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# Zic2 regulates the expression of Sert to modulate eye-specific refinement at the visual targets

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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 April 2010

Thank you very much for submitting your paper for consideration to The EMBO Journal editorial office.
As you will see from the enclosed reports referee #3 as very much supportive of your study. Though also appreciating the interest and technical quality of the study, both refs#1 and #2 ask for considerable further extension and controls that seem however feasible during a single round of major revisions. These should particularly rule out premature retraction (ref#1) and attempt knockdown that should be sufficient to verify phenotypes in target refinement by (at least partial) loss-of function (ref#2) and thus greatly improve impact and scientific value of the study. All in all, I am very happy to offer you the possibility to revise the study accordingly.
I also have to remind you that it is EMBO_J policy to allow a single round of major revisions only. Thus, the final decision on acceptance or rejection entirely depends on the content and strength of the final version of your manuscript that will be assessed from some of the original referees!

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

Yours sincerely,

Editor
The EMBO Journal

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

 

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http://www.nature.com/emboj/about/process.html

REFFEREE REPORTS

Referee #1 (Remarks to the Author):

This study seeks to uncover the role of Zic2 in Retinal Ganglion Cell (RGC) axonal refinement at its target regions, namely the Superior Culliculus (SC) and the dorsal lateral geniculate nucleus (dLGN). Specifically, they claim that the transcription factor Zic2 acts through SERT (the serotonin transporter) to modulate axonal refinement, linking the previously independent roles of Zic2 in mediating ipsilateral pathfinding at the optic chiasm and the serotonin-dependent axonal pruning of RGCs at the SC and dLGN. While this is a significant progress in understanding the molecular mechanisms of ganglion cells axonal refinement, some issues must be addressed in this manuscript before publication:

Mayor issues:
- In Figure 3 and the associated data analysis, the authors claim that due to E13.5 electroporation of Zic2, they see an increase in SERT mRNA via RT-PCR. From this they conclude that Zic2 is sufficient to induce SERT. However, the increase in SERT may be entirely in the VT cells already expressing Zic2 and SERT. A more rigorous assessment of sufficiency would be to, 3 days after electroporation, to stain the retina for Zic2 and SERT (like they did in figure 2a-b), showing that in electroporated central retina that is induced to express Zic2, SERT expression is induced (in addition to an electroporation control).

- In Figure 4, for their negative control in the luciferase assay, the authors used the luciferase gene without any upstream activating region, but since the gene contains a TATA box-like sequence, that is most likely required for any binding by the RNA polymerase independent of activation or repression by transcription factors. Thus, the better negative control would be to also test a construct that only has the TATA-like domain of the 130bp consensus sequence upstream of the luciferase. Should the expression of this plasmid have the same induction by Zic2 as that with the GLI domain deletion, then the necessity of that domain for SERT expression would be greatly strengthened.

- The authors use a drug known to inhibit SERT (Fluoxetine, or Prozac) to rescue the Zic2 over expression phenotype in SC elaboration pattern, which is suggestive that Zic2 acts through SERT. However, while this drug is clinically specific, it is not perfect, with reports stating that it has effects on norepinephrine and dopamine transporters as well. In addition, it is considered one of the less specific of the SSRI's. If SERT is how Zic2 acts, a similar phenotype should be observed with just a SERT over expression, which should also be rescued by the drug. The authors mention this kind of analysis was difficult since SERT over expression does not cause an induction of ipsilateral projection in central RGCs, however, the contralateral phenotype with Zic2 could be replicated with SERT over expression. The authors claim that the contralateral phenotype was difficult to score, probably due to the small number of processes that projected more caudally in the contra lateral SC. However, it seemed like the images were taken with a wide-field but low numerical aperture objective in all images in order to capture the full extent of the SC or dLGN, thus sacrificing resolution for being able to image the entire specimen. Analyzing the contra lateral phenotype with a higher numerical aperture objective (and possibly also confocal microscopy) would probably allow for the differences in process elaboration to be more easily scored, thus allowing for the contralateral phenotype to be examined in both the Zic2 over expression and the SERT over expression.

- Throughout the study, the authors claim that the Zic2 over expression phenotype is a "lack of refinement" or lack of elaboration of dense processes in the ipsilateral SC. However, they do not rule out that the refinement occurred but prematurely retracted. To do so, they should, in Zic2-overexpressing animals, looks the ipsilateral SC not only at E16.5 but also at intermediate time points between E13.5 and E16.5 to compare the normal elaboration of ipsilateral fibers without Zic2 over expression to the ones of ectopic Zic2-overexpressing axons.

- It is unclear what is done in figure 7. Figure a-c is labeled as "E13-P15" but the text claims it was done via P0 electroporations (page 13, line 21). Also, the contralateral EGFP only is shown as well

as the ipsilateral Zic2. The ipsilateral EGFP only as well as the contralateral Zic2 must also be shown with and without the drug rescue, even if no apparent phenotype can be seen. Again, as stated above, the contralateral phenotype may be elucidated with higher resolution microscopy.

Minor issues:
- In page 12, line 12, the authors claim that "axons projecting and arborizing in the medial areas are those that express EGFP but do not co-express Zic2". This is unlikely, since co-electroporation rates of 90-95% or more have been extensively reported in a variety of in vivo contexts including retinal electroporations in the embryo in vivo and in vitro as well as in vivo postnatal retina and brain. If the authors have a reason to claim low co-expression in their system that is not due to using low plasmid concentrations (ie less that 1ug/uL of DNA), they should elaborate further.

- In page 13, line 8, the authors say "axons that aberrantly project to caudal areas in fluoxetine-treated mice, although not significantly, tend to decrease in comparison with saline-treated animals (data not shown)." The use of the word "significantly" is misleading. If there was no statistically significant decrease, there was no decrease. If they meant the change was only subtle but still appeared to be statistically significant, they should change their phrasing.

-The materials and methods should include a section on how the images were obtained stating which microscope and objectives were used.

Referee #2 (Remarks to the Author):

The findings of the manuscript are interesting and novel and add significantly to the field. In addition, the work will be interesting to a number of readers outside the direct field. With a few minor issues (see below), the technical quality is very high especially given the challenging nature of some of the experiments and clearly a lot or work and care has gone into the design and implementation of the experiments. However there are several major issues discussed below.

Major Comments:
1) The manuscript is difficult to read. It does not flow well and is disjointed in places. I found it difficult to understand the meaning of a sentence at several points. The main issues are emphasis and clarity. For example, given that SERT has been previously shown to regulate refinement of visual targets the novelty of the manuscript is that Zic2 regulates refinement of targets by controlling the expression of SERT and it does so independently of its role in midline crossing. This emphasis does not come across well in the introduction. Also, clarity or lack of links into and out of each section makes it difficult to read. The poor clarity of some sentences or sections also hinders the understanding of the very interesting findings.

2) Page 10: The authors use overexpression of Zic2 (and therefore SERT) to provide evidence that Zic2 regulates target refinement.

Although I agree with the arguments used by the authors on page 10 against using the Zic2 knockout or making a conditional line the conclusions would be significantly strengthened by a knockdown approach. Knockdown of Zic2 could be achieved by using the electroporation assay. The advantage of this is that a partial knockdown is likely meaning that axons may still project ipsilaterally but may still be knocked down enough to regulate targeting. Similarly, a knockdown of SERT in the cells of interest (rather than global ablation or pharmacological inhibition) would also greatly strengthen the conclusions.

Figure 3 f'. There is a lot of background staining of SERT (red) - compare with b' (control). How do the authors know that this is not genuine SERT signal. If the authors can not clarify this issue using this technique qPCR on explants would serve as an alternative.

Minor issues:
Figure 1: define kd in figure legend text

For clarity, it would be helpful to have the headings on the figure as well as in the text (probe ID, gene symbol, average fold, p values)

Supplementary figure1: the text is not clear.

Figure 2 : Sert is mRNA so should be in italics

Figure2c: would be better to have a confocal image here to see co expression, expression as the image is out of focus. There also needs even higher power to see cell-by-cell resolution of co-expression.

Figure 3f' Sert staining is mRNA so should be indicated in the figure.

Page 8: SERT expression was assayed 4 days after electroporation with Zic2. If Zic2 is directly regulating SERT as the authors claim, it would be better to assay after 12-24 hours to support the idea that it is direct transcription

Page 12 'it is likely that
axons correctly projecting and arborizing in the medial areas are those that express EGFP but do not co-express Zic2;'
I disagree with this, they could be expressing a lower level and therefore exerting a different effect.

Referee #3 (Remarks to the Author):

This paper from GarcÌa-Frigola and Herrera addresses an important question in visual system development (and generally in axon guidance) of how transcription factors impact the expression of 'effector' genes that directly control wiring of brain circuitry. The authors show that the transcription factor, Zic2 impacts expression of serotonin transporter SERT (which is associated with the ipsi RGCs) and in turn shapes connections between the eyes and the brain.

They provide mRNA and protein expression data to show a spatial and temporal correspondence between Zic2 and SERT expressing RGCs, both in normal and Zic2 overexpression cases. They also show SERT is in the ipsi projection (an important clarification that was made unclear in previous studies focused on which RGCs express SERT). They then use genomic analysis, and in vivo gain of function to show that Zic2 controls RGC axon targeting through SERT and independent of chiasm trajectory. The fluoxetine experiments are a terrific "rescue" to counter potential criticisms about overexpression .

Overall this is an elegant and major step forward in the field of visual system development. Few studies employ so many techniques and level of analysis and for this the authors should be commended. Based on their data (and previous work focused on EphBs) one can now draw a "flow diagram" from Zic2 to chiasm pathfinding (EphB1) and axon targeting (SERT) in the brain.

My only minor criticisms relate to:

1) The use of "eye specific targeting in the title and text.
I see the results as impacting RGC axonal refinement, but little evidence is shown about eye-specific targeting in the traditional sense. I suggest staying closer to the data and describing "axonal refinement" in the title, and elsewhere.

2) The writing.

The results are fine, but the abstract and intro include many awkward words and phrases that make it a difficult read. For instance "ipsilaterality" (abtract) is a mouth full not common to the field. They also repeatedly refer to "at the target" instead of "in the target". This is not a trivial distinction, because it confuses target pathfinding/selection and axonal refinement. There are also many run-on sentences that require commas and there are numerous punctuation errors. Indeed, many sentences fail to end in a period. Maybe this was a formatting error but in any case, some focused editing is needed to match the beautiful experiments and data with adequately clear text in the abstract, intro and discussion.

Referee #1 (Remarks to the Author):

This study seeks to uncover the role of Zic2 in Retinal Ganglion Cell (RGC) axonal refinement at its target regions, namely the Superior Culliculus (SC) and the dorsal lateral geniculate nucleus (dLGN). Specifically, they claim that the transcription factor Zic2 acts through SERT (the serotonin transporter) to modulate axonal refinement, linking the previously independent roles of Zic2 in mediating ipsilateral pathfinding at the optic chiasm and the serotonin-dependent axonal pruning of RGCs at the SC and dLGN.

*We thank the reviewer for appreciating the relevance of our work. We also thank him/her for the suggestions since we believer they have improved the quality of the paper.*

Mayor issues:

In Figure 3 and the associated data analysis, the authors claim that due to E13.5 electroporation of Zic2, they see an increase in SERT mRNA via RT-PCR. From this they conclude that Zic2 is sufficient to induce SERT. However, the increase in SERT may be entirely in the VT cells already expressing Zic2 and SERT.

*Maybe the reviewer missed the description in the Methods section of the original version of the manuscript indicating that the qRT-PCR assays performed to compare SERT mRNA levels in the Zic2 versus EGFP electroporated retinas, only the central (electroporated) portion of the retina was taken. RGCs in the ventrotemporal peripheral retinal region are the only ones endogenously expressing Zic2 and SERT but they were dissected out for the experiment. Therefore it is impossible that the increase in SERT detected by qRT-PCR is due to cells already expressing Zic2 and SERT*

A more rigorous assessment of sufficiency would be to, 3 days after electroporation, to stain the retina for Zic2 and SERT (like they did in figure 2a-b), showing that in electroporated central retina that is induced to express Zic2, SERT expression is induced (in addition to an electroporation control).

*We tried to do this experiment. However, colorimetric in situ hybridization is less sensitive than qRT-PCR and could not be used for proper quantification. This is why we choose this method. The qRT-PCR results performed at 72h after electroporation and also the new ones performed at 24h after electroporation, are highly significant and reproducible, so we are really confident with these data.*

- In Figure 4, for their negative control in the luciferase assay, the authors used the luciferase gene without any upstream activating region, but since the gene contains a TATA box-like sequence, that is most likely required for any binding by the RNA polymerase independent of activation or repression by transcription factors. Thus, the better negative control would be to also test a construct that only has the TATA-like domain of the 130bp consensus sequence upstream of the luciferase. Should the expression of this plasmid have the same induction by Zic2 as that with the GLI domain deletion, then the necessity of that domain for SERT expression would be greatly strengthened.

*The reviewer is correct. In fact, that was our first option as a control. Initially, we included in our luciferase assays a plasmid containing the TATA-like and the CG-rich sequence. However, the result was that this sequence alone was not enough to induce luciferase activity. When this plasmid was transfected together with Zic2 plasmids, luciferase levels didn´t increase either. We have prepared a graph to show the referee these results. In addition we have added the following paragraph in the text: "We also tested the activity of the TATA-like domain. However this sequence alone was not able to induce luciferase activity showing that the rest of the elements in the 130 bp fragment located upstream of the SERT TSS are essential to induce transcription (data not shown). We prefer to keep the figure simple and do not show these data since these results do not add much to the main message of this figure.*

- The authors use a drug known to inhibit SERT (Fluoxetine, or Prozac) to rescue the Zic2 over expression phenotype in SC elaboration pattern, which is suggestive that Zic2 acts through SERT. However, while this drug is clinically specific, it is not perfect, with reports stating that it has effects

on norepinephrine and dopamine transporters as well. In addition, it is considered one of the less specific of the SSRI's. If SERT is how Zic2 acts, a similar phenotype should be observed with just a SERT over expression, which should also be rescued by the drug. The authors mention this kind of analysis was difficult since SERT over expression does not cause an induction of ipsilateral projection in central RGCs, however, the contralateral phenotype with Zic2 could be replicated with SERT over expression. The authors claim that the contralateral phenotype was difficult to score, probably due to the small number of processes that projected more caudally in the contra lateral SC. However, it seemed like the images were taken with a wide-field but low numerical aperture objective in all images in order to capture the full extent of the SC or dLGN, thus sacrificing resolution for being able to image the entire specimen. Analyzing the contra lateral phenotype with a higher numerical aperture objective (and possibly also confocal microscopy) would probably allow for the differences in process elaboration to be more easily scored, thus allowing for the contralateral phenotype to be examined in both the Zic2 over expression and the SERT over expression.

*Following the reviewer´s suggestion we have performed new quantifications on high magnification pictures of the caudal portions of contralateral SC from EGFP and Zic2 electroporated animals. We have also compared high maginification images of the non-refined axons in the contralateral side of Zic2 electroporated mice treated with saline or fluoxetine. Using this method we have found that in fact, there are statistically significant differences between EGFP-electroporated and Zic2-electroporated caudal collicular regions as well as between saline and fluoxetine-treated samples. We have included representative images of these experiments and the corresponding quantification graphs in Figure6 and Figure7.*

*We think that performing the same experiments, but electroporating SERT, instead of Zic2, would be redundant with previous experiments from other groups and will only demonstrate that fluoxetine blocks SERT, something that is already accepted.*

- Throughout the study, the authors claim that the Zic2 over expression phenotype is a "lack of refinement" or lack of elaboration of dense processes in the ipsilateral SC. However, they do not rule out that the refinement occurred but prematurely retracted. To do so, they should, in Zic2-overexpressing animals, looks the ipsilateral SC not only at E16.5 but also at intermediate time points between E13.5 and E16.5 to compare the normal elaboration of ipsilateral fibers without Zic2 over expression to the ones of ectopic Zic2-overexpressing axons.

*We probably did not explain the experiment properly and, as a result, the reviewer is confused. First, the axons cannot prematurely retract since the phenotype we describe for RGCs ectopically expressing Zic2 is that they do not retract at all. Second, we do not analyze the phenotype at E16.5 but at P15, once the refinement process has finished. However based on the referee´s comment we thought that if we show the evolution of the phenotype throughout development, the refinement defect in Zic2 positive axons will be more evident. For this reason we have also analyzed the projection pattern of Zic2 positive axons and EGFP control axons at intermediate developmental stages. We show these new results in Figure 6. Data show that both, EGFP alone and Zic2 positive axons, reach the most caudal region of the SC at P0 and P4. However by P15, EGFP axons have refined and project in central collicular regions while Zic2 positive axons are still found at the caudal colliculus, clearly demonstrating a defect in the refinement process. We have also changed the text in the corresponding section and Figure legend to include the new data.*

- It is unclear what is done in figure 7. Figure a-c is labeled as "E13-P15" but the text claims it was done via P0 electroporations (page 13, line 21). *The electroporation was always performed at E13 and these electroporated animals were sacrificed at P15. Fluoxetine treatment started at P0 in animals that were electroporated at E13 and continued until they were sacrificed at P15. We have changed the sentence: "We administered daily doses of fluoxetine from birth to P15 to mice electroporated with Zic2/EGFP in the center of the retina at E13", to: "...*we electroporated E13.5 embryos in the central retina and waited until the mice were born. Then, we administered a daily dose of fluoxetine until they were analyzed at P15". We think this point is clearer now.

Also, the contralateral EGFP only is shown as well as the ipsilateral Zic2. The ipsilateral EGFP only as well as the contralateral Zic2 must also be shown with and without the drug rescue, even if no

apparent phenotype can be seen. Again, as stated above, the contralateral phenotype may be elucidated with higher resolution microscopy.

*We did not find targeted axons at all in the ipsilateral colliculus of mice electroporated with EGFP alone. This is shown in Figure 6 and it is why we do not show it again in Figure 7. In the revised Figure 7 we have included images and data from the contralateral side of mice electroporated with Zic2/EGFP treated with saline or fluoxetine*

Minor issues:

- In page 12, line 12, the authors claim that "axons projecting and arborizing in the medial areas are those that express EGFP but do not co-express Zic2". This is unlikely, since co-electroporation rates of 90-95% or more have been extensively reported in a variety of in vivo contexts including retinal electroporations in the embryo in vivo and in vitro as well as in vivo postnatal retina and brain. If the authors have a reason to claim low co-expression in their system that is not due to using low plasmid concentrations (ie less that 1ug/uL of DNA), they should elaborate further.

*The reviewer is correct about the co-electroporation rates. In fact this percentage is exactly what we have previously reported for the in utero electroporation method (García-Frigola et al., 2007, García-Frigola et al., 2008). However, since retinal axons form very elaborated and ramified arbors after adopting their mature shape at the colliculus, it is likely that few axons look like many. We recognize, however, that it is possible, as suggested by referee 2 (see below), that these central-projecting contralateral axons are neurons that have incorporated less Zic2 plasmids but still express certain Zic2 levels. To consider this possibility we have changed the sentence to*: "Since we co-electroporated two plasmids (pCAG-Zic2 and pCAG-EGFP) it is possible that axons correctly projecting and arborizing in the medial areas are those that express EGFP but do not co-express Zic2 or express it at low levels*"*.

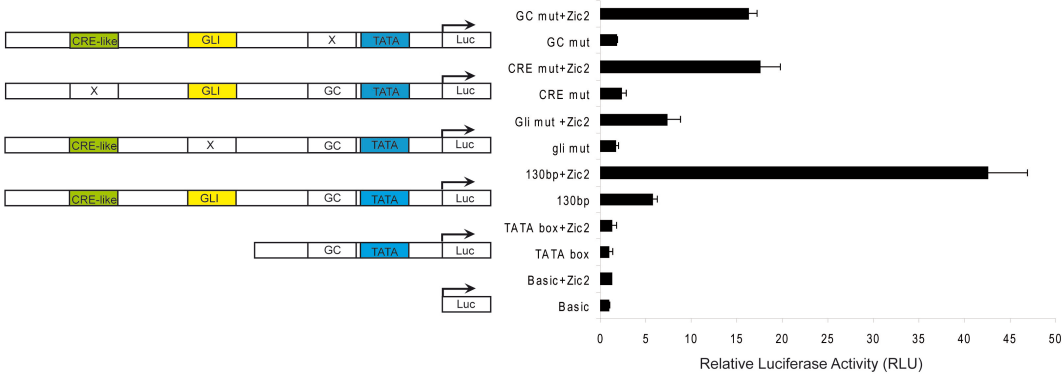
- In page 13, line 8, the authors say "axons that aberrantly project to caudal areas in fluoxetine-treated mice, although not significantly, tend to decrease in comparison with saline-treated animals (data not shown)." The use of the word "significantly" is misleading. If there was no statistically significant decrease, there was no decrease. If they meant the change was only subtle but still appeared to be statistically significant, they should change their phrasing.

*According to our new data showing that the difference between the animals treated with saline or with fluoxetine is significant in images analyzed at higher magnification, we changed the sentence to say: "*In the contralateral SC of P15 mice electroporated with Zic2/EGFP, we focused our analysis in the caudal collicular areas (Figure 7d,e), that contain the axons with a defect in refinement. In this case the amount of axonal projections that aberrantly occupied caudal areas was also significantly reduced in fluoxetine-treated mice in comparison with control saline-treated animals (Figure 7 f)"

-The materials and methods should include a section on how the images were obtained stating which microscope and objectives were used

*We have included this information in the methods section.*

Figure for Referees only:



Referee #2 (Remarks to the Author):

The findings of the manuscript are interesting and novel and add significantly to the field. In addition, the work will be interesting to a number of readers outside the direct field. With a few minor issues (see below), the technical quality is very high especially given the challenging nature of some of the experiments and clearly a lot or work and care has gone into the design and implementation of the experiments. However there are several major issues discussed below.

*We thank the reviewer for the nice compliments and for his/her comment on the quality and novelty of our work. We also thank him for the constructive cristicisms because we believe that the suggestions have enhanced the quality of the paper.*

Major Comments:

1) The manuscript is difficult to read. It does not flow well and is disjointed in places. I found it difficult to understand the meaning of a sentence at several points. The main issues are emphasis and clarity. For example, given that SERT has been previously shown to regulate refinement of visual targets the novelty of the manuscript is that Zic2 regulates refinement of targets by controlling the expression of SERT and it does so independently of its role in midline crossing. This emphasis does not come across well in the introduction. Also, clarity or lack of links into and out of each section makes it difficult to read. The poor clarity of some sentences or sections also hinders the understanding of the very interesting findings.

*We apologize for the language mistakes in our first submission. As the referee suggested we have reorganized the introduction section to emphasize the major findings of the paper. In addition we have asked a native English colleague to revise the new version of the manuscript and as a result of these changes we think the reading of the manuscript has significantly improved.*

2) Page 10: The authors use overexpression of Zic2 (and therefore SERT) to provide evidence that Zic2 regulates target refinement. Although I agree with the arguments used by the authors on page 10 against using the Zic2 knockout or making a conditional line the conclusions would be significantly strengthened by a knockdown approach. Knockdown of Zic2 could be achieved by using the electroporation assay. The advantage of this is that a partial knockdown is likely meaning that axons may still project ipsilaterally but may still be knocked down enough to regulate targeting. Similarly, a knockdown of SERT in the cells of interest (rather than global ablation or pharmacological inhibition) would also greatly strengthen the conclusions.

*We agree with the reviewer that a loss-of-function assay would be the ideal experiment. Unfortunately the experiment that the reviewer suggests is very technically chanllenging. It is very difficult to target the peripheral VT retina. We have repeatedly tried to achieve this, not only for this work but also other projects in our lab. Unfortunately so far, we have been unable to get the peripheral VT region electroporated. On the other hand, our previous work already tells us that knocking-down Zic2 produces a change in the laterality of the axons: the Zic2 mutant mice analyzed in Herrera et al., 2003 were knockdown not null mutants and they showed a near elimination of the ipsilateral projection. Nevertheless we think that the facts that: a) SERT is only expressed in ipsilateral axons, b) SERT expression in Zic2 gain- and loss- of function situations is induced and reduced respectively, and c) that Zic2 positive axons but not Zic2 negative axons respond to fluoxetine, together with previous reports demonstrating the role of the serotonin signaling in axonal refinement, clearly demonstrate that Zic2 regulates SERT and that SERT is involved in axonal refinement at the visual target.*

Figure 3 f'. There is a lot of background staining of SERT (red) - compare with b' (control). How do the authors know that this is not genuine SERT signal. If the authors cannot clarify this issue using this technique qPCR on explants would serve as an alternative.

*We have replaced the images in Figure3. We believe that the new images clearly show that there is a strong reduction of SERT mRNA levels in Zic2 mutant retinas compared to widltype littermates. As the referee pointed out there is some residual staining in the SERT (red) image in the Zic2 mutant. However the remaining staining perfectly matches with the residual staining of Zic2*

*(green). It is important to remember that these are not Zic2 null mice but hypomorphic mice and they still have residual levels of Zic2. The reviewer suggests to perform qRT-PCR. We think this would be redundant with the microarrays results (Figure 1) that already show in a very clear quantitative and clean manner that the levels of SERT mRNA are downregulated 3.4 fold in the Zic2 mutant samples. We have reduced the complexity of this figure removing the Islet2 staining because is not essential for the main message of the figure. The figure legend has been changed accordingly.*

Minor issues:

Figure 1: define kd in figure legend text

*We have done this*

For clarity, it would be helpful to have the headings on the figure as well as in the text (probe ID, gene symbol, average fold, p values)

*We have done this*

Supplementary figure1: the text is not clear.

*We have explained this Figure in more detail*

Figure 2: Sert is mRNA so should be in italics

*We have mended this in the figure and in the rest of the paper.*

Figure2c: would be better to have a confocal image here to see co expression, expression as the image is out of focus. There also needs even higher power to see cell-by-cell resolution of co-expression.

*Figure 2c is not out of focus. The problem is that it is very difficult to appreciate co-expression combining inmunofluorescent staining for Zic2 (in the nucleus) with fluorescent in situ hybridization for SERT (in the cytoplasm). For this reason, we decided to include a high mag. image of the VT region of a retinal section in which double staining for SERT and Zic2 was performed using colorimetric reactions instead of fluocescence. Perhaps the referee has not noticed that Fig2d shows a high magnification picture of the VT region of an E16 retinal section. In this image it can be clearly appreciated that SERT mRNA (purple) is surrounding the Zic2 positive nuclei (brown). We have inserted arrowheads to highlight all the Zic2 positive cells that are surrounded by SERT mRNA and have included higher magnification of two cells in which the colocalization is particularly evident.*

Figure 3f' Sert staining is mRNA so should be indicated in the figure.

*We have corrected this in the corresponding figure*

Page 8: SERT expression was assayed 4 days after electroporation with Zic2. If Zic2 is directly regulating SERT as the authors claim, it would be better to assay after 12-24 hours to support the idea that it is direct transcription

*We have performed this experiment as the reviewer suggested and have included the new results in Figure 4. Data shows that Zic2 activates SERT as soon as 24h after electroporation supporting once again a direct control of Zic2 over SERT expression. We describe these new results in the text.*

Page 12 'it is likely that axons correctly projecting and arborizing in the medial areas are those that express EGFP but do not co-express Zic2; I disagree with this, they could be expressing a lower level and therefore exerting a different effect.

*As we discuss in our respnse to referee 1, it is possible that the central-projecting contralateral axons are neurons that have incorporated less Zic2 plasmids than the ones that changed laterality,*

*but still express certain Zic2 levels. Thus, we have changed the sentence accordingly in the text.*

Referee #3 (Remarks to the Author):

This paper from García-Frigola and Herrera addresses an important question in visual system development (and generally in axon guidance) of how transcription factors impact the expression of 'effector' genes that directly control wiring of brain circuitry. The authors show that the transcription factor, Zic2 impacts expression of serotonin transporter SERT (which is associated with the ipsi RGCs) and in turn shapes connections between the eyes and the brain.

They provide mRNA and protein expression data to show a spatial and temporal correspondence between Zic2 and SERT expressing RGCs, both in normal and Zic2 overexpression cases. They also show SERT is in the ipsi projection (an important clarification that was made unclear in previous studies focused on which RGCs express SERT). They then use genomic analysis, and in vivo gain of function to show that Zic2 controls RGC axon targeting through SERT and independent of chiasm trajectory. The fluoxetine experiments are a terrific "rescue" to counter potential criticisms about overexpression.

Overall this is an elegant and major step forward in the field of visual system development. Few studies employ so many techniques and level of analysis and for this the authors should be commended. Based on their data (and previous work focused on EphBs) one can now draw a "flow diagram" from Zic2 to chiasm pathfinding (EphB1) and axon targeting (SERT) in the brain.

*We thank the reviewer for the very supportive and nice comments.*

My only minor criticisms relate to:

1) The use of "eye specific targeting in the title and text.

I see the results as impacting RGC axonal refinement, but little evidence is shown about eye-specific targeting in the traditional sense. I suggest staying closer to the data and describing "axonal refinement" in the title, and elsewhere.

*To remove the term "eye-specific" from the title as the referee suggest w could change the tittle to: "* Zic2 regulates the expression of SERT to modulate axonal refinement at the visual targets" *but we are reluctan to do so at least that it is considered strictly necessary by the editor. We would prefer to keep the tittle as it is. Changing the tittle would imply that the refinement of all RGCs is affected by alterations in the serotonin signalling, which is not true. Zic2 and SERT are specifically expressed in ipsilateral axons and blocking SERT does not affect the refinement of contralateral axons, affects only those expressing Zic2 and SERT. We would like to point out that we never use in the text "eye-specific targeting", but "eye-specific segregation" or" eye-specific domains".*

2) The writing. The results are fine, but the abstract and intro include many awkward words and phrases that make it a difficult read. For instance "ipsilaterality" (abtract) is a mouth full not common to the field. They also repeatedly refer to "at the target" instead of "in the target". This is not a trivial distinction, because it confuses target pathfinding/selection and axonal refinement. There are also many run-on sentences that require commas and there are numerous punctuation errors. Indeed, many sentences fail to end in a period. Maybe this was a formatting error but in any case, some focused editing is needed to match the beautiful experiments and data with adequately clear text in the abstract, intro and discussion.

*We apologize for these mistakes. We believe that these problems have been addressed in the new version.*

1st Editorial Decision 24 June 2010

Your revised manuscript has now been re-assessed by one of the original referees whose comments are enclosed. Going back to ref#1's notion that if Zic2 functions via SERT, simple SERT expression should elicit similar phenotypes that would be rescued by flouoxetine. If not actually performed by you (still an option that would strengthen the results in your system and compensate for the not really approachable loss function), you should minimally refer to related reports in the literature, thus putting your findings into the more general context. Furthermore, though presentation has already improved, a few changes would make particularly the results section flow better.
Overall, I kindly ask you to incorporate these points as well as the specific remarks from the referee below in preparing the ultimate version of your study for eventual acceptance.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

This manuscript demonstrates that in addition to its known function in regulation axon guidance at the optic chiasm, the transcription factor ZIC2 regulates axonal refinement at central targets. This is achieved by direct regulation of the serotonin transporter, SERT.

The manuscript in interesting, novel and moves the field significantly forward.

Minor comments:
Figure 3:
The images of Zic2 immunofluorescence in the in the Zic2 kd are very poor (Figure 3d and d'). This figure does not support the claim in the text that a low level of Zic2 is detectable in the Zic2kd mice resulting in residual SERT expression in these embryos. If there is another transcription factor independently regulating low levels of SERT it does not detract from the findings of the manuscript but the authors should clarify their data on this.

Figure 5:
If the schematic were to be laterally inverted, it would be easier to read the figure at first glance.

Page 5, second to bottom line

Exchange the word 'perfectly' for something like 'consistent with'.

2nd Revision - Authors' Response 29 June 2010

*We have addressed your suggestions and Referee 2's comments as follows:*

Referee #2
Minor comments:
Figure 3:
The images of Zic2 immunofluorescence in the Zic2 kd are very poor (Figure 3d and d'). This figure does not support the claim in the text that a low level of Zic2 is detectable in the Zic2kd mice resulting in residual SERT expression in these embryos. If there is another transcription factor independently regulating low levels of SERT it does not detract from the findings of the manuscript but the authors should clarify their data on this.

*To avoid this issue we have removed the sentence about the residual expression of Zic2 and Sert in the mutant. The sentence states now: "Sert mRNA levels were highly reduced in the VT retina*

*of Zic2kd/kd embryos compared to WT littermates". We have also increased the magnification of these figures to make even more evident the high decrease in Sert expression in the Zic2 mutant. Figure legend has been changed accordingly.*

Figure 5:
If the schematic were to be laterally inverted, it would be easier to read the figure at first glance.

*We have done this.*

Page 5, second to bottom line
Exchange the word 'perfectly' for something like 'consistent with'.

*We have done this. The new sentence reads: "Consistent with Zic2 expression Sert mRNA was detected in the VT retina at this age".*

Editor comments:

*We have included the requested references.*
*We have also made few changes in the results section to improve the flow of the text.*