Pou2f1 and Pou2f2 cooperate to control the timing of cone photoreceptor production in the developing mouse retina

Awais Javed, Pierre Mattar, Suying Lu, Kamil Kruczek, Magdalena Kloc, Anai Gonzalez-Cordero, Rod Bremner, Robin R. Ali and Michel Cayouette
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First decision letter

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MS TITLE: Pou2f1 and Pou2f2 cooperate to control the timing of cone photoreceptor production in the developing mouse retina

AUTHORS: Awais Javed, Pierre Mattar, Suying Lu, Kamil Kruczek, Magdalena Kloc, Anai Gonzalez-Cordero, Rod Bremner, Robin R Ali and Michel Cayouette

As you will see, the Reviewers came to quite different conclusions about the manuscript with Reviewer 1 supportive and Reviewer 2 much less so. Given the absence of a report form Reviewer 3 and the differences in opinion, I asked both reviewers to look at both reviews and provide me with feedback that would help me make a decision on how to proceed. After doing this, Reviewer 1 agreed that there could be some improvement in the organization of the manuscript but disagreed with the tone of the review. He/she agreed that some of the supplemental data should be in the main paper, more information provided on antibodies and how/when they were used, some graphs/charts could have improved legibility.
She/he considered that the other referee missed some information that you did include about numbers and labelling – but you should check carefully to make sure all such information is complete. The referee didn't feel qualified to comment on issues relating to mouse tools/cone biology raised by the other referee. Overall, however, Reviewer 1 remains enthusiastic about publication of a revised version of the manuscript in Development. Reviewer 2 sent in a briefer response indicating that the other referee's comments and detailed suggestions were all good ones. She/he considered that if both sets of reviews were addressed then the resulting paper would likely be much stronger.

After considering the reviews and Reviewer feedback I am happy to give you the chance to revise your manuscript. If you are able to reviserevise the manuscript along the lines suggested,I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision. It seems likely that you will need to do further experiments to address the reviewer concerns andthat you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to

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discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

Reviewer 1

Advance summary and potential significance to field

The cascade of molecular steps governing neuron formation during development and its universality is an important and open question. Some evidence suggests that a temporal sequence of genes, similar to what occurs in fly neuroblasts, operates in vertebrates. Foundational work from the Cayouette lab provided evidence that Ikaros, a mammalian homologue of the fly gene hunchback, could provide early temporal identity to retinal progenitor cells and stimulate the production of some early-born retinal neurons (e.g., ganglions, some amacrines and horizontals) but not others (e.g., cones). In this study Javed et al now identify Pou2f1 and Pou2f2 as mammalian homologues of the fly Pdm genes (known to act after hb and promote later neuroblast fate) and study how they regulate cone photoreceptor development. This is a very nice study, providing both in vivo and in vitro evidence that Pou2f1 regulates Pou2f2 and that Pou2f2 represses rod fate and enables cone fate to predominate. Moreover, the authors provide evidence that Pou2f1/2 act downstream of Ikaros, filling in an important gap in the temporal cascade for molecular determination of cell types in the retina. I enjoyed reading this study. Thank you.

Comments for the author

There are some minor issues and questions listed below for the authors to address to make the paper even stronger.

1) Are Pou2f1 and Pou2f2 expressed in other places in the developing CNS? Is it possible that this system works similarly in other parts of the brain, not necessarily governing photoreceptor production but timing the production of other types of neurons?
2) In paragraph 2 of the introduction, the authors summarize the heterochronic grafting and in vitro work suggesting that environmental cues are not sufficient to alter fate output but ignore the work from the Calof group (e.g., Kim et al., 2005) and others who have shown that environmental signals can act in negative feedback loops to limit the number of specific types of neurons. I know that point of the paper is the intrinsic temporal cascade of transcription factors that governs cell fate/cell development, but in my view, better to not completely oversimplify.
3) In Figure 1, the authors show expression of Pou2f1 and Pou2f2. The images in A-A'', and D-D'' look like maybe these transcription factors are expressed at low levels in all RPCs and then are later expressed at higher levels in specific cell types. To make this more clear a Western blot showing simply a timecourse of expression of these two genes in the retina would help clarify this and help buttress the authors claims of how/when these proteins act to promote cone cell differentiation.
4) The qPCR experiments are central to the paper. From the supplemental table, it seems like the authors only used a single reference gene, GAPDH. This is really no longer sufficient as metabolism and metabolic gene expression can change over time during development. It is now standard to have multiple reference/normalization genes. The authors need not repeat every single qPCR experiment with multiple reference genes, but it would make this reviewer more comfortable if at least the really central experiments (like those shown in Figures 5 and 6) were repeated with other reference/control genes.
5) I appreciate that all of the experiments using cultured electroporated retinae are challenging and require the sacrifice of animals, but I also appreciate that the numbers are quite

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small. Have the authors performed power testing or some other statistical analysis to confirm that a n=3 is really enough.
6) In Figure 5 (and supplemental Figure 5), the authors present qPCR data which suggest that Pou2f1 is an immediate target of Ikaros. Does Ikaros bind to DNA the same way that hunchback does? That is to say, do Ikzf1 binding sites exist in the enhancer/promoter of Pou2f1 (and not Pou2f2)?
7) Supplementary Figure 5 should be incorporated in the main Figure 5.
8) When discussing Supplemental Figure 6 (on page 30), the authors suggest that Onecut may be acting in parallel or in concert with Pou2f1 and Pou2f2 to regulate Thrb gene expression. Since the ChIP-Seq data suggest that the Thrb cis-regulatory module is activated indirectly by Pou2f1/2, the authors could strengthen their study by asking whether Onecut does regulate expression from this same CRM (and also examine whether Onecut acts downstream or in parallel with Pou2f1/2).
9) The data presented in Figure 6 support the conclusion that Pou2f2 repress expression of Nrl. To buttress the results from the electroporation experiments, the authors perform ChIP-PCR to show that Pou2f2 binds to the POU-site in the Nrl promoter but they miss the opportunity to show that Pou2f2 binding goes away when the POU-site is mutated (though they do include an intronic control).
10) Supplemental Figure 7 and Figure 7, at least the way I read the paper, present data that seem to belong toward the beginning of the paper as they are really showing when during development these two transcription factors are acting to promote cone photoreceptor fate. Then the rest of the paper looks at the more molecular details of how. Perhaps the authors could consider reorganizing the flow of the manuscript.

Reviewer 2

Advance summary and potential significance to field

Javed and colleagues present an evaluation of Pou2f1 and Pou2f2 genes and their roles in regulating mouse cone photoreceptor fate. Such a link has never been made in the scientific literature and the novel findings would extend the field significantly. Unfortunately the work is poorly presented and suffers multiple problems of uneven quality, potentially because it attempted to be broad rather than a deep, thoughtful study. Key information, well-supported by 50 years of cone photoreceptor biologic studies, was not considered by the authors. Other issues such as poor choice of Cre driver, under evaluation of key markers and M cone photoreceptors, plus the inability of another lab to ever replicate these findings (based on information provided) all substantially weakened an interesting study.

Comments for the author

This was far and away the most difficult manuscript this reviewer has reviewed over the past 20 years. It is horribly formatted with Supplemental figures presented ahead of primary data, and all figures and legends awkwardly inserted into the body of the manuscript. Just following the text required an extraordinary amount of patience and willingness to be dragged through a data scavenger hunt. The authors are urged to use established formatting for presenting their study for peer evaluation. Supplemental data goes at the end, it is optional. Papers should stand on their text and primary data. This manuscript could be a journal club for beginning graduate students on how NOT to submit scientific findings for publication.

2. The asymmetrical distribution of S cones was not considered by the authors and this omission likely skewed many results (1). Applebury et al beautifully showed a dorsal-ventral difference in the distribution of S Cones, which is more profound in mice with a C57BL/6 background, on which the Pou2f2 floxed mice are propagated according to JAX database. Moreover, Thrb2 expression and M cones contribute to the asymmetric distribution of S cones in the mouse (2) and spatial distribution of Thrb2 and M cone were not evaluated here.

3. It further concerning that the dorsal-ventral bias of the alpha-Cre reporter was not taken into account(3). To perform the conditional mutant phenotypic analysis with this tool, only those cells autonomously expressing the IRES-GFP cassette in alpha-Cre or flox-stop lineage reporter should have been evaluated. This reviewer's opinion is the wrong cre driver was used. It will be crucial

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for the authors to use a second, relevant Cre driver to validate FIgure 4, Suppl Fig 4. Coronal section images showing dorsal and ventral retina should be analyzed, and per the convention of other papers using alpha-Cre only the distal 1/4 to 1/3 of each retinal section is relevant since this tool does not express in the central optic cup/retina. Most egregious are the flat mounts (Fig 4F), which are inappropriate. There is another Cre line that would uniformly delete Pou2f2 (Rax-Cre) or perhaps the Bac Tg Crx-Cre mouse (4).

4. M Opsin cones were not evaluated in Pou2f1/2 knockdown, overexpression or conditional mutants. The only L/M cone data presented is for human organoids. Nrl mutants lack rods and produce excess S cones; and Thrb2 mutants lose M cones but also produce excess S cones. More remarkably, when Thrb2 was knocked into the Nrl locus (5), rods were lost but M cones were instead produced. These studies point to tripotential precursors which select rod fate first, then each cone subtype. Authors propose that Pou2f1 and Pou2f2 act upstream of both Nrl and Thrb2, but this reviewer is not convinced changes in Thrb2 transcriptional activity (Suppl Fig 6) result in fate changes consistent with the Thrb2 literature. There is a very high quality antibody from Doug Forrest's group that should have been used in nearly every figure to show spatial expression changes, then quantified.

5. Otx2 is not a "photoreceptor-specific" marker. There are numerous papers about the Otx2-Cre lineage and that Otx2 postnatal retinal phenotypes that include bipolar neurons. Perhaps the authors are confused about the genes Otx2 and Crx? It is further disappointing that there was no evaluation of Crx expression here.

6. Which antibodies were validated in Suppl Fig 1, and used in each of the data figures? Which antibodies listed were used for IHC, westerns or ChIP? How can the work in this study be replicated by others? Which data figures used the discontinued Santa Cruz reagents? What were the secondary antibodies used throughout this paper? The authors are urged to include Antibody registry ID numbers for all primary antibodies used.

7. In every graph presented, what does n= ?? There is no description in the legend or Methods. In fact there are no statements about biologic replicates analyzed anywhere in this paper.

8. Incorrect y-axis labeling of every graph, and/or incorrect cell counting methodologies used. One example in Figs 2F and 2G: The percentage of GFP+marker+ cells is stated on y-axis. What is the denominator? Fig 2M is also confusing: The top of the graph states S-Opsin+EdU+ cells (presumably birthdated S Cones), what is the relationship to the percent of GFP+ on y-axis? What are numerator and denominator here? To this reviewer, Edu+GFP+/GFP+ population are relevant cohort to evaluate for the 3 genotypes (Control, Pou2f1, Pou2f2) which is not what could be interpreted was done from the presentation of the images or the graph. The information Fig 2N is murky regarding its relevance. MANY OTHER FIGURES suffer same problems.

9. Figure 3D, 3E. What does "percent of clones" on y axis mean? Only cells in a clone are marked with GFP expression, how do the authors know GFP-neg cells are "in the clone"? This makes no sense. Isn't it statistically relevant that uneven numbers of clones were evaluated (per figure legend)?

10. Because the central hypothesis of this work is about temporal regulation every figure should have a timeline.

More minor issues:

11. There is no mention that Pou2f1 and Pou2f2 were originally termed Oct-1 and Oct-2. These genes have a rich history which was ignored. This reviewer could only find such information online at MGI.

12. Diagram showing location of guide RNAs is missing

13. Genomic coordinates for regions of Thrb2 gene (CRMs) that were cloned and tested is missing.

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14. Fig 6 + Suppl Fig 6 the fuzzy green or red patches of expression were presumably sorted from the rest of the retina by flow cytometry? Whole retina evaluations are not as meaningful but if this is how the experiment was performed the authors must own up to it and discuss why that weakened their findings.

15. The model described on pg 41 could easily be tested by the authors using validated Olig2 antibody.

16. Orientation of all retinal section data presented omitted.

References:

1. Applebury et al Neuron 2000 Vol 27:513-523 (Figure 5 in particular); Szel et al J Comparative Neurology 1992 Vol 325: 327-342; Szel et al J. Comparative Neurology 1993 Vol 331:564-577;

2. Ng et al Nature Genetics 2001 Vol 27: 94-98; Roberts et al PNAS 2006 Vol 103:6218-6223; Applebury et al Dev Dynamics 2007 Vol 236: 1203-1212.

3. Marquardt et al Cell 2001 Vol 106:43-55 ; Baumer et al Development 2002 Vol 129:4535-4545; Kammandel et al Dev Biology 1999 Vol 205:79-97.

4. Prasov et al Dev Biology 2012 Vol 368:214-230.

5. Ng et al J Neuroscience 2011 Vol 31:11118-11125

First revision

Author response to reviewers' comments

We would like to thank the reviewers for their insightful comments on our manuscript (DEVELOP/2020/188730). Over the past few months, we have worked to address their concerns and we think the paper is now much improved. Although the pandemic has limited our ability to carry out additional experiments for more than 2 months, we have been able to return to the lab part-time recently, allowing us to complete experiments that were initiated before the shut-down and that we think will address the reviewers' concerns. Perhaps the most important change is the addition of a new Pou2f2 cKO using a different Cre driver mouse line, which confirms our original conclusion. We have also made a number of clarifications in the text. We hope the reviewers will agree that the paper is now ready for publication.

Reviewer 1:
The cascade of molecular steps governing neuron formation during development and its universality is an important and open question. Some evidence suggests that a temporal sequence of genes, similar to what occurs in fly neuroblasts, operates in vertebrates. Foundational work from the Cayouette lab provided evidence that Ikaros, a mammalian homologue of the fly gene hunchback, could provide early temporal identity to retinal progenitor cells and stimulate the production of some early-born retinal neurons (e.g., ganglions, some amacrines and horizontals) but not others (e.g., cones). In this study, Javed et al now identify Pou2f1 and Pou2f2 as mammalian homologues of the fly Pdm genes (known to act after hb and promote later neuroblast fate) and study how they regulate cone photoreceptor development. This is a very nice study, providing both in vivo and in vitro evidence that Pou2f1 regulates Pou2f2 and that Pou2f2 represses rod fate and enables cone fate to predominate. Moreover, the authors provide evidence that Pou2f1/2 act downstream of Ikaros, filling in an important gap in the temporal cascade for molecular determination of cell types in the retina. I enjoyed reading this study. Thank you.

We thank the reviewer for their positive comments on this study, which represents six years of hard work. We are glad they enjoyed reading it.

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1) Are Pou2f1 and Pou2f2 expressed in other places in the developing CNS? Is it possible that this system works similarly in other parts of the brain, not necessarily governing photoreceptor production but timing the production of other types of neurons?

Yes, they are expressed in other regions and this is entirely possible. We had a section in the original Discussion about the possibility of the role of Pou2f1 and Pou2f2 in other parts of the developing CNS, and cited the original papers showing expression of these genes in other parts of the CNS in the results. But we now make this point more prominent in the revised version (Page 16 line 477-491), which we hope the reviewer will appreciate.

2) In paragraph 2 of the introduction, the authors summarize the heterochronic grafting and in vitro work suggesting that environmental cues are not sufficient to alter fate output but ignore the work from the Calof group (e.g., Kim et al., 2005) and others who have shown that environmental signals can act in negative feedback loops to limit the number of specific types of neurons. I know that point of the paper is the intrinsic temporal cascade of transcription factors that governs cell fate/cell development, but in my view, better to not completely oversimplify.

The reviewer is correct, there are many studies showing that environmental cues can function as feedback inhibitory signals (GDF11 and Shh are the main ones). It was not our intention to ignore these papers. Our point was that evidence for instructive environmental cues is sparse. But we agree that we should discuss the feedback signals in the introduction to provide a more balanced view. We have changed the text accordingly (Page 3 line 60-64).

3) In Figure 1, the authors show expression of Pou2f1 and Pou2f2. The images in A-A'', and D-D'' look like maybe these transcription factors are expressed at low levels in all RPCs and then are later expressed at higher levels in specific cell types. To make this more clear, a Western blot showing simply a timecourse of expression of these two genes in the retina would help clarify this and help buttress the authors claims of how/when these proteins act to promote cone cell differentiation.

The issue with a time-course expression by western blots from whole retinal extracts is that it is difficult to discern which specific cell types contributes to the expression. Moreover, sorting RPCs for western blot analysis would be difficult because of low protein yields, especially from embryonic stages (we tried many times without success). Instead, to answer the reviewer's question, in Fig. S2B, we looked through published scRNA-seq to assess whether these genes are expressed at lower levels in late RPCs compared to early RPCs. As suggested by the reviewer, we find that both Pou2f1 and Pou2f2 expression is lower in late RPCs and expressed in fewer cells, with very few co-expressing both Pou2f1 and Pou2f2, consistent with the idea that these proteins act during early stages of retinal development, when cones are being specified. We have made changes in the text to make this point clearer (Page 5-6 line 134136).

4) The qPCR experiments are central to the paper. From the supplemental table, it seems like the authors only used a single reference gene, GAPDH. This is really no longer sufficient as metabolism and metabolic gene expression can change over time during development. It is now standard to have multiple reference/normalization genes. The authors need not repeat every single qPCR experiment with multiple reference genes, but it would make this reviewer more comfortable if at least the really central experiments (like those shown in Figures 5 and 6) were repeated with other reference/control genes.

We agree with the reviewer that using multiple reference genes would be ideal. However, to follow the reviewer's suggestion and carry out the qPCR again using a different reference gene would require us to redo all the experiments presented in figures 5 and 6, as we ran out of cDNA (sorting out electroporated cells yield very small amount of RNA, allowing us to do only a limited number of experiments). Alternatively, we think the results of our pilot control experiments could be useful here. Indeed, we had the same worry as the reviewer and wanted to make sure there were no changes in GAPDH expression over time during retinal development. To do this, we analysed GAPDH mRNA levels by RT-qPCR at different times after electroporation. We electroporated P0 retinas with a vector expressing GFP, cultured the retinal explants for 2, 7 or

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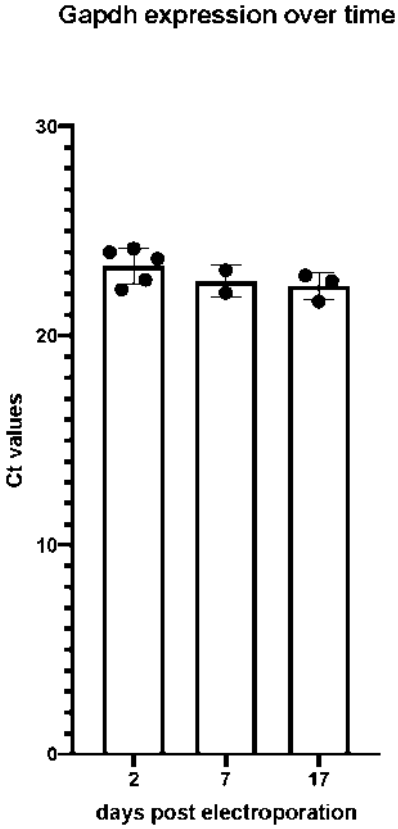
17 days and sorted 25,000 GFP+ cells from each timepoint. When we compared raw Ct values with the same number of cells sorted between the three time points, we found no difference. This suggests that GAPDH mRNA level do not vary significantly over time in our experimental setup and represents a good reference gene for qPCR quantitation.



Nonetheless, as the reviewer was essentially making this point to help us reinforce one of the central conclusion in the paper, which is that Pou2f2 downregulates Nrl, we thought about an alternative method to do this without relying on qPCR. We therefore overexpressed Pou2f2 and used immunofluorescence to study Nrl expression 6 days later. As shown in new Fig. 6C-E, we find that Nrl protein levels are reduced after Pou2f2 expression, complementing our qPCR results and making the additional point that protein levels are also reduced, not only transcripts. We hope the reviewer will agree that this additional data strengthen our conclusions.

5) I appreciate that all of the experiments using cultured electroporated retinae are challenging and require the sacrifice of animals, but I also appreciate that the numbers are quite small. Have the authors performed power testing or some other statistical analysis to confirm that a n=3 is really enough.

There were indeed a few experiments in which we had a n=3, but it is important to note that this represents biological replicates, not the number of explants analyzed. For example, an experiment with a n=3 means that explants prepared from three different animals were used to obtain the data, but more than one explant of each animal is used to quantify. We could instead use the number of explant to represent the n, but we feel it is more accurate to list biological replicates because sometimes the number of electroporated cells that can be counted in one explant is not high enough to provide an accurate representation of the data. We have made this point clear in the revised paper (Page 22, line 611-612). Nonetheless, as suggested by the reviewer, we have added biological replicates to experiments with low n values. We also conducted power testing of our data. Using a criterion of at least a 20% difference between the control and experimental conditions, we require a minimum sample size of n=3, based on power value of 0.8 with a 0.05 significance p-value. We have added this information in the statistics section of the methods.

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6) In Figure 5 (and supplemental Figure 5), the authors present qPCR data which suggest that Pou2f1 is an immediate target of Ikaros. Does Ikaros bind to DNA the same way that hunchback does? That is to say, do Ikzf1 binding sites exist in the enhancer/promoter of Pou2f1 (and not Pou2f2)?

It has been shown that the mouse Ikaros protein binds to the consensus sequence 'GGGAA' in mouse thymocytes (Schjerven et al., 2013). We analysed the genomic regions upstream of the transcriptional start sites of Pou2f1 and Pou2f2 and found multiple regions containing the 'GGGAA' consensus sequence. Whether Ikaros binds to these regions in the retina will require further investigation, but we agree with the reviewer that this is an interesting observation that we have included this in the Discussion (Page 14, line 417-420).

7) Supplementary Figure 5 should be incorporated in the main Figure 5.

We agree with the reviewer and have done as they suggested.

8) When discussing Supplemental Figure 6 (on page 30), the authors suggest that Onecut may be acting in parallel or in concert with Pou2f1 and Pou2f2 to regulate Thrb gene expression. Since the ChIP-Seq data suggest that the Thrb cis-regulatory module is activated indirectly by Pou2f1/2, the authors could strengthen their study by asking whether Onecut does regulate expression from this same CRM (and also examine whether Onecut acts downstream or in parallel with Pou2f1/2).

That is a very good suggestion. CRMs of *thrb* were characterised in the chick retina (Emerson et al., 2013), and the authors showed that Onecutl regulates the thrbCRMI element (equivalent to mouse thrbPR2 in our study). Whether this is actually the case in the mouse retina remains to be shown, but considering sequence similarity, we anticipate that Onecut1 most likely regulates the same element. We did not investigate the role of Onecut1 in regulating the thrb elements in the mouse retina, as it would likely require a whole study in and of itself, but it would be interesting to follow up and assess the relationship between Pou2f1/2 and Onecut1 to regulate *thrb* expression. We have added a mention of these points in the revised Discussion (Page 15-16, line 453-461).

9) The data presented in Figure 6 support the conclusion that Pou2f2 repress expression of Nrl. To buttress the results from the electroporation experiments, the authors perform ChIP-PCR to show that Pou2f2 binds to the POU-site in the Nrl promoter but they miss the opportunity to show that Pou2f2 binding goes away when the POU-site is mutated (though they do include an intronic control).

We agree with the reviewer that this would be the ideal experiment, but it is technically very challenging. As all our ChIP-qPCR experiments are done from in vivo samples, this would require us to develop a mouse model in which the Nrl promoter mutation is knocked-in and then conduct ChIP-qPCR from retinal extracts. This would likely take 18 months to do in the best conditions. This is why we decided instead to carry out promoter reporter assays in explants, which clearly show that Pou2f2 is no longer able to repress Nrl promoter activity when the POU binding motif is mutated (Fig. 6J-L").

10) Supplemental Figure 7 and Figure 7, at least the way I read the paper, present data that seem to belong toward the beginning of the paper as they are really showing when during development these two transcription factors are acting to promote cone photoreceptor fate. Then the rest of the paper looks at the more molecular details of how. Perhaps the authors could consider reorganizing the flow of the manuscript.

We considered this change to improve the flow of the manuscript but we decided against it because we wanted to clearly distinguish the role of Pou2f1 in progenitors to regulate the temporal identity genes (Figure 5) and Pou2f2 in post-mitotic cells to promote cones (Figure 6). We found that re-arranging the manuscript in the way suggested by the reviewer would make this main point of the paper less clear. In the current version, functional analysis is done in figures 2,3 and 4, whereas mechanistic analysis is done in figures 5 and 6, which we hope the reviewer

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will appreciate.

Reviewer 2
Javed and colleagues present an evaluation of Pou2f1 and Pou2f2 genes and their roles in regulating mouse cone photoreceptor fate. Such a link has never been made in the scientific literature and the novel findings would extend the field significantly. Unfortunately the work is poorly presented and suffers multiple problems of uneven quality, potentially because it attempted to be broad rather than a deep, thoughtful study. Key information, well-supported by 50 years of cone photoreceptor biologic studies, was not considered by the authors. Other issues such as poor choice of Cre driver, under evaluation of key markers and M cone photoreceptors, plus the inability of another lab to ever replicate these findings (based on information provided) all substantially weakened an interesting study.

This was far and away the most difficult manuscript this reviewer has reviewed over the past 20 years. It is horribly formatted with Supplemental figures presented ahead of primary data, and all figures and legends awkwardly inserted into the body of the manuscript. Just following the text required an extraordinary amount of patience and willingness to be dragged through a data scavenger hunt. The authors are urged to use established formatting for presenting their study for peer evaluation. Supplemental data goes at the end, it is optional. Papers should stand on their text and primary data. This manuscript could be a journal club for beginning graduate students on how NOT to submit scientific findings for publication.

We thank the reviewer for acknowledging the novelty of our work. We were disappointed to read that, unlike Reviewer 1, this reviewer did not enjoy reading the paper, largely due to the formatting. We simply followed journal's recommendation for initial submissions to embed all figures (including supplemental) within the text in the order they are cited. This is supposed to help the reviewer by avoiding the back and forth flipping of pages between the results section and figures placed at the end. We are sorry this was not helpful to this reviewer.

We respectfully disagree with the reviewer's statement that supplemental data go at the end as "it is optional". We view supplementary figures as equally important, as they provide key controls and complementary experiments that help substantiate the main results. Nonetheless, we hope the reviewer will now like this version of the paper better, which was prepared according to the journal's final submission guidelines, with supplementary data included in a separate section.

We are well-aware of the many studies on cone biology that the reviewer is alluding to and it was certainly not our intention to disregard this work. But these studies deal with a different problem, namely the mechanisms of cone differentiation and patterning of the cone opsin gradient. In contrast, our study deals with the cone specification process, which takes place before opsin is expressed. We are not claiming a role for Pou2f1/2 in cone subtype differentiation (S vs. M opsin), but instead argue that Pou2f1/2 regulate RPC competence to allow cone production specifically during early stages of retinogenesis. This point is further discussed below.

2. The asymmetrical distribution of S cones was not considered by the authors and this omission likely skewed many results (1). Applebury et al beautifully showed a dorsal-ventral difference in the distribution of S Cones, which is more profound in mice with a C57BL/6 background, on which the Pou2f2 floxed mice are propagated according to JAX database. Moreover, Thrb2 expression and M cones contribute to the asymmetric distribution of S cones in the mouse (2) and spatial distribution of Thrb2 and M cone were not evaluated here.

We agree with the reviewer that the cone opsin expression gradient can lead to misinterpretation of the data when cone opsins are used as markers of cone fate. This is precisely why we used generic cone photoreceptor markers to study the Pou2f2 KO mouse, such as RxrY, PNA and Cnga3, which are expressed in all cones irrespective of S/M-opsin expression (Roberts et al., 2005, Biel et al., 1999). S/M opsin-positive cells are clearly patterned in a dorso-ventral gradient, as demonstrated in the beautiful studies cited by the reviewer, whereas generic cone cells (combining all subtypes) are not as drastically patterned (Jeon et al., 1998, Ortin-Martinez et al., 2014). Nonetheless, to avoid any bias in quantification, we always orient the eyes of the animals

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in the same way between the different conditions and systematically analyse the same region. Thus, using three different pan-cone markers, our data indicate that the generic cone population is reduced in the Pou2f2 KO retina. Arr3 was the only marker we used that has a slightly higher expression dorsally than ventrally, but does not have any expression bias in the equatorial axis (nasal to temporal) (Corbo et al., 2007, Fujieda et al., 2009). As will be detailed below, all counts were done in the center equatorial axis of the eye, excluding the dorsal- and ventral-most quadrants to avoid bias due to Cre expression, thereby largely excluding the possibility that difference in Arr3-positive cells is due to sampling errors. We now make these important subtleties more clear in the paper (Page 21, line 618-628) and have added a diagram in Fig. 4A, G to show where sections were collected for counting. We thank the reviewer for raising this point, as we think these precisions will help the reader.

Regarding the contribution of Thrb2 on the patterning of cone subtypes, this is a process that takes place after the initial cone specification. Our data argue that Pou2f2 regulates a CRE of Thrb involved in cone specification, but not in cone opsin patterning. We never observed M-opsin expression in our *in vivo* gain of function experiments, further reinforcing the idea that Pou2f2 induces Thrb expression to promote cone specification, but not the selection of opsin expression, which is likely under different regulatory mechanisms taking place later. We acknowledge that this point was probably not made clear in the paper and have made changes in the Results to correct this aspect (Page 11, line 325-334).

3. It further concerning that the dorsal-ventral bias of the alpha-Cre reporter was not taken into account(3). To perform the conditional mutant phenotypic analysis with this tool, only those cells autonomously expressing the IRES-GFP cassette in alpha-Cre or flox-stop lineage reporter should have been evaluated. This reviewer's opinion is the wrong cre driver was used. It will be crucial for the authors to use a second, relevant Cre driver to validate FIgure 4, Suppl Fig 4. Coronal section images showing dorsal and ventral retina should be analyzed, and per the convention of other papers using alpha-Cre only the distal 1/4 to 1/3 of each retinal section is relevant since this tool does not express in the central optic cup/retina. Most egregious are the flat mounts (Fig 4F), which are inappropriate. There is another Cre line that would uniformly delete Pou2f2 (Rax-Cre) or perhaps the Bac Tg Crx-Cre mouse (4).

The reviewer rightly points out the importance of being aware of the dorso-ventral bias of Cre expression in the alpha-Pax6-Cre driver, which we indeed considered, as stated in the Material and Methods (Statistical and Quantitative analysis section, 2nd paragraph). We always make sure to orient the retinas in a way that allows direct comparisons between controls and mutants. As suggested by the reviewer, we also only analysed the 1/3rd distal-most region of the retina spanning the nasal and temporal part where Cre is expressed. We never analyse the central region of the retina because previously published work, as well as our own lineage tracing experiments, have shown that Cre expression in progenitors is low/absent in the middle part of the dorso-ventral quadrant in the alphaPax6-Cre line. Given that comparisons are systematically made from the same regions in controls and mutants, we feel our data on the Pou2f2 KO retina is solid and well-controlled. With this in mind, we disagree with the reviewer that flatmounts are not appropriate. The images shown in Fig 4G', G" were taken from the exact same temporal region of the retina in the control and Pou2f2 cKO. We agree, however, that this should have been made clear and have now added a diagram pointing to the exact region where these images were taken (Fig. 4G). We would also like to point out that the Pou2f2 inactivation phenotype was confirmed using three additional approaches (shRNA, CRISPR, and Cre electroporations in Pou2f2 flox/flox retinas), which all gave the same results (reduced cone numbers). Importantly, these three different methods allow us to precisely focus our analysis on the electroporated cells, which directly addresses the cell- autonomous function of Pou2f2, as suggested by the reviewer.

Considering the points above, we feel that our conclusion that Pou2f2 is at least partially required for cone production has been extensively substantiated. Nonetheless, in prevision for future studies, we had generated a pan-RPCs, tamoxifen-inducible Pou2f2 cKOs, by crossing the Pou2f2 fl/fl mice with the Chx10- Cre^{ERT2} BAC transgenic line (https://knowledge.brc.riken.jp/resource/animal/card?lang=en&brc_no=RBRC06574) and have now been able to analyse cone production in this line before and after the research shut-down due to the pandemic. We first crossed this line with a Rosa-YFP reporter line and confirmed widespread Cre activity after tamoxifen injection at E11 (see new Fig. S4K-M'). We next crossed

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the Pou2f2 fl/fl mice with the Chx10-CreERT2 line to generate Chx10CreERT2; Pou2f2 fl/fl mice and Chx10CreERT2; Pou2f2fl/+ control mice. We injected tamoxifen in these animals at E11 and assessed cone numbers (RxrY-positive cells in the apical-most part of the retina) at E17.5. Consistent with our results using shRNA, CRISPR, Cre electroporation, and Pax6-Cre cKO, we observed a significant decrease in cone numbers (Fig. 4H-J). We hope these new results will help convince the reviewer that Pou2f2 plays a part in cone production.

4. M Opsin cones were not evaluated in Pou2f1/2 knockdown, overexpression or conditional mutants. The only L/M cone data presented is for human organoids. Nrl mutants lack rods and produce excess S cones; and Thrb2 mutants lose M cones but also produce excess S cones.
More remarkably, when Thrb2 was knocked into the Nrl locus (5), rods were lost but M cones were instead produced. These studies point to tripotential precursors which select rod fate first, then each cone subtype. Authors propose that Pou2f1 and Pou2f2 act upstream of both Nrl and Thrb2, but this reviewer is not convinced changes in Thrb2 transcriptional activity (Suppl Fig 6) result in fate changes consistent with the Thrb2 literature. There is a very high quality antibody from Doug Forrest's group that should have been used in nearly every figure to show spatial expression changes, then quantified.

This comment highlights that we have probably failed to provide a clear interpretation of our results. What the reviewer is referring to here is the role of Thrb in S/M cone subtype selection and we agree that our results are not consistent with a role for Pou2f2 in this process. We are aware that one of the proposed models suggests the existence of a precursor that selects between the S-cone or M-cone fate under the control of Thrb. But another model suggests an additional role for Thrb at an earlier stage of development, before opsin expression, to promote the cone fate in Olig2+ RPCs using a specific cis- regulatory module (Emerson et al., 2013, Fujieda et al., 2009, Hafler et al., 2012). What we propose is that Pou2f2 functions to promote Thrb expression transiently in these RPCs to promote cone specification. To support this hypothesis, we have now included an experiment where we electroporated P0 retinal explants with control GFP, Pou2f1 and Pou2f2 and sorted GFP+^{ve} cells 14 days later for RT- qPCR analysis. We find that Pou2f1 and Pou2f2 expression no longer leads to upregulated *Thrb2* mRNA levels at this stage, while they do at 6 days (new Fig. S5J). Additionally, Pou2f1/2 can upregulate the cis-regulatory module of Thrb2 only transiently (active for 72 hours). Finally, we show that Pou2f2 is not required for *thrb2* expression, as there is no change in mRNA level in Pou2f2 knockout retinas. Due to all of these reasons, we did not assess S or M-cones in the Pou2f2 mouse knockout and do not claim that these factors regulate S- or M-opsin expression through Thrb2. We have made text changes to better reflect these conclusions and avoid confusion (Page 11, line 325-334). We have also changed our model Figure 7 along these lines.

5. Otx2 is not a "photoreceptor-specific" marker. There are numerous papers about the Otx2-Cre lineage and that Otx2 postnatal retinal phenotypes that include bipolar neurons. Perhaps the authors are confused about the genes Otx2 and Crx? It is further disappointing that there was no evaluation of Crx expression here.

We thank the reviewer for pointing this oversight. What we meant is that Otx2 specifically labels photoreceptors in the ONL, although the reviewer is right that it also labels bipolars in the INL. We have clarified this in the figure and the text (Page 6, line 164-165). The antibody we used for this analysis recognises both Otx2 and Crx. Thus, our stainings using the Otx2 antibody likely also picks up Crx in the ONL because both proteins are present in this layer at the stage we did the stainings.

6. Which antibodies were validated in Suppl Fig 1, and used in each of the data figures? Which antibodies listed were used for IHC, westerns or ChIP? How can the work in this study be replicated by others? Which data figures used the discontinued Santa Cruz reagents? What were the secondary antibodies used throughout this paper? The authors are urged to include Antibody registry ID numbers for all primary antibodies used.

We were surprised by this comment because our paper included the vast majority of this information in Table S2, including catalog numbers. We have added mention of the secondary antibodies used and application of the antibodies to this table as requested by the reviewer.

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7. In every graph presented, what does n= ?? There is no description in the legend or Methods. In fact there are no statements about biologic replicates analyzed anywhere in this paper.

The number of replicate (n) stated in all experiments refer to biological replicates. We have clarified the text and added more information in the statistical and quantitative analysis part of the materials and methods section.

8. Incorrect y-axis labeling of every graph, and/or incorrect cell counting methodologies used. One example in Figs 2F and 2G: The percentage of GFP+marker+ cells is stated on y-axis. What is the denominator? Fig 2M is also confusing: The top of the graph states S-Opsin+EdU+ cells (presumably birthdated S Cones), what is the relationship to the percent of GFP+ on y-axis? What are numerator and denominator here? To this reviewer, Edu+GFP+/GFP+ population are relevant cohort to evaluate for the 3 genotypes (Control, Pou2f1, Pou2f2) which is not what could be interpreted was done from the presentation of the images or the graph. The information Fig 2N is murky regarding its relevance. MANY OTHER FIGURES suffer same problems.

We have now made sure graph axis labeling is clear in all figures.

9. Figure 3D, 3E. What does "percent of clones" on y axis mean? Only cells in a clone are marked with GFP expression, how do the authors know GFP-neg cells are "in the clone"? This makes no sense. Isn't it statistically relevant that uneven numbers of clones were evaluated (per figure legend)?

The labeling should read percent of clones containing each cell type. Of course, GFP-negative cells cannot be analyzed in retroviral lineage tracing experiments. We have now changed the labelling of the graphs to make this clearer. Analysing an uneven number of clones is not a problem because the data is shown as percentages.

10. Because the central hypothesis of this work is about temporal regulation every figure should have a timeline.

Many of our figures already include timelines for the key experiments (e.g. Figs. 2, 5, 6), but we have added more reference to time wherever essential and where it does not overly crowd the figure.

More minor issues:

11. There is no mention that Pou2f1 and Pou2f2 were originally termed Oct-1 and Oct-2. These genes have a rich history which was ignored. This reviewer could only find such information online at MGI.

As per journal guidelines, we used the new gene nomenclature throughout the paper, but we have added a mention of the original gene names in parentheses upon first use of Pou2f1 and Pou2f2 (Page 4, line 93).

12. Diagram showing location of guide RNAs is missing

We have added a diagram with the location of the guide RNAs (New Fig.S1G).

13. Genomic coordinates for regions of Thrb2 gene (CRMs) that were cloned and tested is missing.

The reviewer might have missed the genomic location presented in Fig. S6A and sequences of the primers used for the generation of the constructs of Thrb2 CRMs, which we thought was sufficient, but we have also added mm9 genomic coordinates in Table S1 next to the primers.

14. Fig 6 + Suppl Fig 6 the fuzzy green or red patches of expression were presumably

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sorted from the rest of the retina by flow cytometry? Whole retina evaluations are not as meaningful but if this is how the experiment was performed the authors must own up to it and discuss why that weakened their findings.

We are not sure what the reviewer is referring to here. The images are not showing sorted cells by flow cytometry, but rather flatmounts of retinal explants, as indicated in the figure legend, to demonstrate the activity of the CRM of *thrb2* and promoter of *Nrl* with GFP or dsRed reporters, respectively. If the reviewer is referring to the sorted cells in the qPCR experiments, it is important to note that in all our qPCRs we only sorted out GFP+ cells for analysis, rather than whole retinal cell populations, thereby excluding confounding effects of bystander cells.

15. The model described on pg 41 could easily be tested by the authors using validated Olig2 antibody.

That is a good suggestion, but we think outside the scope of this study. This section offers speculation about the possible interconnection between Pou2f1/2 and Olig2 and is not meant to propose a model based on data. We have made this clearer.

16. Orientation of all retinal section data presented omitted.

All retinal sections are oriented according to standard cell biology conventions, which state that apical side should be facing up. We think that adding a mention of this in every figure would unnecessarily crowd up the figures. Instead, we have made this clear in the material and methods.

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Second decision letter

MS ID#: DEVELOP/2020/188730

MS TITLE: Pou2f1 and Pou2f2 cooperate to control the timing of cone photoreceptor production in the developing mouse retina

AUTHORS: Awais Javed, Pierre Mattar, Suying Lu, Kamil Kruczek, Magdalena Kloc, Anai Gonzalez-Cordero, Rod Bremner, Robin R Ali, and Michel Cayouette

I am sure you will be happy to hear that the referees are happy with your revision and there is just one tiny comment for you to consider before publication. 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.

Reviewer 1

Advance summary and potential significance to field

This revised study, which representa extensive and careful work, provides critical new insights about the temporal specification of different types of neurons in the retina, with implications for other parts of the nervous system too. By using a combination of knock-out, over-expression, down-regulation, and genomic expression analysis, this paper from Javed and colleagues uncovers that the transcription factors Pou2f1/2 act sequentially and downstream of Ikzf1 to time the production of cones in the mammalian retina. Essentially, this manuscript illustrates the similarities between the fly neuroblast temporal cascade and the timing of neuron production in vertebrates. Walter Gehring would be stoked.

Comments for the author

Thank you for addressing all of the reviewer comments so completely. I have no further concerns/questions.

Reviewer 2

Advance summary and potential significance to field

The revised paper by Javed and colleagues integrates concepts of temporal patterning and identity to the specification of cone photoreceptors, in the vertebrate eye. This study is a significant advance in our understanding of the principles of neurogenesis, plus clarifies models of retinal competence. The authors are to be commended for thoughtfully and thoroughly addressing all reviewer concerns, during this trying period of lost productive time in the lab. The new data, particularly use of a second Cre driver, further strengthens this study. The revised manuscript presents a very clear message and interpretation of the data within it.

Comments for the author

I have one minor suggestion to globally change the gene/protein name Rxr"gamma" to Rxrg, thereby eliminating a greek symbol that did not appear in the manuscript and in keeping with the newer naming convention in MGI.

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Second revision

Author response to reviewers' comments

REVIEWER 1
This revised study, which representa extensive and careful work, provides critical new insights about the temporal specification of different types of neurons in the retina, with implications for other parts of the nervous system too. By using a combination of knock-out, over-expression, down-regulation, and genomic expression analysis, this paper from Javed and colleagues uncovers that the transcription factors Pou2f1/2 act sequentially and downstream of Ikzf1 to time the production of cones in the mammalian retina. Essentially, this manuscript illustrates the similarities between the fly neuroblast temporal cascade and the timing of neuron production in vertebrates. Walter Gehring would be stoked. Thank you for addressing all of the reviewer comments so completely. I have no further concerns/questions.

We thank the reviewer for their insightful comments during the review process, which have helped make this paper better. Their comment about Walter Gehring made our day!

REVIEWER 2
The revised paper by Javed and colleagues integrates concepts of temporal patterning and identity to the specification of cone photoreceptors, in the vertebrate eye. This study is a significant advance in our understanding of the principles of neurogenesis, plus clarifies models of retinal competence. The authors are to be commended for thoughtfully and thoroughly addressing all reviewer concerns, during this trying period of lost productive time in the lab. The new data, particularly use of a second Cre driver, further strengthens this study. The revised manuscript presents a very clear message and interpretation of the data within it. I have one minor suggestion to globally change the gene/protein name Rxr"gamma" to Rxrg, thereby eliminating a greek symbol that did not appear in the manuscript and in keeping with the newer naming convention in MGI.

We are pleased the reviewer appreciated the improvements made in the paper and thank them for their comments, which helped us reinforce the conclusions. We have now changed the nomenclature of Rxrgamma to Rxrg throughout the main text and figures to align with MGI convention, which we agree is important.

Third decision letter

MS ID#: DEVELOP/2020/188730

MS TITLE: Pou2f1 and Pou2f2 cooperate to control the timing of cone photoreceptor production in the developing mouse retina

AUTHORS: Awais Javed, Pierre Mattar, Suying Lu, Kamil Kruczek, Magdalena Kloc, Anai Gonzalez-Cordero, Rod Bremner, Robin R Ali, and Michel Cayouette
ARTICLE TYPE: Research Article

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

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