Expression status transition of *NOTCH1* accompanies chromatin remodeling in human early retinal progenitor cells

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Abstract

Objective: The regulation of receptor expression is crucial for fine-tuned signal transduction. Notch signaling is a key signaling pathway involved in retinal development. Although the involvement of this signaling pathway in the differentiation of retinal ganglion cells has been documented, less is known about its involvement in earlier stages of retinal progenitor cell differentiation. To clarify the timing of Notch receptor expression in undifferentiated retinal progenitor cells and elucidate the possible involvement of chromatin remodeling in the regulation of Notch receptor expressions, we re-analyzed publicly available human fetal retina single-cell RNA-seq and ATAC-seq data.

Results: On days 59, 74, and 78, we observed *NOTCH1* mRNA expression in early retinal progenitor cells, which diminished at later stages of differentiation. Integration of single-cell RNA-seq and ATAC-seq revealed that chromatin remodeling in part of the *NOTCH1* locus was accompanied by transitions in its mRNA expression. Importantly, chromatin accessibility in the region upstream of *NOTCH1* depended on the differentiated cell types. These results suggest that chromatin remodeling may be involved in the differential expression of *NOTCH1*, although another type of Notch mRNA

expression regulation may exist.

Keywords: differentiation, epigenetics, eye development, single-cell ATAC-seq, single-cell RNA-seq

Introduction

Signal transduction depends on the expression of receptors regulated at multiple levels. These regulations include chromatin remodeling and DNA-binding proteins, such as transcription factors and transcriptional repressors. Because fine-tuned signal transduction is necessary for development, it is important to clarify the regulatory mechanisms of receptor expression.

Notch signaling in mammals is dependent on the binding of five canonical DSL ligands and four Notch receptors [1]. It has been suggested that Notch loci are subject to chromatin remodeling under both normal [2-7] and pathological [8-16] conditions. In terms of developmental biology, the retina is a good model for investigating cellular differentiation involving Notch signaling [17]. The developed retina is composed of multiple cell types, including retinal ganglion cells, bipolar cells, photoreceptors, amacrine cells, horizontal cells, and Müllar glial cells, all of which originate from the retinal progenitor cells (RPCs). RPC is characterized by the expression of *Lhx2*, *Pax6*, *Rax*, and *Vsx2* while there are some additional marker genes for developing horizontal cells/retinal ganglion cells (*Onecut1/2*) [18], developing amacrine cells (*Elavl* genes) [19], developing photoreceptors/amacrine cells/Müllar glial cells (*Eef1a1*) [20], developing retinal ganglion cells (*Meis2*) [21], developing horizontal cells (*Lhx1*,

Ptf1a) [22], and glial cells (*Pax2*) [23]. Of the four Notch receptors in mammals, Notch1, has been suggested to be involved in the maintenance of RPCs and differentiation into retinal ganglion cells [24]. However, it is unclear when and how Notch receptor expression is switched on and off in RPCs during early differentiation. Here, we re-analyzed a public multi-omics dataset of single-cell RNA-seq and single-cell ATAC-seq from three human fetal retinas [25] to clarify the timing of Notch receptor expression and examine the involvement of chromatin remodeling in this receptor expression switch.

Methods

A single-cell multi-omics dataset (GSE183684) [25] was downloaded from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/); data from days 59, 74, and 78 in the dataset were selectively used because they contained many undifferentiated retinal progenitor cells. The data were processed in Seurat version 5.1.0 [26] and Signac version 1.14.0 [27] pipelines in R version 4.4.1 on the Ubuntu 22.04.4 LTS environment. Pseudotime analysis and integration of the single-cell RNA-seq data and the single-cell ATAC-seq data was conducted by using the “FindTransferAnchors” function of Signac and Monocle3 version 1.3.7 [28], respectively. The conditions used in the analysis are provided in the GitHub repository.

Results

73 **Re-analysis day 59 human fetal retina**

74 First, early gestational stage samples were characterized (day 59). UMAP analysis of single-cell RNA-
75 seq identified 13 clusters (Figure 1A), which were further characterized by marker gene expression
76 (Figure 1B). This sample primarily contained RPCs with various differentiation statuses except for
77 *PAX2*-expressing glial cells. Early RPCs (RPC1-3 and *MKI67*-expressing Proliferating RPC) were
78 characterized by *LHX2*, *PAX6*, *RAX*, and *VSX2*. In addition, pseudotime analysis suggested that these
79 RPCs differentiated into either *ONECUT1*/*2*-expressing, *ELAVL2*/*4*-expressing, or *EEF1A1*-
80 expressing RPCs (Figure 1C). Next, we examined Notch mRNA expression and found that *NOTCH1*-
81 *3* was expressed primarily in early RPCs, with *NOTCH1* and *NOTCH3* being the most prominent
82 genes (Figure 1D). Then, we re-analyzed single-cell ATAC-seq data for this day 59 sample, which was
83 mathematically integrated with its single-cell RNA-seq data by using the "FindTransferAnchors"
84 function of Signac (Figure 1E). In the early RPCs (RPC1/2 and *MKI67*-expressing Proliferating RPC),
85 we observed peaks between Chr9 136510000-136520000 of the *NOTCH1* gene, which diminished in
86 the other RPC clusters. These results imply that Notch expression transition during RPC differentiation
87 might be associated with the chromatin remodeling of *NOTCH1* (Figure 1F). However, we noted that
88 chromatin accessibility of the upstream region of the *NOTCH1* locus remained high in *ONECUT*-
89 expressing RPCs. In contrast to these populations, chromatin accessibility of the *NOTCH1* locus in
90 *ELAVL2/4*-expressing RPCs was very low, which was consistent with the low mRNA expression in

these RPC populations. In addition, the chromatin accessibility of the *NOTCH3* locus remained unchanged during RPC differentiation, although its mRNA expression diminished (Figure 1G). Since *NOTCH2* and *NOTCH4* were less prominent, the feature plots of the marker genes and the dot plots of the Notch genes from single-cell RNA-seq along with coverage plots of *NOTCH2* and *NOTCH4* from single-cell ATAC-seq are available in "Additional_file_1."

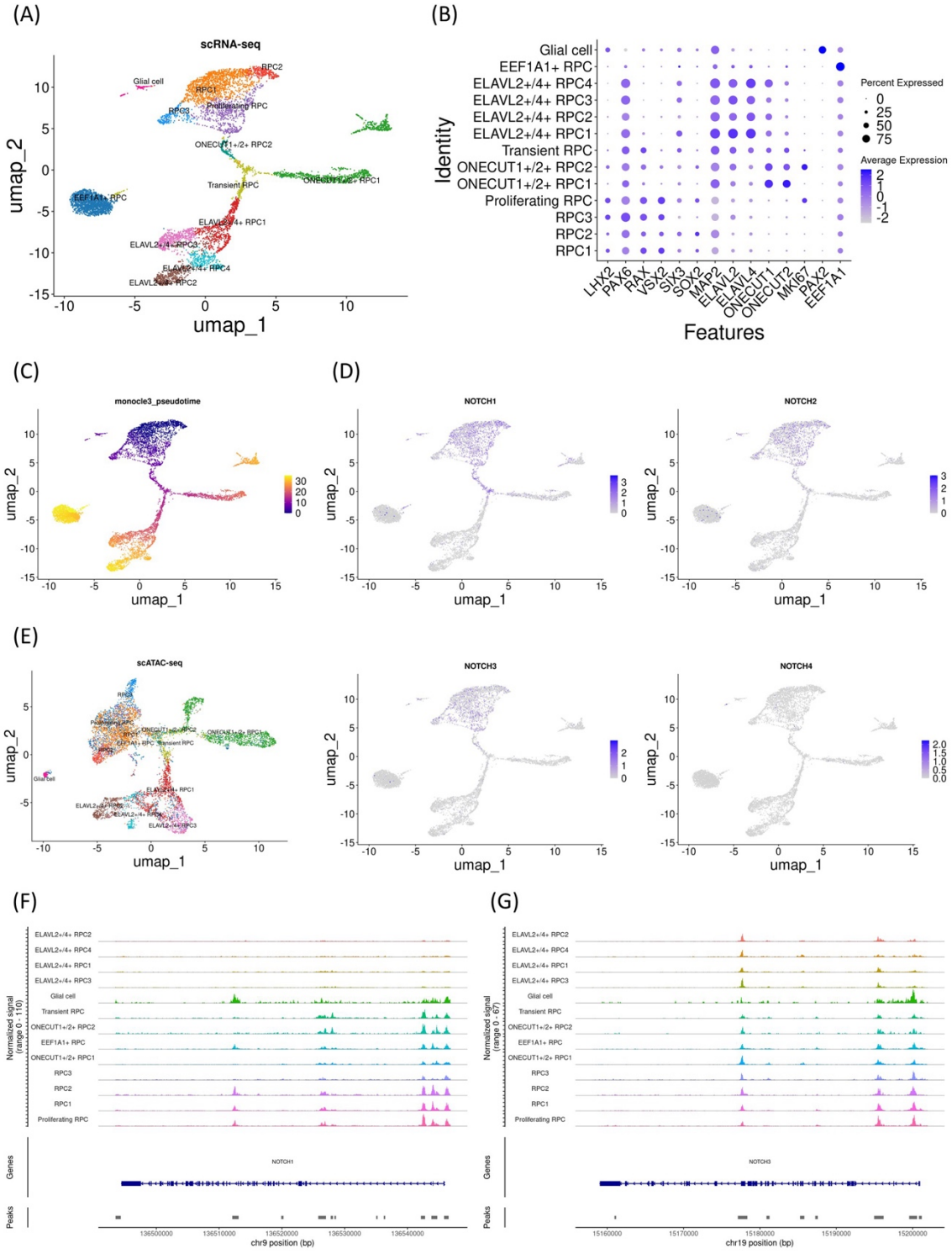


Figure 1 *NOTCH1* mRNA expression decrease and concomitant chromatin remodeling in the day 59 sample. (A) UMAP analysis of the single-cell RNA-seq data identified 13 clusters. (B) Dot

100 plot of marker genes. Note that early RPC clusters expressed *LHX2*, *PAX6*, *RAX*, and *VSX2*. (C)
101 Monocle3 pseudotime analysis for clarifying the differentiation status. (D) Feature plot of *NOTCH1-*
102 *4*. Note that *NOTCH1* and *NOTCH3* expression was prominent in the early RPC clusters. (E) UMAP
103 analysis of the single-cell ATAC-seq data which was integrated with the single-cell RNA-seq data. (F)
104 Coverage plot of the *NOTCH1* locus showing the chromatin accessibility. Note that the upstream of
105 the gene is on the right. (G) Coverage plot of the *NOTCH3* locus. Note that the upstream of the gene
106 is on the right.
107
108 **Re-analysis day 74 human fetal retina**
109 To examine whether the changes in Notch mRNA expression and chromatin accessibility of the Notch
110 loci in the day 59 sample were representative, we investigated another early gestational stage sample
111 (day 74). UMAP analysis identified 15 clusters (Figure 2A) that were further characterized by marker
112 gene expression (Figure 2B). This sample primarily contained RPCs with various differentiation
113 statuses. Early RPCs (RPC1-6) were characterized by *LHX2*, *PAX6*, *RAX*, and *VSX2*. In addition,
114 pseudotime analysis suggested that these RPCs differentiated into either *ELAVL2*/*4*-expressing,
115 *ONECUT1*/*MEIS2*-expressing, or *ONECUT2*-expressing RPCs (Figure 2C). *ONECUT1*/*MEIS2*-
116 expressing RPCs differentiated into RPCs that markedly expressed *VSX2*. Next, we examined Notch
117 mRNA expression and found that *NOTCH1-3* was expressed primarily in early RPCs, with *NOTCH1*

118 and *NOTCH3* being the most prominent genes, similar to the day 59 sample (Figure 2D). We then re-
119 analyzed the single-cell ATAC-seq data for this sample, which were integrated with the single-cell
120 RNA-seq data (Figure 2E). In the early RPCs (RPC1-3), we observed peaks between Chr9 136510000-
121 136520000 of the *NOTCH1* gene, which diminished in the other RPC clusters. These results confirmed
122 that Notch expression transition during RPC differentiation might be associated with the chromatin
123 remodeling of *NOTCH1* (Figure 2F). However, we noted that the chromatin accessibility of the
124 upstream region of the *NOTCH1* locus remained high in *ONECUT*-expressing RPCs. In contrast to
125 these populations, the chromatin accessibility of the *NOTCH1* locus in *ELAVL2/4*-expressing RPCs
126 was very low, which was consistent with the low mRNA expression in these RPC populations. In
127 addition, the chromatin accessibility of the *NOTCH3* locus remained unchanged during RPC
128 differentiation (Figure 2G). In summary, similar to the day 59 sample, in the day 74 sample, a
129 concomitant mRNA decrease and chromatin remodeling in *NOTCH1* was observed, while chromatin
130 accessibility in the upstream region of the *NOTCH1* locus remained high in *ONECUT*-expressing
131 RPCs. The feature plots of the marker genes and dot plots of the Notch genes from single-cell RNA-
132 seq along with coverage plots of *NOTCH2* and *NOTCH4* from single-cell ATAC-seq are available in
133 "Additional_file_2."
134

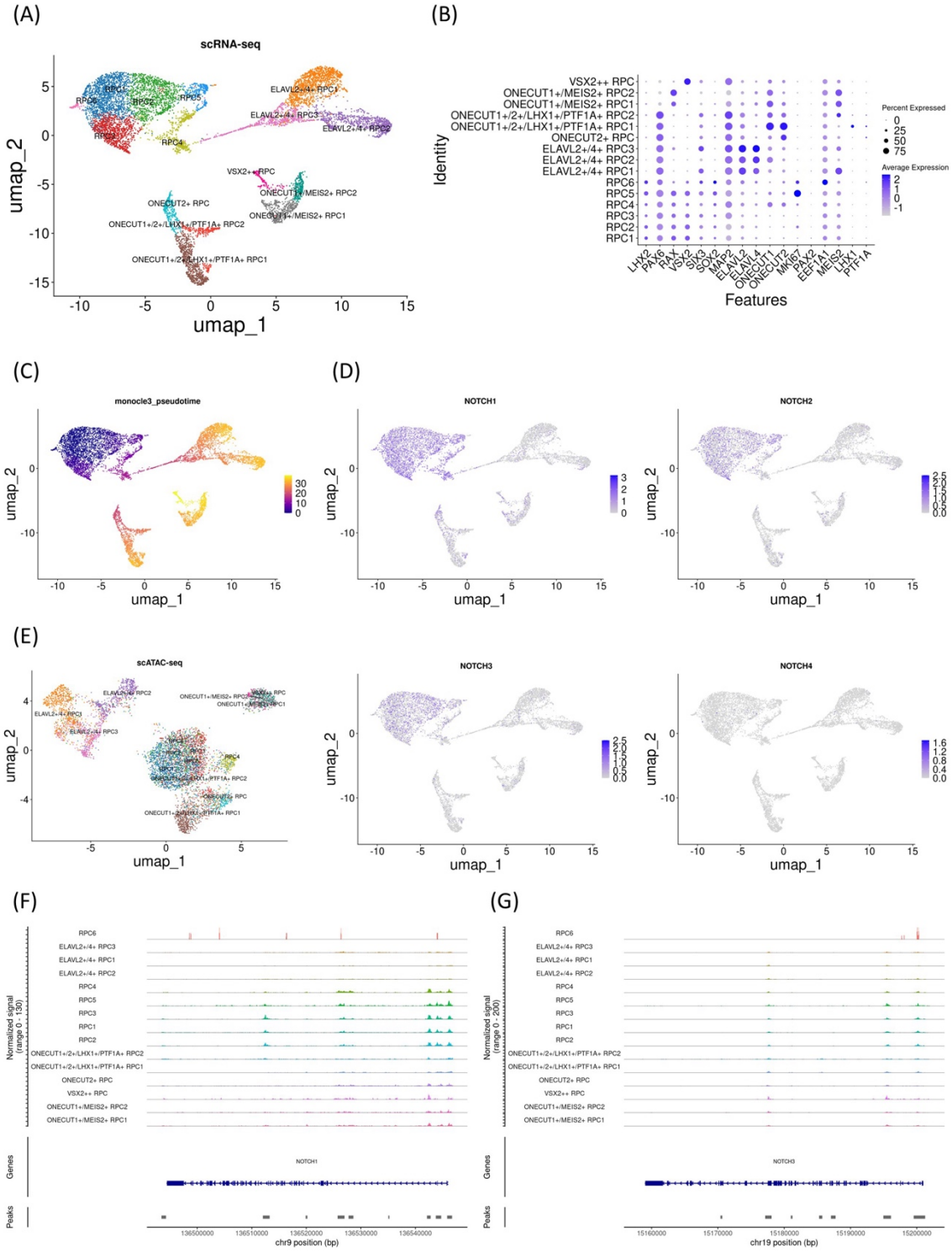
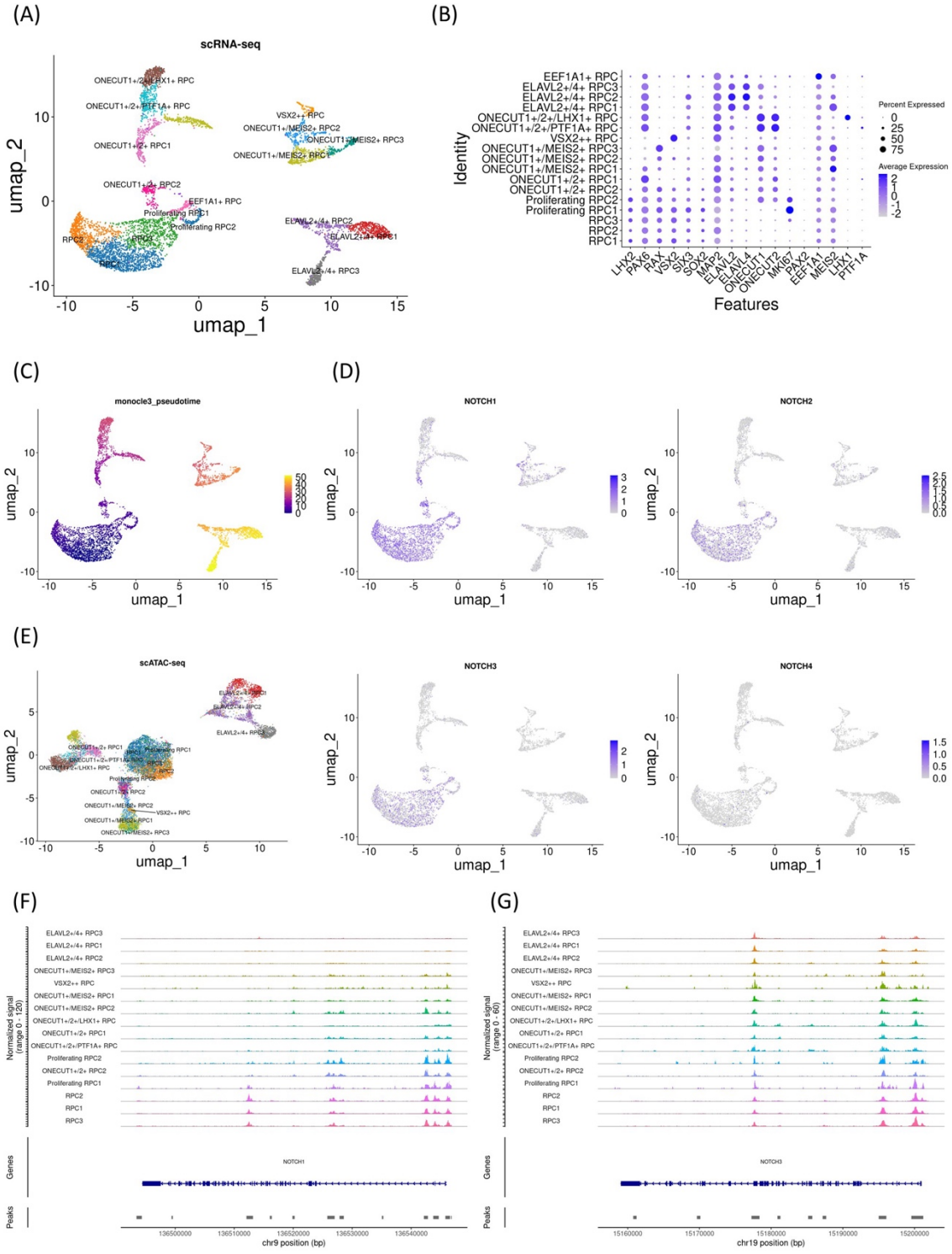


Figure 2 *NOTCH1* mRNA expression decrease and concomitant chromatin remodeling of the day 74 sample. (A) UMAP analysis of the single-cell RNA-seq data identified 15 clusters. (B) Dot

138 plot of marker genes. Note that early RPC clusters expressed *LHX2*, *PAX6*, *RAX,* and *VSX2*. (C)
139 Monocle3 pseudotime analysis. (D) Feature plot of *NOTCH1-4*. Note that *NOTCH1* and *NOTCH3*
140 expression was prominent in the early RPC clusters. (E) UMAP analysis of the single-cell ATAC-seq
141 data which was integrated with the single-cell RNA-seq data. (F) Coverage plot of the *NOTCH1* locus.
142 (G) Coverage plot of the *NOTCH3* locus.
143
144 **Re-analysis day 78 human fetal retina**
145 To further confirm the developmental changes in Notch mRNA expression and chromatin accessibility
146 at the Notch loci, we investigated an additional early gestational stage sample (day 78). UMAP
147 analysis identified 17 clusters (Figure 3A) that were further characterized by marker gene expression
148 (Figure 3B). The sample primarily contained RPCs with various differentiation statuses. Early RPCs
149 (RPC1-3 and *MKI67*-expressing Proliferating RPC1-2 cells) were characterized by *LHX2*, *PAX6*, *RAX*,
150 and *VSX2*. In addition, pseudotime analysis suggested that these RPCs differentiated into
151 *ONECUT1*/*2*-expressing RPCs, which later differentiated into *PTF1A* and *LHX1*-expressing RPCs,
152 *MEIS2*-expressing RPCs, or *ELAVL2/4*-expressing RPCs (Figure 3C). Next, we examined Notch
153 mRNA expression and found that *NOTCH1-3* was expressed primarily in early RPCs, with *NOTCH1*
154 and *NOTCH3* being the most prominent genes (Figure 3D). We then re-analyzed the single-cell ATAC-
155 seq data for the day 78 sample, which was integrated with the single-cell RNA-seq data (Figure 3E).

156 In the early RPCs (RPC1-3), we observed peaks between Chr9 136510000-136520000 of the
157 *NOTCH1* gene, which diminished in the other RPC clusters (Figure 3F). However, we noted that the
158 chromatin accessibility of the upstream region of the *NOTCH1* locus remained high in *ONECUT*-
159 expressing RPCs. In contrast to these populations, the chromatin accessibility of the *NOTCH1* locus
160 in *ELAVL2/4*-expressing RPCs was very low, which was consistent with the low mRNA expression in
161 these RPC populations. In addition, the chromatin accessibility of the *NOTCH3* locus remained
162 unchanged during RPC differentiation (Figure 3G). In summary, examination of all three independent
163 samples suggested that *NOTCH1* mRNA expression decreased, which was concomitant with
164 chromatin remodeling in Chr9 136510000-136520000 of the *NOTCH1* locus. The feature plots of the
165 marker genes and the dot plots of the *NOTCH* genes from single-cell RNA-seq along with coverage
166 plots of *NOTCH2* and *NOTCH4* from single-cell ATAC-seq are available in "Additional_file_3"
167



168

169 **Figure 3 *NOTCH1* mRNA expression decrease and concomitant chromatin remodeling of the**

170 **day 78 sample.** (A) UMAP analysis of the single-cell RNA-seq data identified 17 clusters. (B) Dot

171 plot of marker genes. Note that early RPC clusters expressed *LHX2*, *PAX6*, *RAX*, and *VSX2*. (C)
172 Monocle3 pseudotime analysis. (D) Feature plot of *NOTCH1-4*. Note that *NOTCH1* and *NOTCH3*
173 expression was prominent in early RPC clusters. (E) UMAP analysis of the single-cell ATAC-seq data
174 which was integrated with the single-cell RNA-seq data. (F) Coverage plot of the *NOTCH1* locus. (G)
175 Coverage plot of the *NOTCH3* locus.
176

177 **Discussion**

178 The involvement of Notch signaling in cell fate choices is well documented, including in *Drosophila*
179 neurogenesis [29] and mammalian biliary development [30]. Although the regulation of Notch
180 receptor expression is necessary for these processes, to the best of our knowledge, few studies have
181 used genome-wide investigations of the underlying molecular mechanisms. To examine chromatin
182 remodeling in such regulatory mechanisms, we re-analyzed a single-cell RNA-seq and ATAC-seq
183 dataset from developing retinas in which differentiation trajectories were well characterized. By re-
184 analyzing three independent samples, we observed chromatin remodeling in part of the *NOTCH1* locus,
185 concomitant with changes in its mRNA expression during RPC differentiation.
186 Transcriptional regulation occurs at many levels, including DNA-binding proteins and miRNAs, as
187 well as chromatin remodeling. Importantly, the chromatin accessibility of the upstream regions of the
188 *NOTCH1* locus was unaffected in *ONECUT*-expressing RPCs. Therefore, the observed mRNA

189 changes might be driven by pathways other than chromatin remodeling. Indeed, *NOTCH3* mRNA
190 expression also diminished during differentiation, although we observed no chromatin remodeling in
191 the *NOTCH3* locus. Because chromatin accessibility in *ELAVL2*/*4*-expressing RPCs was very low, this
192 ensured low mRNA expression of *NOTCH1*.
193 An ophthalmological study revealed that the epigenetic landscape of cell type-specific enhancers
194 shifted during differentiation of RPCs [31]. For example, in the single-cell ATAC-seq data from
195 embryonic day 14.5 mouse retina, motif enrichment for *Lhx2*, *Rax* and *Pax6* in the early RPCs were
196 observed, and footprinting analysis validated binding of those transcription factors to their motifs.
197 These high chromatin accessibilities decreased as they differentiated into retinal ganglion cells and
198 non-retinal ganglion cells. Although that study is excellent in providing comprehensive and in-depth
199 insights, ours is unique in focusing on the Notch loci for clarifying the regulatory mechanisms in view
200 of Notch signaling biology.
201
202 **Limitations**
203 This study elucidated the chromatin regions in which chromatin accessibility differed among RPC
204 subsets. We identified the transition of Notch receptor expression and the accompanying chromatin
205 remodeling of Notch loci in the early stages of RPC differentiation. Further investigations, such as a
206 large deletion of these regions, will be needed to evaluate the contribution of these regions to the

differential expression of Notch receptors in RPC subsets.

Abbreviations

RPC: Retinal progenitor cell

UMAP: Uniform manifold approximation and projection

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

A single-cell multiomics dataset (GSE183684) [18] was downloaded from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/). The code used in the present study is available at our GitHub repository (https://github.com/Yoshitokky/Notch_multiomics_retina/tree/v1.0) that was archived using Zenodo

(https://doi.org/10.5281/zenodo.14058572).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y. W. performed the analyses and wrote the original manuscript. S.K. assisted in writing the manuscript. T.N. designed the study. S.T. revised and supervised the study. M.Y. designed the study, performed analyses, wrote the original manuscript, and acquired funding.

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323

Supplementary file

File name: Additional_file_1

File format: JPG

Title of data: Supplementary Figure for day 59 data

Description of data: (A) Feature plot of the marker gene mRNA expression. (B) Dot plot showing Notch mRNA expression. (C) Chromatin accessibility at the *NOTCH2* locus as revealed by coverage plots. (D) Coverage plot of the *NOTCH4* locus.

File name: Additional_file_2

File format: JPG

Title of data: Supplementary Figure for day 74 data

Description of data: (A) Feature plot of the marker gene mRNA expression. (B) Dot plot showing Notch mRNA expression. (C) Chromatin accessibility at the *NOTCH2* locus as revealed by coverage plots. (D) Coverage plot of the *NOTCH4* locus.

File name: Additional_file_3

File format: JPG

Title of data: Supplementary Figure for day 78 data

342 Description of data: (A) Feature plot of the marker gene mRNA expression. (B) Dot plot showing

343 Notch mRNA expression. (C) Chromatin accessibility at the *NOTCH2* locus as revealed by coverage

344 plots. (D) Coverage plot of the *NOTCH4* locus.

345

346 **Figure legends**

347

348 **Figure 1 *NOTCH1* mRNA expression decrease and concomitant chromatin remodeling of the**

349 **day 59 sample**

350 (A) UMAP analysis of the single-cell RNA-seq data identified 13 clusters. (B) Dot plot of marker

351 genes. Note that early RPC clusters expressed *LHX2*, *PAX6*, *RAX*, and *VSX2*. (C) Monocle3

352 pseudotime analysis for clarifying the differentiation status. (D) Feature plot of *NOTCH1-4*. Note that

353 *NOTCH1* and *NOTCH3* expression was prominent in the early RPC clusters. (E) UMAP analysis of

354 the single-cell ATAC-seq data, which was integrated with the single-cell RNA-seq data. (F) The

355 coverage plot of the *NOTCH1* locus showing the chromatin accessibility. Note that the upstream of

356 the gene is on the right. (G) The coverage plot of the *NOTCH3* locus. Note that the upstream of the

357 gene is on the right.

358

359 **Figure 2 *NOTCH1* mRNA expression decrease and concomitant chromatin remodeling of the**

360 **day 74 sample**

361 (A) UMAP analysis of the single-cell RNA-seq data identified 15 clusters. (B) Dot plot of marker

362 genes. Note that early RPC clusters expressed *LHX2*, *PAX6*, *RAX*, and *VSX2*. (C) Monocle3

363 pseudotime analysis. (D) Feature plot of *NOTCH1-4*. Note that *NOTCH1* and *NOTCH3* expression

364 were prominent in the early RPC clusters. (E) UMAP analysis of the single-cell ATAC-seq data which

365 was integrated with the single-cell RNA-seq data. (F) The coverage plot of the *NOTCH1* locus. (G)

366 The coverage plot of the *NOTCH3* locus.

367

368 **Figure 3 *NOTCH1* mRNA expression decrease and concomitant chromatin remodeling of the**

369 **day 78 sample**

370 (A) UMAP analysis of the single-cell RNA-seq data identified 17 clusters. (B) Dot plot of marker

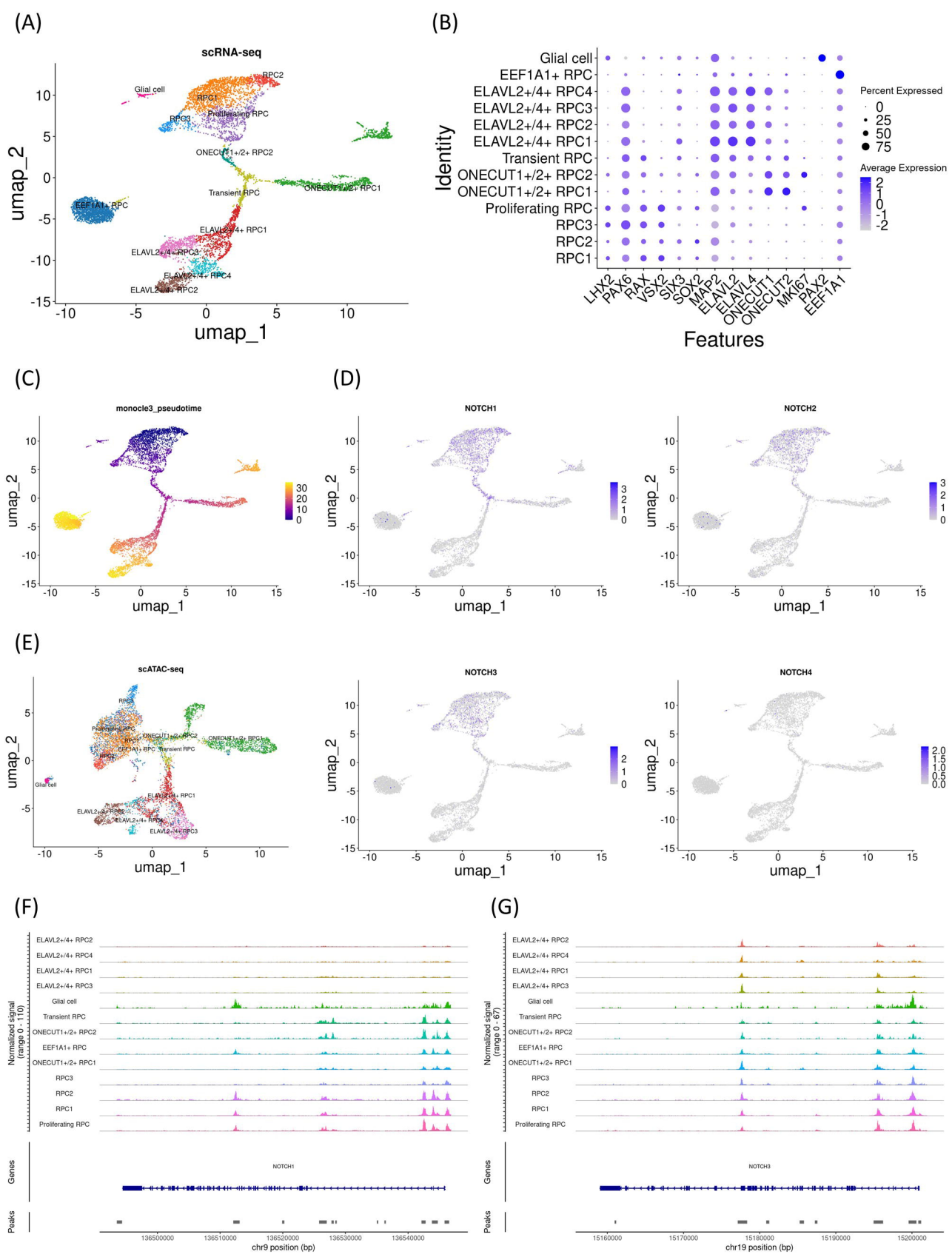
371 genes. Note that early RPC clusters expressed *LHX2*, *PAX6*, *RAX*, and *VSX2*. (C) Monocle3

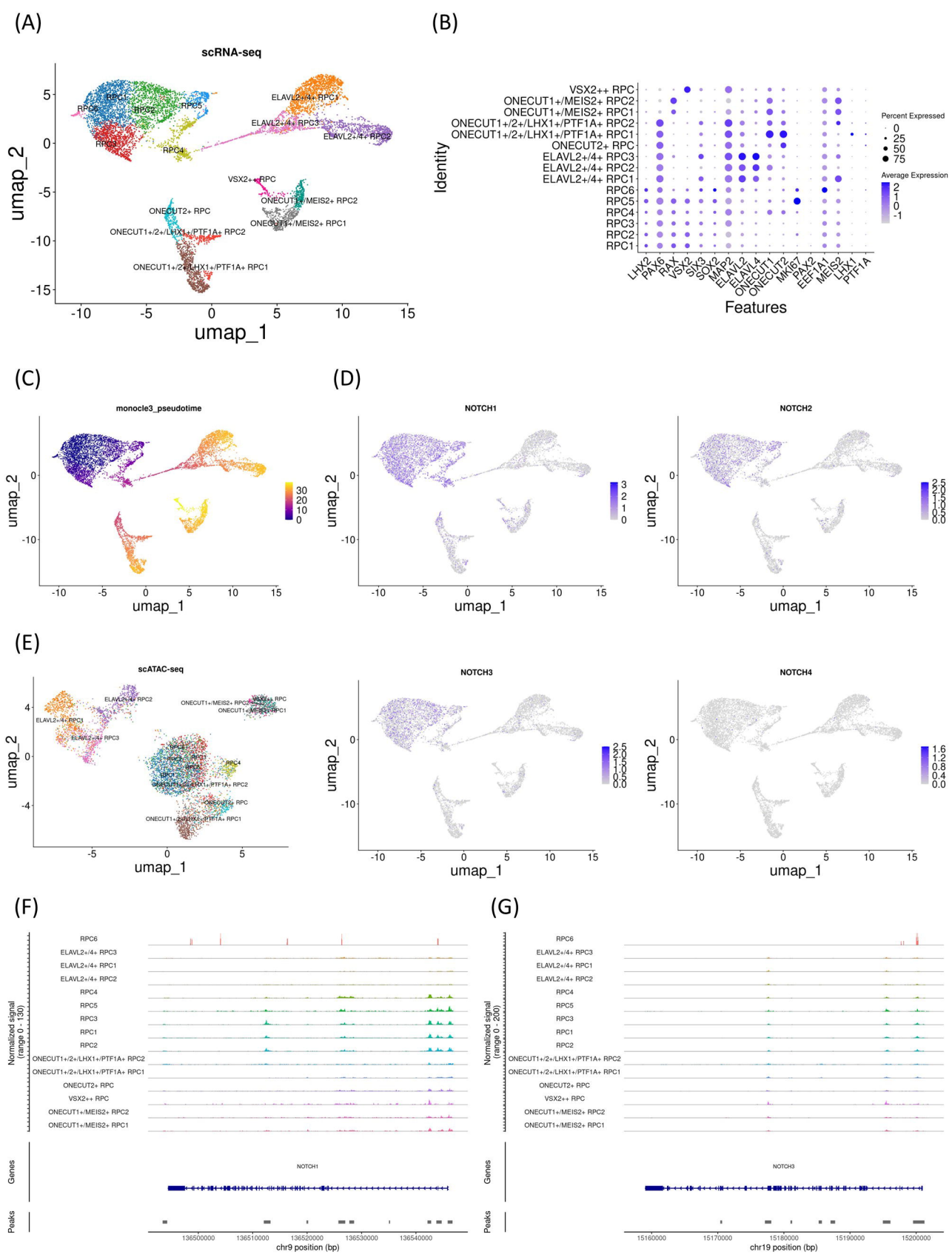
372 pseudotime analysis. (D) Feature plot of *NOTCH1-4*. Note that *NOTCH1* and *NOTCH3* expression

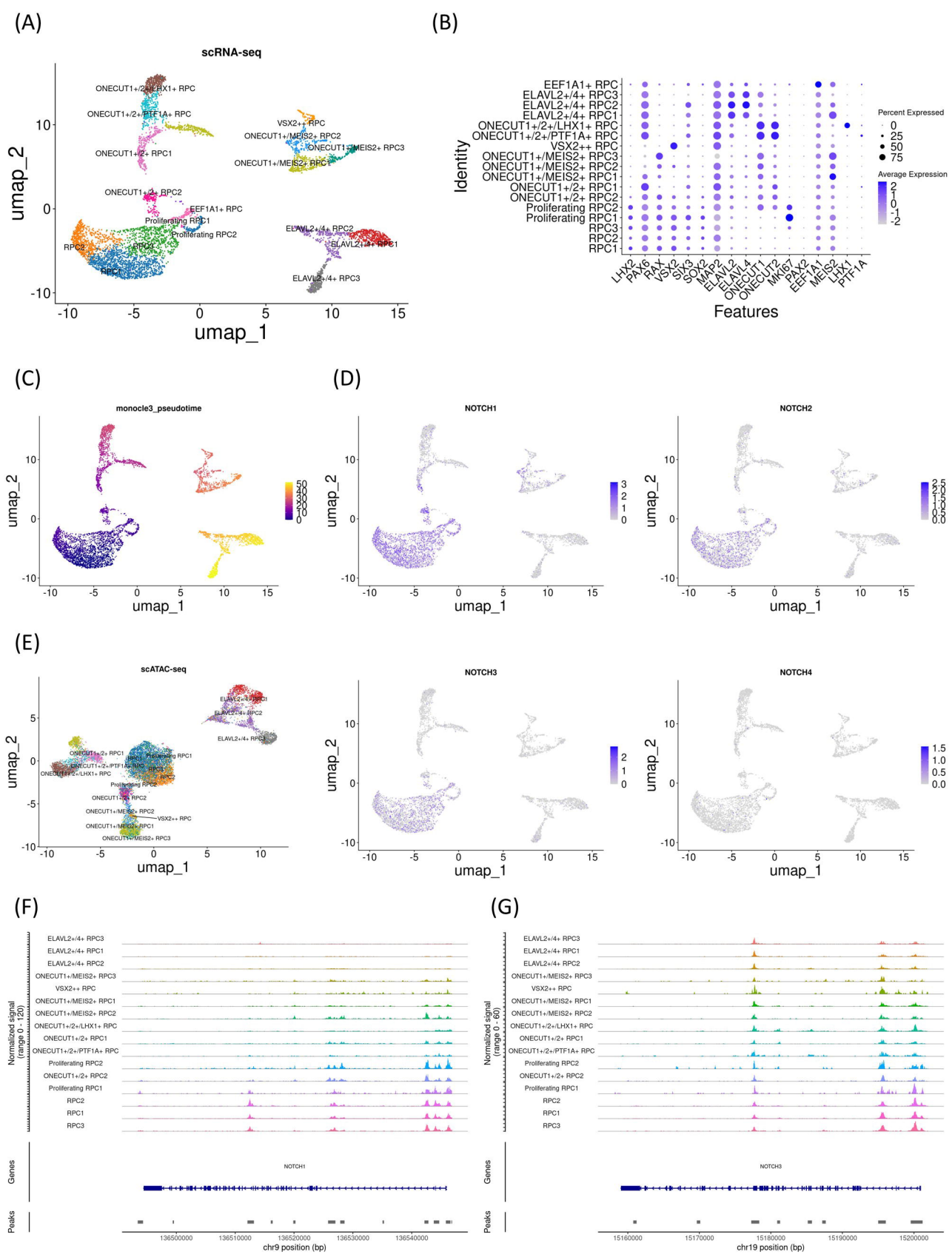
373 were prominent in early RPC clusters. (E) UMAP analysis of the single-cell ATAC-seq data which

374 was integrated with the single-cell RNA-seq data. (F) The coverage plot of the *NOTCH1* locus. (G)

375 The coverage plot of the *NOTCH3* locus.


(A)
scRNA-seq
umap_1
umap_2
Glial cell
RPC2
RPC1
RPC3
Proliferating RPC
ONECUT1+/2+ RPC2
Transient RPC
ONECUT1+/2+ RPC1
EEF1A1+ RPC
ELAVL2+/4+ RPC1
ELAVL2+/4+ RPC3
ELAVL2+/4+ RPC4
ELAVL2+/4+ RPC2
(B)
Identity
Glial cell
EEF1A1+ RPC
ELAVL2+/4+ RPC4
ELAVL2+/4+ RPC3
ELAVL2+/4+ RPC2
ELAVL2+/4+ RPC1
Transient RPC
ONECUT1+/2+ RPC2
ONECUT1+/2+ RPC1
Proliferating RPC
RPC3
RPC2
RPC1
LHX2
PAX6
RAX
VSX2
SIX3
SOX2
MAP2
ELAVL2
ELAVL4
ONECUT1
ONECUT2
MKI67
PAX2
EEF1A1
Features
Percent Expressed
0
25
50
75
Average Expression
2
1
0
-1
-2
(C)
monocle3_pseudotime
umap_1
umap_2
30
20
10
0
(D)
NOTCH1
NOTCH2
umap_1
umap_2
(E)
scATAC-seq
NOTCH3
NOTCH4
umap_1
umap_2
(F)
Normalized signal (range 0 - 110)
ELAVL2+/4+ RPC2
ELAVL2+/4+ RPC4
ELAVL2+/4+ RPC1
ELAVL2+/4+ RPC3
Glial cell
Transient RPC
ONECUT1+/2+ RPC2
EEF1A1+ RPC
ONECUT1+/2+ RPC1
RPC3
RPC2
RPC1
Proliferating RPC
Genes
NOTCH1
Peaks
136500000
136510000
136520000
136530000
136540000
chr9 position (bp)
(G)
Normalized signal (range 0 - 57)
Genes
NOTCH3
Peaks
15160000
15170000
15180000
15190000
15200000
chr19 position (bp)


(A)
scRNA-seq
umap_1
umap_2
RPC1
RPC2
RPC3
RPC4
RPC5
RPC6
ELAVL2+/4+ RPC1
ELAVL2+/4+ RPC2
ELAVL2+/4+ RPC3
VSX2++ RPC
ONECUT1+/MEIS2+ RPC2
ONECUT1+/MEIS2+ RPC1
ONECUT2+ RPC
ONECUT1+/2+/LHX1+/PTF1A+ RPC2
ONECUT1+/2+/LHX1+/PTF1A+ RPC1
(B)
Identity
Features
Percent Expressed
Average Expression
LHX2
PAX6
PAX
VSX2
SIX3
SOX2
MAP2
ELAVL2
ELAVL4
ONECUT1
ONECUT2
MKI67
PAX2
EEF1A1
MEIS2
LHX1
PTF1A
(C)
monocle3_pseudotime
(D)
NOTCH1
NOTCH2
(E)
scATAC-seq
NOTCH3
NOTCH4
(F)
Normalized signal (range 0 - 100)
Genes
Peaks
chr9 position (bp)
(G)
Normalized signal (range 0 - 200)
chr19 position (bp)


(A)
scRNA-seq
ONECUT1+/2+/LHX1+ RPC
ONECUT1+/2+/PTF1A+ RPC
ONECUT1+/2+ RPC1
ONECUT1+/2+ RPC2
EEF1A1+ RPC
Proliferating RPC1
Proliferating RPC2
RPC2
RPC1
RPC3
VSX2++ RPC
ONECUT1+/MEIS2+ RPC2
ONECUT1+/MEIS2+ RPC3
ONECUT1+/MEIS2+ RPC1
ELAVL2+/4+ RPC2
ELAVL2+/4+ RPC1
ELAVL2+/4+ RPC3
umap_1
umap_2
(B)
Identity
Features
Percent Expressed
Average Expression
LHX2
PAX6
RAX
VSX2
SIX3
SOX2
MAP2
ELAVL2
ELAVL4
ONECUT1
ONECUT2
MKI67
PAX2
EEF1A1
MEIS2
LHX1
PTF1A
(C)
monocle3_pseudotime
umap_1
umap_2
(D)
NOTCH1
NOTCH2
(E)
scATAC-seq
NOTCH3
NOTCH4
(F)
Normalized signal (range 0 - 120)
Genes
Peaks
NOTCH1
chr9 position (bp)
(G)
Normalized signal (range 0 - 60)
NOTCH3
chr19 position (bp)