# Regulation of retinal amacrine cell generation by miR-216b and Foxn3

Huanqing Zhang, Pei Zhuang, Ryan M. Welchko, Manhong Dai, Fan Meng and David L. Turner
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**Original submission**

First decision letter

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MS TITLE: Regulation of retinal amacrine cell generation by miR-216b and Foxn3

AUTHORS: Huanqing Zhang, Pei Zhuang, Ryan M Welchko, Manhong Dai, Fan Meng, and David L. Turner

I have now received all the referees reports on the above manuscript, and 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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to the last (and final) comment of reviewer #3. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that 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 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.

Reviewer 1

*Advance summary and potential significance to field*

In this study, the authors screened for miRNAs that were differentially expressed in wild-type vs Ptf1a mutant embryos, which generate fewer amacrine cells. They identified several

downregulated miRNAs in the same genomic cluster, including miR-216b and miR-216a, both of which were expressed in amacrine cells. Gain of function analysis revealed that miR-216a and miR-216b increased amacrine cell production by P0 RPCs. The authors used Argonaute PAR-CLIP to identify miR-216a/b bound genes. One of the predominant targets was the 3'UTR of Foxn3, which they confirmed was a miR-216b-5p target. They went on to show that Foxn3 knockdown increases amacrine cell genesis using shRNAs and CRISPR. Conversely, Foxn3 overexpression inhibits amacrine cell genesis. Overall, this is a very nice and thorough study and I have only a few minor concerns.

*Comments for the author*

General comment:
The authors should change all bar graphs to show individual data points for each experiment. It is not always clear what the N values were, and this data should be in the figure legends and shown in the graphs as individual data points to see the spread.

Specific comments:
1. Line 146-147 " .. we observed a decrease in GFP-labeled cells in the outer part of the INL (Fig. 2C,D,F), suggesting a decrease in bipolar neurons.."
2. line 148-150 – the authors state: "While antibody markers specific for bipolar cells did not work well at P7, additional experiments described below show that miR-216b does decrease the number of bipolar cells among the electroporated cells when scored at P12."
This conclusion from Fig. 2 is not that convincing and the number of GFP+ cells in the upper and lower INL should be quantitated to make this statement.
It is not clear why the authors say the bipolar marker did not work here (in Fig. 2) when it worked well in Fig. 5. I wonder if references to bipolar cells should just be removed here for simplicity as the main focus is on amacrine cells.
Furthermore, the reduction in GFP cells in the upper INL could be because there is a change in Muller glia number. In Fig. 8, the authors quantify Muller glia. Why was it not quantitated in Fig. 5 and Fig. 2.

Reviewer 2

*Advance summary and potential significance to field*

In the manuscript by Zhang et al microRNA miR-216a, miR-216b and 217 were found to be downregulated in the Ptf1a-null retina which lack amacrine cells. These miRNA are expressed in developing amacrine cell and when miR-216a and miR-216b are overexpressed, promote amacrine cell differentiation at the expense of bipolar cells. Foxn3 was identified as a target for these miRNA by PAR-CLIP and its overexpression and knockdown lead to a loss of amacrine cells/increase in bipolar cells and increase in amacrine cells/loss of bipolar cells respectively. Overall, the findings identify novel molecular interactions during retina that will be of interest to those studying retinal development and other aspects of neural development.

Strengths:

Overall the paper is well- and thoughtfully written. Much of the data are sound and convincing e.g. (i) miR-216 and 217 are reduced in the Pft1a-null retina. The miRNA-seq and qPCR data are reasonably convincing, despite criticism below. (ii) ectopic expression of miR-216 increases formation of amacrine cells. The cell counting data is pretty convincing (even though it was incorrectly analyzed using t-test). The effect size (four-fold difference) is convincing. (iii) bipolar cells are lost with ectopic expression of miR-216. The effect size is reasonably convincing (two-fold difference) despite concerns about the statistical test used.

*Comments for the author*

Weaknesses:
There are major concerns with the lack of key pieces of data that greatly diminish the impact and significance of the findings. There are also major concerns with experimental design and with the statistical analyses. These are discussed below:
1. The major weakness of the paper is the lack of miR-216a and miR-216b loss of function. As the authors are aware and discuss, in the miR-216 overexpression experiment, overexpression is taking place in retinal progenitor cells. In contrast, miR-216a and miR-216b only appear to be elevated in post-mitotic amacrine cells (downstream of Ptf1a). Therefore, the miR-216 overexpression phenotype is likely an artificial/out-of-context phenotype that may not reflect the post-mitotic role of miR-216a and miR-216b downstream of Ptf1a. The critical experiment to reveal the role of miR-216a/b is a loss of function experiment. The authors have the necessary expertise to do the experiment so it is something that should be done.
There are many ways in which a loss of function experiment could be performed.
The most direct and desired approach to test the authors' model would be to target the miR-216a and 216b microRNA recognition elements within the Foxn3 3'utr. This approach removes the caveat seen in microRNA knockdown experiments which is that all targets of the microRNA will be affected, which makes it difficult to determine whether the phenotype is due to an effect of the target of interest and/or other targets. Similarly, the miR-216 sites 1 and 2 in Foxn3 could also be shielded from miR-215 repression using a Target Protector approach which would simple require synthesis of a short RNA sequences to span the site 1 and 2.
A miR-216a and miR-216b knockdown would still be worthwhile as it addresses a different, but still relevant question for the paper. A CRISPR-based approach could be used to target both miR-216a and miR-216b but this may be challenging to due to the large ~100kb distance between the two, and perhaps the lack of convenient PAM sites to generate sgRNA. In additions, there are other plasmid based approaches that could be used to knockdown miR-216a and miR-216b such as target decoys Tough Decoy (TuD), antagomirs and miRZip.

2. The second big weakness is the lack of an antibody to detect Foxn3 protein. This is critical for many reasons. First, Foxn3 mRNA is present in amacrine layer + bipolar region implying that that if is being targeted by miR-216a/b, it would be inhibiting translation and not RNA degradation. Determine whether or not FOXN3 protein is absent from the amacrine cell region where miR-216a/b are expressed is critical for supporting the claims of the paper. Although there aren't any useful antibodies for immunolabeling, is this the case for detecting mouse protein for western blot? If so, one would be able to perform a western blot on Ptf1a-null retinas as these would be predicted to have elevated FOXN3 protein. Furthermore, the lack of FOXN3 antibody makes it impossible to determine whether the miR-216 overexpression phenotype is due, in part, to a downregulation of FOXN3.

3. The claim that miR-216 regulates Foxn3 through its 3'UTR is not entirely satisfying:
(i) Though Foxn3 was identified as a candidate target by PAR-CLIP, this approach is known to produce a high false-positive rate.
(ii) The luciferase data are not compelling:
(a) It is mentioned that it was generated by piecing together regions containing sites 1 and site 2. Taking sections of the 3'utr out of their normal position/context may change their function. Is site 1 in the same relative position to the luciferase stop codon as it is to the Foxn3 stop codon? This is important because microRNA recognition element proximity to the stop codon has been suggested to impact their functionality - sites close to the stop codon tend not to be strong.
(b) The effect sizes are a bit small, especially given that miR-216 is being overexpressed.
(c) The reported error is very low for this type of assay, raising concern that pseudoreplication was used. If data was pseudoreplicated by using technical replicates as "independent samples", then any p-value based on that data is not meaningful. The approach used to generate the data in this panel therefore needs to be described in detail. Individual data points for each "n" (not technical replicate) should be plotted along the mean and error (preferably 95% C.I.).
(d) Finally, analyzing this data using multiple t-tests without correcting for multiple comparisons is not appropriate. An approach that accounts for multiple measurements would be more appropriate, such as ANOVA followed by Tukey's Honest Significant Difference (HSD) Test.
4. CRISPR experiments. These experiments are lacking in detail and as presented, which make it difficult for meaningful conclusions to be made from the data presented:

(i) First, aside from the sequence listed for the sgRNA, there is no detail outlining what part of the gene is being targeted and the rationale for targeting that region(s).
(ii) The results section should indicate what types of modifications were introduced and sequence data should be shown in supplemental figure 8 to back this up.
(iii)The sample size is not presented.
(iv) In keeping with the shRNA knockdown experiments, the number of bipolar cells should also be quantitated.

5. The authors show that Foxn3 overexpression keeps cells in cell cycle. They should also examine using EdU, whether the knockdown of Foxn3 promotes premature cell cycle exit. This is an important experiment for the conclusions and model put forward in the paper. Given that bipolar cell birth does not peak until P3, if Foxn3 knockdown at P0 causes cells to prematurely exit the cell cycle, the loss of bipolar fates and increase of amacrine fates may simply arise indirectly, due to the fact that newly post-mitotic cells are not fully competent to become bipolar cells.

6. Controls for miR-216b-5p in situ hybridization are missing but are fairly important and should be included. A meaningful negative control would preferable be on a retinal or another tissue that doesn't express miR-216 by RT-PCR. This would help to determine whether there is low levels of expression in retinal progenitor cells which would potentially change the authors' model. A positive control should be done to show the veracity of the labeling that is observed in the figure. An example of a positive control might be to do an in situ after electroporating the miR-216 overexpression constructs.

7. Similar to the Foxn3 shRNA knockdown experiments, miR-216 overexpression in P19 cells should be performed and endogenous Foxn3 examined by RT-PCR. Although miR-216 overexpression may not lead to degradation of the mRNA and only affect protein levels this is important to determine given that this is what appears to be happening in the retina.

8. The nucleotide positions for miR-216 sites 1 and 2 in the 3'utr are not list. This information should be included in the text and figure.

9. Analyzing this data throughout the manuscript using multiple t-tests is not appropriate. An approach that accounts for multiple comparisons would be more appropriate, such as ANOVA followed by Tukey's HSD Test, or t-tests with a Bonferroni Correction or the Benjamini-Hochberg procedure. This may explain why some results are "significant" even though the effect sizes are tiny (ex: Fig 4B, Fig 8L).

10. Sample sizes are not indicated for many figures. (ex: Fig 2G, 4B, 6D). For graphs, individual data points should be plotted on the graph, instead of just the mean and S.D. This provides a more transparent and easily understandable representation of the data variation. For increased transparency and completeness of data, the authors should indicate total number of cells were counted for retinal transfection experiments.

11. The authors state that the cell counting analysis was not blinded. Though I appreciate the honesty, why was this not done? This is a trivial step to include and is critical for addressing bias. In particular, this raises a great deal of concern for the results in Figs 7J and 8L, where the observed effect sizes are very small. To eliminate the possibility of experimenter bias, images should be blinded and recounted.

Typos, minor editing suggestions
Abstract: "Disruption of Foxn3 by CRISPR in embryonic retinal explants also reduces amacrine cell formation." It leads to an increase in the number of amacrine cells.
Line 137 –"Forced expression of miR-216a, miR-216b, or all three miRNAs together led to a significant increase in the frequency of amacrine cells among GFP-labeled cells at P7" Sentence not 100% (not obvious that referring to miR-217)
Line 205 – "We…" - might be a good place to have a paragraph break to introduce begin luc experiment
Line 219 – Could include at end of paragraph a summary sentence of the experiment like – "show that in vitro, both site 1 and site 2 appear to contribute to repression..", which for some readers might not be as obvious as it is to others.

Line “54 “We observed an increased number…” replace with “increased proportion”
Line 347 “A potential functional requirement for the miR-216a/miR-216b/miR-217 genomic cluster in the retinal development has not been evaluated,..” remove “the”

Reviewer 3

*Advance summary and potential significance to field*

In their manuscript titled “Regulation of retinal amacrine cell generation by miR-216b and Foxn3,” Zhang et al. provide new insights into the specification of amacrine interneurons in the developing retina. This is a particular important topic because little is known about amacrine neuron development and the diversity of amacrine subtypes makes this particularly difficult to study. The authors start with miRNA sequencing in Ptf1a wild type and deficient littermates. This was an ideal starting point because Ptf1a is required for amacrine cell development and in Ptf1a-/- retina, amacrine cells are reduced. The miRNA profiling showed that the miR-216b cluster is downregulated in the Ptf1a-/- retinae and they showed that miR-216b is expressed in amacrine cells by in situ hybridization. To demonstrate that miR-216b is important for amacrine cell development, ectopic expression studies were carried out and amacrine cells were increased. However, this was not sufficient to overcome the defect in Ptf1a-/- retinae suggesting that miR-216b acts through Ptf1a or downstream. To identify direct targets, Argonaute PAR-CLIP was performed. Among the >14,000 miRNA targets, 404 were putative miR216a/b targets with 57 having both target sequences. The gene with the most miR216 targets was Foxn3 and this was validated with a luciferase reporter gene and the Foxn3 3’ UTR. This was an interesting and important discovery because Foxn4 is thought to promote amacrine cell formation while they suggested that Foxn3 blocks amacrine cell genesis. Expression studies confirmed that Foxn3 is expressed in cells of the INL that are not amacrine cells consistent with the idea that Foxn3 is a negative regulator of amacrine cell fate specification. Subsequent studies showed that ectopic expression of Foxn3 led to reduced amacrine cell formation and reduced retinal progenitor cell proliferation. It was also able to reverse effects of miR216a/b. Overall this is a well done and rigorous study and the manuscript is well written.

*Comments for the author*

The manuscript would benefit from scRNA-seq of some of the key experiments such as ectopic Foxn3 expressing cells. While it is not absolutely required, it would significantly strengthen the study.

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**First revision**

Author response to reviewers' comments

**Summary of changes and response to reviewers**
We revised Fig. 2, adding quantitation of changes in GFP+/AP2alpha- cells in the INL.

We revised Fig. 4, adding additional reporter data and an improved schematic of the reporters.

We added Supplemental Fig. S3 to show that miR-216b in situ hybridization can detect ectopic miR-216b expression from the electroporated miR-216b vector in rods, cells which do not normally express this miRNA.

We replaced Supplemental Fig. S9 (formerly Fig. S8) with a new figure that includes a schematic and additional information about the Foxn3 CRISPR sequencing analysis. We reanalyzed the Foxn3 CRISPR Illumina sequencing data using the more recent CRISPResso2 software. We added a new Supplemental Table S6 with the target sequence alignments from the analysis. Our conclusions from the experiment are unchanged.

We now use one-way or two-way ANOVA with appropriate tests for statistical analysis of multiple comparisons. For most comparisons, this did not change the significance of the results. However, the significance of some comparisons in Fig. 5K and Fig. 8I, L, M are changed. We have updated the Results and Discussion to reflect these changes. We do not think that the changes alter the major conclusions in the manuscript.
We now show individual data points in addition to mean and standard deviation on bar graphs. N values are indicated in all figure legends.

In addition to changes to the text, methods, and figure legends because of the revised figures and analyses, we made various changes to wording to improve clarity or correct errors, and to reduce total word count.

In some places in the manuscript, the age of the Ptf1a mutant retinas used for RNA isolation in Fig. 1 was incorrectly listed as E16.5 instead of E18.5. Reporter assays were performed in HEK293 cells, but some places incorrectly referred to P19 cells. The partial Foxn3' UTR reporter in Fig. 4 was listed as positions 1-296 of the Foxn3 3' UTR, when it is positions 1-266.

Figure 6 originally showed a single representative control image and two representative CRISPR images. However, the CRISPR1 retina samples and control retinas for CRISPR1 were generated in a separate experiment from the CRISPR2 retinas and its control retinas. We therefore added a representative image for a CRISPR2 control retina to Fig. 6 and reorganized the figure. The omission of the second control image from the figure does not affect our analysis or conclusions, which were based on the quantitative data in Fig. 6 that correctly compared each set of CRISPR samples with the appropriate control samples.

Sequences for several oligonucleotide primers and a gBlock used for plasmid construction have been added to Supplemental Table S7 (formerly Table S6), as well as additional oligos for the new Luciferase reporters in Fig. 4.

We added a citation for a recent report on regulation of the Foxn3 3' UTR by the miR-182 miRNA in non-retinal cells to the discussion.

We thank the reviewers for their helpful comments. Specific responses to reviewer comments are below **(**our responses are in **bold).**

Reviewer 1 Comments for the Author:
General comment:
The authors should change all bar graphs to show individual data points for each experiment. It is not always clear what the N values were, and this data should be in the figure legends and shown in the graphs as individual data points to see the spread.

**Response: bar graphs now show individual data points. The omitted N values are now included in the figure legends.**

Specific comments:
1. Line 146-147 " .. we observed a decrease in GFP-labeled cells in the outer part of the INL (Fig. 2C,D,F), suggesting a decrease in bipolar neurons.."

2. line 148-150 – the authors state: "While antibody markers specific for bipolar cells did not work well at P7, additional experiments described below show that miR-216b does decrease the number of bipolar cells among the electroporated cells when scored at P12."

This conclusion from Fig. 2 is not that convincing and the number of GFP+ cells in the upper and lower INL should be quantitated to make this statement.

It is not clear why the authors say the bipolar marker did not work here (in Fig. 2) when it worked well in Fig. 5. I wonder if references to bipolar cells should just be removed here for simplicity as the main focus is on amacrine cells.

Furthermore, the reduction in GFP cells in the upper INL could be because there is a change in Muller glia number. In Fig. 8, the authors quantify Muller glia. Why was it not quantitated in Fig. 5 and Fig. 2.

**Response: the ages of the retinas differ between Fig 2 (P7) and Fig. 5/8 (P12). The bipolar marker we used works poorly to detect newly formed bipolar cells at P7, but it labels bipolar cells at P12. We added quantitation for the change in the number of GFP+/AP2alpha- cells within the INL at P7 (Fig. 2H). There is a significant decrease in these cells with miR-216b. While these INL cells include both presumptive bipolar and Müller cells (which are also difficult to identify with antibody markers at P7), our analysis at P12 indicates that miR-216b expression reduced bipolar cells but did not significantly alter the number of Muller glia (Fig. 8).**

Reviewer 2 Advance Summary and Potential Significance to Field:
In the manuscript by Zhang et al microRNA miR-216a, miR-216b and 217 were found to be downregulated in the Ptf1a-null retina which lack amacrine cells. These miRNA are expressed in developing amacrine cell and when miR-216a and miR-216b are overexpressed, promote amacrine cell differentiation at the expense of bipolar cells. Foxn3 was identified as a target for these miRNA by PAR-CLIP and its overexpression and knockdown lead to a loss of amacrine cells/increase in bipolar cells and increase in amacrine cells/loss of bipolar cells respectively. Overall, the findings identify novel molecular interactions during retina that will be of interest to those studying retinal development and other aspects of neural development.

Strengths:
Overall the paper is well- and thoughtfully written. Much of the data are sound and convincing e.g. (i) miR-216 and 217 are reduced in the Pft1a-null retina. The miRNA-seq and qPCR data are reasonably convincing, despite criticism below. (ii) ectopic expression of miR-216 increases formation of amacrine cells. The cell counting data is pretty convincing (even though it was incorrectly analyzed using t-test). The effect size (four- fold difference) is convincing. (iii) bipolar cells are lost with ectopic expression of miR-216. The effect size is reasonably convincing (two-fold difference) despite concerns about the statistical test used.

Reviewer 2 Comments for the Author: Weaknesses:
There are major concerns with the lack of key pieces of data that greatly diminish the impact and significance of the findings. There are also major concerns with experimental design and with the statistical analyses. These are discussed below:

1. The major weakness of the paper is the lack of miR-216a and miR-216b loss of function. As the authors are aware and discuss, in the miR-216 overexpression experiment, overexpression is taking place in retinal progenitor cells. In contrast, miR-216a and miR-216b only appear to be elevated in post-mitotic amacrine cells (downstream of Ptf1a). Therefore, the miR-216 overexpression phenotype is likely an artificial/out-of-context phenotype that may not reflect the post-mitotic role of miR-216a and miR-216b downstream of Ptf1a. The critical experiment to reveal the role of miR-216a/b is a loss of function experiment. The authors have the necessary expertise to do the experiment so it is something that should be done.

There are many ways in which a loss of function experiment could be performed.

The most direct and desired approach to test the authors' model would be to target the miR-216a and 216b microRNA recognition elements within the Foxn3 3'utr. This approach removes the caveat seen in microRNA knockdown experiments which is that all targets of the microRNA will be affected, which makes it difficult to determine whether the phenotype is due to an effect of the target of interest and/or other targets. Similarly, the miR-216 sites 1 and 2 in Foxn3 could also be shielded from miR-215 repression using a Target Protector approach which would simple require synthesis of a short RNA sequences to span the site 1 and 2.

A miR-216a and miR-216b knockdown would still be worthwhile as it addresses a different, but still relevant question for the paper. A CRISPR-based approach could be used to target both miR-216a

and miR-216b but this may be challenging to due to the large ~100kb distance between the two, and perhaps the lack of convenient PAM sites to generate sgRNA. In additions, there are other plasmid based approaches that could be used to knockdown miR-216a and miR-216b such as target decoys Tough Decoy (TuD), antagomirs and miRZip.

**Response: we agree that loss of function analyses for miR-216a/b in the retina would be of interest. However, to date we have not been able to perform a satisfactory loss of function experiment for these miRNAs in retinal somatic cells. The Mir216A and Mir216B genes (precursors) are separated in the mouse genome by ~11kb, making efficient combined CRISPR deletion in somatic cells difficult. In contrast to protein coding genes in which a single indel generated by CRISPR can disrupt the translated protein, disruption of miRNA genes requires deletion of the miRNA precursor with two sgRNAs. We have attempted to delete the precursors for miR-216b and miR-216a (either miR-216b only, or both miRNAs together) in retinas, using pairs of sgRNAs and CRISPR/Cas9. We did not detect a retinal phenotype in GFP+ cells after electroporation of the CRISPR plasmids, but we could not confirm efficient miRNA gene deletions. Since we cannot distinguish absence of a biological phenotype from inefficient homozygous miRNA deletion or another technical failure, we did not include those experiments in this manuscript. We have not found that TuDs or other vector-based miRNA competitive inhibition strategies are sufficiently effective at blocking miRNA repression in our hands. We also investigated making partial deletions of the Foxn3 3' UTR with CRISPR, to remove the miR-216b target sites, but there are few SpCas9 sgRNAs that can be synthesized by RNA pol III near the Foxn3 stop codon, and we could not achieve effective deletion with sgRNA pairs or HDR replacement in tests. We have not attempted the use of Target Protector morpholinos.**

**As discussed in the manuscript, we think it is likely that the endogenous miR-216a/b miRNAs do not regulate the formation of amacrine cells, since they are upregulated in postmitotic amacrine cells (downstream of Ptf1a). We think that the miRNAs likely regulate target gene expression in differentiated amacrine cells, but ectopic/premature expression increases amacrine formation, probably through premature downregulation of targets. If that model is correct, the loss of the endogenous miR-216a/b miRNAs would not be expected to change the initial generation of amacrine cells. While loss of miR-216a/b could lead to later defects in amacrine cell differentiation, such defects might be subtle and difficult to identify, especially if only some amacrine subtypes are impacted. In addition, as discussed in the manuscript, loss of function analyses are complicated by the possibility of redundant miRNA regulation of Foxn3 (by miR-216a and miR-216b, and/or by other miRNAs expressed in the retina). Analysis of mice with genetic loss of function alleles for the miR-216a/b miRNAs is likely to be the best approach to these questions, but that analysis is beyond the scope of the present manuscript.**

2. The second big weakness is the lack of an antibody to detect Foxn3 protein. This is critical for many reasons. First, Foxn3 mRNA is present in amacrine layer + bipolar region implying that that if is being targeted by miR-216a/b, it would be inhibiting translation and not RNA degradation. Determine whether or not FOXN3 protein is absent from the amacrine cell region where miR-216a/b are expressed is critical for supporting the claims of the paper. Although there aren't any useful antibodies for immunolabeling, is this the case for detecting mouse protein for western blot? If so, one would be able to perform a western blot on Ptf1a-null retinas as these would be predicted to have elevated FOXN3 protein. Furthermore, the lack of FOXN3 antibody makes it impossible to determine whether the miR-216 overexpression phenotype is due, in part, to a downregulation of FOXN3.

**Response: 10-25% decreases in the abundance of mammalian miRNA target mRNAs or in the expression of reporters in response to repression through a single miRNA binding sites are common, although targets with multiple sites can show larger changes. The Foxn3 reporters, with two target sites, show 30-40% repression in response to miR-216b. Foxn3 mRNA expression is detectable but not abundant in the INL. Unfortunately detecting less than two-fold changes by in situ hybridization (or immunohistochemistry) is difficult. We observe high-level expression of Foxn3 in a small subset of amacrine cells (specifically cholinergic amacrine cells). Foxn3 mRNA expression is likely regulated by additional mechanisms (e.g. transcription), so high level Foxn3 expression in a subset of amacrine cells is not inconsistent with regulation by miR-216b in amacrine cells. We do show that co-expression of Foxn3 partially reverses the effects of miR-216b overexpression (Fig. 8), which is consistent with Foxn3 repression as part**

**of the mechanism for the miR-216b phenotype.**

3. The claim that miR-216 regulates Foxn3 through its 3'UTR is not entirely satisfying:
(i) Though Foxn3 was identified as a candidate target by PAR-CLIP, this approach is known to produce a high false-positive rate.
**Response: while PAR-CLIP has limitations, it is not known for a high false positive rate. Unlike the original CLIP methods that generated broad read peaks near miRNA binding sites, PAR-CLIP has single nucleotide resolution because of T to C substitutions at crosslink sites, allowing mapping of seed sites near crosslinks. Our analysis applied stringent criteria for identifying miRNA/Argonaute binding sites in the PAR-CLIP data: we focused on mRNA crosslinks present at identical positions in multiple independent PAR-CLIP retina libraries, but not in control libraries. Crosslinks were present in all 5 independent Argonaute libraries near the initial Foxn3 miR-216 site that we identified. The use of 4SU for RNA-Argonaute crosslinking does constrain crosslinking to sites with a U sufficiently near the binding site to allow crosslinking (and the precise location of the crosslinked U may modulate crosslinking efficiency). Our PAR-CLIP analysis likely missed or undercounted some functional miRNA target sites because of the lack of a nearby U suitable for efficient crosslinking. Nonetheless, we saw strong enrichment of retinal miRNA seed matches within a few bases of consistent crosslink sites (Fig. S5), indicating that most crosslinked sites are miRNA binding sites. PAR-CLIP provides biochemical evidence for Argonaute binding at one of the Foxn3 miR-216b target sites, and in combination with the reporter assays, including new data on a full-length Foxn3 3' UTR reporter (see below), we think that our data provides strong evidence that Foxn3 is a target of miR-216b.**

(ii) The luciferase data are not compelling:
(a) It is mentioned that it was generated by piecing together regions containing sites 1 and site 2.

Taking sections of the 3'utr out of their normal position/context may change their function. Is site 1 in the same relative position to the luciferase stop codon as it is to the Foxn3 stop codon? This is important because microRNA recognition element proximity to the stop codon has been suggested to impact their functionality - sites close to the stop codon tend not to be strong.

**Response: the original UTR reporter was not pieced together or taken out of context, but rather is a truncation that includes only the 5' end of the Foxn3 3' UTR (bases 1-266), facilitating mutational analysis of the two candidate miR-216 target sites (seed matches). We have added a schematic of the Foxn3 3' UTR to Fig. 4A and updated the text to clarify both the reporter design and the location of the miR-216 sites within the UTR. The reporter inserts the Foxn3 3' UTR immediately after the Luciferase coding region (there is ~25 nt of polylinker in between).**

**In addition to the original truncated Foxn3 3' UTR reporter, we now have constructed a full-length Foxn3 3' UTR reporter that includes the complete ~6.2 kb Foxn3 3' UTR + the endogenous Foxn3 polyadenylation site inserted after Luciferase. Data on miR-216b repression for both the full length 3' UTR reporter and the full-length reporter with two miR-216 sites mutated are now included in Fig. 4. The full-length reporter shows similar repression to the partial reporter when co-expressed with miR- 216b, and the repression depends on an intact miR-216b seed sequence in the miRNA and the two miR-216 sites in the 3' UTR.**

(b) The effect sizes are a bit small, especially given that miR-216 is being overexpressed.

**Response: the effect sizes are typical of miRNA repression for many mammalian sites: ~30-40% reporter repression with two non-adjacent sites (too far apart for strong cooperativity), up to ~25% repression with one site. In addition, when we mutated the target sites, we used sequences complementary to the miR-216b-mut miRNA. That allows us to show that the reporters with mutated sites (both partial and full-length) have reduced repression by miR-216b, but it also allows repression of the mutant reporters by miR-216b-mut. The use of compensatory changes to confirm the specificity of the miRNA target site repression adds to our confidence in the sites (validation with compensating mutations is not often done when testing miRNA target sites). The repression of the mutant reporters by miR-216b-mut also confirms that the miR-216b-mut pre-miRNA, which we use as a control for functional analyses,**

**is processed into a functional mature miRNA.**

(c) The reported error is very low for this type of assay, raising concern that pseudoreplication was used. If data was pseudoreplicated by using technical replicates as "independent samples", then any p-value based on that data is not meaningful. The approach used to generate the data in this panel therefore needs to be described in detail. Individual data points for each “n” (not technical replicate) should be plotted along the mean and error (preferably 95% C.I.).

**Response: in both the original manuscript and the current revision, the luciferase data show independent transfections (biological replicates). Luciferase assays were performed in duplicate on each sample (technical replicates). In the revised Fig. 4, the individual data points included on the graphs are the mean of the technical assay replicates, and we include data from 6 biological replicates for each reporter. We have revised the methods section to further clarify the details of the assays.**

(d) Finally, analyzing this data using multiple t-tests without correcting for multiple comparisons is not appropriate. An approach that accounts for multiple measurements would be more appropriate, such as ANOVA followed by Tukey’s Honest Significant Difference (HSD) Test.

**Response: significance for the Luciferase reporter data is now determined by two-way ANOVA with Tukey’s multiple comparison test.**

4. CRISPR experiments. These experiments are lacking in detail and as presented, which make it difficult for meaningful conclusions to be made from the data presented:
(i) First, aside from the sequence listed for the sgRNA, there is no detail outlining what part of the gene is being targeted and the rationale for targeting that region(s).
(ii) The results section should indicate what types of modifications were introduced and sequence data should be shown in supplemental figure 8 to back this up.
(iii) The sample size is not presented.
(iv) In keeping with the shRNA knockdown experiments, the number of bipolar cells should also be quantitated.

**Response: N values are now indicated in the figure legend (N=3 samples for each CRISPR construct, and N=3 or 4 for the controls).**

**We did not analyze bipolar cells in the CRISPR retinal explants because the explants were collected at the equivalent of either E18 (2 DIV) or P4 (8 DIV), prior to substantial expression of differentiated bipolar cell markers.**

**To provide additional information on the Foxn3 CRISPR experiments, we have replaced Supplemental Figure S9 (Figure S8 in the original manuscript) with a new figure that includes a schematic of the Foxn3 gene showing the locations of the sgRNA targets within the Foxn3 coding region, in Fig. S9A. We also reanalyzed the CRISPR Illumina sequencing data with the newer CRISPesso2 program. The results were almost identical to the original CRISPResso analysis, and the conclusions from the experiments are unchanged. Indel percentages and total numbers of reads for each sample/sgRNA are shown in Fig. S9B. We include the CRISPesso2 sequencing alignments for the sgRNA target sites in the new Supplemental Table S6.**

5. The authors show that Foxn3 overexpression keeps cells in cell cycle. They should also examine using EdU, whether the knockdown of Foxn3 promotes premature cell cycle exit. This is an important experiment for the conclusions and model put forward in the paper. Given that bipolar cell birth does not peak until P3, if Foxn3 knockdown at P0 causes cells to prematurely exit the cell cycle, the loss of bipolar fates and increase of amacrine fates may simply arise indirectly, due to the fact that newly post-mitotic cells are not fully competent to become bipolar cells.

**Response: we do not see a loss of Müller glia after miR-216b expression (Fig. 8), another late generated cell type, which would suggest that the loss of bipolar cells is specific and not just depletion of retinal progenitors. While Foxn3 can affect the timing of cell cycle exit, we do not think that assessing continued proliferation would distinguish whether the effect of Foxn3 is**

**direct (modulation of bipolar or amacrine fates) or indirect (fate changes only through modulation of proliferation). We propose that Foxn3 may function by inhibiting activation of genes by Foxn4 (although other mechanisms are possible, as we discuss). Foxn4 is a temporal competence factor (Liu et al. 2020) that regulates generation of amacrine cells (and other cell types), but also regulates proliferation (Li et al. 2004), potentially making it difficult to separate cell fate determination from changes to the timing of cell cycle exit.**

6. Controls for miR-216b-5p in situ hybridization are missing but are fairly important and should be included. A meaningful negative control would preferable be on a retinal or another tissue that doesn't express miR-216 by RT-PCR. This would help to determine whether there is low levels of expression in retinal progenitor cells which would potentially change the authors' model. A positive control should be done to show the veracity of the labeling that is observed in the figure. An example of a positive control might be to do an in situ after electroporating the miR-216 overexpression constructs.

**Response: we have added a new figure, Fig. S3. miR-216b is not expressed in cells in the ONL, but ectopic mature miR-216b can be detected in rod photoreceptors by in situ hybridization after electroporation of the miR-216b expression vector. The specificity of the in situ hybridization method also has been described in previous papers (Deo et al. 2006, Zhuang et al. 2020).**

7. Similar to the Foxn3 shRNA knockdown experiments, miR-216 overexpression in P19 cells should be performed and endogenous Foxn3 examined by RT-PCR. Although miR-216 overexpression may not lead to degradation of the mRNA and only affect protein levels this is important to determine given that this is what appears to be happening in the retina.

**Response: we think that the Luciferase reporter assays address the ability of miR-216b to regulate the Foxn3 3' UTR more directly and with better controls, since it is feasible to mutate the specific miR-216 target sites in the reporter as a specificity control, as we have done in Fig. 4.**

8. The nucleotide positions for miR-216 sites 1 and 2 in the 3'utr are not list. This information should be included in the text and figure.

**Response: that information is now incorporated into the schematic in Fig. 4A. Sequences of oligonucleotides used for reporter construction are included in Supplemental Table S7.**

9. Analyzing this data throughout the manuscript using multiple t-tests is not appropriate. An approach that accounts for multiple comparisons would be more appropriate, such as ANOVA followed by Tukey's HSD Test, or t-tests with a Bonferroni Correction or the Benjamini-Hochberg procedure. This may explain why some results are "significant" even though the effect sizes are tiny (ex: Fig 4B, Fig 8L).

**Response: all multiple comparisons in the paper are now analyzed with ANOVA and appropriate multiple testing corrections. Pairwise comparisons are analyzed with t-tests as indicated.**

10. Sample sizes are not indicated for many figures. (ex: Fig 2G, 4B, 6D). For graphs, individual data points should be plotted on the graph, instead of just the mean and S.D. This provides a more transparent and easily understandable representation of the data variation. For increased transparency and completeness of data, the authors should indicate total number of cells were counted for retinal transfection experiments.

**Response: we have corrected the figure legends to indicate the sample sizes where they were omitted. We added the number of GFP+ cells counted per vector in retinal electroporation experiments to the methods under "Cell counts, graphs, and statistics".**

11. The authors state that the cell counting analysis was not blinded. Though I appreciate the honesty, why was this not done? This is a trivial step to include and is critical for addressing bias. In particular, this raises a great deal of concern for the results in Figs 7J and 8L, where the observed effect sizes are very small. To eliminate the possibility of experimenter bias, images should be blinded and recounted.

**Response: we did not utilize masked (blinded) cell counting because in most cases altered cell numbers among the GFP labeled cells in specific retinal layers were discernable by eye, allowing identification of the phenotype prior to counting. We have added a statement on this to the methods.**

Typos, minor editing suggestions
Abstract: "Disruption of Foxn3 by CRISPR in embryonic retinal explants also reduces amacrine cell formation." It leads to an increase in the number of amacrine cells.
Line 137 –"Forced expression of miR-216a, miR-216b, or all three miRNAs together led to a significant increase in the frequency of amacrine cells among GFP-labeled cells at P7" Sentence not 100% (not obvious that referring to miR-217)
Line 205 – "We…" - might be a good place to have a paragraph break to introduce begin luc experiment
Line 219 – Could include at end of paragraph a summary sentence of the experiment like – "show that in vitro, both site 1 and site 2 appear to contribute to repression..", which for some readers might not be as obvious as it is to others.
Line "54 "We observed an increased number…" replace with "increased proportion"
Line 347 "A potential functional requirement for the miR-216a/miR-216b/miR-217 genomic cluster in the retinal development has not been evaluated,.." remove "the"

**Response: all have been changed or corrected in the revised manuscript.**

Reviewer 3 Advance Summary and Potential Significance to Field:
In their manuscript titled "Regulation of retinal amacrine cell generation by miR-216b and Foxn3," Zhang et al. provide new insights into the specification of amacrine interneurons in the developing retina. This is a particular important topic because little is known about amacrine neuron development and the diversity of amacrine subtypes makes this particularly difficult to study. The authors start with miRNA sequencing in Ptf1a wild type and deficient littermates. This was an ideal starting point because Ptf1a is required for amacrine cell development and in Ptf1a-/- retina, amacrine cells are reduced. The miRNA profiling showed that the miR-216b cluster is downregulated in the Ptf1a-/- retinae and they showed that miR-216b is expressed in amacrine cells by in situ hybridization. To demonstrate that miR-216b is important for amacrine cell development, ectopic expression studies were carried out and amacrine cells were increased. However, this was not sufficient to overcome the defect in Ptf1a-/- retinae suggesting that miR-216b acts through Ptf1a or downstream. To identify direct targets, Argonaute PAR-CLIP was performed. Among the >14,000 miRNA targets, 404 were putative miR216a/b targets with 57 having both target sequences. The gene with the most miR216 targets was Foxn3 and this was validated with a luciferase reporter gene and the Foxn3 3' UTR. This was an interesting and important discovery because Foxn4 is thought to promote amacrine cell formation while they suggested that Foxn3 blocks amacrine cell genesis. Expression studies confirmed that Foxn3 is expressed in cells of the INL that are not amacrine cells consistent with the idea that Foxn3 is a negative regulator of amacrine cell fate specification. Subsequent studies showed that ectopic expression of Foxn3 led to reduced amacrine cell formation and reduced retinal progenitor cell proliferation. It was also able to reverse effects of miR216a/b.
Overall this is a well done and rigorous study and the manuscript is well written.

Reviewer 3 Comments for the Author:
The manuscript would benefit from scRNA-seq of some of the key experiments such as ectopic Foxn3 expressing cells. While it is not absolutely required, it would significantly strengthen the study.

**Response: we agree with the reviewer that single cell RNA-seq could provide valuable insights into this system. Unfortunately, such experiments would require substantial additional resources and analysis, so we think that they are beyond scope of the present manuscript.**

Second decision letter

MS ID#: DEVELOP/2021/199484

MS TITLE: Regulation of retinal amacrine cell generation by miR-216b and Foxn3

AUTHORS: Huanqing Zhang, Pei Zhuang, Ryan M Welchko, Manhong Dai, Fan Meng, and David L. Turner
ARTICLE TYPE: Research Article

I have now received all the referees reports on the above manuscript, and 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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to the last (and final) comment of reviewer #3. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

In this manuscript, the authors identify a group of miRNAs regulated by Ptf1a, a critical regulator of amacrine cell genesis. They show that overexpression of miR-216a and miR-216b promotes amacrine cell genesis. The authors then use Argonaute PAR-CLIP to identify Foxn3 as a miR-216b target. Overexpression of Foxn3 has the opposite effect - reducing amacrine cell genesis. This is a well written manuscript with well controlled experiments. The findings represent a key advance in the field and expand our knowledge of the molecular regulators of amacrine cell genesis.

*Comments for the author*

The authors have addressed all of my concerns. The manuscript is suitable for publication.

Reviewer 2

*Advance summary and potential significance to field*

The authors have identified microRNA (miR-216a and miR-216b) that are specifically upregulated in amacrine cells and the gene Foxn3 as one of their targets. Although ectopic expression of miR-216a and miR-216b in retinal progenitor cells promotes amacrine cell differentiation at the expense of bipolar cells, it is not thought that these miRNA normally play a role in amacrine cell fate determination. Given their expression pattern, it is thought that they function downstream of amacrine cell formation to regulate genes such as Foxn3, but this downstream function is not specifically addressed in the paper. Foxn3 overexpression and knockdown lead to a loss of amacrine cells/increase in bipolar cells and increase in amacrine cells/loss of bipolar cells, respectively, which is a novel finding that adds to our knowledge of amacrine cell fate determination. Overall the paper provides insight into two novel regulatory mechanisms that are involved in amacrine cell development.

*Comments for the author*

As a follow to reviewer point 6, in the manuscript, the authors state that “At P0, the three miRNAs were detected in the inner retina, but were not present in the neuroblast layer.” However, the data in Figure 1 show weak miR-216b-5p labeling in the NBL at P0 (Fig 1F) in contrast to the ONL at P12 (Fig 1I) which mostly devoid of labeling. Rather than making a definitive statement that these miRNA are not present in the NBL, the authors should comment on this weak labeling. Does it represent expression in newly born amacrine cells, if so, is it lost absent in Ptf1 null mice at E18.5? Alternatively, can low levels of miR-216 in progenitor cells be excluded?

The model presented in Fig 8N suggests that in a miR-216b/a knockout, Ptf1a would be repressed and there would be a loss of amacrine cells. However, this is not what the authors predict. They think that miR-216b/a functions downstream of amacrine formation and predict that loss of miR-216a/b miRNAs would not change the initial generation of amacrine cells. To provide clarity and avoid confusion about the predicted roles of miR216b/c and Foxn3, the authors should break their model into 2 schematics (A and B) both of which have the developmental timeline incorporated. e.g. (A) normal development showing that miR-216 is upregulated after amacrine cells are generated and predicted to regulate downstream genes (including Foxn3) involved in amacrine differentiation, (B) mR-216 gain of function showing what happens when miR216 is ectopically expressed in progenitors, and how it is predicted to target mRNAs (including Foxn3) that lead to an increase in amacrine cells.

Reviewer 3

*Advance summary and potential significance to field*

This is an important and interesting study on the development of amacrine cells in the retina.

*Comments for the author*

The authors have addressed all of my comments and the manuscript is now suitable for publication.

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**Second revision**

Author response to reviewers' comments

Reviewers 1 and 3 had no concerns.

Reviewer 2 Comments for the Author:
*As a follow to reviewer point 6, in the manuscript, the authors state that "At P0, the three miRNAs were detected in the inner retina, but were not present in the neuroblast layer." However, the data in Figure 1 show weak miR-216b-5p labeling in the NBL at P0 (Fig 1F) in contrast to the ONL at P12 (Fig 1I) which mostly devoid of labeling. Rather than making a definitive statement that these miRNA are not present in the NBL, the authors should comment on this weak labeling. Does it represent expression in newly born amacrine cells, if so, is it lost absent in Ptf1 null mice at E18.5? Alternatively, can low levels of miR-216 in progenitor cells be excluded?*

Response: while the miR-216 miRNAs are much more highly expressed in the INL/GCL than the neuroblast layer of the retina, we agree that low level miR-216 expression in retinal progenitor cells is possible. We have revised the text (Results page 5 and Discussion page 13) and Supplemental Figure legend S3 to acknowledge this possibility. Our working model remains that miR-216b functions primarily in differentiating amacrine cells and not in progenitor cells.

*Reviewer 2: The model presented in Fig 8N suggests that in a miR-216b/a knockout, Ptf1a would be repressed and there would be a loss of amacrine cells. However, this is not what the authors predict. They think that miR-216b/a functions downstream of amacrine formation and predict that loss of miR-216a/b miRNAs would not change the initial generation of amacrine cells. To provide clarity and avoid confusion about the predicted roles of miR216b/c and Foxn3, the authors should break their model into 2 schematics (A and B) both of which have the developmental timeline incorporated. e.g. (A) normal development showing that miR-216 is upregulated after amacrine cells are generated and predicted to regulate downstream genes (including Foxn3) involved in amacrine differentiation, (B) mR-216 gain of function showing what happens when miR216 is ectopically expressed in progenitors, and how it is predicted to target mRNAs (including Foxn3) that lead to an increase in amacrine cells.*

Response: We thank the reviewer for the suggestion. We have separated the schematic in Fig. 8 into two panels. The first panel (Fig. 8N) shows our model of sequential gene expression and regulation during normal amacrine cell formation. The second panel (Fig. 8O) shows a schematic of our model for the effects of ectopic/premature expression of miR-216b. We think this organization makes the model clearer, and it distinguishes the proposed normal miR-216 expression from ectopic expression.

Additional changes to the manuscript:
In addition to the changes above, minor changes were made to improve clarity/grammar and reduce the total word count to meet Development requirements. We made minor clarifications/corrections in the Methods, and we corrected a grammatical error in the Abstract. Additions or changes to the text and figure legends are highlighted (word deletions/rewording to reduce length and formatting changes are not).

Additional changes to the supplemental figure legends and tables:
We corrected typos in Fig. S9 legend and Table S7. Minor wording changes were made to the Figure S5 legend to improve clarity/grammar.

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Third decision letter

MS ID#: DEVELOP/2021/199484

MS TITLE: Regulation of retinal amacrine cell generation by miR-216b and Foxn3

AUTHORS: Huanqing Zhang, Pei Zhuang, Ryan M Welchko, Manhong Dai, Fan Meng, and David L. Turner
ARTICLE TYPE: Research Article

Dear Dr. Turner

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. Where referee reports on this version are available, they are appended below.