of specific neurons.

In the manuscript currently under review by EMBO J, the authors claim the same (which is true), but state that this is the first publication that does so, which is not true. For example in the abstract they state that they uncover a "novel" function of sox-2, at the end of the introduction they state "by showing - for the first time - that Sox2 can also have a role in a terminal neuronal differentiation" and in the discussion they state "This is the first time that sox-2 is implicated in specific terminal neuron differentiation programs rather than earlier roles in neurogenesis.". These claims should be

amended and refer to the Vidal et al paper.

We have amended these claims and referred to the Vidal et al paper in the Abstract, Introduction (p.5), and Discussion (p.21).

The authors do refer to the Vidal et al. Development paper, when they mention the deletion allele, the expression pattern and in a paragraph in the results, where they briefly discuss that the ky707 allele has a very cell-specific effect, which differs from the effect of the null mutant. I'm surprised this manuscript was not included as a related manuscript in the original submission.

I must stress though that the current EMBO J manuscript goes way beyond the analysis provided in the Development paper and significantly increases our molecular and mechanistic understanding of how sox-2 regulates terminal differentiation in a cell specific manner.

We appreciate the positive comment.

Advisor:

I find it surprising that the authors made no mention of their previous Development paper while this one was at consideration at The EMBO Journal. I also note that nowhere in this paper do they mention that they previously already implicated SOX-2 and SOX-3 as terminal selector factors (for other neuron types) in their previous paper. They refer to it for alleles and expression patterns but that's about it. Moreover, they include this statement as the last sentence of the Intro: "Our paper significantly broadens the perspective of Sox2 by showing for the first time that Sox2 can also have a role in a terminal neuronal differentiation, through direct regulation of target genes that define the differentiated state of a neuron."

While it is true that their previous paper did not get into the mechanisms of SOX2 regulation of terminal differentiation genes, given the previous paper it is definitely not true that this is the first demonstration of a SOX factor acting as a terminal selector.

We have changed the last sentence of the Introduction to the following:

Together with another recent report (Vidal et al, 2015), our paper significantly broadens the perspective of Sox2 by showing that Sox2 can also have a role in a terminal neuronal differentiation, through direct regulation of target genes that define the differentiated state of a neuron.

In addition, we have amended the claims and added the following sentences in the Discussion:

Previous work has demonstrated a role of *sox-2* in controlling the terminal differentiation of postmitotic neuron types (Vidal et al, 2015), yet the mechanistic basis for how *sox-2* fulfills such function has been unclear. We define here a competition mechanism by which two distinct homeodomain factors can compete for cooperation with *sox-2* to drive neuron-type specific gene expression programs.

Having said that, this current paper does take the mechanism quite far.Leaving aside the above issue, the paper is very detailed and comprehensive and is quite well done.

We thank the advisor for the positive comment.

While this turns out not to be the first report of a SOX factor's role in terminal differentiation, it is still a very good paper. However, they need to tone down statements like the above, and cite and discuss the previous manuscript extensively in the context of this work. But beyond that, I think this will really be an Editorial decision.

We have toned down the statements, and cited and discussed the Development paper (Vidal et al, 2015) extensively in the context of this work as suggested by the advisor.

