Enhancer transcription identifies *cis*-regulatory elements for photoreceptor cell types

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MS TITLE: Enhancer transcription identifies *cis*-regulatory elements for photoreceptor cell types

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My apologies for the long time it has taken me to collect the referees' reports on your manuscript. I have now received the reports of three referees and I have reached a decision. The reports are appended below and you can access them online: please go to BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees are enthusiastic about your work but they also have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they comment on the need to quantify the enhancer assays or alternatively remove the quantitative comments in your description of the assays, and they request that you compare more thoroughly your findings with those of prior studies characterising retinal enhancers by other approaches. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

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

This is a truly excellent paper by Moskowitz and colleagues describing a detailed analysis of transcription factor-dependent non-coding RNA profiling and cross-referencing region and cell-specific ATACseq, RNAseq and other genome-wide data to identify active cis-regulatory elements in retinal photoreceptors, allowing insights into the mutually repressive networks underpinning rods and cones. A particular strength of this paper is the quantitative outputs correlating proximal gene expression to enhancer ncRNA levels. The work demonstrates the superiority of the approach to high throughput enhancer assays (often limited by use of irrelevant cell types), and to assays limited to chromatin accessibility alone. Additional insights suggest that TF-dependent ncRNA profiling in mature cells can identify developmental enhancers which may establish epigenetic memory, and identify TFs previously unknown to have rod/cone-specific functions. The data generated provide a refined resource for interrogation of rod and cone networks at the level of functional enhancer. This is a scholarly article that will be of specific interest to retinal biologists, and of broad relevance to transcriptional regulation. The embedded information has longer term relevance to therapeutic interventions to ameliorate retinal disease. I support publication after revisions.

Comments for the author

1. Page 7: paragraph 2, line 21: something missing from this sentence.

2. Would it be possible to quantify enhancer assays with a select number of elements including normalisation for transfection frequency. I think this would strengthen this section.

3. Is the model supported by the phenotype of CRX knockout mice?

4. In modelling, how do you distinguish between loss of positive specification of one cell fate and de-repression of the alternative cell fate, eg. in Nrl mutants. I imagine that in may cases the output is not so clean.

Reviewer 2

Advance summary and potential significance to field

This manuscript by Perez-Cervantes and colleagues examines the expression of ncRNAs from mouse retina tissue. By profiling all ncRNAs from wild type and Nrl-null tissue, the authors defined RNAs associated with rod and cone cells, based on the requirement of Nrl for rod formation. By intersecting the expression of Nrl-activated and Nrl-repressed ncRNA transcripts with ATAC-seq peaks from whole retina, rod, and cone tissue, the authors successfully and accurately predict enhancer elements associated with rods and cones. Several interesting bioinformatics analyses are presented, including an intersection of ncRNA expression as a surrogate for CREs with ChIP-seq datasets for NRL and for CRX (a pan-photoreceptor [rods and cones] transcription factor), which supports a combinatorial model for cell type specification in the retina.

This is a convincing study that supports the notion that ncRNA expression at regions of accessible chromatin can be used to identify cell type-specific cis-regulatory elements in the retina and, more generally, for identification of active enhancers. This study is appropriate for publication in Development and will be interesting to a wide segment of its readership. In two instances, the authors present conclusions that cannot be concluded from the data presented. Prior to publication, the authors should fully address my concerns about these two issues prior to publication.

Comments for the author

1) In the enhancer validation experiments presented in Fig. 4 (particularly in Fig. 4C), the authors make quantitative conclusions based on qualitative data. In these experiments, alkaline

phosphatase activity under the control of predicted CREs is examined visually in electroporated retinal explants, and the authors draw conclusions about which enhancers have the most activity, even suggesting the strongest enhancers in a sort of rank-order (e.g. “the three most active sites”). These data do not support a quantitative assessment. The authors must quantify AP activity and then express the data as a ratio of CRE-directed AP activity to the mCherry fluorescence generated from the control reporter (as a measure of electroporation efficiency) for a minimum of five electroporated retinas for each construct and conduct statistical analyses on the resultant data in order to draw conclusions about which elements are stronger than others. Alternatively, the authors should adjust the text to indicate that these data are qualitative and they should be careful to only conclude which enhancers are active (and in which cell types) and which are not. Indeed, I would prefer that the authors simply adjust the text to be more precise about their conclusions rather than adding the suggested experimental data, unless the authors already have the data in hand.

2) In the Discussion section on page 13, the authors discuss combinatorial regulation of CREs and state the following “…as demonstrated through our analysis of the effect of combinatorial TF action (Figure 3).” This is too strong an interpretation of the authors’ data. The authors never examine TF “action”. Rather TF binding is analyzed in the referenced experiments. TF action implies something about function beyond binding, especially since it is well known that many TF binding events are “non-productive”. I suggest changing the word “action” to “binding” in order to more accurately reflect what was actually examined in these studies.

Reviewer 3

Advance summary and potential significance to field

This is a descriptive study using ncRNA (enhancer RNA) expression in the retina to define enhancers in the developing mouse photoreceptors. Overall the work is well done and clearly presented; however, it is not clear that this has much improvement over other methods, such as ChIP for histone modifications or even accessibility alone for identifying putative enhancers.

This study would benefit greatly from a more detailed comparison with prior studies that identified photoreceptor enhancers with other methods to demonstrate that there are advantages/differences using ncRNA expression.
Specific concerns related to this and other issues are detailed below:

Comments for the author

Specific concerns related to this and other issues are detailed below:

1. The authors validated regions by showing motif enrichment for rod and cone specific TFs, but this has been previously demonstrated using accessibility alone and so it is not clear what has been gained by adding the ncRNAseq information. It would be useful to compare the enriched motifs from ATACseq or DNase1 alone versus overlapping the accessible regions with ncRNAseq data.

2. The authors state that additional TF sites were also found that were not previously reported in rods and cones, but these are less well enriched and are possibly just lower down the list in other reports. The authors could go back to the previous analyses of TF enrichment in ChIP-seq (Otx2/Crx/Nrl) and ATACseq for rods and cones and retinal DNase1 to see if these same motifs are present.

3. Are any of the ncRNA regions not present in the Crx ChIPseq?

4. The authors "observed that ncRNAs from regions with NRL and CRX binding in the wild-type retina had a larger decrease in ncRNA expression in the Nrl-/- retina compared to those from regions with either NRL or CRX binding alone." It seems like this statement is based on knowing which of these regions binds Nrl but the authors have assessed this indirectly by the changes in the Nrl -/- mouse; however, Nrl ChIPseq is available and they need to test this statement directly by comparing the regions with the Nrl ChIPseq.

5. The authors analyze putative Otx2 enhancer regions; a very similar analysis was done by Wilken et al (Wilken et al. DNase I hypersensitivity analysis of the mouse brain and retina identifies region-specific regulatory elements. Epigenetics Chromatin. 2015 Feb 28;8:8. doi: 10.1186/1756-8935-8-8. eCollection 2015. PubMed PMID: 25972927; PubMed Central PMCID: PMC4429822) using DNAse1 accessibility data several years ago. The authors should compare the results from this study with their own results to determine whether their results confirm the earlier study. they might also be able to show they get a better "hit" rate with the ncRNA approach in predicting functional enhancers that using DNAse1 accessibility alone, but this needs to be shown directly.

Minor comments:

1. The authors refer to regions that gain ncRNA expression in Nrl-/- retinas as "Nrl-repressed". Perhaps they can find another term for this since it implies direct repression, and the repression may instead be due to Nr2e3 (downstream of Nrl)

2. page 7, second to the last sentence on the page has some issues: accessibility is spelled incorrectly and the sentence seems to be missing some words.

First revision

Author response to reviewers' comments

November 8, 2019

Re: Reviewer response for manuscript MS ID#: DEVELOP/2019/184432

Dear Dr. Guillemot,

Thank you for your comments and careful consideration of our manuscript “Enhancer transcription identifies cis-regulatory elements for photoreceptor cell types”. We were pleased by the very positive feedback from the Reviewers. As requested, please find a point-by-point response to the Reviewer comments below. Addressing their comments with the inclusion of new analysis and editing of the text has significantly improved the manuscript.

Reviewer response for manuscript MS ID#: DEVELOP/2019/184432
Enhancer transcription identifies *cis*-regulatory elements for photoreceptor cell types

Thank you for your comments and careful consideration of our manuscript. We were pleased by the very positive feedback.

Comments regarding enhancer assay quantification:
Reviewer 1: “Would it be possible to quantify enhancer assays with a select number of elements including normalization for transfection frequency. I think this would strengthen this section.”
And
Reviewer 2: “In the enhancer validation experiments presented in Fig. 4 (particularly in Fig. 4C), the authors make quantitative conclusions based on qualitative data. In these experiments, alkaline phosphatase activity under the control of predicted CREs is examined visually in electroporated retinal explants, and the authors draw conclusions about which enhancers have the most activity, even suggesting the strongest enhancers in a sort of rank-order (e.g. “the three most active sites”). These data do not support a quantitative assessment. The authors must quantify AP activity and then express the data as a ratio of CRE-directed AP activity to the mCherry fluorescence generated from the control reporter (as a measure of electroporation efficiency) for a minimum of five electroporated retinas for each construct and conduct statistical analyses on the resultant data in order to draw conclusions about which elements are stronger than others. Alternatively, the

authors should adjust the text to indicate that these data are qualitative and they should be careful to only conclude which enhancers are active (and in which cell types) and which are not. Indeed, I would prefer that the authors simply adjust the text to be more precise about their conclusions rather than adding the suggested experimental data, unless the authors already have the data in hand."

Response:
We appreciate these comments and agree with both Reviewers that the data presented are qualitative and do not support quantitative conclusions. In fact, there are multiple concerns involved in the quantification of these enhancer assays that are difficult to control, beyond controlling for transfection rate. Perhaps the most important one is that the enhancer constructs are not tested in their endogenous genomic context. Therefore, it is inappropriate to compare enhancer activity between different enhancers assessed by different transfections or to a separate electroporation control, as they reflect distinct biological contexts. Following the suggestion from Reviewer 2 and the Editor, we have adjusted the text to address these concerns by describing our conclusions qualitatively and removing quantitative comments concerning relative enhancer activity.

Reviewer 1:
1. "Page 7: paragraph 2, line 21: something missing from this sentence."

Response:
Thank you for noting this error. We have corrected the text.

2. "Is the model supported by the phenotype of CRX knockout mice?"

Response:
We have previously shown that CRX deficient mice do not develop photoreceptor outer segment structures and have an extremely diminished ERG response (Furukawa et al, 1999). Therefore, because of the major decrement in mature photoreceptor cells in CRX mutants, analysis meaningful to our model cannot be performed in the CRX mutant.

3. "In modelling, how do you distinguish between loss of positive specification of one cell fate and de-repression of the alternative cell fate, eg in Nrl mutants. I imagine that in many cases the output is not so clean."

Response:
This interesting point is not well considered in the literature or in our manuscript. Our experiments identify a set of regulatory elements that nominate a subset of gene regulatory networks in adult photoreceptors, well after the rod vs cone decision. We have declined to model the fate determination events here, as our data do not provide specific insight into this fascinating process. The functional developmental perturbations and developmental analysis required to address this question are of great interest. Our previous work and that of others indicates that both repressive and activating events are necessary for cell fate specification events in the retina. For example, we analyzed developmental gene regulatory networks in the rod vs bipolar decision and observed feedback and feedforward transcriptional regulatory interactions (e.g. Wang et al, Dev Cell, 2014). We anticipate that the rod vs cone decision will be equally interesting and complex, and have begun such analyses (Emerson et al. Dev. Cell 26:59-72 (2013), and will follow up on this in future investigations.

Reviewer 2:
1. "In the Discussion section on page 13, the authors discuss combinatorial regulation of CREs and state the following "…as demonstrated through our analysis of the effect of combinatorial TF action (Figure 3)." This is too strong an interpretation of the authors' data. The authors never examine TF "action". Rather TF binding is analyzed in the referenced experiments. TF action implies something about function beyond binding, especially since it is well known that many TF binding events are "non-productive". I suggest changing the word "action" to "binding" in order to more accurately reflect what was actually examined in these studies."

Response:
We agree with this comment. We have changed the corresponding text in the results and discussion.

Reviewer 3:
1. “The authors validated regions by showing motif enrichment for rod and cone specific TFs, but this has been previously demonstrated using accessibility alone and so it is not clear what has been gained by adding the ncRNAseq information. It would be useful to compare the enriched motifs from ATACseq or DNase1 alone versus overlapping the accessible regions with ncRNAseq data.”
And
2. “The authors state that additional TF sites were also found that were not previously reported in rods and cones, but these are less well enriched and are possibly just lower down the list in other reports. The authors could go back to the previous analyses of TF enrichment in ChIP-seq (Otx2/Crx/Nrl) and ATACseq for rods and cones and retinal DNase1 to see if these same motifs are present.”

Response for both points:
We thank the Reviewer for both of these important points. To address them, we performed motif enrichment analysis on available DNase datasets (ENCFF040EOQ (7days) and ENCFF976MAY (8wks) and whole retina ATAC-seq in the wild-type and Nrl-/- conditions (Mo et al., 2016; Mouse et al., 2012). While most TF families are shared between these datasets, the addition of the ncRNA analysis specifically revealed the presence of the CUT and Six6 (TATCA, Helix-turn-Helix) motifs. The CUT motif protein Onecut1 (HNF6) has been identified as enriched in green cone promoters, however our study is the first to report its enrichment in retinal CREs (Hughes et al., 2017). We have added these results to the manuscript as Supplemental table 1.
Furthermore, we performed differential motif analysis between the open chromatin at ncRNA putative promoter regions versus open chromatin alone (datasets listed above). This analysis showed significant enrichment for the Six6 motif in the ncRNA-defined group, but not in the open chromatin datasets alone. These observations indicate that the ncRNA approach identified motifs whose cognate family members are novel candidates for participation in a relevant gene regulatory network. These results have been added to the results of the manuscript and in a new Supplemental Table 1.
3. “Are any of the ncRNA regions not present in the CRX ChIPseq?”

Response:
Yes, there are ncRNA regions not present in the CRX ChIPseq. We have clarified this in Figure 3 legend (explicitly describing the “none” bin, grey) as well as in the text (page 10). Consistent with our model, locations without CRX ChIP showed no clear trend in ncRNA expression, in contrast to sites that are bound by CRX in either the wild type or *Nrl*$^{-/-}$.

4. “The authors "observed that ncRNAs from regions with NRL and CRX binding in the wild-type retina had a larger decrease in ncRNA expression in the Nrl-/- retina compared to those from regions with either NRL or CRX binding alone." It seems like this statement is based on knowing which of these regions binds Nrl, but the authors have assessed this indirectly by the changes in the Nrl -/- mouse; however, Nrl ChIPseq is available and they need to test this statement directly by comparing the regions with the Nrl ChIPseq.”

Response:
In Figure 3C, we had utilized the NRL ChIP to describe binding categories for the ncRNAs. These categories take into account both NRL and CRX ChIP datasets in order to describe quantitative ncRNA output and deepen our understanding of the combinatorial interactions between NRL and CRX. We have edited the figure legend to illustrate this point more clearly.

5. “The authors analyze putative Otx2 enhancer regions; a very similar analysis was done by Wilken et al (Wilken et al. 2015) using DNAse1 accessibility data several years ago. The authors should compare the results from this study with their own results to determine whether their results confirm the earlier study. they might also be able to show they get a better "hit" rate with the ncRNA approach in predicting functional enhancers that using DNAse1 accessibility alone, but this needs to be shown directly.”

Response:
Indeed, Wilken et al. pioneered the use of DNase I HS data in the retina to identify active CREs, including at the Otx2 locus. Using an overlap of such regions with the presence of enhancer marker P300, the authors tested 7 regions for activity and identified 3 elements active in different cell populations of the retina. To our knowledge, the precise sequences of the regions tested are not available. Furthermore, the authors tested these regions at a later stage of development, P0, thus a direct comparison with our ncRNA-defined CREs is difficult as our analysis was conducted on embryonic tissue. None-the-less, based on the approximate coordinates and Figure 5 in Wilken et al., it is possible that our ncRNA1 corresponds to the DHS-2 element (active in photoreceptors) and ncRNA3 corresponds to the DHS-4 element (active in "the progenitor zone"). These data further corroborate the hypothesis that ncRNAs are enriched in enhancers active throughout development. We have updated the text to reflect this earlier work and have cited these results in our discussion.

6. "The authors refer to regions that gain ncRNA expression in Nrl-/- retinas as "Nrl-repressed". Perhaps they can find another term for this since it implies direct repression, and the repression may instead be due to Nr2e3 (downstream of Nrl)."

Response:
This is a critical distinction and we thank the Reviewer for pointing out that our previous language was not specific enough. We have clarified this in the text, by explicitly noting that "Nrl-repressed" includes transcripts that are reduced due to direct or indirect activities of NRL.

7. "Page 7, second to the last sentence on the page has some issues: accessibility is spelled incorrectly and the sentence seems to be missing some words."

Response:
We thank the Reviewer for noting this error. We have edited the text accordingly.

While we have edited the entire manuscript for clarity, we have highlighted regions in the revised manuscript that directly correspond to reviewer comments.

Thank you

Second decision letter

MS ID#: DEVELOP/2019/184432

MS TITLE: Enhancer transcription identifies *cis*-regulatory elements for photoreceptor cell types

AUTHORS: Carlos Perez-Cervantes, Linsin A. Smith, Rangarajan D. Nadadur, Andrew E. O. Hughes, Sui Wang, Joseph C. Corbo, Constance Cepko, Nicolas Lonfat, and Ivan P. Moskowitz
ARTICLE TYPE: Techniques and Resources Article

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