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A Single-Cell Transcriptome Atlas of the Adult Human Retina

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1st Editorial Decision 19th Mar 2019

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from their comments the referees find the analysis interesting and are overall supportive. They raise a number of constructive comments that I would like to ask you to address in a revised version. Let me know if we need to discuss any of them in more detail.

REFEREE REPORTS:

Referee #1:

This paper provides an extremely interesting dataset for neuroscientists and eye researchers. The authors generated a human neural retina transcriptome atlas by single cell RNA sequencing. The technical quality is very high and the paper is well written.

I have only two minor points:

1. I would advise the authors to tone down their assumptions that the longer post mortem culture time of the retinas reflect a similar process as human retinal degeneration by aging or gene mutations. Therefore, the MALAT1 story as mirror of rod degeneration should be described more carefully as "poitentially2 etc.

2. The microglia dataset needs a bit more attention. There is a new paper on brain microglia: Masuda et al, Nature. 2019 Feb 13. doi: 10.1038/s41586-019-0924-x.Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution.

The authors could discuss this paper in light of the retinal dataset.

Referee #2:

In this manuscript, the authors report the generation of a human neural retina transcriptome atlas based on single cell RNA sequencing (scRNA-seq) of 20000 cells from three donor retinas. Clustering of the transcripts identified from these cells identified 18 transcript clusters, 16 of which correspond to 9 retinal cell types. Seven of the cell types identified had single transcript clusters, wheras there were 6 clusters for rod photoreceptor cells, and 3 for bipolar cells. The majority of cells identified were rods (70%), consistent with prior reports. The 6 rod photoreceptor clusters could be condensed into two groups based on the level of expression of the long non-coding RNA MALAT1. The level of this RNA was shown to be reduced in association retinal samples with longer post-mortem times, suggesting it could be used as a marker of photoreceptor cell health. To demonstrate the value of the transcript atlas, the authors compared the transcript profile of photoreceptor cells with that of iPSC-derived cones reported in the literature. This showed that iPSC-derived cones are more similar to fetal cones than adult cones. The authors also compared the transcript profiles of glial cells with that of an established human Muller cell line, for which they generated additional scRNA-seq data. This comparison showed that the putative Muller cell line is more similar to astrocytes than Muller cells. The data presented will be of interest to the vision research community. Some experimental limitations need further consideration in the manuscript.

Specific comments
1. Via sequencing of 5 libraries from the three donor retinas, an average of 40000 sequence reads were obtained per cell, derived from approximately 1600 unique transcripts per cell. The authors indicate they are aware that this is a low depth of sampling. For example, data from the GTEx consortium and other large scale transcriptome studies show that individual cells typically express tens of thousands of transcripts. With 1600 transcripts detected from a relatively small number of reads, it appears the depth of sampling in the reported studies is low. It would be helpful for this issue to be discussed in the manuscript, both with regard to results and conclusions.

2. Along the same lines, further explanation regarding why 6 rod and 3 bipolar cell clusters were identified would be helpful. Is this due to under sampling of the transcripts, or does it reflect true diversity of the cell types? Were other clustering approaches tested?

3. The finding that the 6 rod photoreceptor clusters segregate into two groups based on MALAT1 levels is interesting, but also potentially problematic. The reduction in MALAT1 levels at 15 hours postmortem suggest that this length of post-mortem time is too long, with cell degeneration already in progress. How does this affect interpretation of all data, and the atlas created, since postmortem time ranged from 6 to 14 hours in the three samples that make up the core data set? This issue needs to be discussed in the manuscript.

4. The hypothesis that MALAT1 levels decrease in association with rod cell degeneration in the post-mortem period is interesting, and as a central part of the manuscript, warrants additional validation. It would be helpful to determine if the same is true in other models of rod cell death, either genetically or environmentally induced.

5. The single transcript cluster of cones appears to have some micro-heterogeneity, related to cone sub-type. The identification of genes with preferential expression in the cone sub-types is also potentially of interest, but also warrants further validation, at least for some of the differentially expressed cone sub-type genes.

6. The cell surface markers identified could be useful, as indicated. How did the ones identified correspond to prior reports regarding protein location? For example, RLBP1 is expressed in the retinal pigment epithelium (RPE) and Müller cells, and thus is not specific to Muller glia.

7. In order to make this data available to the wider community it would be helpful if the data could be presented in a more accessible manner than the raw data deposited in Array Express (this reviewer was not able to access the data at Array Express). For example, a supplementary table depicting the post analysis data per gene could allow readers to look up individual genes and assess

their contribution to the separation of the clusters.

8. The authors used Seurat for informatic analyses, from QC/QA to all downstream data processing and figure generation. However, there is not enough information provided to reproduce the results. It would be helpful to include more detailed descriptions of the steps used in the transcriptome analyses, such as how the QC/QA steps were performed, and how normalization was applied. Further, it is not clear how gene expression was assessed using STAR, which is an aligner.

Referee #3:

The study by Lukowski and co-authors describes the transcriptome analysis of single cell RNA sequencing on 20,009 cells isolated from three cadaveric retinae. The authors suggest that the data generated by this analysis could be used as a reference transcriptome atlas to compare stem-cell derived or primary retinal cells. They also suggest that their data identified a novel role of MALAT1 in rod degeneration. There are various issues that need to be considered in relation to the data analyses presented:
The description of the data is repetitive throughout the manuscript. It is also scattered throughout. This makes the reader going backwards and forwards to the same Figs and Tables, yet it is the same data represented in different forms. An example of this is the description of similarities between donor samples and library preparations (3rd paragraph results, page 4), which refers to the data shown in Suppl Table 2, Suppl Figs 3 and 4, and again in Fig 1B and Suppl Figs 3A and 3B. These Figs and Tables should be organized in a more simplified manner.
On page 5, 2nd paragraph, the authors describe the grouping of genes within cell types by performing differential gene expression analysis to identify marker genes for each cluster identified. They extracted 'membrane related proteins' from GO annotations, which they suggest could be used to identify surface markers for isolation of primary retinal cells, and is presented in Suppl Table 3. However, a large number of genes listed on this table are not membrane related proteins. This should be revised.
On the 2nd paragraph of page 5, the authors also state that they assessed the gene expression of a panel of common markers of amacrine and bipolar cells, which is shown in Suppl Figs 6A and 6B. Although some of the genes shown in these figures have been associated with amacrine and bipolar cells, they are not commonly known markers for these cells as described. Some of these genes are more commonly associated to retinal progenitors (ie, VSV2, OTX2 and SOX6). Other genes identified as specific markers of amacrine and bipolar cells, such as SERPINI1 and CASP7, code for proteases associated to many other cells, not only retinal cells. This should also be reviewed.
The authors state that they identified `a novel role for the MALAT1 gene in rod degeneration'. However, the expression of this gene is shown to be highly expressed in the INL and RGC layers. As judged by Figs 3D,E and 4, there also appears to be differences in its expression within these layers. Yet, the authors do not comment on the importance of this expression anywhere within the manuscript. In addition, the fact that the expression of MALAT1 appears to be associated with the length of time lapsing between death and post-mortem retina harvesting, it is not reasonable to suggest that this gene has a role in rod degeneration without doing functional studies.
Finally, as there are noticeable differences in the expression of genes between the three tissue donors analysed, it is not reasonable to suggest that the data obtained from these specimens can be used as a 'reference transcriptome atlas' to compare stem-cell derived or primary retinal cells'. More data with consistent gene expression would be needed for this purpose.

1st Revision - authors' response 14th Jun 2019

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I have only two minor points:

1. I would advise the authors to tone down their assumptions that the longer post mortem culture time of the retinas reflect a similar process as human retinal degeneration by aging or gene mutations. Therefore, the MALAT1 story as mirror of rod degeneration should be described more carefully as "potentially" etc.

[Response:] Thank you very much for this helpful suggestion. We have now modified the manuscript to tone down the description that longer post mortem culture potentially reflects rod degeneration.
The new modifications were made in the following sections:

- Summary section (page 2): *Notably, our data captured molecular profiles for healthy and putative early degenerating rod photoreceptors, and revealed the loss of MALAT1 expression with longer post-mortem time, which potentially suggested a novel role of MALAT1 in rod photoreceptor degeneration.*

- Results section (page 7): *As we utilised post-mortem retinal samples in this study, we reasoned that MALAT1-lo subpopulation may potentially reflect the early stages of post-mortem degeneration in rod photoreceptors…..Together, these results demonstrated that MALAT1 is a novel marker for healthy photoreceptors with loss of expression potentially preceding putative cell degeneration.*

- Discussion section (page 11): *Our results demonstrated the loss of MALAT1 expression in rod photoreceptors following longer post-mortem time with putative degeneration…*

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Masuda et al, Nature. 2019 Feb 13. doi: 10.1038/s41586-019-0924-x.Spatial and temporal heterogeneity of mouse and human microglia at single-cell resolution. The authors could discuss this paper in light of the retinal dataset.

[Response:] We thank the reviewer for pointing out this recent study. As suggested, we have incorporated new discussion of recent scRNAseq studies in the revised manuscript on page 12:

'With the identification of surface markers for these retinal cell types in this study, this work lays the foundation for future research using selection and enrichment (Shekhar et al, 2016) of these and other retinal cell types to improve the resolution of the human neural retina transcriptome atlas. Two recent studies have reported the use of surface marker to preselect or enrich for microglia (Masuda et al, 2019) and bipolar cells (Peng et al, 2019) in human tissues prior to scRNA-seq, which provided a feasible strategy to increase sensitivity to profile cell types less frequently represented.'

Referee #2:

In this manuscript, the authors report the generation of a human neural retina transcriptome atlas based on single cell RNA sequencing (scRNA-seq) of 20000 cells from three donor retinas. Clustering of the transcripts identified from these cells identified 18 transcript clusters, 16 of which correspond to 9 retinal cell types. Seven of the cell types identified had single transcript clusters, whereas there were 6 clusters for rod photoreceptor cells, and 3 for bipolar cells. The majority of cells identified were rods (70%), consistent with prior reports. The 6 rod photoreceptor clusters could be condensed into two groups based on the level of expression of the long non-coding RNA MALAT1. The level of this RNA was shown to be reduced in association retinal samples with longer post-mortem times, suggesting it could be used as a marker of photoreceptor cell health. To demonstrate the value of the transcript atlas, the authors compared the transcript profile of photoreceptor cells with that of iPSC-derived cones reported in the literature. This showed that iPSC-derived cones are more similar to fetal cones than adult cones. The authors also compared the transcript profiles of glial cells with that of an established human Muller cell line, for which they generated additional scRNA-seq data. This comparison showed that the putative Muller cell line is more similar to astrocytes than Muller cells. The data presented will be of interest to the vision research community. Some experimental limitations need further consideration in the manuscript.

Specific comments

1. Via sequencing of 5 libraries from the three donor retinas, an average of 40000 sequence reads were obtained per cell, derived from approximately 1600 unique transcripts per cell. The authors indicate they are aware that this is a low depth of sampling. For example, data from the GTEx consortium and other large scale transcriptome studies show that individual cells typically express tens of thousands of transcripts. With 1600 transcripts detected from a relatively small number of reads, it appears the depth of sampling in the reported studies is low. It would be helpful for this issue to be discussed in the manuscript, both with regard to results and conclusions.

[Response:] We thank the reviewer for this comment and we have addressed this point in the Discussion. Following the manufacturer's guidelines, we believe the sequencing depth we obtained is sufficient to robustly classify cell types by their gene expression signatures.

The number of transcripts (UMIs) and genes detected per cell is highly dependent on the sample and cell type mRNA content. The number of expressed transcripts reported in GTEx reflects the average expression of a large number of cells in a bulk sample; however, in single cells, the number of expressed transcripts is in fact much lower as not all genes are expressed at once. This is why the number of transcripts appears low and is not necessarily due to the sequencing depth. The biological reasons underlying this, as opposed to technical, are linked to transcriptional bursting, cell cycle, and mRNA abundance and stability. Most cells in the retina are not highly transcriptionally active and are non-proliferative (e.g. postmitotic retinal neurons). For our retina data, we do not expect to see high numbers of UMIs or genes detected per cell. In studies using scRNA-seq to investigate the properties of induced pluripotent stem cells, for example, the cells are more actively transcribing and this is reflected in the UMI and number of genes relative to the read depth (Nguyen and Lukowski et al 2018).

We acknowledge that our explanation regarding this was unclear and have amended the Discussion text to reflect this. Specifically, we have modified the following text in the Discussion on page 10:

"We obtained a mean sequencing depth of 40,232 reads per cell across 23,000 cells, which enabled us to confidently classify the majority of cell types in a complex tissue like the retina. We confirmed that this sequencing depth is sufficient to identify the major cell types. For less transcriptionally distinct cell types, including amacrine and retinal ganglion cells, the ability to resolve subtypes might be improved by increased sample size, greater cell numbers or ultra-deep sequencing of those populations."

In our manuscript, we note there is also the potential for confusion between the expected read depth for different scRNA-seq platforms, such as between the Fluidigm C1 and the 10x Genomics Chromium. In the literature, Fluidigm-based studies have reported > 100,000 reads per cell, which is necessary for the Fluidigm technology since it captures the full length transcripts and requires deeper sequencing. This is different for the 3'-based assays that only sequence a small section of the 3' end. As such, we also removed the words *'relatively low level of sequencing depth'* from the Discussion.

2. Along the same lines, further explanation regarding why 6 rod and 3 bipolar cell clusters were identified would be helpful. Is this due to under sampling of the transcripts, or does it reflect true diversity of the cell types? Were other clustering approaches tested?

[Response:] We thank the reviewer and agree that this should be explained in greater detail. In our analysis, we identified the six rod clusters were subject to donor-related batch effects. We performed canonical correlation analysis (CCA) to overcome this batch effect, which then reduced the rod clusters to three - two major clusters and one minor cluster. The two major clusters, delineated by MALAT1 expression (MALAT1-high or -low), did not show any evidence of donor-related technical effects. The minor cluster (CCA10) represents low quality cells with high mitochondrial gene expression and is excluded from further analysis. Further examination of the MALAT1 expression in the rod clusters, CCA0, CCA1 and CCA10 showed all populations have robust and abundant MALAT1 expression, albeit at differing levels (Figure 3E in the manuscript), but also have some cells with low (near-zero) expression. The strongly detected MALAT1 signal, both in terms of abundance and number of positive cells, in our data indicates sufficient sequencing depth

was obtained. As a result, the near-zero expression is not related to undersampling from shallow sequencing, but rather real stochastic and/or inter-individual expression differences.

Regarding the three bipolar cell clusters, these clusters were not separated due to donor-related technical effects but due to gene expression profiles, as they are well represented in all donor samples (Figure 1A, B). There are at least 10 subtypes of mammalian retinal bipolar cells (Euler et al, PMID 25158357) and we believe that the three populations identified in our data globally represent these subtypes. Using known genetic markers for bipolar cells (Shekhar *et al* 2016 PMID 27565351), we examined the 3 bipolar cell populations to see if there was true diversity. We found distinct transcriptional profiles for these bipolar cells, C6 represents OFF-bipolar cells (GRIK1^{+}), C8 and C11 represents ON-bipolar cells (ISL1^{+}). Further analysis showed that C8 represents rod bipolar cells based on the marker PRKCA, while C11 express the marker TTR corresponding to a subtype of diffuse bipolar cells (DB4) (Peng et al. 2019 PMID 30712875). These data reflects the true diversity of the bipolar populations as opposed to undersampled transcriptomes. We also acknowledged that increasing the number of profiled bipolar cells would allow more comprehensive classification of bipolar subtypes. We have added this new data (Figure EV1) and new discussion on page 5:

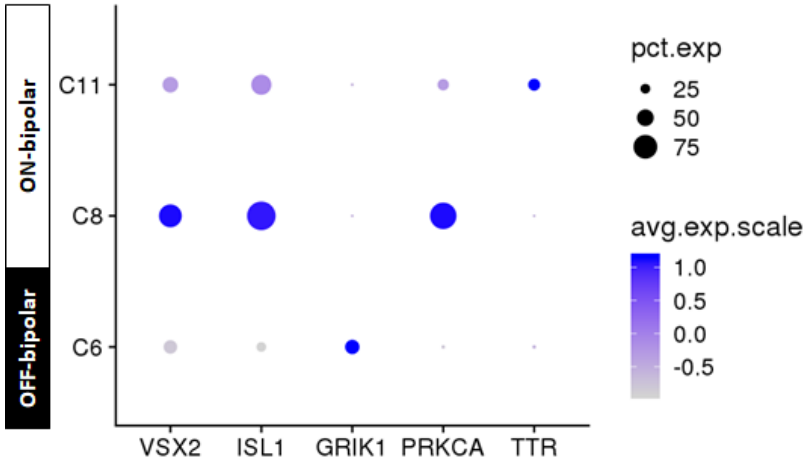

Figure EV1: Bipolar marker gene expression in the compiled human neural retina transcriptome atlas (20,009 cells), as shown by feature expression heatmap of *VSX2* (pan-bipolar), *ISL1* (ON-bipolar), *GRIK1* (OFF-bipolar), *PRKCA* (rod bipolar cells) and *TTR* (DB4 bipolar).

New discussion (page 5).

'In particular, the bipolar clusters can be classified as OFF-bipolar cells (GRIK1^{+}: C6) and ON-bipolar cells (ISL1^{+}: C8, C11). Further analysis showed that C8 represents rod bipolar cells based on the marker PRKCA, while C11 expresses the marker TTR corresponding to a diffuse bipolar subtype DB4 (Figure EV1A).'

In addition to the current graph-based clustering approach implemented in Seurat, we used unsupervised hierarchical clustering in the ascend pipeline. Hierarchical clustering was not able to resolve the cell types in our data, therefore, we did not pursue this analysis further.

3. The finding that the 6 rod photoreceptor clusters segregate into two groups based on MALAT1 levels is interesting, but also potentially problematic. The reduction in MALAT1 levels at 15 hours postmortem suggest that this length of post-mortem time is too long, with cell degeneration already in progress. How does this affect interpretation of all data, and the atlas created, since postmortem time ranged from 6 to 14 hours in the three samples that make up the core data set? This issue needs to be discussed in the manuscript.

[Response:] We thank the reviewer for this constructive feedback and we have added new discussion to clarify this issue. We observed comparable level of cell viability across 5 retinal samples retrieved within 4-15 hours post-mortem (Appendix Figure S1A), therefore we used 15 hours as a cutoff retrieval time, and 3 retinal samples with 6, 11 and 14 hours postmortem are used for scRNAseq. With the exception of the rod photoreceptors, it is important to note that clusters for all the major retinal cell types are well represented for all 3 donor retina. This suggested that at the transcriptome levels there are no obvious variations (i.e. degeneration) in most of the retinal cell types in retina retrieved from 6-14 hours (except rods), which supported the quality of our dataset.

On the other hand, our data indicated differences in rod photoreceptors isolated at different post-mortem time, which potentially reflects various degrees of rod degeneration. Interestingly, this potentially suggested that the rod photoreceptors are more sensitive to post-mortem degeneration compared to other retinal cell types. Our FISH results showed that with short retrieval time the majority of rod photoreceptors express high levels of MALAT1; in contrast, using the same retinal sample, we showed that loss of MALAT1 expression is observed in rod photoreceptors with longer retrieval time (Figure 4 in manuscript). These results supported that MALAT1 expression can be used to identify putative healthy rod photoreceptors (MALAT1-high) or early degenerating rod photoreceptor post-mortem (MALAT1-low).

It is important to note that all 3 retinal samples used for scRNAseq contained putative healthy rod photoreceptors (MALAT1-high, ranging from ~40-90%, Figure EV3B below), thus the core dataset reported here is representative of rod photoreceptors from 3 individuals. To simplify the transcriptome analysis for bona fide rod photoreceptors, we performed canonical correlation analysis to eliminate donor or batch variations, and identified 1 cluster of bona fide rod photoreceptors (CCA1, MALAT1-high, Figure 3 in manuscript) which is represented in 3 retinal samples. We also identified another 1 cluster of putative degenerating rod photoreceptors (CCA0, MALAT1-low), as well as another rod photoreceptor cluster (CCA10) consisted of low quality cells which was excluded from further analysis.

In the revised manuscript, we have added the following discussion related to this topic (page 10):

'Also regarding post-mortem time for the donor retina, we found that at the transcriptome levels there are no obvious variations in all major cell types in retina retrieved from 6-14 hours postmortem, with the exception of rod photoreceptors. This potentially suggested that the rod photoreceptors are more sensitive to putative post-mortem degeneration compared to other retinal cell types. Further studies to optimise methods to preserve donor retinal tissues will help to minimize post-mortem effects prior to scRNA-seq processing.'

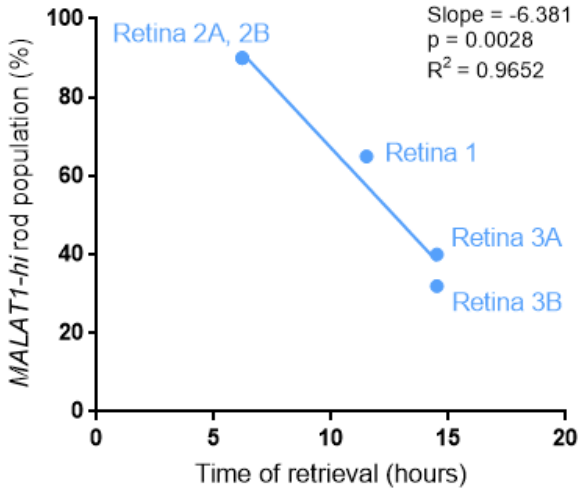

Figure EV3B: Proportion of MALAT1-high rod photoreceptors are negatively correlated with longer retrieval time in 3 retinal samples (5 libraries) used for the core scRNAseq dataset.

4. The hypothesis that MALAT1 levels decrease in association with rod cell degeneration in the post-mortem period is interesting, and as a central part of the manuscript, warrants additional

validation. It would be helpful to determine if the same is true in other models of rod cell death, either genetically or environmentally induced.

[Response:] Thank you very much for this helpful feedback. We agree that MALAT1's potential role in rod cell degeneration is an interesting finding and warrant further research beyond the scope of this study.
This is in part due to the fact that primary donor retina samples with short retrieval time are not readily available in Australia, in particular retinas from patients with genetic diseases that cause photoreceptor degeneration are extremely rare (e.g. retinitis pigmentosa). Also, retinal samples with short retrieval time would be required to environmentally induced rod degeneration, thus ruling out the possibilities of seeking delivery of retinal samples outside of Australia.

Therefore, as suggested by reviewer 1 (Q1) to 'tone down the assumptions that the longer post mortem culture time of the retinas reflect a similar process as human retinal degeneration by aging or gene mutations', we have revised the manuscript to better describe our findings that the loss of MALAT1 in rods with longer post-mortem time (Figure 4), which potentially reflects rod cell degeneration.

The new modifications were made in the following sections:

- Summary section (page 2): *Notably, our data captured molecular profiles for healthy and putative early degenerating rod photoreceptors, and revealed the loss of MALAT1 expression with longer post-mortem time, which potentially suggested a novel role of MALAT1 in rod photoreceptor degeneration.*

- Results section (page 7): *As we utilised post-mortem retinal samples in this study, we reasoned that MALAT1-lo subpopulation may potentially reflect the early stages of post-mortem degeneration in rod photoreceptors…..Together, these results demonstrated that MALAT1 is a novel marker for healthy photoreceptors with loss of expression potentially preceding putative cell degeneration.*

- Discussion section (page 11): *Our results demonstrated the loss of MALAT1 expression in rod photoreceptors following longer post-mortem time with putative degeneration…*

5. The single transcript cluster of cones appears to have some micro-heterogeneity, related to cone sub-type. The identification of genes with preferential expression in the cone sub-types is also potentially of interest, but also warrants further validation, at least for some of the differentially expressed cone sub-type genes.

[Response:] We agree that the observed heterogeneity in the cone population may represent cone subtypes and that determining the nature of this cluster would be interesting. The 3 cone subtypes can be identified by opsin expression. We are able to identify S-cones based on OPN1SW expression, however we are unable to distinguish distinguish L and M cones, since OPN1LW and OPN1MW are highly similar in sequence and cannot be readily distinguished using 3' scRNA-seq.

One approach for dissecting this microheterogeneity is to isolate the cone cells, recluster them and perform differential expression between the new cone sub-clusters. This should allow a higher resolution insight into the composition of cone cells. We performed an additional analysis on the cone cell subset to examine the microheterogeneity of the cone cluster and we detected 4 clusters within the cone population (Figure A). Although we performed differential expression analysis between each cluster and remaining cells, we could not confidently further classify the cone subpopulations, possibly due to the limited number of profiled cones in this study (564 cones).

[Figure for reviewers removed]

Regarding validation of cone subtype genes identified in this study, we have added new analysis and discussion in the revised manuscript (page 8):

'We compared this list of cone subtype genes to those identified in scRNA-seq studies of the macaque and mouse retina (Macosko et al. 2015; Peng et al. 2019), and showed that a number of

the cone subtype genes in human are conserved in macaque and mouse, including S-cone marker CCDC136 and L/M-cone marker THRB. Interestingly, CCDC136 is located next to the OPN1SW locus in human and could possibly be co-regulated at the transcriptional level. The thyroid hormone receptor THRB is required for the development of M-cones in mice (Ng et al. 2001) and L/M cones in humans as determined by pluripotent stem cell model (Eldred et al. 2018). Notably, there are two known receptor isoforms for THRB (TRβ1 and TRβ2) and further research to determine the roles of THRB isoforms in subtype specification of human cones would be of great interest. Moveover, the transcription factor TBX2 has been implicated in subtype specification of Sws1-cones in zebrafish and chicken (Alvarez-Delfin et al. 2009; Enright et al. 2015). In support of these studies, our data showed that TBX2 marks the S-cones in human which is also conserved in macaque (Peng et al. 2019).'

Given the known developmental role for *THRB* in specification of L/M cones in mammals, we also performed several immunostaining attempts to validate *THRB* as a L/M cone marker in human. *THRB* encodes for two known isoforms of thyroid hormone receptor: TRβ1 and TRβ2. We performed immunostaining using an established antibody against TRβ1 (Thermo Scientific, #MA1216) and we did not detect any expression for TRβ1 in human retina (Figure B). On the other hand, there is no commercially available antibody specifically for TRβ2. Thus it is possible that TRβ2 is the expressed isoform of *THRB* in cone photoreceptors and further research beyond the scope of this study would be needed to determine the precise roles of the two *THRB* isoforms in specifying cone subtypes in human. We have added new discussion of the two THRB isoforms to the revised manuscript.

[Figure for reviewers removed]

6. The cell surface markers identified could be useful, as indicated. How did the ones identified correspond to prior reports regarding protein location? For example, RLBP1 is expressed in the retinal pigment epithelium (RPE) and Müller cells, and thus is not specific to Muller glia.

[Response:] Thank you for this helpful comment. Indeed, our dataset on the neural retina samples excluded the RPE, and RLBP1 was identified to be differentially expressed in Muller glia compared to other cell types in the neural retina. As the reviewer pointed out, indeed RLBP1 is known to be expressed in RPE too. The membrane-related markers we identified included known retinal cell markers (e.g. Rod: RHO, ROM1, CNGA1, CNGB1; cone: SLC24A2, OPN1LW; Muller glia: RGR, RLBP1; Bipolar cells: TRPM1, GRM6; Microglia: CD74, TYROBP; RGC: YWHAH), as well as new marker genes that are not well studied in retina.

We acknowledge that some cell types will require a combination of multiple markers to accurately distinguish them. The ability to identify new cell type/state markers using unbiased approaches is one of the strengths of scRNA-seq. For highly similar cell (sub)-types, or for cell types that share expressed protein markers (e.g. RPE and Muller glia), a single protein or gene marker is unlikely to provide sufficient classification power. Classifying cells based on gradient expression of multiple known or novel gene or protein markers provides the desired level of granularity. In practice, this could be achieved using RNA-FISH coupled with immunohistochemical methods.

7. In order to make this data available to the wider community it would be helpful if the data could be presented in a more accessible manner than the raw data deposited in Array Express (this reviewer was not able to access the data at Array Express). For example, a supplementary table depicting the post analysis data per gene could allow readers to look up individual genes and assess their contribution to the separation of the clusters.

[Response:] We thank the reviewer for pointing out the accessibility of the ArrayExpress data and apologise for not providing proper access at the time of review. The data we uploaded consists of raw (FASTQ) and processed data (count matrices). In the revised manuscript, we have included 2 new supplementary datasets summarising the Average Gene Expression per Cluster for each gene in the dataset (Dataset EV1 and EV2).

8. The authors used Seurat for informatic analyses, from QC/QA to all downstream data processing and figure generation. However, there is not enough information provided to reproduce the results. It

would be helpful to include more detailed descriptions of the steps used in the transcriptome analyses, such as how the QC/QA steps were performed, and how normalization was applied. Further, it is not clear how gene expression was assessed using STAR, which is an aligner.

[Response:] We thank the reviewer for this helpful comment and, where possible, have clarified the text in the Methods section. We have modified the Methods section to include more detail in the steps we used for our analysis. This includes descriptions of the QC, filtering and normalization steps. With regards to the STAR software, since it is provided by 10x Genomics in the cellranger software package, it was only used within the standard processing pipeline as the read alignment tool. It was not used to assess gene expression. We have amended the Bioinformatics processing section in the Methods to clarify these points.

Referee #3:

The study by Lukowski and co-authors describes the transcriptome analysis of single cell RNA sequencing on 20,009 cells isolated from three cadaveric retinae. The authors suggest that the data generated by this analysis could be used as a reference transcriptome atlas to compare stem-cell derived or primary retinal cells. They also suggest that their data identified a novel role of MALAT1 in rod degeneration.

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The description of the data is repetitive throughout the manuscript. It is also scattered throughout. This makes the reader going backwards and forwards to the same Figs and Tables, yet it is the same data represented in different forms. An example of this is the description of similarities between donor samples and library preparations (3rd paragraph results, page 4), which refers to the data shown in Suppl Table 2, Suppl Figs 3 and 4, and again in Fig 1B and Suppl Figs 3A and 3B. These Figs and Tables should be organized in a more simplified manner.

[Response:] Thank you very much for this constructive feedback. As requested, we have reorganised the figures to improve readability in the revised manuscript. We have selected 5 supplementary figures that are more important and organized them into Expanded View Figures (Figure EV 1-5), the rest of supplementary figures went to the appendix. Also, two supplementary figures have been combined (suppl figure 3 + 4 into new Figure EV3) and reorganised. we have removed a figure (previously suppl figure 3B) as it's a different graphical representation of Appendix figure S3A. We have also modified the text to simplify references to figures and Appendix figures, including notable changes in first result section (page 4) and last results section (page 9-10)

2. On page 5, 2nd paragraph, the authors describe the grouping of genes within cell types by performing differential gene expression analysis to identify marker genes for each cluster identified. They extracted 'membrane related proteins' from GO annotations, which they suggest could be used to identify surface markers for isolation of primary retinal cells, and is presented in Suppl Table 3. However, a large number of genes listed on this table are not membrane related proteins. This should be revised.

[Response:] We thank the reviewer for this comment and we have modified Appendix table S3 (previously Suppl Table 3) to address this comment. In our analysis, we extracted membrane-related genes from the GO annotation. Based on the reviewer's comment, we re-examined these genes in the context of their utility as cell type-specific markers and acknowledge that several genes are not specific transmembrane proteins. We also noted that the second column in Appendix table S3 refers to 'identified surface markers'. Our re-examination of the genes in this category clarified that, in addition to cell surface proteins, it may also include proteins interacting with the plasma membrane or those involved in membrane trafficking. In Appendix table S3, we determined that up to 8 out of 49 unique proteins may have this alternative role but be classified as 'membrane-related'. These specific proteins have been marked in the table with an asterisk to denote this.

3. On the 2nd paragraph of page 5, the authors also state that they assessed the gene expression of a panel of common markers of amacrine and bipolar cells, which is shown in Suppl Figs 6A and 6B. Although some of the genes shown in these figures have been associated with amacrine and bipolar

cells, they are not commonly known markers for these cells as described. Some of these genes are more commonly associated to retinal progenitors (ie, VSV2, OTX2 and SOX6). Other genes identified as specific markers of amacrine and bipolar cells, such as SERPINI1 and CASP7, code for proteases associated to many other cells, not only retinal cells. This should also be reviewed.

[Response:] Thank you for this helpful comment. We acknowledge that some cell types, particularly those with different subtypes, will require multiple markers to accurately distinguish them. There is a growing need to use combinatorial marker classification, including specifying cell with low or high expression of shared markers. We have added the following text to the Discussion on page 12:

"Furthermore, we determined that multiple genetic markers, based on binary and/or gradient expression profiles, were required to improve the classification of clustered cell populations. More detailed classification of highly similar cell types may be possible through the combination of single cell mRNA and protein measurements using barcoded antibodies, as implemented in the CITE-seq method."

We have also performed new analysis to further classify the bipolar clusters (Reviewer 1, Q2). Using known genetic markers for bipolar cells (Shekhar *et al* 2016 PMID 27565351), we examined the 3 bipolar cell populations to see if there was true diversity. We found distinct transcriptional profiles for these bipolar cells, C6 represents OFF-bipolar cells (GRIK1$^+$), C8 and C11 represents ON-bipolar cells (ISL1$^+$). Further analysis showed that C8 represents rod bipolar cells based on the marker PRKCA, while C11 express the marker TTR corresponding to a subtype of diffuse bipolar cells (DB4) (Peng et al. 2019 PMID 30712875).

We have added this new data (Figure EV1) and new discussion on page 5:

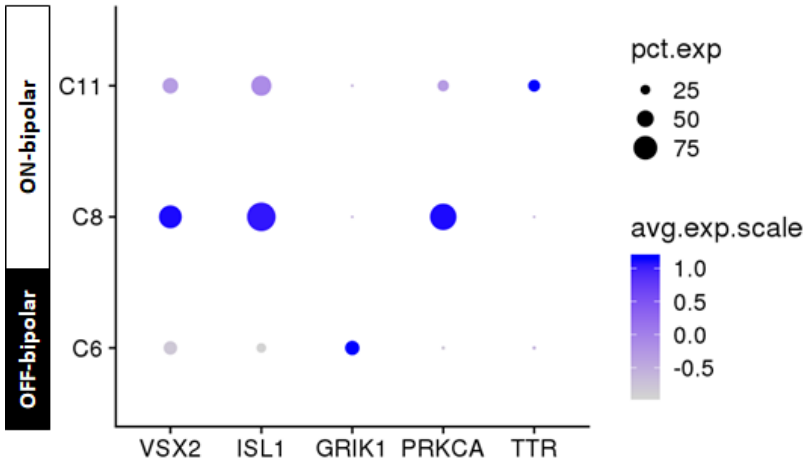

Figure EV1A: Bipolar marker gene expression in the compiled human neural retina transcriptome atlas (20,009 cells), as shown by feature expression heatmap of *VSX2* (pan-bipolar), *ISL1* (ON-bipolar), *GRIK1* (OFF-bipolar), *PRKCA* (rod bipolar cells) and *TTR* (DB4 bipolar).

New discussion (page 5).

'In particular, the bipolar clusters can be classified as OFF-bipolar cells (GRIK1$^+$: C6) and ON-bipolar cells (ISL1$^+$: C8, C11). Further analysis showed that C8 represents rod bipolar cells based on the marker PRKCA, while C11 expresses the marker TTR corresponding to a diffuse bipolar subtype DB4 (Figure EV1A).'

4. The authors state that they identified `a novel role for the MALAT1 gene in rod degeneration'. However, the expression of this gene is shown to be highly expressed in the INL and RGC layers. As judged by Figs 3D,E and 4, there also appears to be differences in its expression within these

layers. Yet, the authors do not comment on the importance of this expression anywhere within the manuscript. In addition, the fact that the expression of MALAT1 appears to be associated with the length of time lapsing between death and post-mortem retina harvesting, it is not reasonable to suggest that this gene has a role in rod degeneration without doing functional studies.

[Response:] Thank you for this comment, we have included new discussions related to these topics.

Indeed, our results indicated there is some heterogeneous MALAT1 expression in other retina cell types. The function of MALAT1 in retina is largely understudied. Previous studies have demonstrated a role of *MALAT1* in regulating the survival of retinal ganglion cells (Li *et al*, 2017) and in pathogenesis of retinal pigment epithelium cells (Yang *et al*, 2016). Future studies are warranted to investigate the functional role of *MALAT1* in different retinal cell types, including rod photoreceptors. We have included this discussion in the revised manuscript (page 10).

'We also noted that there is some heterogenous MALAT1 expression in other retinal cell types in human, albeit to a lesser extent compared to rod photoreceptors. Previous studies have demonstrated a role of MALAT1 in regulating the survival of retinal ganglion cells (Li et al, 2017) and in pathogenesis of retinal pigment epithelium cells (Yang et al, 2016). However, the functional role of MALAT1 in photoreceptors remained unclear. Our results demonstrated that loss of MALAT1 expression in rod photoreceptors following longer post-mortem time with putative degeneration, and suggests MALAT1 as a potential target to enhance rod photoreceptor survival and retinal preservation. Future studies are warranted to investigate the functional role of MALAT1 in photoreceptors, as well as other retinal cell types in human.'

In relation to *MALAT1*'s potential role in rod degeneration, we believe this is an interesting finding that warrant further research beyond the scope of this study. This is in part due to the fact that primary donor retina samples with short retrieval time are not readily available in Australia, in particular retinas from patients with genetic diseases that cause photoreceptor degeneration are extremely rare (e.g. retinitis pigmentosa). Also, retinal samples with short retrieval time would be required to environmentally induced rod degeneration, thus ruling out the possibilities of seeking delivery of retinal samples outside of Australia.

Therefore, as suggested by reviewer 1 (Q1) to 'tone down the assumptions that the longer post mortem culture time of the retinas reflect a similar process as human retinal degeneration by aging or gene mutations', we have revised the manuscript to better describe our findings that the loss of MALAT1 in rods with longer post-mortem time (Figure 4), which potentially reflects rod cell degeneration.

The new modifications were made in the following sections:

- Summary section (page 2): *Notably, our data captured molecular profiles for healthy and putative early degenerating rod photoreceptors, and revealed the loss of MALAT1 expression with longer post-mortem time, which potentially suggested a novel role of MALAT1 in rod photoreceptor degeneration.*

- Results section (page 7): *As we utilised post-mortem retinal samples in this study, we reasoned that MALAT1-lo subpopulation may potentially reflect the early stages of post-mortem degeneration in rod photoreceptors…..Together, these results demonstrated that MALAT1 is a novel marker for healthy photoreceptors with loss of expression potentially preceding putative cell degeneration.*

- Discussion section (page 11): *Our results demonstrated the loss of MALAT1 expression in rod photoreceptors following longer post-mortem time with putative degeneration…*

We also include new discussion related to post-mortem time in the Discussion section on page 10:

'Also regarding post-mortem time for the donor retina, we found that at the transcriptome levels there are no obvious variations in all major cell types in retina retrieved from 6-14 hours postmortem, with the exception of rod photoreceptors. This potentially suggested that the rod photoreceptors are more sensitive to putative post-mortem degeneration compared to other retinal

cell types. Further studies to optimise methods to preserve donor retinal tissues will help to minimize post-mortem effects prior to scRNA-seq processing.'

5. Finally, as there are noticeable differences in the expression of genes between the three tissue donors analysed, it is not reasonable to suggest that the data obtained from these specimens can be used as a 'reference transcriptome atlas' to compare stem-cell derived or primary retinal cells'. More data with consistent gene expression would be needed for this purpose.

[Response:] We agree with the reviewer that the optimal transcriptome atlas would contain many samples for a large number of donors. However, our data is of unprecedented resolution, consisting of >20,000 transcriptomes from individual cells and is currently the largest adult human retina dataset. Importantly, we found that at the transcriptome levels there are no obvious variations in all major cell types in the retina from all 3 donors, with the exception of rod photoreceptors. The donor variation is only observed in rod photoreceptors, which we could correct computationally using canonical correlation analysis. For benchmarking external datasets, we believe our dataset is a powerful and accurate resource. We anticipate that additional donors and improved capture technologies will vastly improve the atlas by allowing more accurate cell type classification and greater statistical power for determining differences between populations.

We have added new discussion related to this topic on page 12:

'Future studies to increase the donor sample size, number of profiled cells with improved capture technologies will further improve the resolution of this human retina transcriptome atlas, allowing more accurate cell type classification and greater statistical power to determine molecular differences between cell populations.'

2nd Editorial Decision 24th Jul 2019

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by the original referee #3 and I have now received the comments back. As you can see below. the referee appreciates the introduced changes and before proceeding with the formal acceptance there are just a few things to sort out.

REFEREE REPORTS:

Referee #3:

The manuscript is interesting and presents important data that can be of benefit to other investigators in the field. The manuscript has significantly improved after corrections suggested by Reviewers.

2nd Revision - authors' response 29th Jul 2019

The authors performed the requested editorial changes.

3rd Editorial Decision 31st Jul 2019

Thanks for sending me the revised version. Everything looks good and I am pleased to accept the manuscript for publication here.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Raymond Wong
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2018-100811

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The core scRNASeq dataset was generated using 5 libraries from 3 retina donor samples, which would allow us to assess donor variations. Thie final analysed dataset comprised 20,009 cells, each of which has its individual transcriptome sequenced.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	n/a
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	n/a
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	n/a
For animal studies, include a statement about randomization even if no randomization was used.	n/a
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	We used an unbiased and unsupervised clustering method to group the cells into their respective populations. The algorithms used were part of the Seurat package used for analysis, published as Butler et al 2018, Nature Biotechnology.
4.b. For animal studies, include a statement about blinding even if no blinding was done	n/a
5. For every figure, are statistical tests justified as appropriate?	n/a
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The scRNA-seq data fit a negative binomial distribution. The tests used in this analysis, including differential expression, took this into account, and elsewhere, non-parametric tests were used for analysis.

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Is there an estimate of variation within each group of data?	yes, the identified cell populations (clusters) captured a minimum of ~40 cells - ~4000 cells. For gene expression, we used a feature expression heatmap to display the proportion of cells expressing a particular gene.
Is the variance similar between the groups that are being statistically compared?	n/a

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	n/a
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	MIO-M1 was purchased from XIP and was cultured for several passages. Given that it was not culture for prolonged period, no authentication or mycoplasma testing was performed.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	n/a
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	n/a
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	n/a

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Collection of patient samples was approved by the Human Research Ethics committee of the Royal Victorian Eye and Ear Hospital (HREC13/1151H) and Save Sight Institute (16/282) and carried out in accordance with the approved guidelines.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes, informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n/a
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n/a
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	n/a

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The raw and processed scRNA-seq files for this analysis are available at ArrayExpress under the accession number E-MTAB-7316.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	The raw and processed scRNA-seq files for this analysis are available at ArrayExpress under the accession number E-MTAB-7316.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n/a
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	n/a

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No