

Materials and Methods

Ethical statement

This study was conducted following the guidelines for the Ethical Treatment of Non-Human Primates and was approved by the Institutional Animal Care and Use Committee of the Institute of Zoology (Chinese Academy of Sciences).

Experiment models and biological samples

All cynomolgus monkeys (*Macaca fascicularis*) were of Southeast Asian origin, and raised at around 25°C on a 12-hour (h) light, 12-h dark schedule at Xieerxin Biology Resource, an accredited primate research facility in Beijing. All animal experiment procedures were performed by certified veterinarians, complying with laws governing animal research. All animals were given commercial monkey diet twice a day and vegetables and fruits once a day with tap water *ad libitum* (Li et al., 2020; Wang et al., 2020; Zhang et al., 2020). None of the animals had a clinical or experimental history potentially affecting the physiological aging process. Retinal and choroidal tissues for scRNA-seq analysis were obtained from 8 young (4-6 years old) and 8 aged cynomolgus monkeys (18-21 years old).

Single cell isolation and collection

To ensure tissue quality, animals were sampled on different days. In brief, the animal was anesthetized and perfused with physiological saline, and the peripheral retinal and choroidal tissues were isolated. The neural retina layer and RPE-choroid layer were dissected and processed separately. Samples were minced and then transferred into 1.5 mL tubes and subjected to 1 mL of digestion buffer containing 1 mg/mL Collagenase, Type IV (Gibco 17104-019) and 1mg/mL dispase II. After incubation at 37°C for 15 min on a shaking thermomixer (1000 rpm), tubes were briefly spun at 500 x g for 8 min at 4°C, and the supernatant was then gently removed. Pellets were resuspended in 1 mL PBS containing 10 % FBS and gently pipetted on ice for 50-100 times to dissociate the cells and the resulting supernatant immediately filtered through 100-µm mesh filters to remove undigested tissues. Debris was removed by fluorescence-activated cell sorting (FACS) (BD FACSAria™ II) and samples were subjected to single-cell collection. The purified cell suspension was then transferred into a 3.5 cm dish and cells were randomly picked by mouth pipette under a dissection microscope. For each animal, approximately 384 cells were picked for the neural retina, and 48-96 cells for the RPE-choroid layer. Each cell was picked into an individual PCR tube with lysis buffer and stored at -80°C for subsequent experiments.

Single-cell RNA-seq library preparation and sequencing

Single cell transcriptome amplification was performed with a modified STRT-seq protocol, as previously described (Dong et al., 2018; Islam et al., 2012). Briefly, single cells were randomly collected by a mouth pipette and transferred to lysis buffer. After cell lysis, released mRNAs were reverse transcribed into cDNA by barcode-reverse transcription primers with 8 bp unique molecular identifiers (UMIs). Then, the second-strand cDNAs were synthesized, followed by cDNA pre-amplification. The amplified cDNAs were pooled together and purified for fragmentation after which 3' ends of fragmented cDNAs were collected for library construction with Kapa Hyper Prep Kit. Then, sequencing was performed on HiSeq 4000 platform with 150 bp paired-end reads.

Hematoxylin and Eosin (H&E) staining

H&E staining was performed according to previous studies (Nichols et al., 2005; Wang et al., 2020; Zhang et al., 2020).

Large-scale three-dimensional retina reconstruction

Tissues were fixed with 2.5% (vol/vol) glutaraldehyde with Phosphate Buffer (PB) (0.1 M, pH 7.4), washed four times in PB. Then the samples were immersed in 1% (wt/vol) OsO₄ and 1.5% (wt/vol) potassium ferricyanide aqueous solution at 4°C for 1 h. After several washes, the samples were incubated in filtered 1% thiocarbohydrazide aqueous solution (Sigma-Aldrich) at room temperature (RT) for 30 min, 1% unbuffered OsO₄ aqueous solution at 4°C for 1 h and 1% UA aqueous solution at 4°C overnight following four rinses in ddH₂O for 10 min each between each step. Next, the samples were dehydrated through graded alcohol (30%, 50%, 70%, 80%, 90%, 100%, 100%, 10 min each step) at 4°C into pure acetone (3×10 min). Samples were infiltrated in a graded mixture (3:1, 1:1, 1:3) of acetone and SPI-PON812 resin (19.6 mL SPI-PON812, 6.6 mL DDSA and 13.8 mL NMA, 1.5% BDMA), then changed with pure resin. The samples were finally embedded in pure resin with 1.5% BDMA and polymerized for 12 h at 45°C, 48 h at 60°C.

Automatic collector of ultrathin sections scanning electron microscopy (AutoCUTS-SEM) was performed as previously described (Li et al., 2017; Zhang et al., 2020). In brief, about 2000 sections were collected by the ultramicrotome (UC7, Leica, Germany) with the AutoCUTS device for each sample. Next, high throughput serial sections were automatically acquired by a Helios Nanolab 600i dual-beam SEM (Scanning Electron Microscope, FEI) with an automated software (AutoSEE), and an image reconstruction program was conducted. The image parameters including accelerating voltage of 2 kV, beam current of 0.69 nA, CBS detector, pixel size of 58.6 nm and dwell time of 5 μs. About 988 and 1121 sections were collected for young and old samples, respectively.

Tissue immunostaining

Immunostaining was performed following the previously published studies (Ma et al., 2020; Wang et al., 2020; Zhang et al., 2020). First, monkey retinal and choroidal tissues were fixed with 4% paraformaldehyde (PFA) at 4°C overnight, soaked in 30% sucrose, embedded in Tissue-Tek[®] O.C.T.[™] Compound (Sakura Finetek, 4583) and frozen. Frozen sections (10 μm) were obtained and stored at -80°C prior to use. Antigen retrieval was performed by microwaving the sections at 98°C in 10 mM sodium citrate buffer (pH 6.0) for three times (5 min each time). After cooling down, the slides were washed three times with PBS, permeabilized with Triton X-100 (0.4% in PBS) for 25 min, blocked with 10% donkey serum in PBS for 1 h at RT, and stained with primary antibodies at 4°C overnight. Then, after washes with PBS, sections were incubated with secondary antibodies for 1 h at RT. Nuclear DNA was stained by Hoechst 33342 (Thermo Fisher Scientific). Next, sections were mounted with VECTASHIELD[®] Antifade Mounting Medium (Vector Laboratories, H-1000), and fluorescent images were obtained using a confocal microscope (Leica TCS SP5 II). The antibodies used in this study are shown in Supplementary material, Table S6.

Lipofuscin fluorescence imaging

The paraffin section was deparaffinized and permeabilized with Triton X-100 (0.4% in PBS) for 25 min, stained with Hoechst 33342 and mounted with VECTASHIELD[®] Antifade Mounting Medium. Next, the lipofuscin fluorescence imaging was performed by using a confocal microscope as previously described (Dorey et al., 1989).

Single-cell RNA-seq data processing

Raw paired scRNA-seq data in each library was split into single cells based on barcode sequences. To obtain clean reads for each cell, the scRNA-seq data was first processed by trimming the template switch oligo (TSO) and poly(A) tail sequences, and then removing the reads with adapters and low-quality bases. Next, clean reads were mapped to the *Macaca fascicularis* reference genome (Ensembl version: *Macaca_fascicularis_5.0*) using *Tophat* (version: 2.0.12) (Trapnell et al., 2009). UMIs within the uniquely mapped reads were counted for each gene using *HTSeq* (Anders et al., 2015), in which process the same UMI sequences were only counted once.

We quantified the gene expression level with transcripts per million (TPM) which was calculated as the number of UMIs of a given gene in a given cell divided by the total number of UMIs of this given cell and then multiplied by 1,000,000. Gene expression levels were then transformed into $\log_2(\text{TPM}/10 + 1)$ since the scRNA-seq library complexity was estimated to be $\sim 100,000$ transcripts.

To obtain high-quality single cells for the downstream analysis, cells that met the following three criteria were retained: the rate of uniquely mapped reads aligned to the reference genome was greater than 10%, the number of detected genes was greater than 800, and the number of detected UMIs was greater than 5,000 (Fig. S1C; Table S1). After this quality filtering process, 6,410 out of 7,461 cells were retained for further analysis. In high-quality single cells, the median rate of mapped reads was 41.8%, and the median number of detected genes and UMIs was 2,439 and 29,869, respectively.

Identification of cell types and cell-type-specific marker genes

Based on the transformed TPM data, 1,126 highly variable genes (HVGs) were identified using R package *Seurat* (Satija et al., 2015) with parameters '*x.low.cutoff* = 1, *x.high.cutoff* = 10, *y.cutoff* = 1' according to the average expression and dispersion, and then these selected HVGs were used to perform principle component analysis (PCA). To exclude the principal components (PCs) which explained very little of variance and improve the signal-to-noise ratio as described in the previous study (Haber et al., 2017), 'significant' components were chosen using function *JackStraw* in *Seurat* based on the permutation test, and 30 significant components (PC1~PC30) were used for *t*-distributed stochastic neighborhood embedding (*t*-SNE) analysis. Unsupervised clustering analysis was performed using function *FindClusters* in *Seurat* with the parameter '*resolution* = 2', and different clusters were labeled by the expression levels of known marker genes.

A standard area under the curve (AUC) classifier was utilized to identify cell-type-specific markers (differentially expressed genes (DEGs) among different cell types) with function *FindAllMarkers* in *Seurat*. Marker genes were chosen only if the average difference of the \log_2 -transformed TPM was greater than 1 with a statistical significance *power* greater than 0.25. Gene ontology (GO) analysis of cell-type-specific marker genes was performed using website tool *Toppgene* with default parameters (Chen et al., 2009).

Pseudotime trajectory analysis of the subpopulation of Müller glial cells

Based on scRNA-seq data in which gene expression levels were quantified with raw UMI count data, pseudotime trajectory analysis was performed using R package *monocle* (version: 2.10.1) (Qiu et al., 2017; Trapnell et al., 2014). To exclude effects from other cells, only specific retinal cell types were considered, including Müller, Rod, Cone, Bipolar and RGC, comprising 4,946 cells in total. HVGs were computed based

on the average expression and dispersion using function *dispersionTable* in *monocle* with default parameters, and 3,057 HVGs were selected with filtering condition '*mean_expression* ≥ 0.01 & *dispersion_empirical* $\geq 1.5 * dispersion_fit$ '. To ensure reproducibility, the random seed was set as '19961109' in this analysis, and functions *reduceDimension* and *orderCells* were used to compute a projection of cells into a lower dimensional space and order cells according to pseudotime, respectively.

Cross-species comparison analysis

The scRNA-seq data of human and mouse retina was collected from two published studies (Heng et al., 2019; Lukowski et al., 2019). For comparison analysis between human being and other species, only six cell types were used, including Rod, Cone, Bipolar, Müller, MG and RGC. The cell type information was directly collected from the corresponding studies. Then, 19,450 and 12,832 single cells in human and mouse were obtained for the downstream analysis, respectively. Only the homologous genes between human and mouse was used, and genes with one to many correspondences were also discarded. Gene expression level was quantified with TPM in these three datasets (monkey, human and mouse), and was transformed into $\log_2(\text{TPM}/10 + 1)$.

Cross-species comparison analysis was performed with the method as same as previous studies (Cui et al., 2019; La Manno et al., 2016). We firstly identified DEGs among selected cell types in human and mouse with function *FindAllMarkers* (a standard AUC classifier was used) in R package *Seurat*, and DEGs were chosen only if the average difference of the \log_2 -transformed TPM was greater than 0.5 with a statistical significance *power* greater than 0.25. Then, 774, 945 and 1,791 DEGs were selected in human, mouse and monkey scRNA-seq. We calculated the average expression level of these DEGs in each cell type of each species, which was utilized in the downstream analysis. We then labelled each gene based on the coefficient of variation (CV) value, in which gene was labelled with 1 if the CV ranged from the 25th percentile to the 75th percentile and otherwise gene was labelled with 0. And therefore, we can exclude the influence of housekeeping genes and 'noisy' genes. These labeled genes make up a new sample as a control, and we calculated the coefficient of correlation between each average sample and control, and then normalized each dataset with that coefficient of correlation. Finally, we obtained the correlation result between any two average samples.

Ageing-associated transcriptional variation analysis

For cells in a given cell type, ageing-associated CV was defined to measure the consistency of gene expression patterns between cells collected from young monkeys and cells from aged monkeys, and this definition reflected the extent of influence on the transcriptome driven by aging, which was described similarly as in previous studies (Salzer et al., 2018; Wang et al., 2020).

As we identified cell types in all filtered cells, 1,126 HVGs were firstly selected based on the average expression and dispersion, and these HVGs were used for the downstream ageing-associated transcriptional variation analysis. For each HVG g in a given cell type c (including num_y cells collected from young monkeys and num_o cells from aged monkeys), g_y and g_o were denoted as the expression level in young and aged monkeys, respectively; and cell pairwise distance d_g was defined as:

$$d_g = |g_y - g_o|, \text{ where } y \in \{1, 2, \dots, num_y\}, o \in \{1, 2, \dots, num_o\}.$$

Arithmetic mean of d_g was denoted as μ_g and standard deviation of d_g was denoted as σ_g , and therefore ageing-associated CV was calculated as standard deviation divided by arithmetic mean for each HVG.

Identification of aging-associated differentially expressed genes

To identify aging-associated DEGs between cells collected from young monkeys and cells from aged monkeys in each cell type, based on the \log_2 -transformed TPM data, we performed the reproducibility optimized test statistic (ROTS) algorithm using R package *ROTS* (Elo et al., 2008; Seyednasrollah et al., 2016). Aging-associated DEGs were chosen only if the average difference of the \log_2 -transformed TPM was greater than 0.5 with a statistical significance P value less than 0.05. GO analysis of aging-associated DEGs was performed using website tool *Metascape* with default parameters (Zhou et al., 2019).

Potentially core transcriptional regulator analysis

Based on scRNA-seq data in which gene expression levels were quantified with raw UMI, co-regulation transcriptional network analysis was performed to identify the potentially core transcriptional regulators in a given cell type with given genes. To construct the upregulated network between aging transcriptional regulators and aging targets of a given cell type, upregulated aging-associated DEGs were used as input for R package *SCENIC* (Aibar et al., 2017; Elo et al., 2008). Since there is no well-established transcriptional regulator database for *Macaca fascicularis*, whose genome highly similar to that of humans, we used the human transcriptional regulator database. Of 1,839 transcriptional regulators in the human database, we detected 1,579 regulators in the *Macaca fascicularis* gene set. The regulator network was then established using function *runGenie3* in *SCENIC* based on \log_2 -transformed UMI data. Only the transcriptional regulator-target connected with a high weight (weight > 0.1) was retained for the downstream analysis. The same process was executed for the downregulated DEG network.

For visualization of the network, the node size indicates the number of connections and the line size indicates the weight of a connection; and nodes are ranked based on node size.

Cell-cell interaction analysis

Based on scRNA-seq data in which gene expression levels were quantified with raw TPM instead of \log_2 -transformed TPM, the cell-cell interaction analysis was performed using *CellPhoneDB* (version: 2.0.0) (Ma et al., 2020; Vento-Tormo et al., 2018; Zheng et al., 2020). For a given cell type, only the ligand and receptor interacting pair satisfying the following three criteria was considered in the downstream analysis: (1) at least 10% cells expressed that pair (set the parameter as '*--threshold 0.1*'), (2) the average expression level was greater than 5, and (3) compared between different cell types, the enrichment of the pair was statistically significant ($P < 0.05$). A ligand-receptor pair detected only in cells collected from young monkeys was considered as a young-specific interaction pair; a ligand-receptor pair detected only in cells from aged monkeys was considered as an aged-specific interaction pair; the rest were considered as common interaction pairs. GO analysis of newborn or disappeared interactions was performed using website tool *Metascape* with default parameters.

Network visualization of high-risk aging-associated differentially expressed genes

The eye disease-associated gene set was collected from two previous studies (Elo et al., 2008; Orozco et al., 2020). Given that several of the same eye diseases were included in these two studies, such as 'retinitis pigmentosa', 'age-related macular degeneration', 'macular degeneration' and 'congenital stationary night blindness', we merged genes

in the same eye diseases in our downstream analysis. GenAge gene set of aging/longevity-related genes was collected from public website (Magalhães et al., 2009): <https://www.genomics.senescence.info/>.

Statistical analysis

All data were statistically analyzed using PRISM software (GraphPad 6 Software). Results were presented as mean \pm SEM. Comparisons were conducted using the one-tailed or two-tailed Student's *t*-test as indicated in figure legend.

The bioinformatics data were statistically analyzed using a two-tailed Student's *t*-test with R language, and *P* values are indicated in each figure.

Data availability

All RNA-seq raw sequencing data and processed data have been deposited in the NCBI Gene Expression Omnibus (GEO) under the accession number GSE113917.

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Supplementary Figure Legends

Figure S1. Quality control information of single-cell RNA-seq data.

- (A) Table showing basic information about the cynomolgus monkey cohort.
- (B) Representative images of ARR3-positive Cones in young and old monkeys. Scale bar, 50 μm .
- (C) Representative H&E stained images of RPE in young and old monkeys. Scale bar, 50 μm .
- (D) Histograms showing the cell distribution with different mapping rate (the ratio of uniquely mapped reads) (left) or different number of genes (right) detected in each single cell.
- (E) *t*-SNE plots showing the single cell distribution in individual monkeys (left) and age groups (right).
- (F) Bar plots showing the expression level of representative known and novel marker genes for various cell types. All expression levels are measured using the same scale. Data are shown as mean \pm SEM.

Figure S2. Subtype identification of Müller glial cells and cross-species comparisons.

- (A) *t*-SNE plot showing the two subtypes of Müller glial cells.
- (B) Left, heatmap showing the gene expression signatures of each Müller glial cell subtype, and the number of DEGs is shown on the left of the heatmap. Right, representative GO terms of the DEGs in each cell subtype. Values in each row are *z*-score scaled. The color key from blue to red indicates low to high gene expression levels.
- (C) Trajectory analysis showing the pseudotime of Müller glial subtype cells and other cells. Arrowhead indicates the pseudotime from early ones to late ones.
- (D) Bar plots showing representative gene signatures of retinal progenitor cells. Two-tailed Student's *t*-test *P* values are indicated.
- (E) Heatmaps showing cross-species comparison correlation between human and human (left), between human and mouse (middle), and between human and monkey (right). The value in each diagonal indicates the correlation coefficient.

Figure S3. Cell identity and transcriptional noise analysis during aging process.

- (A) Dot plot showing the expression level of representative marker genes across different cell types in both young (red dots) and old (blue dots) monkeys. Dot color indicates the scaled gene expression levels, and dot size indicates the percentage of expressed cells.
- (B) Coefficient of variation (CV) analysis showing the transcriptional noise of different cell types.

Figure S4. Transcriptional signatures of genes in the GenAge gene set and retinal disease gene sets.

Heatmaps showing the expression level of genes in the GenAge gene set and retinal disease gene sets in different cell types. Values in each row are *z*-score scaled. The color key from blue to red indicates low to high gene expression levels.

Figure S5. Age-related transcriptional alterations in various cell types.

- (A) Box plots showing SASP score in different cell types from the neural retina layer or choroid layer. Two-tailed Student's *t*-test *P* values are indicated. SASP score is calculated as the average expression level of SASP-related genes in each single cell.
- (B) Dot plot showing the log₂-transformed fold change of SASP-related genes between

cells from aged monkeys and cells from young monkeys. Only genes with statistically significant difference between cells from aged monkeys and cells from young monkeys are shown. Dot color indicates the \log_2 -transformed fold change. Genes which are only upregulated in given cell types are shown in the left (red shadow), and genes which are only downregulated in given cell types are shown in the right (blue shadow).

Figure S6. Integrative analysis of aging-associated differentially expressed genes with genes from GenAge gene set.

Heatmaps showing the overlapping genes between aging-associated DEGs and genes in the GenAge gene set. Left, upregulated aging-associated DEGs; right, downregulated aging-associated DEGs.

Figure S7. Integrative analysis of aging-associated differentially expressed genes with genes from retinal disease gene sets.

(A) Heatmaps showing the overlapping genes between aging-associated DEGs and genes in different retinal disease gene sets. Each panel corresponds to different diseases. Left, upregulated aging-associated DEGs; right, downregulated aging-associated DEGs. (B) Heatmaps showing the overlapping genes between aging-associated DEGs and genes in the retinal disease gene set. This gene set is the union set of all different retinal diseases corresponding to Figure S7A. Top, upregulated aging-associated DEGs; bottom, downregulated aging-associated DEGs.

Figure S8. Age-related cell-cell interaction alterations between monkey RPE and other cells in neural retina or choroid.

Dot plots showing newborn (only existed in aged monkeys, left) and disappeared (only existed in young monkeys, right) ligand-receptor gene pairs in a given cell pair. For a given cell pair, a given gene pair is either a newborn one or a disappeared one in this figure. The dot color and size indicate the average expression level and the statistical significance of gene pairs, respectively. In each row, pink and blue words correspond to ligands and receptors, respectively; in each column, pink words correspond to cell types expressing ligands and blue words correspond to cell types expressing receptors.

Figure S9. Potentially core transcriptional regulators during aging process across different cell types.

(A) Regulatory network showing potentially core transcriptional regulators in upregulated aging-associated DEGs of RPE. The line thickness indicates the weight of a connection, and the dot size indicates the number of connections. Top-ranked 4 nodes (ranked by the number of connections) are colored with red. Only connections with high weights are kept.

(B) Dot plots showing potentially core transcriptional regulators in aging-associated DEGs across difference cell types. The dot size indicates the number of connections. Only connections with high weights are kept.

Supplementary Table Legends

Table S1. Basic quality control information of single-cell RNA-seq dataset.

Table S2. Cell clustering, cell-type-specific marker genes and DEGs between two Müller glial subtype cells.

Table S3. SASP-related genes, the GenAge gene set and retinal disease gene sets.

Table S4. Aging-associated DEGs and corresponding GO terms in each cell type.

Table S5. Cell-cell interaction information between any two cell types.

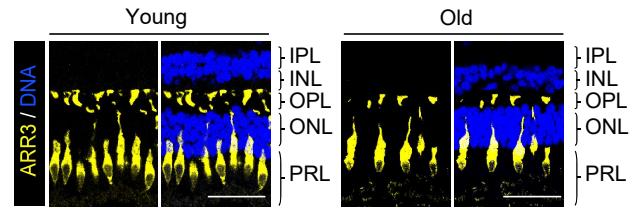
Table S6. Antibodies used in this study.

Figure S1

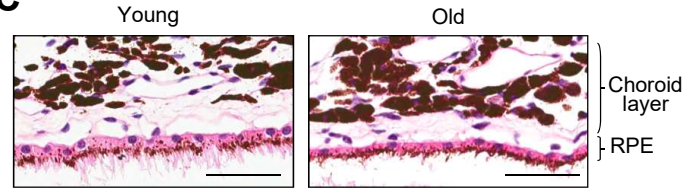
A

No.	Animal symbol	Sex	Age (year)	Number of total cells	Number of cells after filtering
1	YF1	Female	~ 5	412	242
2	YF2	Female	~ 5	472	438
3	YF3	Female	~ 5	479	441
4	YF4	Female	~ 4	473	408
5	YM1	Male	~ 5	480	423
6	YM2	Male	~ 5	430	360
7	YM3	Male	~ 6	447	390
8	YM4	Male	~ 6	480	363
9	OF1	Female	~ 18	480	443
10	OF2	Female	~ 19	460	410
11	OF3	Female	~ 19	480	412
12	OF4	Female	~ 20	480	440
13	OM1	Male	~ 18	455	357
14	OM2	Male	~ 19	475	401
15	OM3	Male	~ 20	479	443
16	OM4	Male	~ 21	479	439

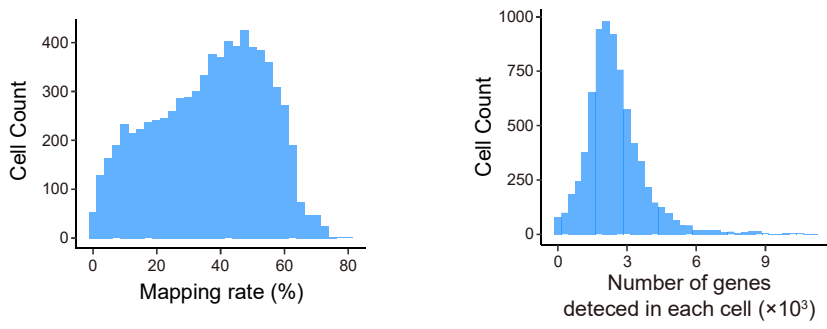
B



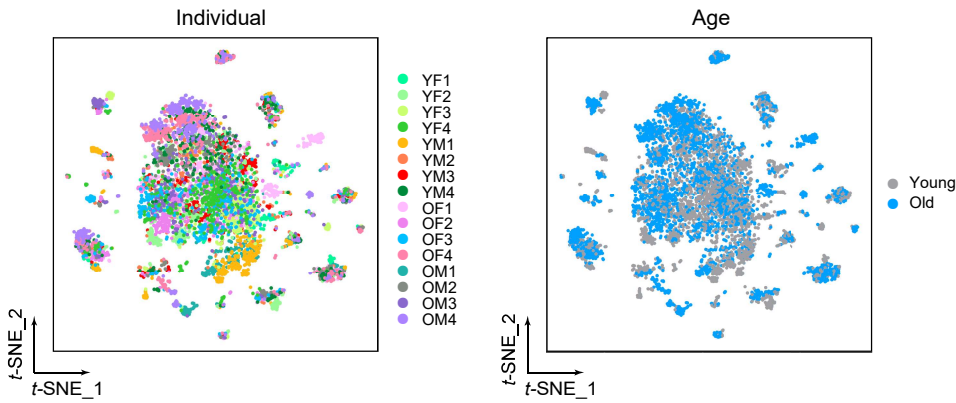
C



D



E



F

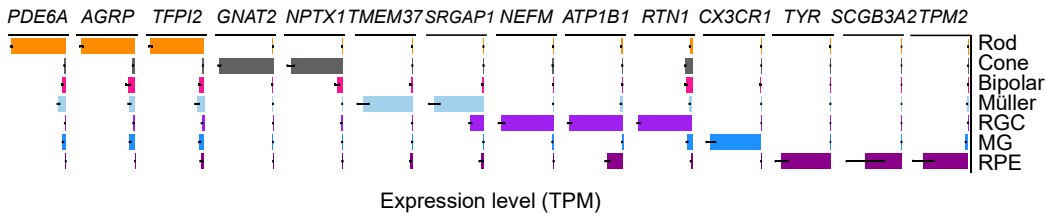
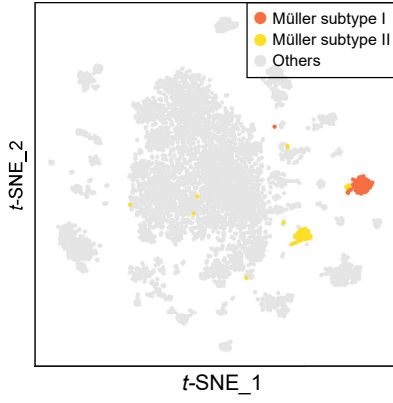
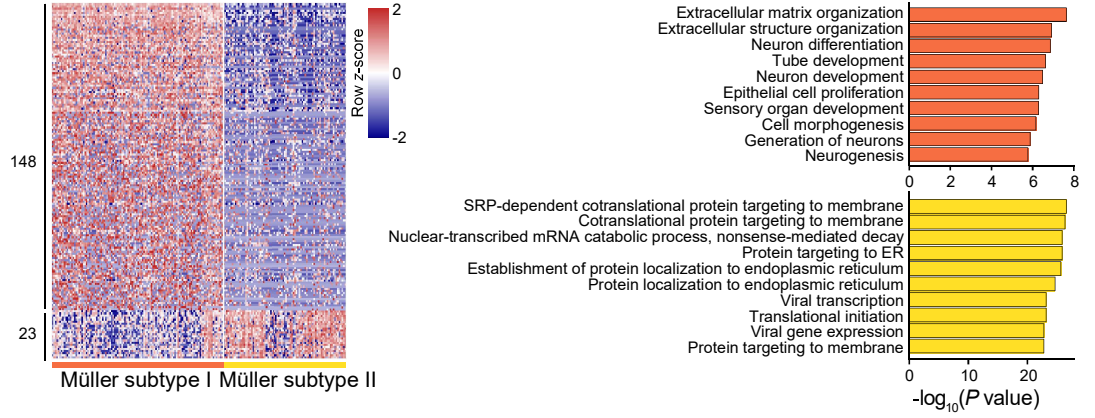


Figure S2

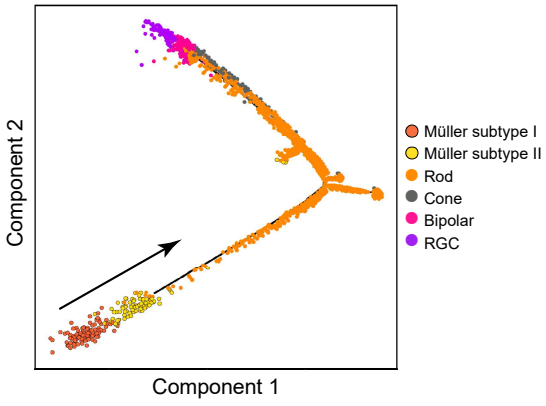
A



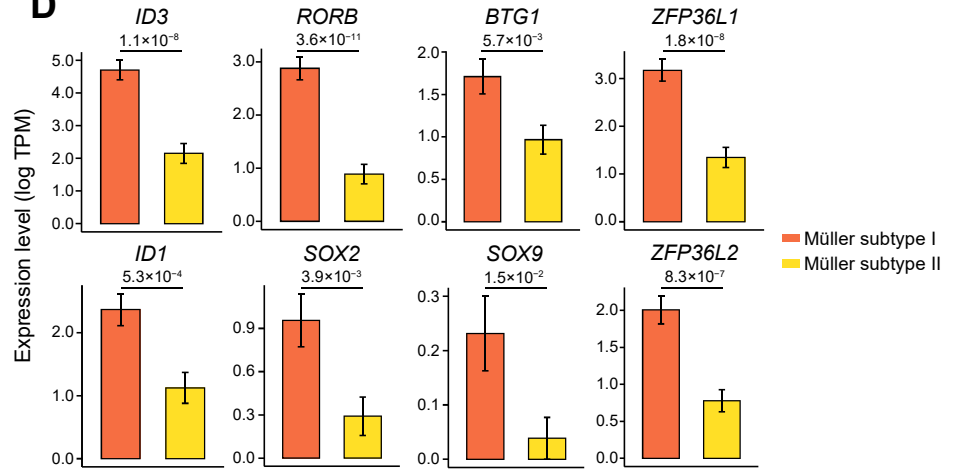
B



C



D



E

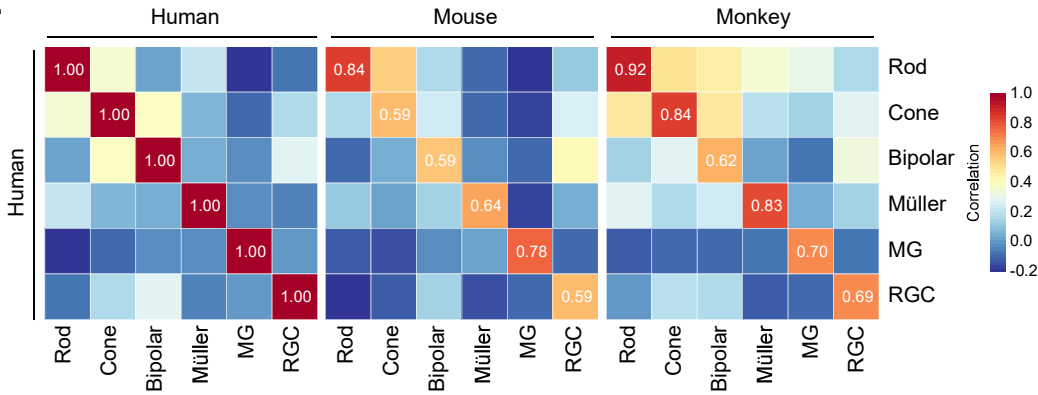
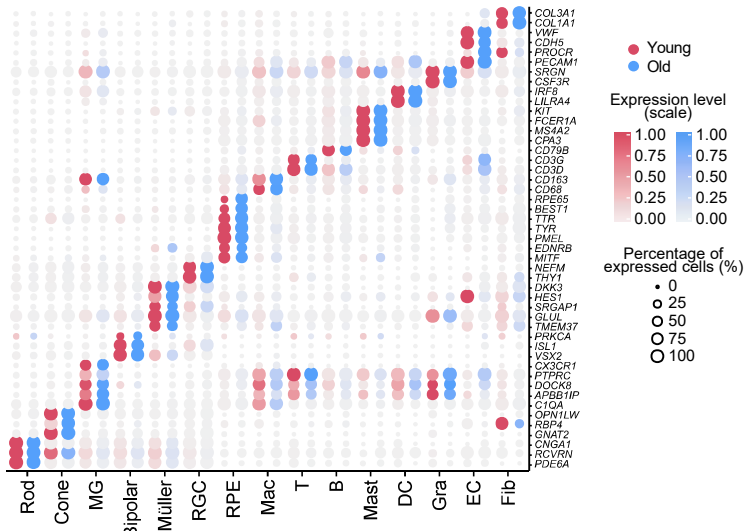


Figure S3

A



B

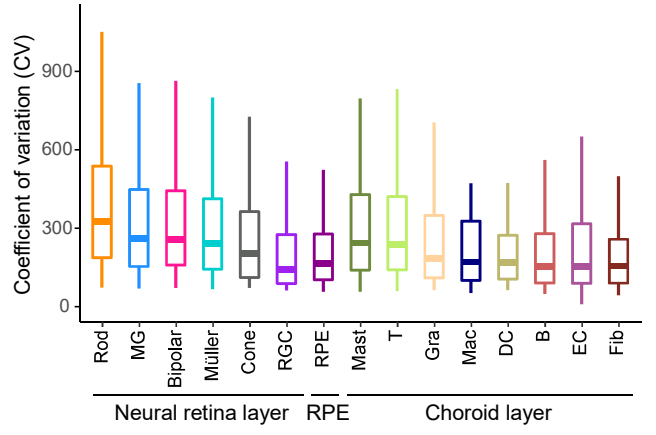


Figure S4

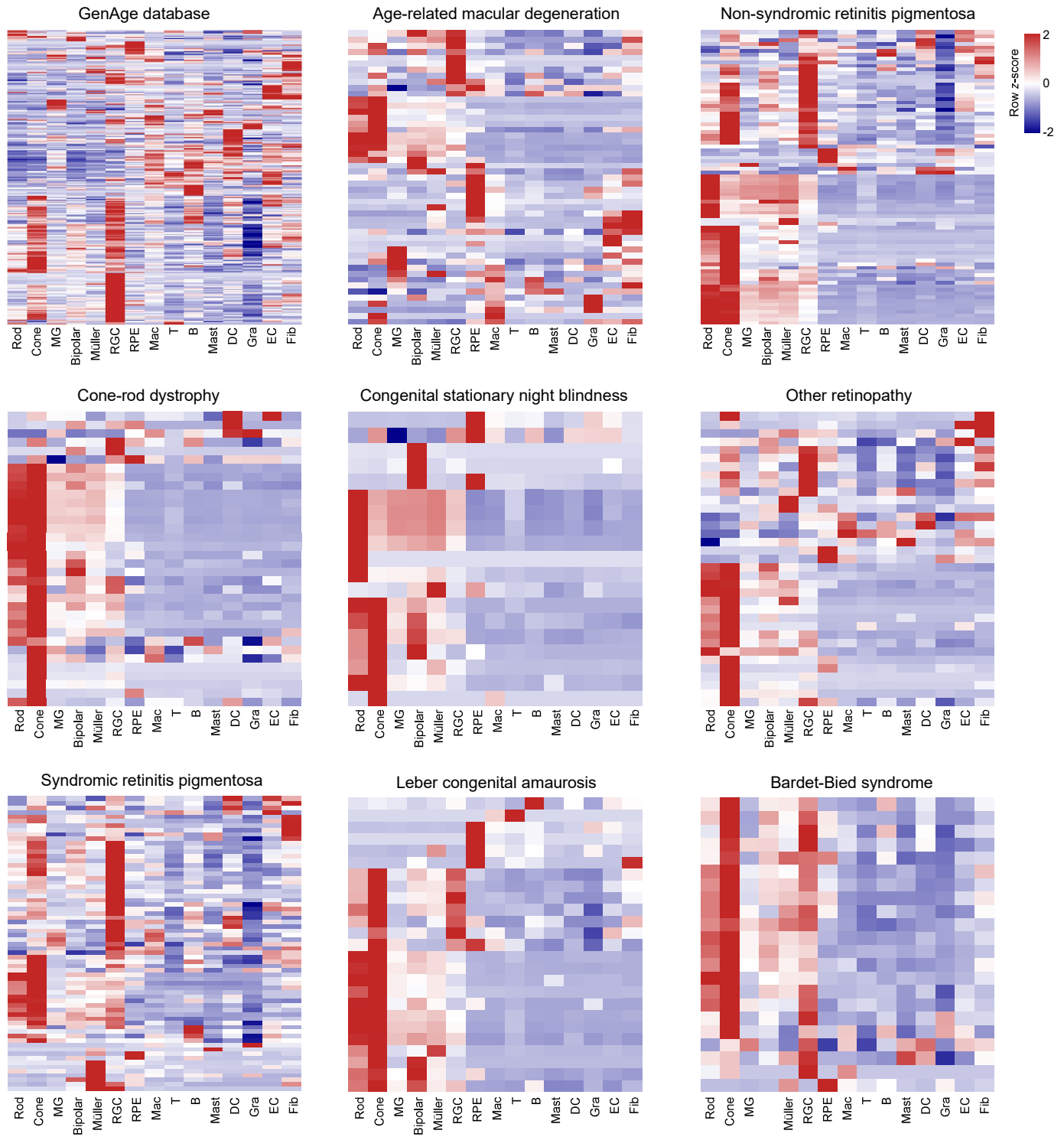
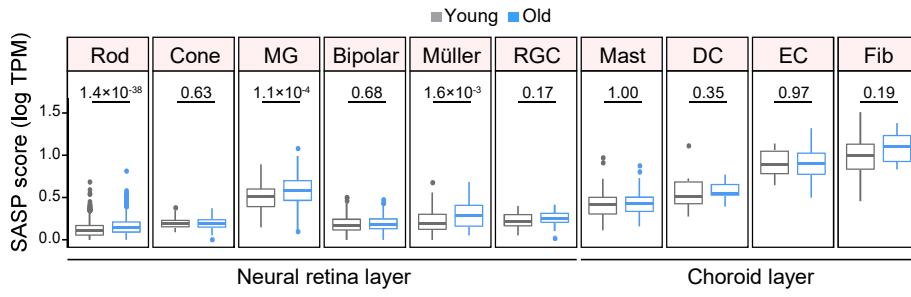


Figure S5

A



B

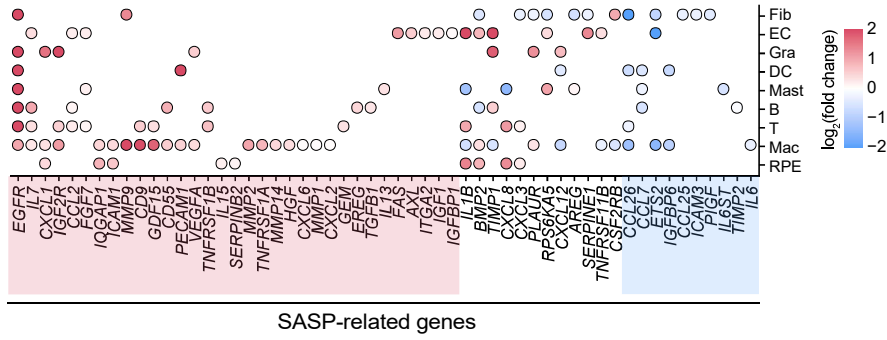
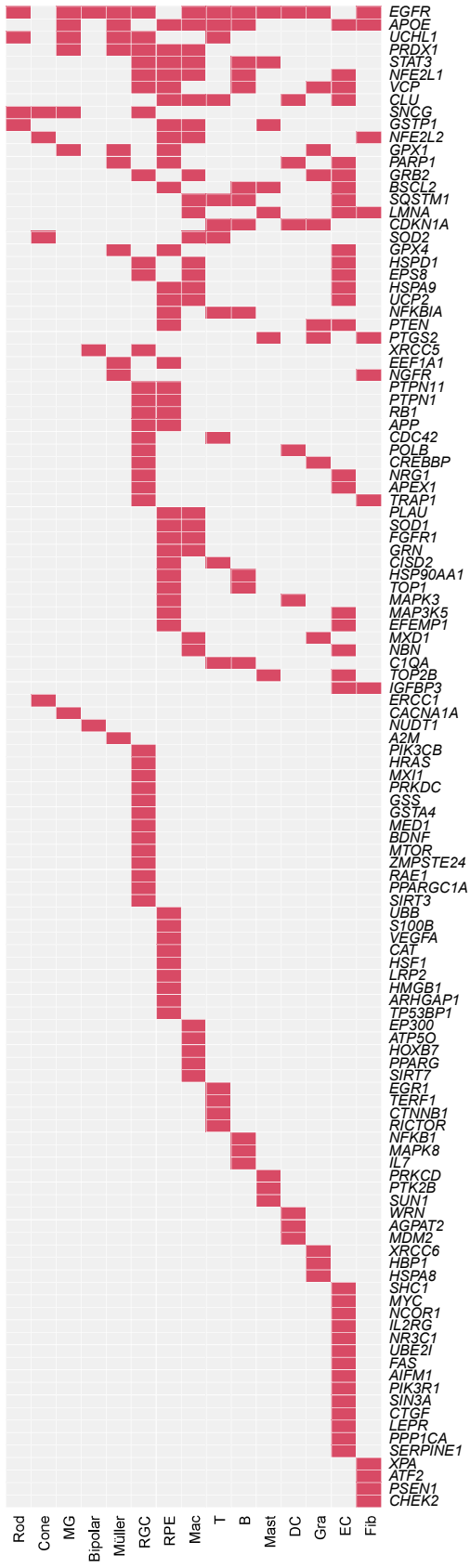
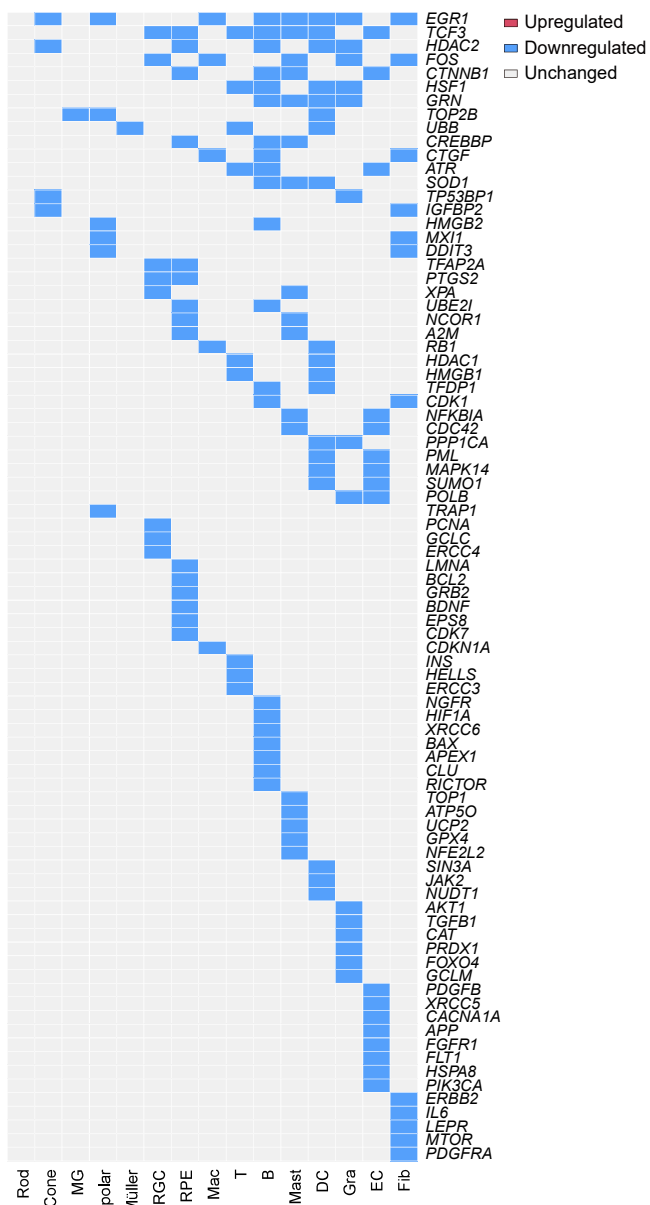


Figure S6

DEGs GenAge gene set



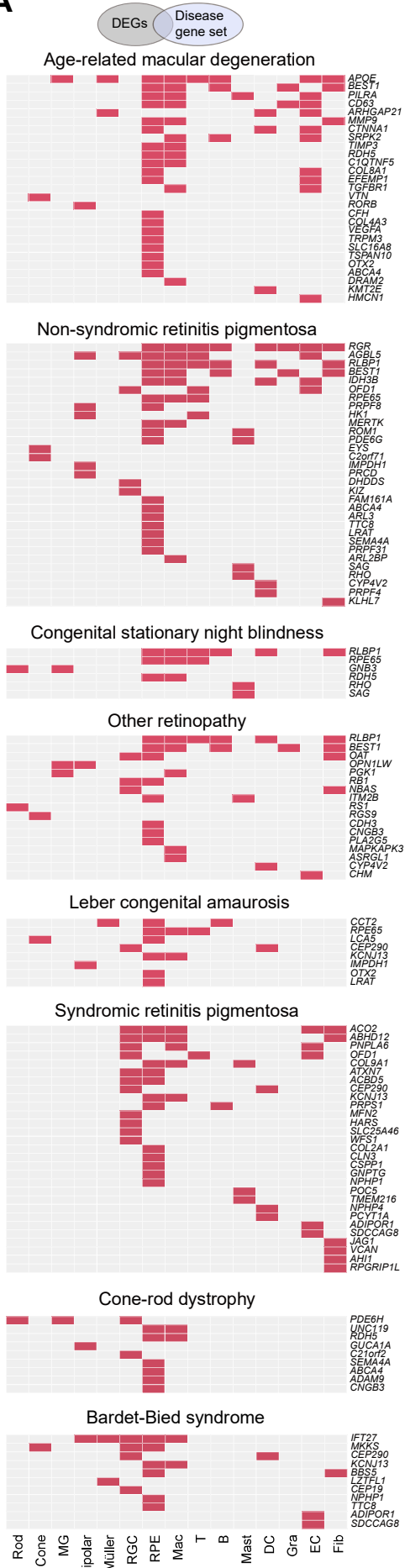
DEGs GenAge gene set



■ Upregulated
■ Downregulated
□ Unchanged

Figure S7

A



B

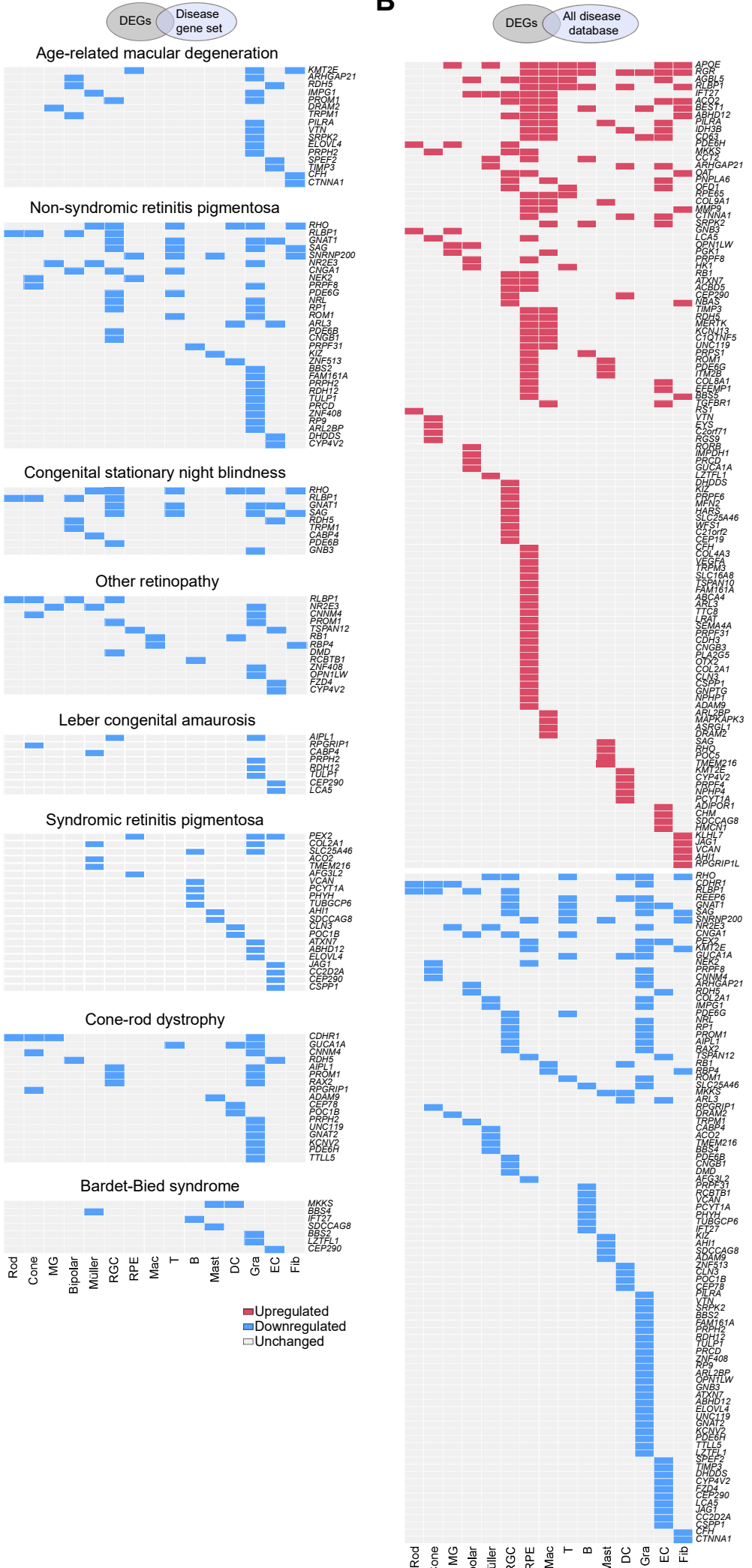
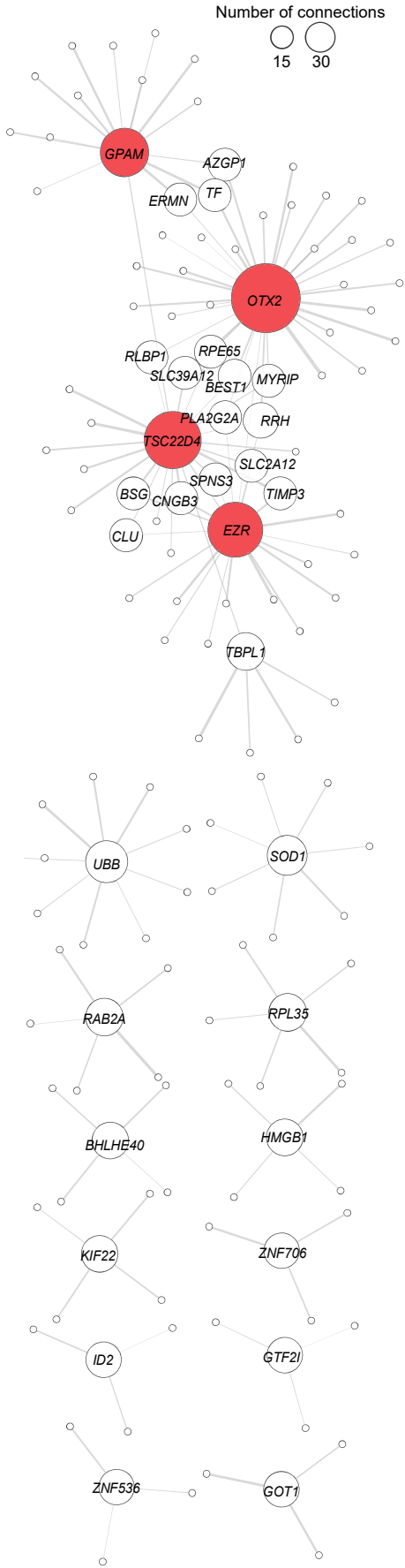


Figure S9

A



B

