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Sox2, Tlx, Gli3 and Her9 converge on Rx2 to define retinal stem cells in vivo

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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 19 December 2014

Thank you very much for considering The EMBO Journal as venue to present your results on Rx2 in the potential regulation of stem cells.

As you will see from the very constructive comments we solicited from two expert scientists, they agree on the technical quality and potential relevance of the subject matter. However, it also becomes obvious that the paper falls short in definitively proving the proposed function of Rx2 as determining factor for retinal stem cells!

Similarly, definitive causal evidence for the reported TF-circuit as to either govern or the maintain Rx2 expression/stemness are apparently lacking from your technically excellent and very concisely presented dataset.

Please notice that we run a policy to only invite revision for papers with rather predictable and timely outcome. This strategy allows authors to decide whether to take their study significantly further OR seek rapid publication in a less stringent publication (I your case possibly Development?), based on thorough estimates of available resources and general commitment to one particular project.

Given the substantial demands, and upon extensive discussions within the editorial team as well as checking with some of the original referees, we found it most appropriate to return the current (much too preliminary) data back to you with the message that we are unable to offer straightforward revisions/publication at this stage.

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Please also note that despite this formal rejection, I would entertain the option to assess a substantially amended version in the future, in case you would take the strong but very constructive demands from our valued referees into account and develop the paper significantly further.

Once again, I am sorry to be unable to transmit more encouraging news.

REFEREE REPORTS

Referee #1:

In this manuscript, the authors use a cis-regulatory element from the Rx2 gene to identify a gene regulatory network involved in the establishment of the stem cell state in the zebrafish retina. They first lineage labelling the cells in which the regulatory element is active to show that Rx2 is expressed by multipotent stem cells in the ciliary marginal zone of the retina. They then identify two transcriptional activators (Sox2 and Tlx) and two repressors (Gli3 and Her9) that control the activity of the Rx2 element in an in vitro assay and demonstrate direct binding of the four factors to the element. They perform conditional mosaic gain of function experiments and find that activation of Tlx or Sox2 in individual retinal neurons is sufficient to drive Rx2 expression and promote cell cycle re-entry. They argue that the combined activity of the four factors controls stemness in the adult neural retina.

The manuscript addresses the important and very topical question of the nature of the transcriptional networks that establish stem cell populations and reports very interesting findings on the regulation of Rx2 and the activity of its upstream regulators. The data is clear and well documented and mostly support the conclusions of the paper. I have a few comments below to improve the manuscript.

1. The model involving the four transcription factors in the regulation of Rx2 expression in the CMZ which is presented at the end of the manuscript, is based on overexpression of the four factors and mutations of Gli3 and Sox2 binding sites in the Rx2 element. The effect of the loss of Tlx and Her9 inputs into the Rx2 element is not examined and therefore important aspects of the model are not tested. Other major features of this model remain speculative. Why is Rx2 expressed in the intermediate CMZ domain and not repressed since Her9 is also expressed there? What distinguishes this Rx2-expressing intermediate RSC domain and the more central Rx2-negative transit amplifying domain, which both co-express Sox2, Tlx and Her9? If either Sox2 or Tlx alone are sufficient to induce the stem cell state in ectopic expression experiments, why are RSCs confined to a territory where the two factors are co-expressed? Their model of Rx2 regulation raises many unanswered questions, suggesting that important players in Rx2 regulation and the RCS fate are still missing. The authors should acknowledge more clearly the limits of their model.

2) The conclusion that the gene regulatory network identified in this study controls stemness is overstated. Ectopic expression of Rx2 and cell cycle re-entry (marked by a single cell cycle gene, PCNA) in retinal neurons are not sufficient criteria to define a stem cell, as the Rx2 gene is not an independent stem cell marker but a direct target of the ectopically expressed factors. Also there is no evidence of de-differentiation of the neurons that ectopically express Sox2 or Tlx â(e.g. loss of expression of terminal differentiation markers), contrary to what is stated in p. 11 and p. 15. This part of the analysis should be re-phrased to more closely reflect the data.

3) The ChIP experiments in Figs 6 and S6 lack controls, eg PCR for the bound regions using the input chromatin (before ChIP) and ChIP with an irrelevant antibody.

Referee #2:

The topic of retinal stem cells is a hot one in the field of stem cell biology as several laboratories are trying to create these from iPSCs for therapeutic use. Much is therefore known about the steps required to make these cells. The ciliary body of mammals and the ciliary marginal zone of fish and frogs have also been studied extensively reported to have intrinsic retinal stem cells. And the early formation of the eye and the genes associated with microphthalmia and anophthalmia are also relevant to retinal stem cells.

In this paper, Reinhard et al characterise the Rx2 positive cells in the ciliary marginal zone (CMZ) of medaka fish as retinal stem cells for the neural retina and the pigment epithelia and then go on to search for the transcription factors that regulate these Rx2 positive stem cells. I have several problems with this paper which I outline below.

1. Rx2 cells as shown in this paper are not all retinal stem cells. The CMZ is thought to have stem cells only at the very tip, yet Rx2 positive cells are throughout the CMZ. When clones are made from single Rx2 cells then there are some ArCoS clones but there are also many small clones that end up in the central retina (see previous Development paper by these authors) showing that, consistent with the distribution of Rx2 cells in the CMZ, that many Rx2 cells are intermediate progenitors. So, although Rx2 is a good marker of the early retina, it is not a selective marker of retinal stem cells. Some Rx2 cells at the edge of the retina are stem cells but others are not. Therefore this entire study is really about the transcription factors that regulate Rx2 rather than the transcription factors that regulate retinal stem cells. In fact, there is no evidence that Rx2 controls stemness as the abstract suggests. Rx2 is essential for normal retinal development. It is an early marker of the eye field and the optic cup. It's expression signals retinal development. But it is not at all clear that it has a role in stemness. Some Rx2 postive cells make ArCoS clones, others do not. This paper should be called "Sox2, Tlx, Gli3 and Her9 converge to regulate Rx2 in the ciliary margin of the medaka retina." It is worth knowing what factors regulate the expression of Rx2, but that is a less interesting question than what regulates stemness.

2. To really understand the regulation of stem cells in the CMZ and the role of Rx2 in stemness, the authors should do experiments that turn on or off stemness by turning on or off Rx2. For example, turning off Rx2 in what would otherwise be stable ArCoS clones developing , should cut the stripe off peripherally. Turning on Rx2 by itself, should start an new ArCoS. Rather they do other kinds of experiment that do not get at the question directly. Over-expression of the negative regulators of Rx2 (Gli3 and Her9) lower PCNA levels in the CMZ. But is that because they lower Rx2? Tlx and Sox2 promote Rx2 expression in clones and lead to occasion ectopic expression of PCNA, but Rx2 expression does not equate to stemness and the occasional ectopic S-phase is not necessarily due to the increased Rx2. It might, for example, be a direct effect of Sox2 on the cell cycle as Sox2 does many things and is a well known stemness factor.

3. Part of the novelty of this paper is the discovery of factors that should be upstream of Rx2. In the story here, it is not clear if these TFs are turning on Rx2 or feeding back on Rx2 to regulate it after it is already expressed. Moreover, there have been several links in the literature between Hh (Gli) signaling and Rx or Rax, between Tll or Tlx and Rx or Rax, between Sox2 and Rx/Rax and Notch signaling through Hairy Enhancer of Split (Hes and Her), so the links here are not so novel. And this work is not discussed in light of the current What is potentially important for understanding Rx2 regulation is whether these genes are the major direct regulators of Rx2 expression. The data on the EMSA look promising for direct binding, but the ChIP data were not done on the native gene.

4. Related to this, the screen looked at 1000+ transcription factors - there are many transcription factors that have been linked to retinal stem cells - such as Pax6, Six3, Six6, Tbx, Wnt effectors, myc genes, BMP effectors, Nodal effectors, etc. Were all these tested? As this is not really a fully unbiased screen, the full list of what transcription factors were tested should be shown. If key factors such as the above clearly have no effect in this screen, that should also be mentioned in the paper.

Appeal 02 February 2015

We were very pleased to see that both referees felt that we are addressing a very topical and important question (ref. 1). and that all of the referees concerns are easily addressed.

I realized that there was an important misconception of referee #2 who stated that Rx2 would be expressed in the entire CMZ. This is not the case, it is exclusively expressed in the distal tip of the CMZ, the domain where stem cells are discussed to be localized.
We may have fallen short to state that clear enough in the manuscript, but once again, this is easily addressable.

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Under the angle of a big conceptual misunderstanding (from our side, due to not clearly stating that Rx2 is only expressed in the distal tip of the CMZ), I can see why the referee was very critical. However there is no basis for that criticism.

On the other hand, referee 2 discusses what would be the best possible experiment in the field in a couple of years if acute conditional knock out will eventually be possible in fish.
I am afraid, even though an interesting exercise, this does not really give justice to the manuscript where we applied available, top notch technology to approach the question today.

Given that crucial misunderstanding of referee 2 and the rather positive and easily addressable points of referee 1 we feel that we could submit a revised version addressing the addressable concerns of the referees.

RESPONSE TO REVIEWS

>> Referee #1:
>>
>> *In this manuscript, the authors use a cis-regulatory element from the Rx2 gene to identify a gene regulatory network involved in the establishment of the stem cell state in the zebrafish retina. They first lineage labelling the cells in which the regulatory element is active to show that Rx2 is expressed by multipotent stem cells in the ciliary marginal zone of the retina. They then identify two transcriptional activators (Sox2 and Tlx) and two repressors (Gli3 and Her9) that control the activity of the Rx2 element in an in vitro assay and demonstrate direct binding of the four factors to the element. They perform conditional mosaic gain of function experiments and find that activation of Tlx or Sox2 in individual retinal neurons is sufficient to drive Rx2 expression and promote cell cycle re-entry. They argue that the combined activity of the four factors controls stemness in the adult neural retina.*
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>> *The manuscript addresses the important and very topical question of the nature of the transcriptional networks that establish stem cell populations and reports very interesting findings on the regulation of Rx2 and the activity of its upstream regulators. The data is clear and well documented and mostly support the conclusions of the paper. I have a few comments below to improve the manuscript.*
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>> *1. The model involving the four transcription factors in the regulation of Rx2 expression in the CMZ which is presented at the end of the manuscript, is based on overexpression of the four factors and mutations of Gli3 and Sox2 binding sites in the Rx2 element. The effect of the loss of Tlx and Her9 inputs into the Rx2 element is not examined and therefore important aspects of the model are not tested. Other major features of this model remain speculative. Why is Rx2 expressed in the intermediate CMZ domain and not repressed since Her9 is also expressed there? What distinguishes this Rx2-expressing intermediate RSC domain and the more central Rx2-negative transit amplifying domain, which both co-express Sox2, Tlx and Her9? If either Sox2 or Tlx alone are sufficient to induce the stem cell state in ectopic expression experiments, why are RSCs confined to a territory where the two factors are co-expressed? Their model of Rx2 regulation raises many unanswered questions, suggesting*
>> *that important players in Rx2 regulation and the RCS fate are still missing. The authors should acknowledge more clearly the limits of their model.*

We can relax the model even further by removing the scheme in the final figure showing the expression patterns of the factors in the CMZ and focus only on the network. Lack of putative Tlx or Her9 TFBSs did not alter Rx2 expression in the CMZ at the stages tested.

>> *2) The conclusion that the gene regulatory network identified in this study controls stemness is overstated. Ectopic expression of Rx2 and cell cycle re-entry (marked by a single cell cycle gene, PCNA) in retinal neurons are not sufficient criteria to define a stem cell, as the Rx2 gene is not an independent stem cell marker but a direct target of the ectopically expressed factors. Also there is no evidence of de-differentiation of the neurons that ectopically express Sox2 or Tlx å(e.g. loss of expression of terminal differentiation markers), contrary to what is stated in p. 11 and p. 15. This*

part of the analysis should be re-phrased to more closely reflect the data.

We can re-phrase this paragraph, again.

>> 3) The ChIP experiments in Figs 6 and S6 lack controls, eg PCR for the bound regions using the input chromatin (before ChIP) and ChIP with an irrelevant antibody.

In this case the controls are included through the primers binding the co-transfected luciferase coding sequence (using input and IP).

>> Referee #2:

>> The topic of retinal stem cells is a hot one in the field of stem cell biology as several laboratories are trying to create these from iPSCs for therapeutic use. Much is therefore known about the steps required to make these cells. The ciliary body of mammals and the ciliary marginal zone of fish and frogs have also been studied extensively reported to have intrinsic retinal stem cells. And the early formation of the eye and the genes associated with microphthalmia and anophthalmia are also relevant to retinal stem cells.
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>> In this paper, Reinhard et al characterise the Rx2 positive cells in the ciliary marginal zone (CMZ) of medaka fish as retinal stem cells for the neural retina and the pigment epithelia and then go on to search for the transcription factors that regulate these Rx2 positive stem cells. I have several problems with this paper which I outline below.
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>> 1. Rx2 cells as shown in this paper are not all retinal stem cells. The CMZ is thought to have stem cells only at the very tip, yet Rx2 positive cells are throughout the CMZ. When clones are made from single Rx2 cells then there are some ArCoS clones but there are also many small clones that end up in the central retina (see previous Development paper by these authors) showing that, consistent with the distribution of Rx2 cells in the CMZ, that many Rx2 cells are intermediate progenitors. So, although Rx2 is a good marker of the early retina, it is not a selective marker of retinal stem cells. Some Rx2 cells at the edge of the retina are stem cells but others are not. Therefore this entire study is really about the transcription factors that regulate Rx2 rather than the transcription factors that regulate retinal stem cells. In fact, there is no evidence that Rx2 controls stemness as the abstract suggests. Rx2 is essential for normal retinal development. It is an early marker of the eye field and the optic cup. It's expression signals retinal development. But it is not at all clear that it has a role in stemness. Some Rx2 postive cells make ArCoS clones, others do not. This paper should be called "Sox2, Tlx, Gli3 and Her9 converge to regulate Rx2 in the ciliary margin of the medaka retina." It is worth knowing what factors regulate the expression of Rx2, but that is a less interesting question than what regulates stemness.
>>
Reviewer 2 misses the point by mixing up the results of different papers. Figure 1 shows Rx2 clearly as a marker for RSCs, selective for multipotent RSCs, while random recombination (eg. hsp-driven) gives the full complement of terminated and continued clones. A well defined role of Rax during eye development does not preclude a key function of Rx2 during adult stages.

>> 2. To really understand the regulation of stem cells in the CMZ and the role of Rx2 in stemness, the authors should do experiments that turn on or off stemness by turning on or off Rx2. For example, turning off Rx2 in what would otherwise be stable ArCoS clones developing , should cut the stripe off peripherally. Turning on Rx2 by itself, should start an new ArCoS. Rather they do other kinds of experiment that do not get at the question directly. Over-expression of the negative regulators of Rx2 (Gli3 and Her9) lower PCNA levels in the CMZ. But is that because they lower Rx2? Tlx and Sox2 promote Rx2 expression in clones and lead to occasion ectopic expression of PCNA, but Rx2 expression does not equate to stemness and the occasional ectopic S-phase is not necessarily due to the increased Rx2. It might, for example, be a direct effect of Sox2 on the cell cycle as Sox2 does many things and is a well known stemness factor.
>>
Again misses the point (probably because reviewer 2 did not get Figure 1, which introduces Rx2 as an novel RSC marker) because it is never stated that Rx2 is the factor responsible for stemness in the post-embryonic CMZ. We show that Rx2 is a reliable proxy for retinal stem cells.

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>> 3. Part of the novelty of this paper is the discovery of factors that should be upstream of Rx2. In the story here, it is not clear if these TFs are turning on Rx2 or feeding back on Rx2 to regulate it after it is already expressed. Moreover, there have been several links in the literature between Hh (Gli) signaling and Rx or Rax, between Tll or Tlx and Rx or Rax, between Sox2 and Rx/Rax and Notch signaling through Hairy Enhancer of Split (Hes and Her), so the links here are not so novel. And this work is not discussed in light of the current What is potentially important for understanding Rx2 regulation is whether these genes are the major direct regulators of Rx2 expression. The data on the EMSA look promising for direct binding, but the ChIP data were not done on the native gene.

Plenty of links between Rax and the four factors (and a lot of other factors) exist in the literature. The link between Rx, post-embryonic stemness, multipotency and the network has not been demonstrated so far. In addition the manuscript provides strong evidence for the direct interaction between Rx2 pCRE and the four factors (again, a novel finding).

>> 4. Related to this, the screen looked at 1000+ transcription factors - there are many transcription factors that have been linked to retinal stem cells - such as Pax6, Six3, Six6, Tbx, Wnt effectors, myc genes, BMP effectors, Nodal effectors, etc. Were all these tested? As this is not really a fully unbiased screen, the full list of what transcription factors were tested should be shown. If key factors such as the above clearly have no effect in this screen, that should also be mentioned in the paper.
>>
Reviewer 2 is concerned about the factors included in the screen. Most of the factors listed above were tested but discarded because they did not show significant changes. We can provide a list of factors tested in the supplement. In addition a lot of these factors are obviously known to be important for eye development (six3, six6, pax6) but have not been directly linked to life-long growth or proliferation in the retina.

1st Revision - authors' response — 21 March 2015

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that important players in Rx2 regulation and the RCS fate are still missing. The authors should acknowledge more clearly the limits of their model.

We agree with the reviewer that the described TFs are not the only players involved in this network, rather that they form a core scaffold to regulate Rx2 expression in the CMZ (Figure 8). We now clearly state this in the revised version of the manuscript.
Nevertheless, we provide compelling evidence for the existence of direct interaction between Sox2, Tlx, Gli3, Her9 and the predicted sites as forming a core scaffold in the regulation of Rx2 and thus balancing between the two types of retinal stem cells.
EMSA and ChIP-PCR show binding of Sox2, Tlx, Gli3 and Her9, while the Sox2 and Gli3 proteins show less affinity for mutated versions of the binding sites. For functional validation, we tested the mutations in luciferase assays and show decreased activation with Sox2 and a loss of repression with Gli3 (Figure 7). In addition, we included dose-response luciferase assays for each factor (Figure 2).

2) The conclusion that the gene regulatory network identified in this study controls stemness is overstated. Ectopic expression of Rx2 and cell cycle re-entry (marked by a single cell cycle gene, PCNA) in retinal neurons are not sufficient criteria to define a stem cell, as the Rx2 gene is not an independent stem cell marker but a direct target of the ectopically expressed factors. Also there is no evidence of de-differentiation of the neurons that ectopically express Sox2 or Tlx å(e.g. loss of expression of terminal differentiation markers), contrary to what is stated in p. 11 and p. 15. This part of the analysis should be re-phrased to more closely reflect the data.

We appreciate the reviewers concern regarding the stem cell potential of cells overexpressing Sox2 or Tlx. We rephrased this part to avoid confusion.
With the new data on the Rx2 mutant we can now state with confidence that Rx2 impacts on the balance the two types of retinal stem cells. In the absence of Rx2, stem cells preferentially contribute to RPE, consistent with the findings of Rx repression (via Gli3) determining the transition of NR to RPE in the analysis of the corresponding binding sites in the Rx2 reporter line.

3) The ChIP experiments in Figs 6 and S6 lack controls, eg PCR for the bound regions using the input chromatin (before ChIP) and ChIP with an irrelevant antibody.

We had avoided the use of unrelated, potentially non-relevant ABs (Schuster et al, 2014) and instead used control primers binding the co-transfected luciferase coding sequence (for both input and IP).
Therefore as a control we tested our specific AB (anti-GFP) in relevant and unrelated regions next to the predicted TFBSs. The qPCR results for the TFBSs were normalized against the control luciferase regions, resulting in the fold-changes as shown in our revised manuscript (Figure 6).

Referee #2:

The topic of retinal stem cells is a hot one in the field of stem cell biology as several laboratories are trying to create these from iPSCs for therapeutic use. Much is therefore known about the steps required to make these cells. The ciliary body of mammals and the ciliary marginal zone of fish and frogs have also been studied extensively reported to have intrinsic retinal stem cells. And the early formation of the eye and the genes associated with microphthalmia and anophthalmia are also relevant to retinal stem cells.

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from single Rx2 cells then there are some ArCoS clones but there are also many small clones that end up in the central retina (see previous Development paper by these authors) showing that, consistent with the distribution of Rx2 cells in the CMZ, that many Rx2 cells are intermediate progenitors. So, although Rx2 is a good marker of the early retina, it is not a selective marker of retinal stem cells. Some Rx2 cells at the edge of the retina are stem cells but others are not. Therefore this entire study is really about the transcription factors that regulate Rx2 rather than the transcription factors that regulate retinal stem cells. In fact, there is no evidence that Rx2 controls stemness as the abstract suggests. Rx2 is essential for normal retinal development. It is an early marker of the eye field and the optic cup. It's expression signals retinal development. But it is not at all clear that it has a role in stemness. Some Rx2 postive cells make ArCoS clones, others do not. This paper should be called "Sox2, Tlx, Gli3 and Her9 converge to regulate Rx2 in the ciliary margin of the medaka retina." It is worth knowing what factors regulate the expression of Rx2, but that is a less interesting question than what regulates stemness.

We appreciate the reviewers criticism and changed the manuscript accordingly. Our revised version of the manuscript emphasizes most clearly that Rx2 is only expressed in the distal-most tip of the CMZ and that only these cells Rx2-positve cells are multipotent, in contrast to cells in the central CMZ which are limited to producing short-lived clones (Centanin et al., 2014).
While Rax (or Rx) genes are well known for their role during embryonic eye development, until now Rx2 has never been demonstrated as a proxy labelling multipotent RSCs. In addition to being a molecular marker for stem cells contributing to the NR or RPE, our mutant analysis shows a functional role for Rx2 in balancing the life-long growth of NR and RPE from same pool of Rx2 positive stem cells.

2. To really understand the regulation of stem cells in the CMZ and the role of Rx2 in stemness, the authors should do experiments that turn on or off stemness by turning on or off Rx2. For example, turning off Rx2 in what would otherwise be stable ArCoS clones developing , should cut the stripe off peripherally. Turning on Rx2 by itself, should start an new ArCoS. Rather they do other kinds of experiment that do not get at the question directly. Over-expression of the negative regulators of Rx2 (Gli3 and Her9) lower PCNA levels in the CMZ. But is that because they lower Rx2? Tlx and Sox2 promote Rx2 expression in clones and lead to occasion ectopic expression of PCNA, but Rx2 expression does not equate to stemness and the occasional ectopic S-phase is not necessarily due to the increased Rx2. It might, for example, be a direct effect of Sox2 on the cell cycle as Sox2 does many things and is a well known stemness factor.

The referee raises an interesting point regarding the role of Rx2. In our revised manuscript we have now incorporated functional data highlighting the role of Rx2 via Rx2 RNA null mutants. We show in clonal analyses that the absence of Rx2 shifts retinal stem cells towards an RPE fate, consistent with the mechanism we proposed for the action of Gli3 in the transition zone between NR and RPE.
Rx2 is thus necessary for balancing the lineages RSC progeny contributing to NR or RPE.

3. Part of the novelty of this paper is the discovery of factors that should be upstream of Rx2. In the story here, it is not clear if these TFs are turning on Rx2 or feeding back on Rx2 to regulate it after it is already expressed. Moreover, there have been several links in the literature between Hh (Gli) signaling and Rx or Rax, between Tll or Tlx and Rx or Rax, between Sox2 and Rx/Rax and Notch signaling through Hairy Enhancer of Split (Hes and Her), so the links here are not so novel. And this work is not discussed in light of the current What is potentially important for understanding Rx2 regulation is whether these genes are the major direct regulators of Rx2 expression. The data on the EMSA look promising for direct binding, but the ChIP data were not done on the native gene.

The reviewer is correct to point out known regulatory interactions between the mammalian Rax (Rx in fish and frog) and other TFs. However, the direct link between Rx, post-embryonic stemness, multipotency and the core scaffold has not been demonstrated so far. Performing ChIP-qPCR in fish is non-trivial and technically challenging considering the number of adult RSCs in the medaka CMZ. Therefore we complemented our in vivo data with cell culture-based assays using medaka genes (Becarri et al., 2011) to support direct regulatory interactions between rx2 and the four TFs forming the core scaffold.

4. Related to this, the screen looked at 1000+ transcription factors - there are many transcription factors that have been linked to retinal stem cells - such as Pax6, Six3, Six6, Tbx, Wnt effectors, myc genes, BMP effectors, Nodal effectors, etc. Were all these tested? As this is not really a fully unbiased screen, the full list of what transcription factors were tested should be shown. If key factors such as the above clearly have no effect in this screen, that should also be mentioned in the paper.

We appreciate the reviewers comments regarding the screen. Our unbiased screen (Souren et al., 2009) tested the ability of a large complement of the medaka TFs to regulate Rx2 expression. However, given the novel role of Rx2 for post-embryonic RSCs it is not surprising that TFs involved in early eye specification (eg. Pax6, Six3) did not result in significant fold-changes, while genes required for NSC proliferation (Sox2, Gli3, Hes1) were identified as the strongest Rx2 regulators.
We have attached an Excel sheet with all validated ENSEMBL IDs of the factors analyzed (due to the not yet perfect annotation of the medaka genome, a fair number of tested factors does not yet have an ENSEMBL ID).
We provide a second Excel sheet representing the results of the transactivation screen. These data are submitted as supplemental tables.

Accept letter

Thank you very much for your revised study that has been carefully assessed based on the new data and the provided responses to the referee critiques.

I am therefore pleased to inform you that we can offer to move ahead with eventual production/publication. For this, we would need the following items before being able to formally accept your study:

-please provide a 2 up to 4 'bullet point' synopsis, that highlights major novelty/advance/significance of your study.

-an integrating figure/graphical abstract in the format 550 x 150 (max 400) pixel would facilitate featuring your study in eToC's and our homepage.

I am very much looking forward to receive the relevant files before acceptance and hopefully timely presentation of your paper in The EMBO Journal.

Yours truly,

Thomas Schwarz-Romond, PhD/MBA
Senior Editor
The EMBO Journal