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Supplemental information

Retinal ganglion cell-specific genetic regulation in primary open-angle glaucoma

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SUPPLEMENTARY FIGURES AND TABLES

Retinal ganglion cell-specific genetic regulation in primary open angle glaucoma

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Figure S1. Breakdown of quality control filtering performed on cells from each participant. Fibroblasts were collected from donors and reprogrammed into iPSCs, and subsequently retinal organoids. Cells from lines were removed as quality control was performed on cell lines, donor genotypes and scRNA-seq quality control. Related to Figure 1.

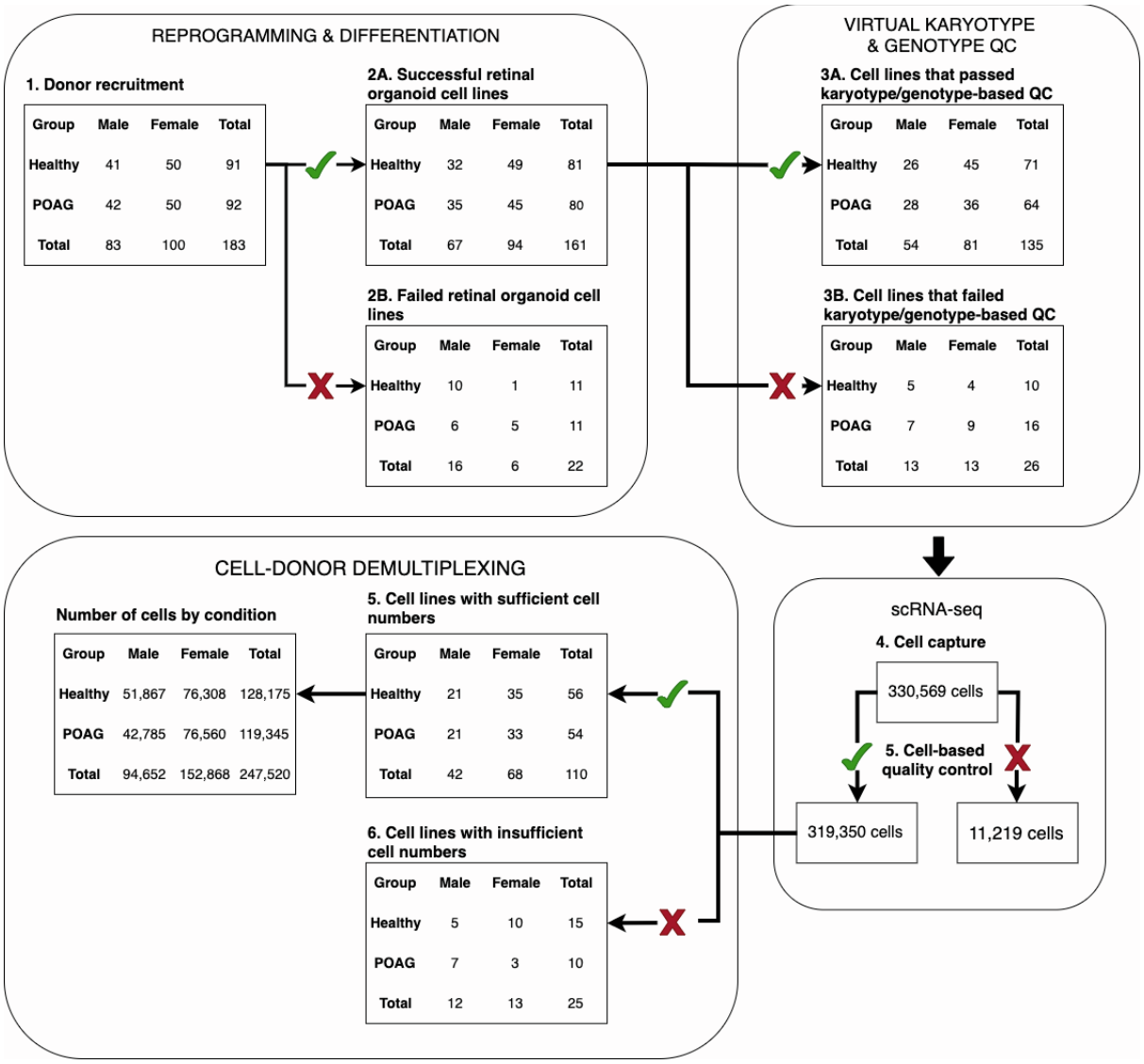
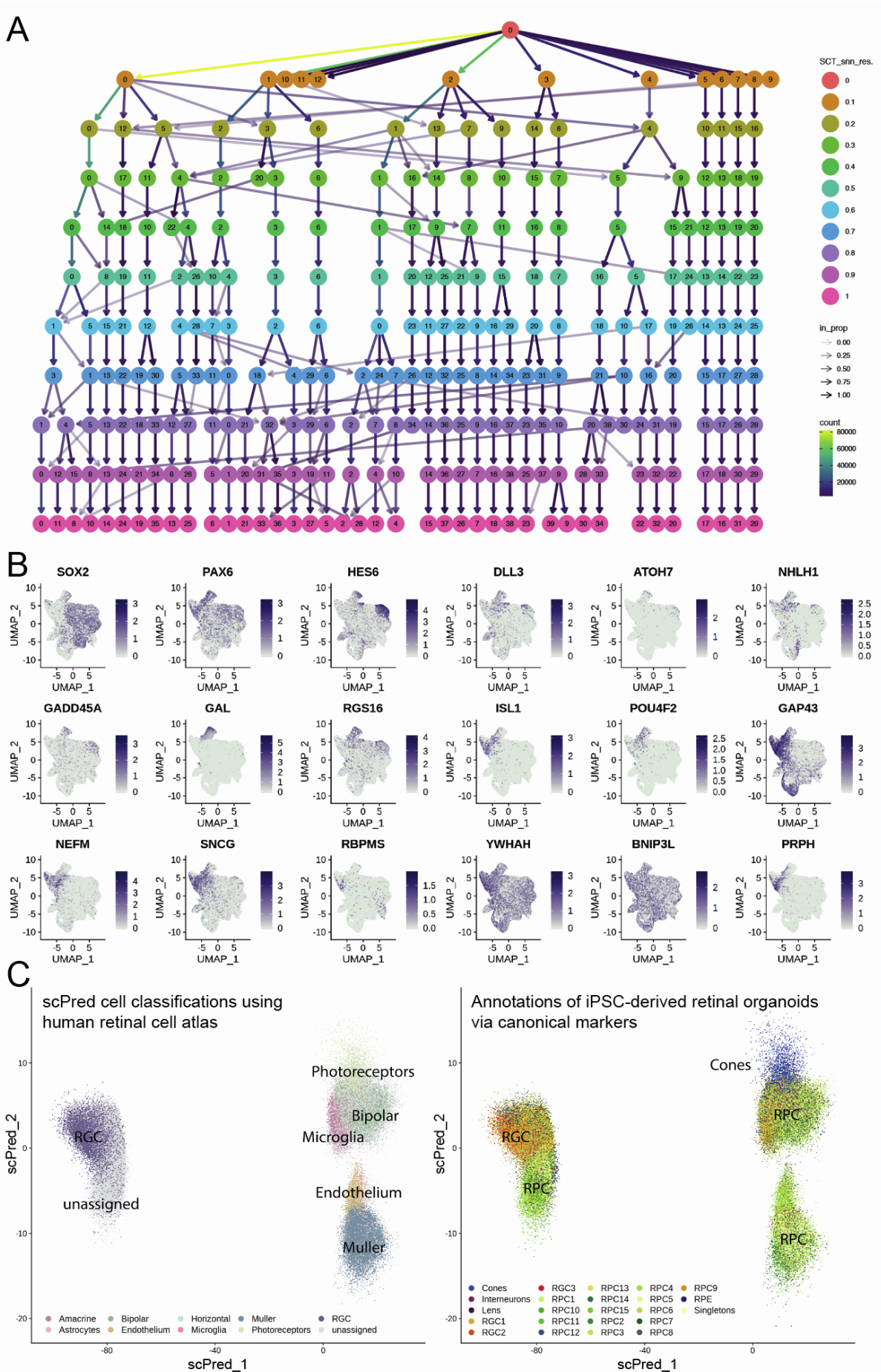


Figure S2. Identification and characterisation of cell subpopulations. (A) “Clustree” representation of graph-based clustering results over range of resolutions from 0 (top row) to 1 (bottom row). Arrows indicate movement of cells from one group to another at each resolution. Minimal movement of cells between groups are indicative of grouping stability. **(B)** Feature plots of selected RPC and RGC markers across all cells. Color scale represents gene expression level in a cell. **(C)** Comparison of our iPSC-derived retinal cell annotations to a previously reported single cell RNAseq dataset generated from adult human retinas. Related to Figure 1.


A
SCT_snn_res.
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count
80000
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B
SOX2
PAX6
HES6
DLL3
ATOH7
NHLH1
GADD45A
GAL
RGS16
ISL1
POU4F2
GAP43
NEFM
SNCG
RBPMS
YWHAH
BNIP3L
PRPH
UMAP_1
UMAP_2
C
scPred cell classifications using human retinal cell atlas
Photoreceptors
Bipolar
Microglia
RGC
unassigned
Endothelium
Muller
Amacrine
Astrocytes
Bipolar
Endothelium
Horizontal
Microglia
Muller
Photoreceptors
RGC
unassigned
scPred_1
scPred_2
Annotations of iPSC-derived retinal organoids via canonical markers
Cones
RPC
RGC
RPC
RPC
Cones
Interneurons
Lens
RGC1
RGC2
RGC3
RPC1
RPC10
RPC11
RPC12
RPC13
RPC14
RPC15
RPC2
RPC3
RPC4
RPC5
RPC6
RPC7
RPC8
RPC9
RPE
Singletons
scPred_1
scPred_2

Figure S3. Variation in cell differentiation between control and POAG samples. (**A**) Distribution of number of cells per cell type, per cell line. Each point represents a donor, with healthy donors shown in grey and donors with POAG shown in red. **(B)** Differentially expressed genes due to % RGC in cell lines, across all cell types. (**C**) Distribution of identified cell types across all donors. Related to Figure 1.

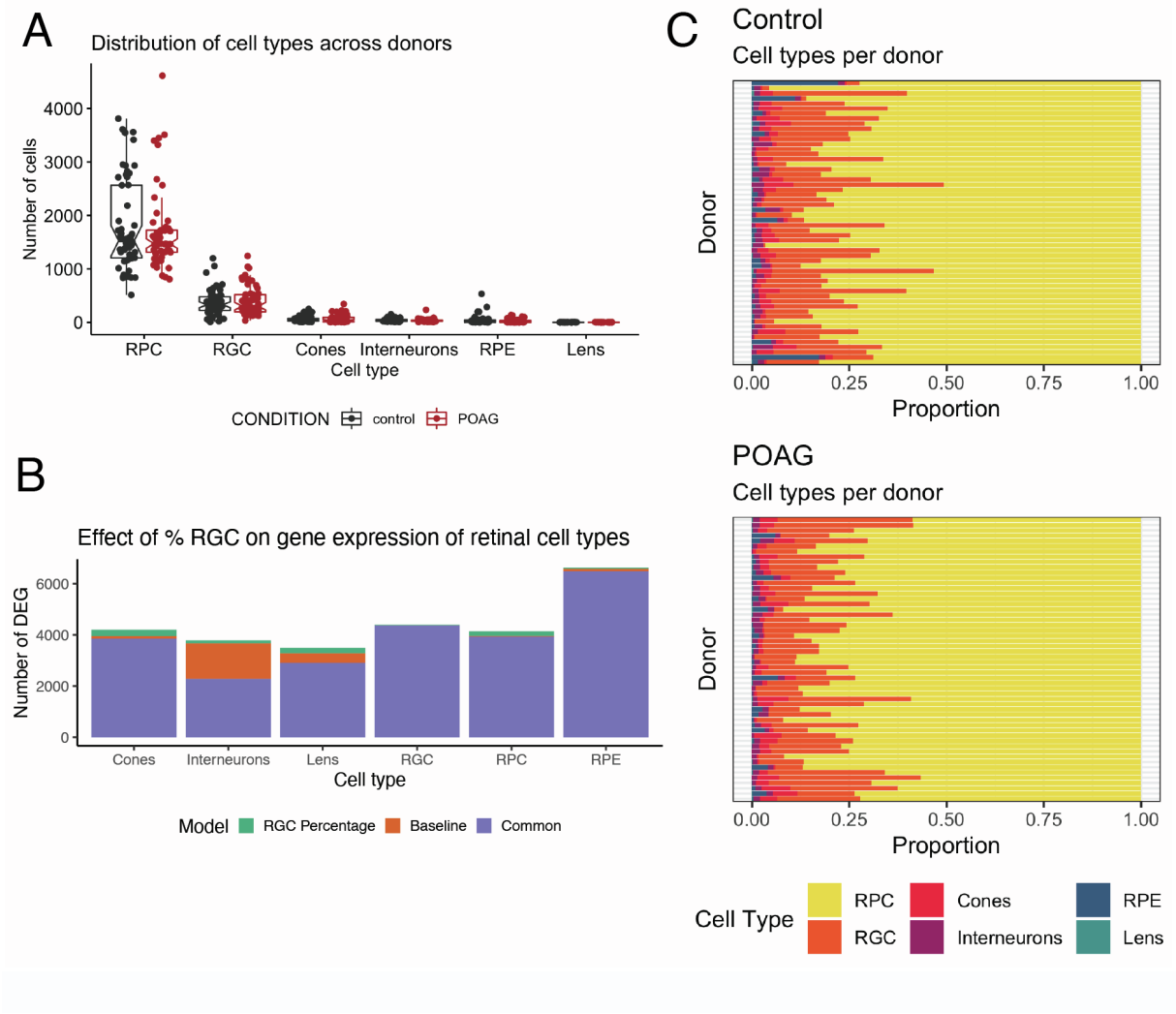

Figure S4. Ancestry composition of study participants. Genotype data of study participants were combined with data from the 1000 Genomes project (1KGP), and the resulting population structure was reduced via PCA and projected on to this plot. Study participants used for the final analysis are labelled by bright red asterix. The dark grey box demarcates the “acceptable” box of +/- 6SD from the European mean in PC1 and PC2. Related to Figure 1.

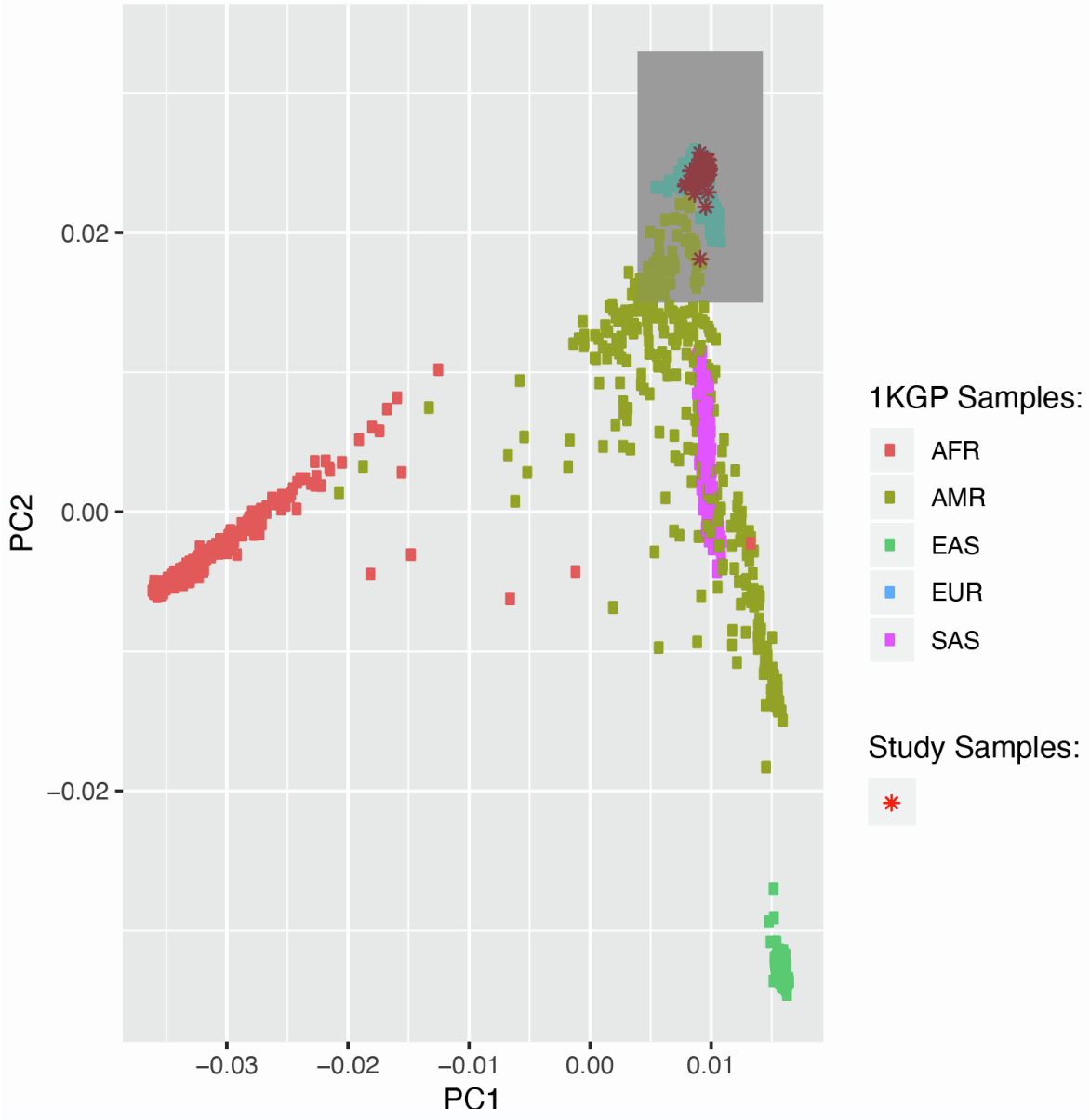

Table S3. Cell-donor deconvolution summary metrics. Cell lines from donors were randomly assigned to 25 pools for scRNA-seq. Healthy and POAG cell lines were combined into pools, and cells were traced back to their cell line using transcriptome and genotype data with *demuxlet*. 289,617 out of 309,223 cells were traced back to donors. 29,733 cells were classified as doublets by *demuxlet* and *scrublet.* 12,786 cells were removed due to reasons outlined in **Figure S1**. Pool 4 had failed as a single cell pool, and was discarded from the study. Related to Figure 1.

Pool	Healthy	POAG	Loaded Individuals	Detected Individuals	Singlets	Doublets
1	3	3	6	5	10,630	1,418
2	2	6	8	7	15,677	909
3	5	3	8	6	10,602	1,027
4	3	5	8	0	0	0
5	3	4	7	5	10,464	1,107
6	3	5	8	8	9,652	485
7	4	4	8	8	11,106	716
8	2	5	7	7	11,586	1,028
9	2	5	7	7	12,553	2,280
10	3	5	8	7	12,725	1,309
11	5	3	8	7	10,794	903
12	3	4	7	7	9,686	919
13	1	7	8	8	13,247	1,135
14	1	7	8	8	13,662	1,014
15	5	2	7	6	13,382	993
16	3	5	8	8	13,748	732
17	6	2	8	8	10,920	686
18	5	3	8	7	14,546	1,314
19	6	2	8	8	13,504	508
20	5	3	8	7	11,402	786
21	4	4	8	8	15,621	856
22	4	4	8	8	8,883	546

Pool	Healthy	POAG	Loaded Individuals	Detected Individuals	Singlets	Doublets
23	6	2	8	8	12,306	728
24	5	1	6	6	14,648	1,061
25	1	5	6	3	8,273	7,273

Table S4. Percentage breakdown of captured cell population. Cells were initially grouped into subpopulations based on Louvain-based clustering. These subpopulations were then annotated using gene markers associated with each known cell type associated with human optic cups. Related to Figure 1.

Cell types	**Proportion (%)**
Retinal progenitor cells	77.4
Retinal ganglion cells	17.0
Retinal pigmented epithelium cells	1.27
Photoreceptors / bipolar cells	2.56
Interneurons	1.66
Lens cells	0.04

Table S6. Comparison of cell type proportions between conditions. The distribution of cell type proportions for each condition were compared using a t-test. Related to Figure 1.

Cell Type	Baseline Prop	Mean Prop Control	Mean Prop POAG	Prop Ratio	T-statistic	p-value	FDR
RPC	0.774	0.773	0.777	0.994	-0.173	0.863	0.874
RGC	0.170	0.168	0.173	0.969	-0.575	0.566	0.849
Cone	0.026	0.025	0.024	1.005	0.159	0.874	0.874
Interneurons	0.017	0.017	0.015	1.162	1.318	0.190	0.661
RPE	0.013	0.017	0.010	1.710	0.784	0.435	0.849
Lens	0.000	0.001	0.000	1.695	1.232	0.220	0.661

Table S11. Disease-associated pathways in RGC3 lineage. Disease Ontology was performed on markers identified in Table S7 via Gene Set Enrichment Analysis (GSEA), as implemented in clusterProfiler. Related to Figure 5.

ONTOLOGY	ID	Description	Gene Ratio	Bg Ratio	pvalue	p.adjust	qvalue	Count
BP	GO:0060560	developmental growth involved in morphogenesis	7/56	257/21081	5.14E-06	9.52E-03	4.21E-03	7
BP	GO:0051402	neuron apoptotic process	7/56	262/21081	5.83E-06	1.08E-02	4.21E-03	7
BP	GO:0070997	neuron death	8/56	440/21081	2.00E-05	3.70E-02	9.61E-03	8

<u>Data S1</u> Description of Delineated Cell Clusters (Related to Figure 1C, Figure 1D)

Subpopulation Zero (35,377 cells, 13.7 % of all cells, 186 conserved markers) consisted of the "orphan" samples that show no relationships at the selected resolution. Genes specific to this subpopulation, such as *SFRP2*, *DAPL1* and *CYP1B1*, *SPP1* are expressed in RPCs [1]. *PLEKHA1* was also highly expressed in this subpopulation. Its expression is controlled by *PAX6* [2] which controls multipotent states of RPCs [3]. This pattern of gene expression suggests an RPC population.

Subpopulation One (29,469 cells, 13.2 % of all cells, 313 conserved markers) was characterised by genes known to be expressed in the retina. Some markers are associated with the cytoskeleton, such as *TAGLNs*, *TUBA1A*, *TUBB2A*, *ACTB*, *MYL6*, *TMSB10*. In particular, genes involved in neuronal growth (for instance *STMN2*, *GAP43*, *NEFM*) and in sensory neurons (such as *PRPH*) were enriched markers of this subpopulation. Further, some specific RGC lineage markers were also present in this cluster, including the combined expression of *STMN2/4*, *GAP43*, *PRPH*, *ISL1*, *NEFM*, *ELAVL4/HuD* and the high expression of genes enriched in RGCs (*GAP43*, *SNCG*, *POU4F1/2*, *POU6F2*, *ISL1*, *NHLH2*, *EBF1*, *EBF3*, *MYC*) [4-8] suggests an RGC lineage. The presence of *STMN2/*4, *PAX6*, *DAPL1, SFRP2*, *SOX2/4/11* also suggests an immature phenotype. Altogether this suggests a RGC subpopulation.

Subpopulation Two (23,588 cells, 10.6 % of all cells, 142 conserved markers) was characterised by expression of *UBE2C*, *PTTG1*, *TOP2A*, *KPNA2*, *CENPF*, *HMGB2*, CCNB1 all of which participate in regulation of cell cycle [9-13]. Combined with the high expression of the proliferation marker *MKI67*, it suggests an RPC subpopulation.

Subpopulation Three (20,425 cells, 9.2 % of all cells, 100 conserved markers) was characterised by genes involved in cell growth and differentiation, which, for many are not specific to the retina, such as *FABP7* (Fatty Acid Binding Protein 7, expressed in radial glial cells and immature astrocytes, as well as in retinal astrocytes and Müller cells upon injury [14]), *C1orf61*, *PTN* or *TMSB4X* (associated with cytoplasmic sequestering of NF-kB). The presence of the transcription factor genes *SOX2* and *SOX3* further suggests a RPC subpopulation [15].

Subpopulation Four (19,648 cells, 8.8 % of all cells, 254 conserved markers) was characterised by its main gene markers being involved in DNA binding, transcriptional activity, and regulation of the cell cycle, such as *HIST1H4C*, *PTTG1*, *HMGB2*, *HMGN2*, *MKI67*, *KIAA0101/PCLAF*, *CKS1B*, *H2AFZ* or *NUSAP1*. This pattern suggests a RPC population.

Subpopulation Five (15,821 cells, 7.1 % of all cells, 226 conserved markers) was characterized by expression of many ribosomal genes, including 46 *RPLs* (Ribosomal protein L), 29 *RPSs* (Ribosomal protein S) and 7 mitochondrially-encoded genes (*MT-ND3*, *MT-ND4*, *MT-CO1*, *MT-CO2*, *MT-CO3*, *MT-ATP6*, *MT-CYB*). The expression of ribosomal genes and mitochondrially-encoded genes has been correlated with

development and maturation [16]. Presence of genes associated with respiratory chain suggests a metabolic switch essential for neurogenesis [17-19]. This pattern of gene expression suggests a RPC population.

Subpopulation Six (14,842 cells, 6.7 % of all cells) identified 288 conserved markers. Many of the most conserved markers of this subpopulation are associated with the cytoskeleton, such as *TAGLN*, *CALD1*, *TPM1*, *TPM2*, *ACTA2*, *ACTG2*, *ACTN1*, *ACTB*. Others are associated with cell differentiation and proliferation, including *LGALS1*, *CTGF/CCN2*, *ANXA2*, *S100A11*. The population expresses high levels of genes known to be expressed in early-stage RPCs (including *DLX1*, *DLX2*, *ONECUT2*, *ATOH7*). This pattern is suggestive of an early RPC subpopulation.

Subpopulation Seven (14,145 cells, 6.4 % of all cells, 149 conserved markers) showed varied conserved markers, with genes involved in metal-binding (*MT1X* and *MT2A*), transcription, cell cycle and proliferation (such as *HES6*, *HMGB2*, *PTTG1*, *TOP2A*). The presence of genes known to be expressed in the retina but not cell type specific as markers of this subpopulation (*CKB*), as well as markers of neuronal differentiation (*NEUROD1*) with markers of RPCs (*SFRP2*, *ASCL1*) and high levels of expression of *DLX1/2* suggests an early RPC subpopulation.

Subpopulation Eight (9,744 cells, 4.4 % of all cells, 298 conserved markers) identified markers of transcriptional activity (*NFIB*, *NFIA*, *BCL11A*), and many neural markers (such as *CNTNAP2*, *LMO3*, *TUBA1A*, *TUBB2A*), involved in retinal differentiation (*SOX4*, *SOX11*), neuronal differentiation (such as *NEUROD6*, *NEUROD2*, *GPM6A*) and neuroendocrine secretion (*RTN1*). The presence of the RPC markers (*CLU, SFRP2*, *NFIA*, *NFIB* and *VIM*) together with RGC markers (*GAP43*, *PRPH*, *ELAVL4, TBR1*), combined with the high expression of genes enriched in late RPC genes (*SOX4*, *NEUROG2*), interneurons (*CALB2*), photoreceptors (*NHLH1*, *RHO*), suggest a late RPC population differentiating into retinal neurons.

Subpopulation Nine (9,652 cells, 4.3 % of all cells, 355 conserved markers) showed conserved markers for nuclear transport (*UBE2C*, *KPNA2*), transcriptional activity and cell cycle (*PTTG1*, *TOP2A*, *CENPF*, *MKI67*, *CCNB1*, *CDK1*) [9-11,13,20]. It was also characterised by the RPC/RGC marker *ATOH7* and RGC gene markers *GAP43*, *PRPH*, *NEFM* and *ELAVL4*. Combined with the high expression of RPC genes (*DLX1/2*, *GAL*, *ONECUT2*, *ATOH7*), this suggests an RPC population differentiating into a RGC population.

Subpopulation Ten (7,843 cells, 3.5 % of all cells, 202 conserved markers) shows expression of markers of neurogenesis including of the retina (*NEUROD1*/*4*, *C8orf46/VXN*), transcription (*PRDM1*), melanogenesis (*DCT*), apoptosis (*PHLDA1*), which suggests a progenitor population [1]. The presence of conserved markers that are RPC markers together with photoreceptor progenitor markers (such as *OTX2* and *CRX*), and with the low expression of genes associated with most cell types of the retina suggests a differentiating RPC population.

Subpopulation 11 (7,693 cells, 3.5 % of all cells, 96 conserved markers) shows conserved marker genes associated with RPCs (*CLU*, *SFRP2, VIM*, *DLX2* and *SOX4* [1]) as well as conserved markers associated with neuronal development (*NNAT*, *MEG3*, *PEG10*, *MEIS2*), neuronal differentiation and growth (*SOX4*, *MLLT11*, *STMN2*, NSG2), neuroendocrine secretion (*RTN1, PCSK1N*), cytoskeleton organization (*TUBA1A*, *STMN1*, *TUBB2A/B*, *MARCKSL*1, *DCX*, TMSB10), telomere maintenance (*TERF2IP*), transport (*VAMP2*). We observed high expression of genes enriched in amacrine cells (ONECUT1/2*, ESRRB*) also known to be expressed by RGCs [8,21]. Together with the high expression of genes enriched in RGCs (*GAP43*, *SNCG*, *POU4F1, POU4F2, POU6F2*, *ISL1*, *NHLH2*, *EBF1/3*, *MYC*), this pattern of expression suggests a RGC population.

Subpopulation 12 (7,455 cells, 3.3 % of all cells, 42 conserved markers) identified conserved markers associated with hormonal activity (*TTR*, *IGFBP7*, *C1orf194*), calcium signaling (*TRPM3*), ciliogenesis (*FAM183A*, *C1orf192/ CFAP126*), cell adhesion/ Wnt signaling (*TPBG*, *PIFO*), retinal development (RP11-356K23.1) or cytoskeleton organization (*TPPP3*). Together with the high expression of genes found in photoreceptor progenitors (*OTX2*, *CRX*) and cone cells (*LHX9*, *ARR3*, *GNGT2*, *GUCA1C*, *DCT*, *LMO4*, *THRB*, *RXRG*), this pattern suggests a cone population.

Subpopulation 13 (7,321 cells, 3.3 % of all cells, 121 conserved markers) identified conserved markers associated with neuronal development (*HOXB4/5/6*, *TAGLN3*), transcriptional regulation (*RP11-834C11.4*, *HOTAIRM1*, *HOXB-AS3*), neuronal growth (*NEFM*, STMN2), synaptic transmission (*SNCG*, *LAMP*5), axon guidance (*NOVA1*), cytoskeletal organization (*TUBB2B, TUBA1A*). Expression of *HOX* genes suggests an RPC population and expression of genes associated with RGCs, (*TAGLN3*, *NEFM*, *STMN2*, *SNCG)* [6,22,23] suggests a RPC population differentiating into RGCs.

Subpopulation 14 (6,962 cells, 3.1 % of all cells, 347 conserved markers) identified genes associated with synaptic transmission (*SST*, *NSG1*, *SNCA/SNCG*), neuronal growth (*STMN2*, *GAP43*, *NEFM*, *ISL1*, *PRPH*, *ELAVL4/HuD*), neuroendocrine secretion (*RTN1*) which are also markers of RGCs. Yet the low level of expression of cell type specific markers suggests a RPC population.

Subpopulation 15 (6,399 cells, 2.9 % of all cells, 137 conserved markers) was characterized by expression of several genes involved in lipid metabolism and trafficking, i.e., *APOA1*, *APOA2*, *APOC1*, *APOC3*, *APOE*, *NPC2*. It was also suggested that apolipoproteins may be important for membrane assembly during cell division [24]. We also detected genes associated with regulation of cell cycle: *KRT8/18* [25], *S100A10* [26], *FTL* (Ferritin light chain)[27], *AFP* [28], *KRT19* [29] and maintenance of open chromatin - *HMGA1* [30]. This pattern of gene expression suggests a RPC population.

Subpopulation 16 (4,429 cells, 2.0 % of all cells, 100 conserved markers) identified genes involved in neuromodulation (*NTS*), neuronal differentiation (*NEUROD6*, *NFIB*, *NFIA*, *CALB2*, *BCL11A*, *GPM6A*, *NEUROD2*, *NSG2*, *GAP43*, *TBR1*),

neuroendocrine secretion (*RTN1*), transport (*FXYD6*, *LY6H*, *VAMP2*), synaptic transmission (*CAMKV*, *GRIA2*, MEF2C), axonal outgrowth/ autophagy (*FEZ1*), cell adhesion (*CNTNAP2*), and cytoskeleton organization (*THSD7A*, *DSTN*]). This indicates a RPC population.

Subpopulation 17 (4,012 cells, 1.8 % of all cells) only identified 34 conserved markers, with some associated with differentiation (*IGFBP5*, *FABP7*, *HES6, STMN2*). The high expression of *TFAP2A/B*, *CALB1* and *CHAT* suggests an interneuron population, such as horizontal and amacrine cells [31].

Subpopulation 18 (3,590 cells, 1.6 % of all cells) showed 32 conserved markers genes, including markers associated with neuronal differentiation (*HES6, FABP7, ANXA2, PCP4, CALB1*), signaling (*CXCL14*, *TTYH1, TPBG, SFRP2*), metabolism (*GATM, CKB, HMGCS1*), or transcriptional activity (*HMGB2, ID2, HMGN2, CKS2, PTTG1, CENPF, NUSAP1, CDK1*), cytoskeleton organization (*TMSB4X, TUBA1B, STMN4*). The high expression of *VSX2* further suggests a RPC population [32].

Subpopulation 19 (3,326 cells, 1.5 % of all cells, 635 conserved markers) identified genes associated with RPE cells, such as *TTR*, *TRPM3*, *IGFBP7*, *CST3, RPE65*, *RBP1/CRBP1*, or *SERPINF1/PEDF*. It is also characterised by genes involved in early retinal development, including the RPE and eye morphogenesis (*SOX4*, *SOX11*, *BMP7*, *GJA1*, *PTN*). Many RPE genes are highly expressed in this population. Altogether, this suggests a RPE population.

Subpopulation 20 (2,936 cells, 1.3 % of all cells, 128 conserved markers) identified genes associated with protein transport such as *CRYAB, HSPA5* and *HSPB1*; iron homeostasis (*FTL*), gene regulation (*NEAT1*) or cytoskeleton regulation (such as *STMN1, TUBB* or *ACTB*). The absence of markers for specific cell types suggests a RPC population.

Subpopulation 21 (2,866 cells, 1.3 % of all cells, 129 conserved markers) identified genes associated with early neural differentiation (*HES4*, *HES6, CLU, PAX6, POU4F2, RORB, DLX1/2*, *SOX11*), axon guidance (*CXCR4*), neurite growth (*MDK, RTN4*), synapse formation (*NPTX2*), synaptic plasticity (*SERPINI1*), synaptic vesicle transport (*SLC18A2, CPLX2*) and neuromodulation (*GAL*, *TRH*). The expression of early RPE genes suggests a RPC population with potential for neuronal and RPE differentiations.

Subpopulation 22 (528 cells, 0.2 % of all cells, 33 conserved markers) is characterised by 12 genes encoding different types of crystallins as well as *LIM2* which is highly expressed in the lens. The presence of *AQP5* and *MIP*, *CYP26A1* all known to play roles in the lens further supports the lens identity of this subpopulation.

Impact of RGC numbers on other populations

Comparing gene expression of samples with high levels of RGCs, the other samples revealed an effect of RGC proportion on both differentially expressed genes in all cell

types (**Figure S3B**), and the detection of eQTL in RGC subpopulations. In particular, RGC numbers mainly influenced gene expression in interneurons, with an upregulation of pathways linked to apoptosis or development (**Table S9**). As RGCs are one of the earliest cell types to arise during retinal development, they can influence retinogenesis by interactions with progenitor cells and other retinal neurons [33]. The variation in gene expression observed in interneurons thus suggests that RGCs influence the differentiation and survival of early interneurons, by upregulated pathways associated with neuronal death, cell growth and differentiation.

Supplementary references

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