Initiation of Otx2 expression in the developing mouse retina requires a unique enhancer and either *Ascl1* or *Neurog2* activity

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DOI: 10.1242/dev.199399

Editor: François Guillemot

Review timeline
Original submission: 6 January 2021
Editorial decision: 11 February 2021
First revision received: 7 April 2021
Accepted: 10 May 2021

Original submission

First decision letter

MS ID#: DEVELOP/2020/199399

MS TITLE: The initiation of Otx2 expression in the developing mouse retina requires a unique enhancer and either Ascl1 or Neurog2 activity

AUTHORS: Michael L Kaufman, Noah B Goodson, Ko Uoon Park, Michael Schwanke, Emma Office, Sophia R Schneider, Joy Abraham, Austin Hensley, Kenneth L Jones, and Joseph A Brzezinski IV

I have now received the reports of three referees on your manuscript and I have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, all the referees express great interest in your work, but they also have some criticisms and recommend a revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, I will be happy to receive a revised version of the manuscript. Referee 2 requests that the enhancer activity of DHS-4 is validated by transgenic analysis in vivo. While this would significantly strengthen the study, it is not absolutely necessary for the revision of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily their concerns. Please also note that Development will normally permit only one round of major revision.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing

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how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Kaufman and colleagues investigates how Otx2 expression in regulated in the developing retina by defining a minimal enhancer element that promotes expression transiently as progenitors exit the cell cycle and identifying its upstream regulators. This study is thorough, carefully designed, and convincingly shows that DHS-4D represents a minimal Otx2 enhancer that is required for embryonic expression of Otx2. Through systematic analysis and mutagenesis, they showed that multiple E-box bHLH factor binding sites are required for enhancer activity and that Ascl1 and Neurog2 regulated enhancer activity and Otx2 expression. The study is rigorous and the findings are clear. Overall, this represents a significant advance in understanding mechanisms regulating Otx2 expression, and the model nicely summarizes the findings.

Comments for the author

Minor comments:
1. Figure 1B is a bit difficult to interpret. First, there are many GFP-labeled cells that do not seem to express the RFP control, which should be a more ubiquitous readout of transfection, so this is puzzling. In addition, the arrow highlights one GFP/Otx2 double labeled cell from the boxed area, but it is difficult to assess how much overlap there is beyond that. This is not a major concern since it was previously published and panel E nicely quantifies this for all constructs.

2. In a related question – Figure 4 shows GFP+ and RFP+ cells being separately sorted. This approach makes sense. But are the GFP+ cells also RFP+? Or a mix?

3. For the single cell analysis, it is difficult from Figure 4C to determine how the clusters relate to the proposed cell classifications. It is surprising that there are so many progenitor clusters. Were default parameters used? Perhaps the parameters could be optimized for this data set. The single cell analysis by Clark et al 2019 defined primary RPCs and neurogenic progenitors. Do those distinctions apply to the clusters defined in this analysis?

Reviewer 2

Advance summary and potential significance to field

The manuscript of Kaufman et al., 2020 provides detailed analysis/characterization of previously identified enhancer element of transcription factor Otx2 (DHS-4) (Winken et al., 2015) in mouse retinal development.
Using reporter assays in electroporated retinal explants the authors identified a potential early minimal enhancer within DHS-4, DHS-4D, that is able to drive reporter gene expression into Otx2-expressing retinal progenitors. This enhancer element was subjected to further analysis to address its importance in initiation of Otx2 expression and identification of potential upstream acting regulators. The authors identified several putative bHLH transcription factor binding sites within DHS-4D and following a mutagenesis approach they analysed their respective contribution to Otx2 retinal expression.
In further search for candidate upstream regulators of DHS-4D, authors employed single cell RNA sequencing approach. Employing the CRISPR/Cas9 Â– mediated gene editing system, the authors were able to demonstrate that the DHS-4D enhancer and the presence of upstream acting bHLH transcription factors Ascl1, Neurog2 and Olig2 are important for embryonic Otx2 retinal expression as their targeting clearly negatively impact on the Otx2 expression in electroporated, explanted retinae.
The manuscript is well written. Experiments are logically organized and well described. The data and analysis provided are detailed and straight forward to follow.

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Comments for the author

Major concerns:
However, the data quality of some of the presented data is not satisfying (impaired retinal morphology or quality of presented immunostainigs) and needs to be improved.

Conceptually, it is not clear what is the contribution of DHS-4D enhancer to Otx2 expression in wider in vivo developmental context. Otx2 is the pivotal regulator of neural/retinal development and identification and validation of such an enhancer would be of broad interest and relevance.

To validate the relevance and wider applicability of their retinal explant assay, the cross validation of critical aspects to the in vivo situation in the mouse embryo is required.

In the light of the fact that other enhancer elements have been identified to regulate the onset of Otx2 expression in the embryonic retina (EELPOT; Muranishi et al., 2011), the manuscript would requires at least an in vivo validation of the contribution of DHS-4D enhancer to Otx2 early retinal expression.

In some figures, the position of retinal layers is indicated, in some it is not. Although in most figures the immunostaining results are clear, for some it is not clear at all. The authors need to make sure that the layers are consistently presented and recognisable throughout the manuscript. A DAPI nuclear staining or indication of corresponding retinal layers (with dashed lines) ois required for the reader to better orient in the presented figures (Fig. S2, Fig. 5C, Fig. 6C).

In their lineage tracing experiment (Fig. S2) the authors characterise the fate of cells with an active DHS-4 enhancer. The authors indicate that they observe RFP-positive amacrine cells and occasional ganglion or Muller glia cells (S2C). This characterization would benefit from description of markers that have been used to identify particular cell types. It should be clear and unambiguous which cells were identified to be amacrine cells and which as Muller glia cells.

Fig. 3: Mutating E boxes 1, 2 and 5 resulted in continued robust GFP expression overlapping with Otx2 expression . The authors refer to Fig. 3 C, D, however, there is no data in D showing it. Those data need to be shown (single E box mutagenesis of 1, 2, 4, 5), at least as supplementary figure? It is notclear in D what was the motivation to show the samples, authors are showing.

Some samples are having strange retinal morphology/Otx2 staining (mut 1-2-3-4-5-6, mut 1-2-5). Is it the artefact caused by sample handling?
• Single cell RNA-sequencing: “These cell type annotations were then mapped back onto the UMAP cluster number they most represented” – authors refer to Fig. S3 instead of S5.
Minor:
• Supplementary Fig. S6 is not mentioned in the text.
• While using CRISPR/Cas9 authors always designed three guides. However, in text they state they used 2-
3 guides. What exactly does it mean, please clarify.
• Often when authors talk about the effect of manipulation on Otx2 expression – they use words blocks/prevents/inhibits. The word “reduce” would suit the data much better.
• Fig. 5C: retinal morphology and immunostaining in control looks strange. Is it the artefact of the sample handling?
• Fig.6C: C is missing in figure and figure legend.
• Fig 6: Authors claim that “some cells” would end up as amacrine cells but they don’t state how many and don’t show any amacrine cell marker.

Reviewer 3

Advance summary and potential significance to field

Kaufman and colleagues report identification of an Otx2 enhancer element and characterization of Ascl1 and Neurog2 dependent activation in the differentiation of retinal cells. This study combines transient enhancer assays with Cas9-based mutation testing in retina using both ex vivo and in vivo models. The primary finding is that characterization of a specific Otx2 enhancer as required for

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transcriptional activation as cells exit the proliferative stage. The authors show that the DHS-4 enhancer, defined in previous work, depends on a minimal core (4D) that contains E-box sites that are responsive to Ascl1 and Neurgo2. They use a combination of GFP labeling and in vivo Cas9 mutagenesis to show that the 4D enhancer activates when retinal progenitors are still Ki67 positive and is critical for initial activation of Otx2 but that at later stages the effect of 4D mutation is reduced. Thus they propose a model where 4D activity is not required for the long term maintenance of Otx2 expression in rods, cones, and bipolar cells. The study leaves many questions unanswered, particularly the continued activity but loss of specificity in the minimal D1 and D2 elements, the somewhat unexpected results from combinatorial E-box mutagenesis, and the proposed hand off of Otx2 regulation to other enhancers. But leaving these questions to be addressed in future work seems fair considering the scope and elegance of this work. I have no major concerns and applaud the authors for the clarity of the manuscript and figures. The value of this study to the field is both regarding retinal development, particularly the mechanisms driving transcriptional control and more generally as an exemplar of more general enhancer function in development.

Comments for the author

No essential major revisions.

First revision

Author response to reviewers' comments

Dear Dr. Guillemot and the Reviewers,

Thank you for your enthusiasm and constructive feedback on our manuscript. We have conducted additional experiments, improved our figures, added new data and figures, and updated the text to strengthen the manuscript and address your concerns.

Before detailing our responses, we wish to highlight some of the new data and figures we added to the manuscript. We added new results to a revised Figure 6 (*in vivo Ascl1/Neurog2* double targeting) and to a revised Supplemental Figure 9 (additional *Olig2* targeting data in combination with *Ascl1* and *Neurog2*. A new Supplemental Figure 5 has been created to show representative images from the entirety of the Core E-box mutagenesis strategy (see Reviewer 2, Major Concern 4). This has changed some of the supplemental figure numbering throughout the manuscript. Although some of these additions were not requested by the reviewers, the new data strengthen the manuscript without significantly altering the original interpretations of our findings.

Below are the point-by-point responses to the reviewer concerns. The reviewer comments are in black text and our responses are in blue text. Some of the reviewer comments are lightly paraphrased or numbered for clarity.

Reviewer 1 Comments for the Author:

Minor comments:
1. Figure 1B is a bit difficult to interpret. First, there are many GFP-labeled cells that do not seem to express the RFP control, which should be a more ubiquitous readout of transfection, so this is puzzling. In addition, the arrow highlights one GFP/Otx2 double labeled cell from the boxed area, but it is difficult to assess how much overlap there is beyond that. This is not a major concern since it was previously published and panel E nicely quantifies this for all constructs.

The original channel-merged image is a bit difficult to see, but we felt that brevity was more appropriate for this figure as the DHS-4 pattern had been previously published. Nonetheless, we have modified the image by increasing the levels of the red and green channels to show a more representative overlap of DHS-4 GFP and the ubiquitously expressed control RFP (Cherry). The co-

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electroporation rate is high, but not 100%. Moreover, some of the red, green, and purple nuclei are bright enough to obfuscate each other's signal and make the overlap appear lower than it actually is. In examining other enhancer sequences and mutations, we rely upon the presence of Cherry+ nuclei to determine that the lack of a GFP signal is meaningful.

2. In a related question – Figure 4 shows GFP+ and RFP+ cells being separately sorted. This approach makes sense. But are the GFP+ cells also RFP+? Or a mix?

The reviewer is correct to point out that the sorting strategy was not clear. The purpose of this sorting strategy was to enrich for the DHS-4D GFP marked cells and to provide OTX2-negative, yet electroporated, cells. We have modified Figure 4 and the text to reflect that there are two populations that were enriched. The first population includes any cells that express GFP, regardless of whether they co-express Cherry (RFP). These would be green or yellow in the sort and represent electroporated cells that activated DHS-4D. The second population includes cells that *only* express Cherry (RFP). These represent electroporated cells that are OTX2-negative. These populations were then pooled at a 40 (GFP) to 60 (Cherry-only) percent ratio for single cell RNA-sequencing.

3a. For the single cell analysis, it is difficult from Figure 4C to determine how the clusters relate to the proposed cell classifications. It is surprising that there are so many progenitor clusters. Were default parameters used?

We agree that it is difficult to assign cell classifications when there are so many clusters that can be attributed as progenitors. However, to be as unbiased as possible, we used default clustering parameters and avoided expression level manipulations, such as regression of cell cycle genes. This is now noted in our revised methods section. By following this unbiased approach, expression variability remains within these populations and results in multiple clusters in the UMAP. The genes supplying these differences can be better observed within Figure S6B, where clusters vary in their levels of progenitor genes (*Vsx2*, *Sox2*, *Pax6*, etc.). In addition, cell cycle markers and Notch pathway genes were also highly variable (data not shown). As the reviewer mentions in Minor Comment 3b, progenitor sub- populations are not uncommonly seen in other single cell RNA-seq data from the developing retina. Classifications of these progenitor cluster differences have been described in Clark et al., 2019 (PMID: 31128945) and more recently in Wu et al., 2021 (PMID: 33674582).

3b. Perhaps the parameters could be optimized for this data set. The single cell analysis by Clark et al 2019 defined primary RPCs and neurogenic progenitors. Do those distinctions apply to the clusters defined in this analysis?

We kept our analysis parameters in the most unbiased format we could, which still allows for pseudotime trajectory analysis. Nonetheless, we agree that the description of the progenitor clusters was not detailed enough to make it readily interpretable. To remedy this, we decided to follow the classifications as described by Clark et al., 2019 (PMID: 31128945). This allowed us to classify progenitors into broad groups. We added the following text to the results section to improve clarity: "Comparing these progenitor clusters to the previously classified populations described by Clark et al., we find that they fall into two broad groups. Clusters 0, 1, and 5 likely represent early/intermediate progenitors due to their relatively high levels of *Fgf15*, *Vsx2*, and *Sox9*. Clusters 2, 6, and 11 can be classified as neurogenic or late neurogenic progenitors based on their expression of *Otx2*, *Ascl1*, *Neurog2*, and *Olig2* (Fig. S6). Differences in the stage of the cell cycle likely underlie the relatively large number of progenitor clusters we identified (Fig. S6)."

Reviewer 2 Comments for the Author:

Major concerns:
1. However, the data quality of some of the presented data is not satisfying (impaired retinal morphology or quality of presented immunostainigs) and needs to be improved. Conceptually, it is not clear what is the contribution of DHS-4D enhancer to Otx2 expression in wider in vivo developmental context. Otx2 is the pivotal regulator of neural/retinal development and identification and validation of such an enhancer would be of broad interest and relevance. To validate the relevance and wider applicability of their retinal explant assay, the cross validation of critical aspects to the in vivo situation in the mouse embryo is required. In the light of the fact that

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other enhancer elements have been identified to regulate the onset of Otx2 expression in the embryonic retina (EELPOT; Muranishi et al., 2011), the manuscript would requires at least an in vivo validation of the contribution of DHS-4D enhancer to Otx2 early retinal expression.

We agree that additional *in vivo* experiments would strengthen our understanding of how *Otx2* is regulated in the retina and beyond. These tools will be valuable to understand redundancy or compensation at the enhancer level as well. We are in the process of building *Otx2* enhancer deletion mouse lines to evaluate DHS-4D and other *Otx2* enhancers. However, this work is beyond the scope of the current manuscript and will be included in a future paper.

2. In some figures, the position of retinal layers is indicated, in some it is not. Although in most figures the immunostaining results are clear, for some it is not clear at all. The authors need to make sure that the layers are consistently presented and recognisable throughout the manuscript. A DAPI nuclear staining or indication of corresponding retinal layers (with dashed lines) ois required for the reader to better orient in the presented figures (Fig. S2, Fig. 5C, Fig. 6C).

We agree that the interpretation of the immunostaining data would be strongly improved by labeling layers in the images. We have revised our figures to include such labels and point out in the revised methods section that all of the images are in the same orientation throughout the manuscript. DAPI stains obfuscate the other staining patterns (see Reviewer 1, Minor Comment 1), so we find it best to omit DAPI and label the layers in the images instead.

3. In their lineage tracing experiment (Fig. S2) the authors characterise the fate of cells with an active DHS-4 enhancer. The authors indicate that they observe RFP-positive amacrine cells and occasional ganglion or Muller glia cells (S2C). This characterization would benefit from description of markers that have been used to identify particular cell types. It should be clear and unambiguous which cells were identified to be amacrine cells and which as Muller glia cells.

We agree that our assessment of amacrine, ganglion, and Müller glia cells was not thoroughly explained in the lineage tracing experiments. We have revised the figure caption and the results section to emphasize that we used markers, laminar position, and cytoplasmic morphology of the RFP staining to determine identity. Figure S2 includes PAX6 and BRN3A stains, which when coupled with laminar position, allow for the discrimination of amacrine and ganglion cell identities. Müller glia were evident as RFP+ cells that span the thickness of the retina. These glia often weakly express PAX6 as well. Amacrines, glia, and ganglion cells are also conspicuous in that they lack OTX2 staining.

4. Fig. 3: Mutating E boxes 1, 2 and 5 resulted in continued robust GFP expression overlapping with Otx2 expression. The authors refer to Fig. 3 C, D, however, there is no data in D showing it. Those data need to be shown (single E box mutagenesis of 1, 2, 4, 5), at least as supplementary figure? It is not clear in D what was the motivation to show the samples, authors are showing.

We agree with the reviewer that the choice of representative images shown for the mutagenesis experiments was not clear or comprehensive. To rectify this, we have revised Figure 3D to include all six of the single E-Box mutants. We have created a new Supplemental Figure 5 where histological examples of all the other E-box mutants are shown. The revisions make the mutagenesis dataset comprehensive and easier to interpret.

5. Some samples are having strange retinal morphology/Otx2 staining (mut 1-2-3-4-5-6, mut 1-2-5). Is it the artefact caused by sample handling?

The reviewer has a good eye. Cultured explants are subject to moderate morphological abnormalities and staining artifacts are seen when there is zero GFP for the antibodies to bind. We also occasionally see minor damage due to electroporation. We have replicated several of the experiments in question and higher-quality images are now used for the revised Figure 3D and new Supplemental Figure 5. These changes make the results of these enhancer mutants visually clear and easier to interpret. Improved images were used to revise some of the other figures as well.

6. Single cell RNA-sequencing: “These cell type annotations were then mapped back onto the UMAP cluster number they most represented” – authors refer to Fig. S3 instead of S5.

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Thank you for pointing out this mistake. It has been corrected and updated to reflect new supplemental figure numbering in the revised manuscript.

Minor:
1. Supplementary Fig. S6 is not mentioned in the text.

Thank you for pointing this out. This has been addressed in the revised manuscript.

2. While using CRISPR/Cas9 authors always designed three guides. However, in text they state they used 2-3 guides. What exactly does it mean, please clarify.

We apologize for the ambiguity. Three guides were indeed designed and utilized to target and disrupt the DHS-4D enhancer element. This was done to increase the probability that deletions between the guide sequences would occur and cause a loss of function. On the other hand, a frameshift mutation is likely on its own to prevent proper expression of a coding gene. While we designed three guides to the various coding targets, we found that two guides were sufficient to efficiently reduce target expression. Ostensibly, this was through 1-2 frame shift mutations or deletions between the two guide sequences. To target two coding genes at once, we have now built plasmids where two guides are expressed from the same PX458 plasmid to reduce issues with co-electroporating large numbers of plasmids. As these were more recently designed, only the *in vivo Ascl1/Neurog2* double targeting experiments and the triple *Ascl1*, *Neurog2*, and *Olig2* targeting experiments used the two-guide single-plasmid approach. We have added new language to a revised methods section to clarify how guides were used (2 vs. 3) and how the two-guide all-in-one plasmids were built.

3. Often when authors talk about the effect of manipulation on Otx2 expression – they use words blocks/prevents/inhibits. The word “reduce” would suit the data much better.

We agree (for the most part) that “reduce” and “inhibit” refer agnostically to the results while “block” or “prevent” imply a mechanism. We have changed our word usage throughout the manuscript to refer to results with “reduce” or “inhibit”, but discuss our findings in the interpretive terms of “block” or “prevent”.

4. Fig. 5C: retinal morphology and immunostaining in control looks strange. Is it the artefact of the sample handling?

In this case, the P0 explanted tissue was electroporated and grown in culture for 3 days. The three-day culturing protocol can lead to more structural abnormalities than what is seen after two days of culture. To overcome this, we have reimaged these samples and revised Figure 5C to improve clarity. Some evidence of culturing effects remain, but we feel that the revised figure is much easier to interpret. We have also annotated the retinal layers to help orient the reader.

5. Fig.6C: C is missing in figure and figure legend.

We apologize for this oversight. We have added the part C label for Figure 6 and updated the corresponding caption.

6. Fig 6: Authors claim that “some cells” would end up as amacrine cells but they don’t state how many and don’t show any amacrine cell marker.

The presence of OTX2-negative round nuclei in the inner aspect of the inner nuclear layer is completely consistent with amacrine cell identity. This amacrine fate shift is consistent with prior *Otx2* perturbations, such as in Nishida et. al 2003 (PMID: 14625556) and more recently with similar methods in Ghinia-Tegla et al. 2020 (PMID: 32347797). This is reflected in the revised results section of the manuscript. Please also see the earlier discussion about lineage tracing experiment interpretation (Major Concern 3).

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Reviewer 3 Comments for the Author:

The study leaves many questions unanswered, particularly the continued activity but loss of specificity in the minimal D1 and D2 elements, the somewhat unexpected results from combinatorial E-box mutagenesis, and the proposed hand off of Otx2 regulation to other enhancers. But leaving these questions to be addressed in future work seems fair considering the scope and elegance of this work. I have no major concerns and applaud the authors for the clarity of the manuscript and figures. The value of this study to the field is both regarding retinal development, particularly the mechanisms driving transcriptional control, and more generally as an exemplar of more general enhancer function in development.

We thank the reviewer for their enthusiasm for our manuscript and their eagerness to learn more about the underlying mechanisms of *Otx2* regulation, enhancer function, and retinal development.

Second decision letter

MS ID#: DEVELOP/2020/199399

MS TITLE: The initiation of Otx2 expression in the developing mouse retina requires a unique enhancer and either Ascl1 or Neurog2 activity

AUTHORS: Michael L Kaufman, Noah B Goodson, Ko Uoon Park, Michael Schwanke, Emma Office, Sophia R Schneider, Joy Abraham, Austin Hensley, Kenneth L Jones, and Joseph A Brzezinski IV
ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

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