



## Supplementary Materials for

### **Aging Fly Cell Atlas identifies exhaustive aging features at cellular resolution**

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#### **The PDF file includes:**

Materials and Methods  
Figs. S1 to S30  
References

#### **Other Supplementary Material for this manuscript includes the following:**

Movies S1 to S3  
MDAR Reproducibility Checklist

## Materials and Methods

### Fly sample and lifespan

Fly head and body samples were from F1 of two wild type fly stains: female *w<sup>1118</sup>* and male Oregon R (*OreR*). Flies were maintained on standard cornmeal medium at 25 °C with 12-hour light–dark cycle. For the lifespan experiment, progenies were collected 3 days after the first fly hatched. Flies were allowed to mate for 3 days. Then male and female flies were separated into different cages (120 flies per cage) for lifespan or sample collection at 25°C. Fly food was changed every 2 days. Dead flies were counted every 2 days. Lifespan curves were generated in Excel.

### Single-nucleus RNA sequencing

Fly heads and bodies dissected at different ages were put into 1.5ml RNAase-free Eppendorf tubes, flash-frozen in liquid nitrogen, and then stored at –80 °C. We put 100 heads or 50 bodies in each tube. Single-nucleus suspensions were prepared following the protocol described in the Fly Cell Atlas study (7). Next, we collect nuclei by FACS. We used the SONY SH800 FACS sorter for collecting nuclei. Nuclei were stained by Hoechst-33342 (1:1000; >5min). Since polyploidy is common for many fly tissues, we observed different populations of nuclei according to DNA content (Hoechst signal). In order to include all cell populations with different nuclear sizes, we have included all nuclear populations from the FACS. Nuclei were collected into a 1.5ml tube with 200ul 1x PBS with 0.5% BSA as the receiving buffer (RNase inhibitor added). For each 10x Genomics run, 100k–400k nuclei were collected. Nuclei were spun down for 10 min at 1000g at 4 °C and then resuspended using 40ul or desired amount of 1x PBS with 0.5% BSA (RNase inhibitor added). 2ul nucleus suspension was used for counting the nuclei with hemocytometers to calculate the concentration. When loading to the 10x controller, we always target 10k nuclei for each channel. We observed that loading 1.5 folds more nuclei as recommended by the protocol allowed us to recover about 10k cells after sequencing. For example, if the concentration is 1500 nuclei per ul for one sample, we treat it as 1000 nuclei per ul when loading to the 10x controller.

Next, we performed snRNA-seq using the 10x Genomics system with 3' v3.1 kits with the following settings. All PCR reactions were performed using the Biorad C1000 Touch Thermal cycler with 96-Deep Well Reaction Module. 13 cycles were used for cDNA amplification and 16 cycles were used for sample index PCR. As per 10x protocol, 1:10 dilutions of amplified cDNA and final libraries were evaluated on a bioanalyzer. Each library was diluted to 4 nM, and equal volumes of 18 libraries were pooled for each NovaSeq S4 sequencing run. Pools were sequenced using 100 cycle run kits and the Single Index configuration. Read 1, Index 1 (i7), and Read 2 are 28 bp, 8 bp and 91 bp respectively. A PhiX control library was spiked in at 0.2 to 1% concentration. Libraries were sequenced on the NovaSeq 6000 Sequencing System (Illumina).

For each age, we sequenced the head for 12 runs (6 for male and 6 for female) and body for 12 runs (6 for male and 6 for female). To collect enough nuclei for 6 runs, we used 100 heads and 50 bodies. For three ages, we sequenced  $24 \times 3 = 72$  runs. We have recently published a protocol describing the single-nucleus RNAseq in *Drosophila* and all the above steps have been detailed (54).



## Sequencing read alignment

The raw FASTQ files were first filtered for the index-hopping reads by using the index-hopping-filter developed by the 10x Genomics (version 1.0.1). Before mapping reads, the *Drosophila melanogaster* genome (FlyBase r6.31) was indexed with a pre-mRNA GTF, which was established by FCA (7), using the Cell Ranger (version 4.0.0). FASTQ files removing the index-hopping reads were mapped to the *Drosophila melanogaster* genome (r6.31) using Cell Ranger Count (version 4.0.0) to generate the count matrix.

## AFCA annotation

In the FCA dataset, there are 251 cell types annotated, many of which were characterized in dissected tissues. For cell types from the head, besides the whole head sequencing, there are only two dissected tissues: antennae and proboscis. So, most cell types from the aging head can be annotated based on the FCA whole head data. For cell types from the body, most cell type annotations are from tissues (only 33 annotated cell types from the FCA whole-body data). To annotate aging body data, we need to transfer annotations from the body and individual tissues (e.g., gut, wing, leg, testis, ovary, fat body, etc). Thus, the aging head and body data were annotated using slightly different strategies and we characterized them separately. For both, we first integrated FCA and AFCA datasets and corrected age and sex differences using Harmony (55). Next, we characterized cell types using two different approaches: co-clustering and supervised machine learning (ML)-based classification. Then, we validated those two approaches using cell type-specific markers and performed manual correction when necessary.

For the head data, FCA and AFCA head cells were co-clustered and 150 principal components (PCs) were selected for clustering. We followed the co-cluster-based approaches utilized in FCA brain-head integration (7) and annotations from the FCA cells were transferred to the clusters that contained at least 15% of cells with the same annotation. Alternatively, if more than 15% of the annotation-specific cells were enriched in the clusters, those clusters were assigned to the corresponding annotations. Different Leiden resolutions were applied for the transfer and the one with the maximum number of transferred annotations was chosen. If annotations from the two approaches were not consistent, cluster-dominant annotations would be assigned to the clusters. Besides cluster-based transfer, we also included a supervised ML method. We utilized the Logistic Regression classifier to transfer the FCA annotations to AFCA cells. FCA cells were used as the training dataset, while AFCA samples were considered as test data. By comparing the annotations from these two approaches, annotations were manually confirmed by examining the expression of cell type-specific genes to prevent the loss of transfer due to aging. Also, annotations were unified and denoised at the cluster level. In total, 91 cell types were annotated, including 17 newly identified neuron types.

To annotate AFCA body cells, FCA and AFCA body cells were first co-clustered using 50 PCs and 33 annotations from the FCA body were transferred to the AFCA body cells using approaches similar to the head transfer. To include annotations from different body tissues, we co-clustered

cells from the FCA body, AFCA body, and all FCA tissues to identify clusters with tissue-specific annotations (**Fig. S4**). The cluster-based method enabled us to quickly identify tissue-specific annotations from integrated body and tissue cells. Clusters with tissue-specific annotations were chosen for further sub-clustering and tissue annotations were transferred similarly using cluster- and supervised ML-based approaches. Due to the similarity between some gut and malpighian tubule cell types, especially for the intestinal stem cell and renal stem cell (**Fig. S6C and S7**), we combined annotations from these two tissues together for the transfer. Some cell types, like muscle cells, were present in multiple tissues and we considered subsetting those cells based on the expression of cell-type markers, instead of selecting the annotation-enriched clusters (**Fig. S5**). After subsetting marker-positive cells, FCA annotations were similarly transferred. After transferring all tissue annotations, 72 cell types were annotated in the AFCA body.

### **Gut cell type case study**

The analysis of the gut cells in the AFCA data was performed using SCANPY and scFates python packages (56, 57). The intestinal stem cell lineage was isolated from the data and includes intestinal stem cells, enteroblasts, adult differentiating enterocytes, enteroendocrine cells, anterior enterocytes, and posterior enterocytes. The pseudotime was generated by combining the young (FCA age 5d sample) and old (AFCA age 50d and 70d samples). To generate this plot, the partition-based graph abstraction (PAGA) function was applied to infer the connectivity of clusters then the ForceAtlas2 algorithm was implemented to spatially overlay the cells onto the connectivity plot generated by PAGA (16, 17). To compare the cell compositions of each age, the cells of different ages were plotted separately next to the pseudotime. The percentages of cells comprising the intestinal stem cell lineage were extracted from the data and then plotted in **Fig 2D**.

The changes in gene expression were generated using the scFates python package (57). Cells were isolated from each age and then run separately in the scFates pipeline. Briefly, scFates uses the EIPiGraph algorithm to learn the topography of the data. Then, the pseudotime was constructed from the graph inferred by the EIPiGraph algorithm. The genes differentially expressed along the trajectory were determined using a cubic spline regression model and then these results were compared to an unrestrained model. The Benjamini-Hochberg correction was then applied to adjust for multiple comparisons. A significantly altered gene along the trajectory between each age, *Rbfox1*, is shown in **Fig S11**.

### **Fat body and indirect flight muscle staining**

To label the fat body tissue with GFP, *cg-GAL4* (Bloomington Drosophila Stock Center (BDSC), #7011) virgins were crossed with *UAS-unc84GFP* (from Dr. Gilbert L. Henry) or *UAS-CD8GFP* (from Dr. Liqun Luo) males to obtain *cg-GAL4>unc84GFP* or *cg-GAL4>CD8GFP* flies. For indirect flight muscle labeling, *Act88F-GAL4* (BDSC #38461) virgins were crossed with *UAS-unc84GFP* to obtain *Act88F-GAL4>unc84GFP* flies. Flies were cultured at 25°C to desired ages.

For fat body dissection, fly abdomen filets were prepared in 1X PBS using Vannas Spring Scissors (FST, 3mm Cutting Edge, 15000-00) and Dumont #55 forceps (FST, 11255-20). For indirect flight

muscle dissection, flies in 1.5ml Eppendorfs tube were snap-frozen in liquid nitrogen and placed on the ice rack. After removing the head, leg, and wing, the abdomen was clamped with forceps and the thorax was cut in the sagittal section by a razor. Tissues were fixed with 4% paraformaldehyde for 20 min at room temperature (RT), washed with 1X PBS, blocked in 5% NGS blocking buffer (1XPBS, 0.3% Triton X-100, 5% NGS) for 2 h at RT, incubated with primary antibodies (in 5% NGS blocking buffer) overnight at 4°C, and then washed 5 times with 0.3% PBST (1XPBS, 0.3% Triton X-100) before incubation with secondary antibodies for 2 h at RT. Tissues were thoroughly rinsed in PBST, stained with DAPI(1:1000) for 15 min, washed with 1XPBS and mounted with SlowFade™ Gold Antifade Mountant (Thermo Fisher, S36936).

Mouse anti-LamC (1:100, Developmental Studies Hybridoma Bank (DSHB), LC28.26), chicken anti-GFP (1:1000, Aves Labs), rabbit anti-pH3 (1:1000, Cell Signaling Technology, #9701), rabbit anti-cleaved-Caspase3 (1:100, Cell Signaling Technology, #9661) were used as primary antibodies. For secondary antibodies, we used Cy™3 AffiniPure Donkey Anti-Mouse IgG (H+L) (1:250, Jackson ImmunoResearch, AB\_2340813), Alexa Fluor® 488 AffiniPure Donkey Anti-Chicken IgY (IgG) (H+L) (1:250, Jackson ImmunoResearch, AB\_2340375), Alexa Fluor™ 647 Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody (1:250, Invitrogen, A31573). Alexa Fluor 647 Phalloidin (1:250, Invitrogen, A22287) was used to stain F-actin in muscle.

Images were obtained with Leica STELLARIS 5 confocal microscope. Images were obtained as Z series with the same interval. Z series images were merged by ImageJ (Image-Stacks-Z projection-Max Intensity), and then the mean fluorescence intensity was measured (Analyze-Measure-Mean gray value) or the nuclear number was counted by Point Tool. Quantification graphs are generated by GraphPad Prism. P-values from unpaired t-test. Error bar, SD.

## **DEG analysis**

To identify genes that are differentially expressed in the aged population, we applied the Wilcoxon Rank Sum test (`rank_genes_groups()` in SCANPY) to compare the gene expression between the aged (30d, 50d, and 70d) and the young population (5d). Genes with FDR lower than 0.05 would be considered to be differentially expressed.

## **Change of cell composition**

Cell numbers of each cell type were counted and separated by age. To get the ratio of one cell type from one age, the cell numbers of a specific cell type and age were further divided by the total cell number of the corresponding age. The cell-type ratios from the aged population (30d, 50d, 70d) were further divided by ratios from the young population (5d) to get the relative ratios in the log<sub>2</sub> scale.

## **GO analysis**

In each cell type, aging DEGs, including up- or down-regulated genes, were applied for the Gene Ontology (GO) analysis using GOATOOLS (version 1.2.3) (58). The gene association file (version FB2022\_04) was downloaded from FlyBase. Redundant GO terms were removed by REVIGO

(59). GO terms from Biological Process (BP) were mainly used for our analysis (**Fig. 3E and 3F**). GOs restricted within 5 cell types were considered as cell-type specific GOs (**Fig. 3E, 3F, and Fig. S20A**).

### **Aging clock analysis**

For aging clock analysis, we focused on 64 head and body cell types that have at least 200 cells at each time point. For each cell type satisfying the condition, we then built aging clock models – regression models that predict age from the transcriptome. In particular, for each cell type we trained the elastic net model (33) by using cells' transcriptome as explanatory (independent) variables and age as the response (dependent) variable. We randomly split the dataset into a train and test dataset where we used 70% of cells to train the model, and 30% of cells to validate the model. We compared the predictions to the true age and measured performance of the model as  $R^2$ , *i.e.*, proportion of the variance for a dependent variable that's explained by an independent variable or variables. We repeated the analysis over five random seeds, resulting in different train and test data splits. To find genes that correlate with age, we first filtered all genes with zero coefficient in the regression model. For the remaining genes, we calculated the Pearson correlation between its value and age and retained those genes for which the correlation coefficient was higher than 0.3. This analysis resulted in 480 genes. We repeated the same analysis for the Mouse Aging Cell Atlas (Tabula Muris Senis) (27).

To compare models between two consecutive stages, we trained three logistic regression models for each cell type to distinguish between (i) 5 days and 30 days old fly, (ii) 30 days and 50 days old fly, and (iii) 50 days and 70 days old fly. We split the data into train and test dataset by using 70% data for training the model and 30% data for evaluation by preserving the percentage of samples for each class (stratified sampling). We evaluated classification performance using accuracy.

### **Identification of possible transcription factor(s) regulating the RP genes**

Head and body regulons identified by SCENIC were obtained from FCA (7, 36). TF regulons containing more than 5 RP genes were considered to be the potential transcription factors. RP genes were removed from the regulon of each transcription factor and the average expression of RP and regulon genes was calculated in each cell type. The average expression of RP and regulon genes was clustered to find regulons showing gene expressions similar to the RP genes.

### **Cell-identity analysis**

Change in cell identity was based on the expression changes of marker genes between different ages. Cell-type markers from young (5d) and old animals (50d) were identified separately using the Wilcoxon Rank Sum test (`rank_genes_groups()` in SCANPY). The top 200 genes that are differentially expressed between cell types were defined as marker genes for each cell type. Expression of marker genes was further compared between young and old populations using the Wilcoxon Rank Sum test to identify differentially expressed markers (FDR < 0.05). Young marker genes with a significant reduction of expression would be considered to decrease the cell-type

identity. On the other hand, old marker genes with a significant increase in expression were also considered to decrease the identity. Together, we used the average of these two ratios to indicate the change in cell identity.

### **AFCA website**

The AFCA Website was developed with the Shiny package (v1.6.0) in R language (v4.0.5). We included three major datasets: Head dataset, Body dataset, and Head and Body dataset, where they contain the snRNA-Seq analytical results from head tissues, body tissues, and combined respectively. For the Cell type and Gene Expression tabs, the data processing and visualization were performed with the Tidyverse (v1.3.0) and ggplot2 (v3.3.3) packages in R. For the customized analysis tab, its features and functions were powered by the ShinyCell (v2.1.0) package.

### **The rank sum of aging features**

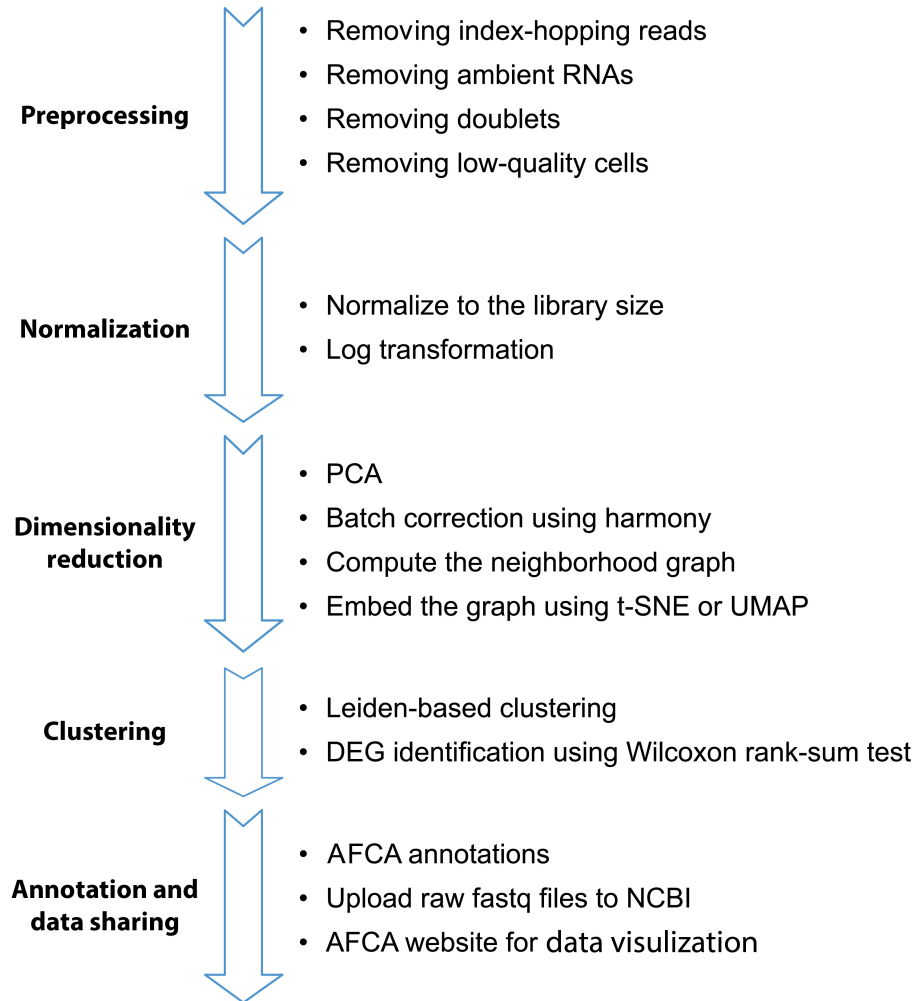
We measured the absolute differences of each aging feature between the young (5d) and old (50d) population and ranked the cell-type differences. High ranks represented high aging differences, while low ranks indicated low differences between young and old populations. To have a better estimation, we focused on cell types with more than 200 nuclei from each age. Correlations of different aging features were compared using Spearman's correlation. To know which cell types showed high aging differences supported by different features, we summed the ranks of five different aging features and ranked the sums. High-rank sums of different aging features represented high aging differences and vice versa.

### **Analysis of alternative polyadenylation (APA) analysis**

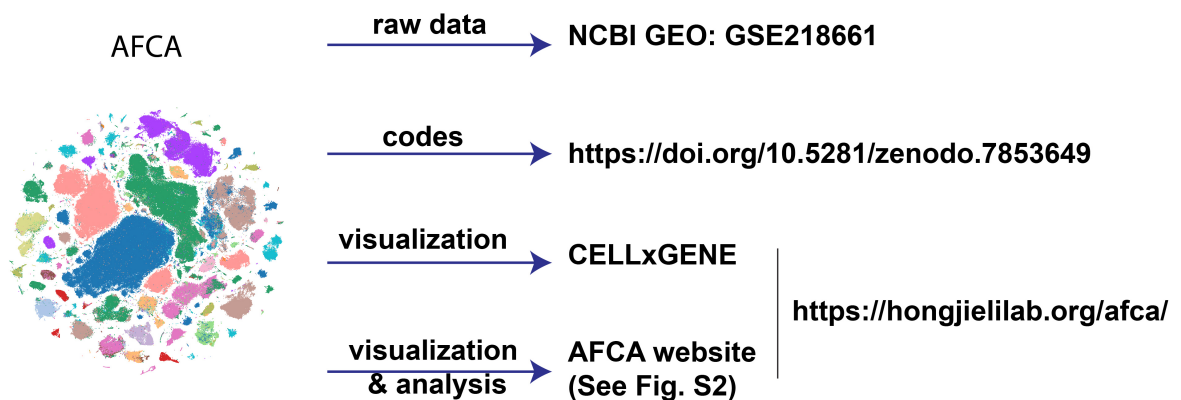
Our overall analysis strategy was based on our recent scAPA study of the FCA dataset (50). We used LABRATsc (<https://github.com/TaliaferroLab/LABRAT>) to quantify APA from the scRNA-seq data with current FlyBase *Drosophila melanogaster* gene annotation (version r6.45). Two modes were used for either single-cell level (cellbycell) or cell-type specific (subsampleClusters) quantification using '--mode' parameter. We used read coverage thresholds of at least 3 or 100 counts per gene for single-cell level or cell-type specific quantification respectively to minimize noise and false positives. LABRAT calculates  $\psi$  values reflecting 3' isoform usage, with "0" indicating exclusive usage of the most proximal pA site, while "1" indicates exclusive usage of the most distal pA site.  $\Psi$  values for each annotated cell-type, and for individual cells, were used for downstream analysis.

**Figs. S1 to S30**

**A** data processing steps



**B** data availability



**Fig. S1. General pipeline of snRNA-seq analysis and data availability.**

A) Data processing steps, including preprocessing, normalization, dimensionality reduction, clustering, annotation, and data sharing. B) Data available from different sources, including NCBI, GitHub, CELLxGENE, and AFCA website.



# Figure S2

## Aging Fly Cell Atlas

<https://hongjielilab.org/afca/>

Afca Home Head Dataset - Body Dataset - Head and Body Dataset - Downloads

**Cell Type (Head)**

Functional Tabs:

- Cell Type
- Gene Expression
- Customized Analysis

**step 1: choose cell type**

Cell Type: All

Dimension Reduction:  UMAP  t-SNE

**\* change settings**

Toggle graphics controls

**step 2: check aging features**

t-SNE by Age **Marker Gene** Aging Feature DE Analysis

Age: 5

Age: 30

Age: 50

Age: 70

**cell type-based analysis**

- cell type from 4 ages
- marker genes
- aging features
  - change of cellular composition
  - # DEG
  - # gene & UMI
- age-related genes

step 1: choose a gene
step 2: choose a cell type or all cells

Gene name: CG17005

Cell Type: outer photoreceptor cell

Age: 5

Age: 30

Age: 50

Age: 70

Show Clustering

**age-related change**

**statistics**

Gene name	P-value	FDR	Log2FC	Cell Type	Comparison
CG17005	0	0	-7.0807315	outer photoreceptor cell	5/30
CG17005	0	0	-4.7280275	outer photoreceptor cell	5/50
CG17005	0	0	-4.1232018	outer photoreceptor cell	5/70

- gene-based analysis**
- expression in all cell types
  - expression in one cell type
  - ranking cell types by changes

Details vs GeneExp
Details vs CellExp
GeneExp vs GeneExp
Gene co-expression
Marker / Single
Proportion plot
Bubble plot / Heatmap

Co-expression of two genes on reduced dimensions

In this tab, users can visualize the co-expression of two genes on low-dimensional representations.

Dimension Reduction:  UMAP  t-SNE

Gene Expression

Gene 1: nompC

Gene 2: rSyb

Toggle plot controls

Color:  Red (Gene1), Blue (Gene2)  Orange (Gene1), Blue (Gene2)  Red (Gene1), Green (Gene2)  Green (Gene1), Blue (Gene2)

Plot order:  Max-Val  Min-Val  Original  Random

Toggle graphics controls

Cell numbers

expression > 5	0	< 5	percent
nompC	1008	1008	0.71
rSyb	1008	1008	0.71
nompC	1008	1008	0.71
rSyb	1008	1008	0.71
note	2016	2016	1.42

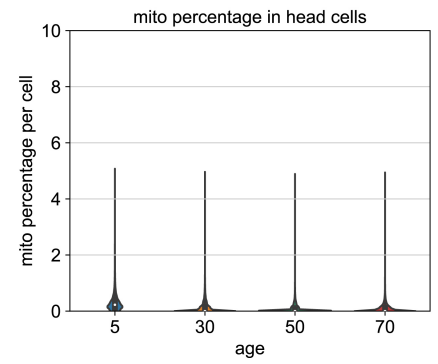
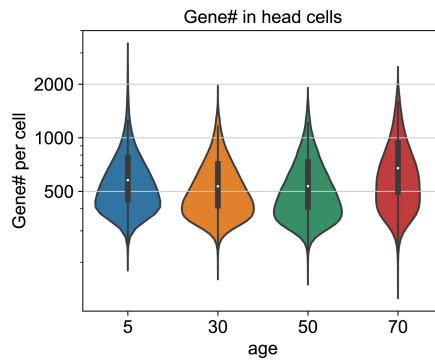
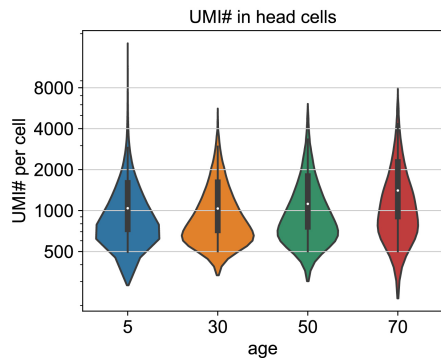
- custom analysis**
- gene co-expression
  - compare cell types
  - compare genes
  - multi-gene plot

**Fig. S2. AFCA web portal for surveying the age-related changes.**

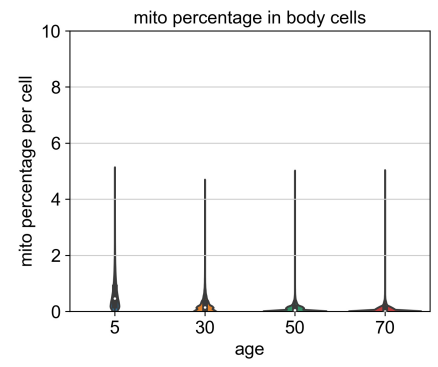
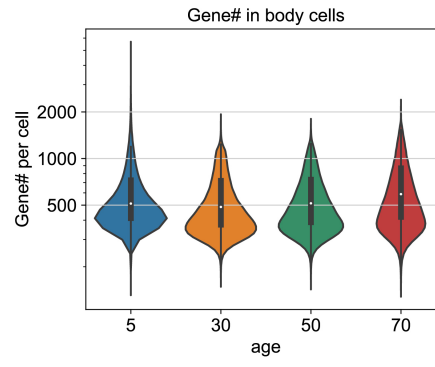
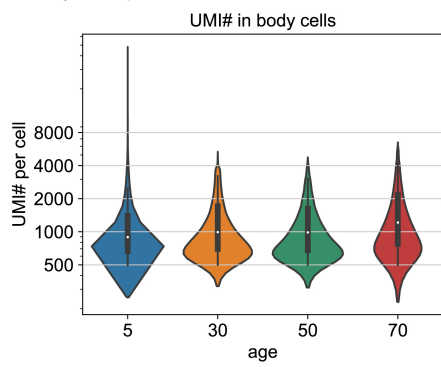
AFCA web portal provides cell type-based, gene-based, and customized analysis.

Figure S3

head 



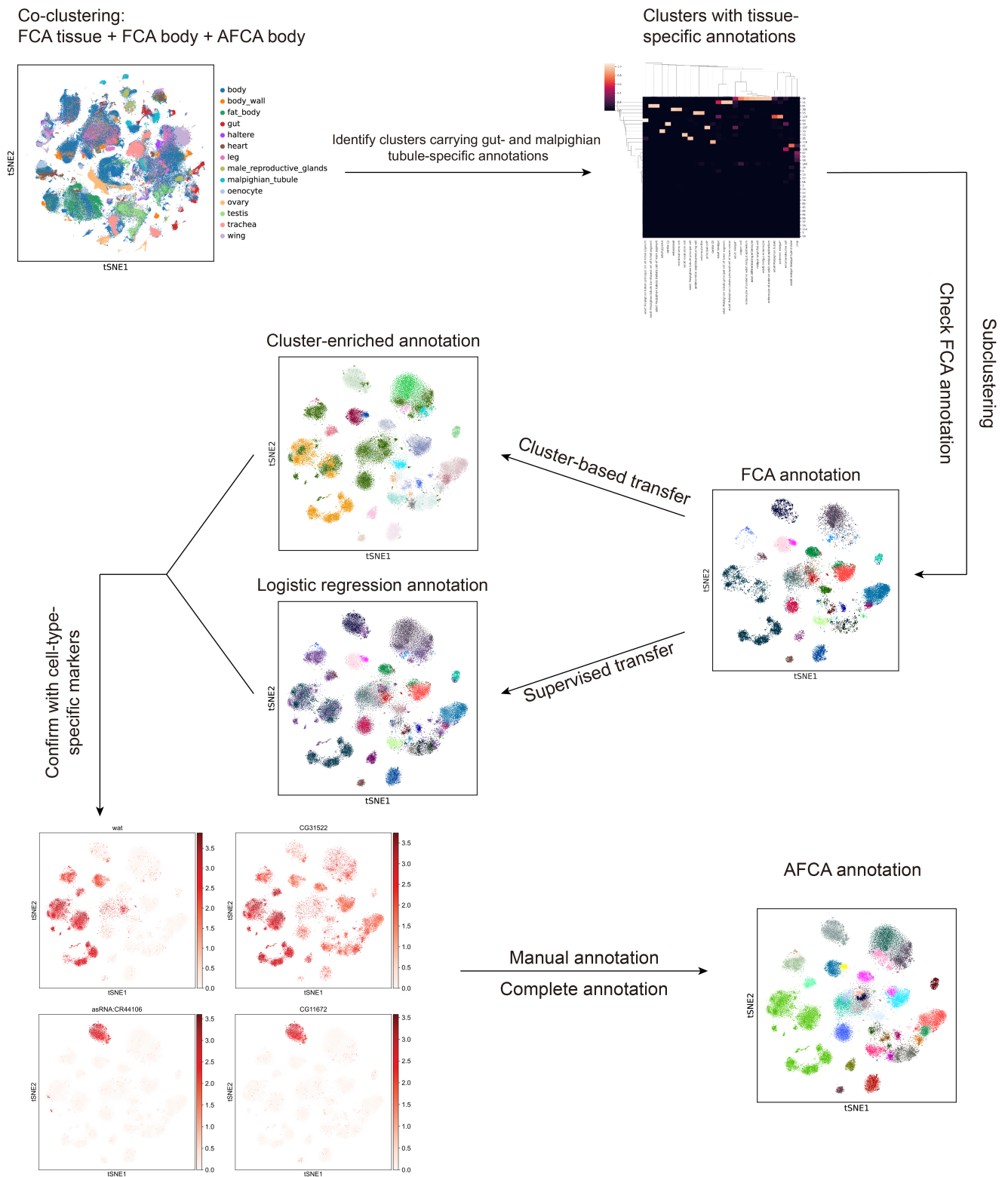
body 



**Fig. S3. General quality of snRNA-seq results.**

UMI number, expressed gene number, and mitochondria transcript ratio from the AFCA data.

Figure S4

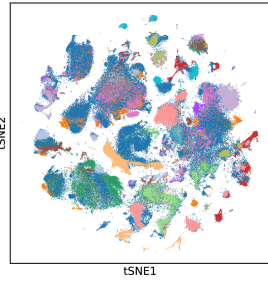


**Fig. S4. Illustration of transferring the FCA annotation to the AFCA cells.**

Flowchart of transferring the annotations from FCA to AFCA. FCA and AFCA data are first co-clustered. Co-clustered data can be further extracted for tissue-specific cell types, e.g. gut- and malpighian tubule-specific cell types. After subclustering targeted cells, FCA annotations are transferred to AFCA cells using cluster-based or supervised machine-learning approaches. Results from two transfer methods are compared and ambiguous transfers are further checked for the expression of the corresponding marker genes. After confirming the expression of the marker genes, annotations are corrected and finalized.

Figure S5

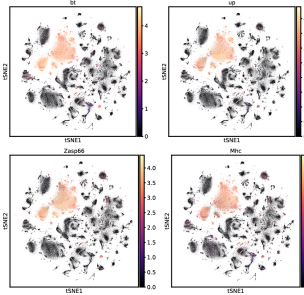
Co-clustering:  
FCA tissue + FCA body + AFCA body



- body
- body\_wall
- fat\_body
- gut
- haltere
- heart
- leg
- male\_reproductive\_glands
- malpighian\_tubule
- oenocyte
- ovary
- testis
- trachea
- wing

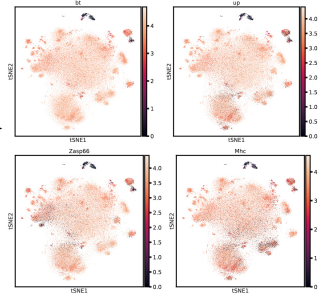
Identify clusters expressing muscle cell markers

Expression of tissue-specific markers

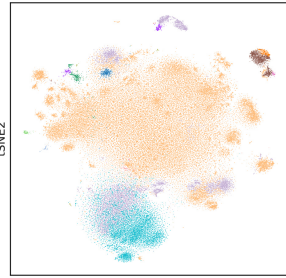


subcluster marker-positive cells

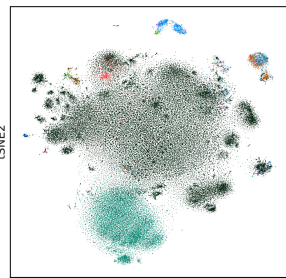
Subclustering



Cluster-enriched annotation

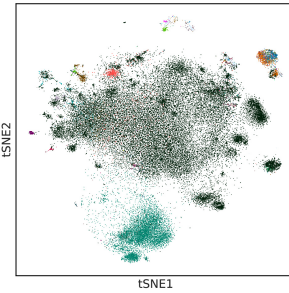


Logistic regression annotation



Confirm with cell-type-specific markers

FCA annotation

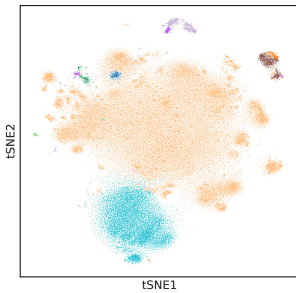


Check FCA annotation

Cluster-based transfer

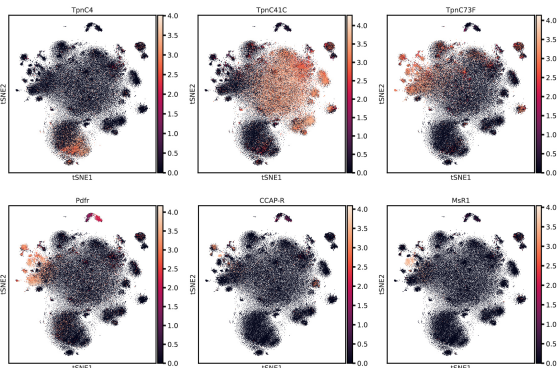
Supervised transfer

AFCA annotation



Manual annotation

Complete muscle cell annotation



**Fig. S5. Illustration of transferring the FCA annotation to the AFCA muscle cells.**

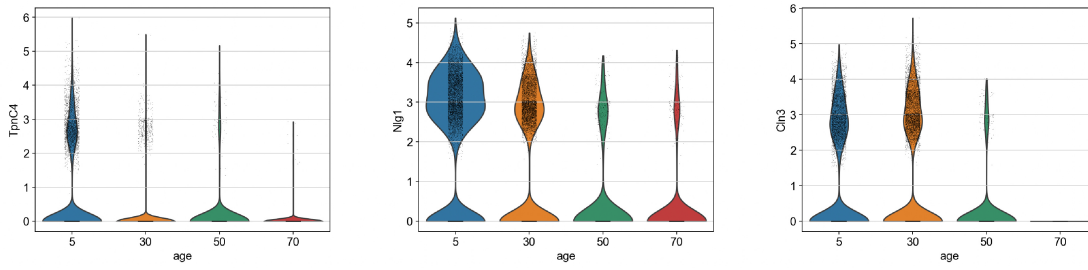
Flowchart of transferring the muscle-type annotations from FCA to AFCA. Some cell types, like muscle cells, can be found in multiple tissues and we consider first extracting those cells based on the expression of general markers of muscle cells. After subclustering the corresponding cells, all remaining analyses are similar to approaches used in **Fig. S4**.



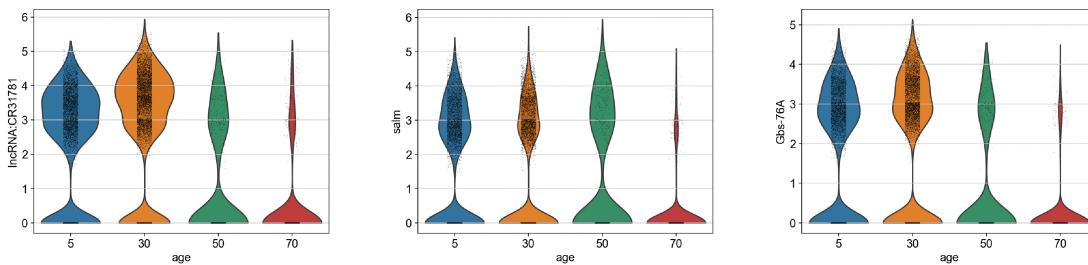
Figure S6

A

Decreased expression of marker genes from the indirect flight muscle

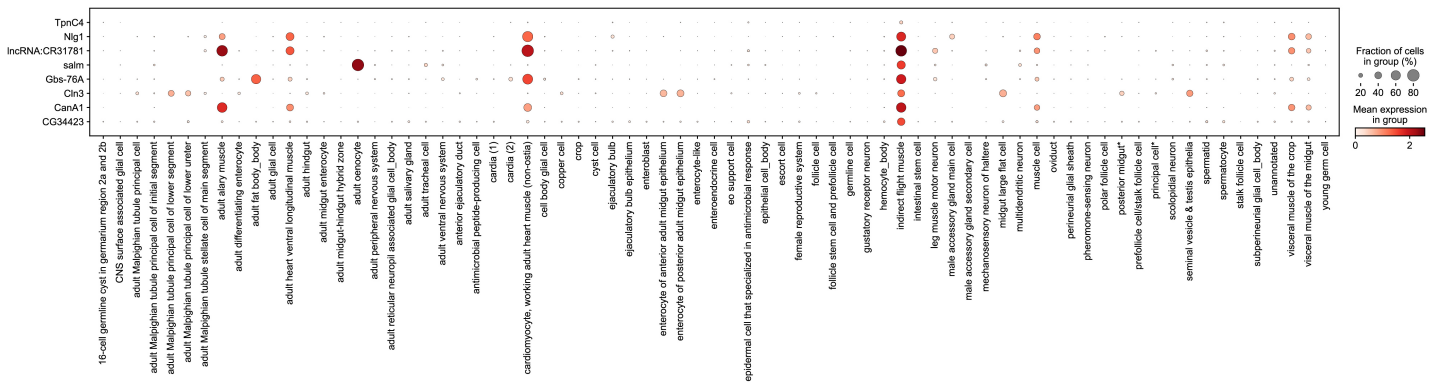


Other marker genes from the indirect flight muscle



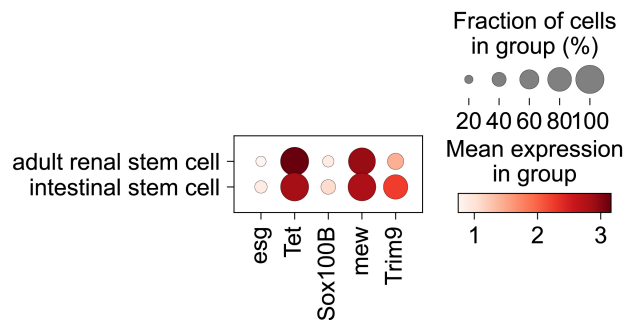
B

Marker genes from the indirect flight muscle



C

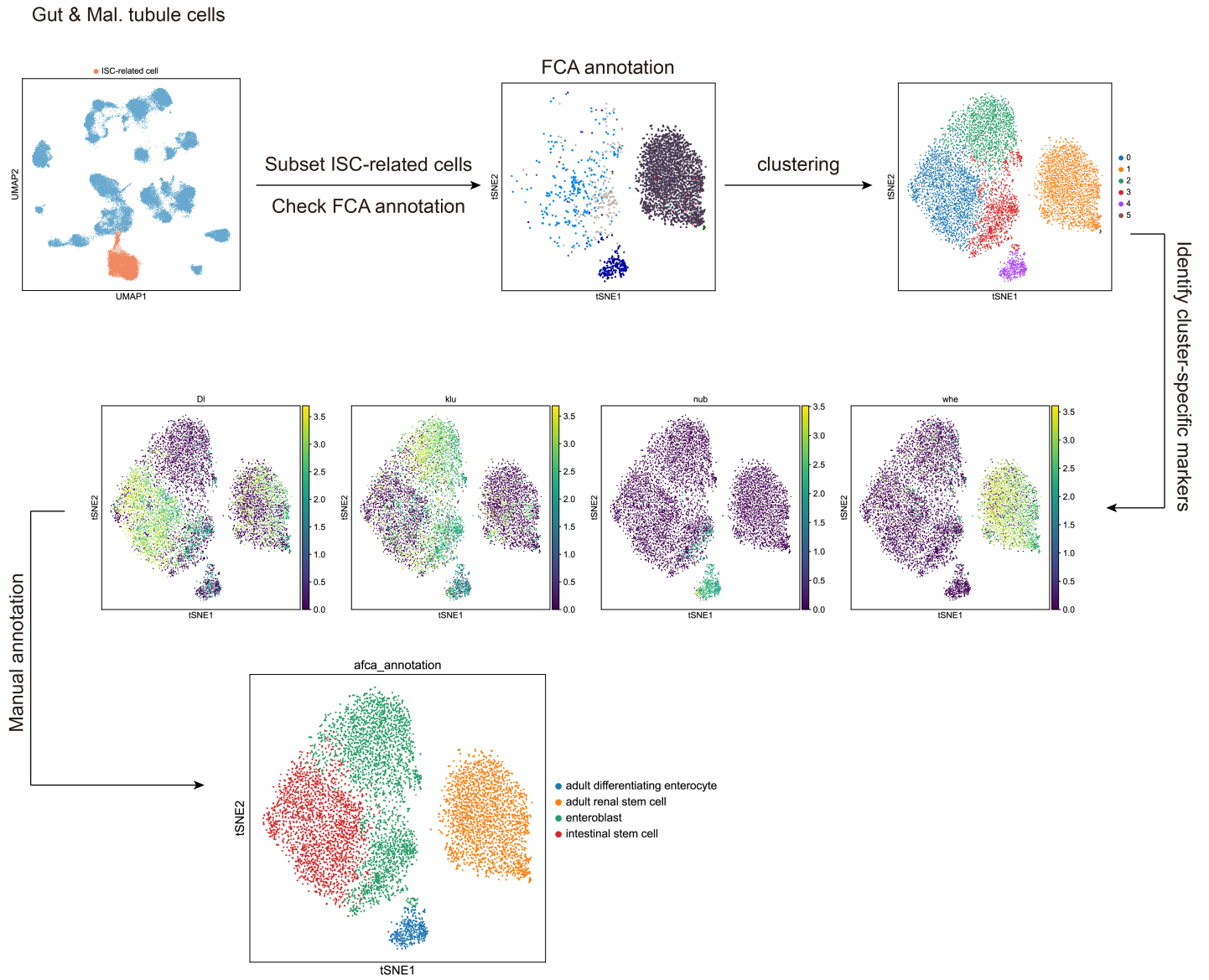
Overlapped marker genes from two stem cells



**Fig. S6. Ambiguous annotations from the aging samples.**

A, B) Marker genes of the indirect flight muscle. A) Loss of marker genes in the indirect flight muscle. B) Markers specific for the indirect flight muscle. C) Overlapped marker genes from renal stem cells and intestinal stem cells.

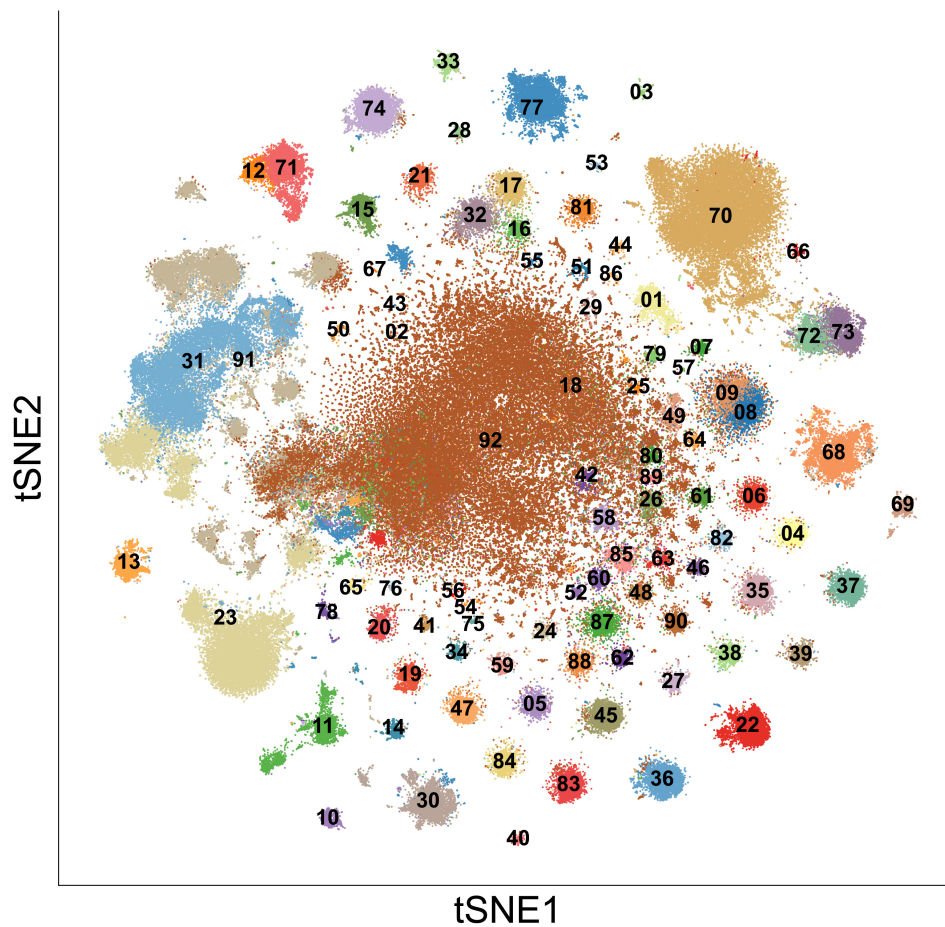
Figure S7



**Fig. S7. Illustration of manually annotating ISCs, EBs, and renal stem cells.**

Flowchart of manual annotation of the stem cell population. ISCs, EBs, and differentiating enterocyte cells are selected from the gut and malpighian tubule dataset shown in **Fig. S4**. Marker genes enriched in each cluster are identified and confirmed.

Figure S8

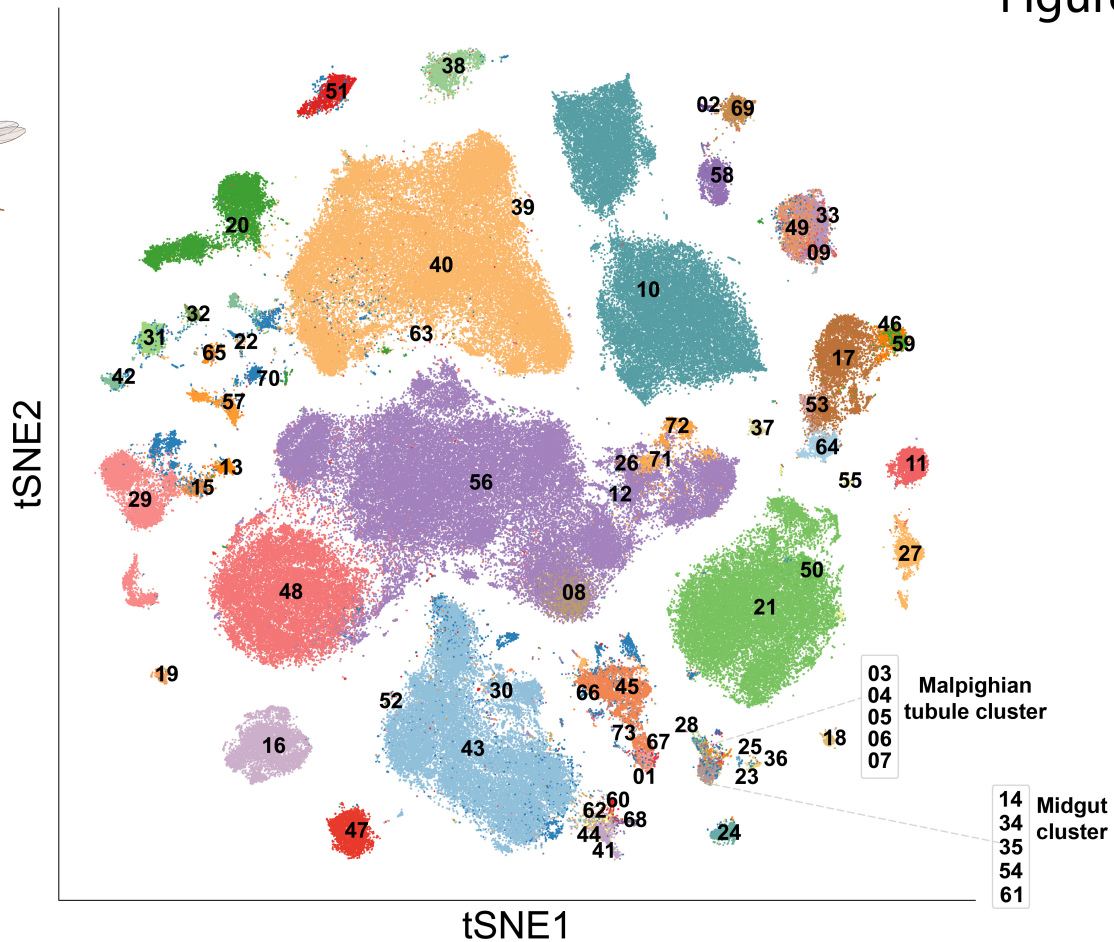


- |  |   |  |
|--|---|--|
| ● 01.Johnston organ neuron                               | ● 32.gamma Kenyon cell                    | ● 63.neuron 7                                |
| ● 02.Kenyon cell   | ● 33.hemocyte_head                        | ● 64.neuron 8                                |
| ● 03.Poxn neuron   | ● 34.lamina intrinsic amacrine neuron Lai | ● 65.neuron 9                                |
| ● 04.T neuron T2   | ● 35.lamina monopolar neuron L1           | ● 66.ocellus retinula cell                   |
| ● 05.T neuron T2a  | ● 36.lamina monopolar neuron L2           | ● 67.octopaminergic/tyraminerbic neuron      |
| ● 06.T neuron T3   | ● 37.lamina monopolar neuron L3           | ● 68.olfactory receptor neuron               |
| ● 07.T neuron T4/T5                                      | ● 38.lamina monopolar neuron L4           | ● 69.optic-lobe-associated cortex glial cell |
| ● 08.T neuron T4/T5a-b                                   | ● 39.lamina monopolar neuron L5           | ● 70.outer photoreceptor cell                |
| ● 09.T neuron T4/T5c-d                                   | ● 40.lamina wide-field 1 neuron           | ● 71.pericerebral adult fat mass             |
| ● 10.adult brain cell body glial cell                    | ● 41.lamina wide-field 2 neuron           | ● 72.photoreceptor cell R7                   |
| ● 11.adult brain perineurial glial cell                  | ● 42.lobula columnar neuron LC10          | ● 73.photoreceptor cell R8                   |
| ● 12.adult fat body_head                                 | ● 43.lobula columnar neuron LC12          | ● 74.pigment cell                            |
| ● 13.adult lamina epithelial/marginal glial cell         | ● 44.lobula columnar neuron LC17          | ● 75.proximal medullary amacrine neuron Pm2  |
| ● 14.adult optic chiasma glial cell                      | ● 45.medullary intrinsic neuron Mi1       | ● 76.proximal medullary amacrine neuron Pm4  |
| ● 15.adult reticular neuropil associated glial cell_head | ● 46.medullary intrinsic neuron Mi15      | ● 77.skeletal muscle of head                 |
| ● 16.alpha'/beta' Kenyon cell                            | ● 47.medullary intrinsic neuron Mi4       | ● 78.subperineurial glial cell_head          |
| ● 17.alpha/beta Kenyon cell                              | ● 48.medullary intrinsic neuron Mi9       | ● 79.transmedullary Y neuron TmY14           |
| ● 18.antennal lobe projection neuron                     | ● 49.neuron 1                             | ● 80.transmedullary Y neuron TmY4            |
| ● 19.auditory sensory neuron                             | ● 50.neuron 10                            | ● 81.transmedullary Y neuron TmY5a           |
| ● 20.centrifugal neuron C2                               | ● 51.neuron 11                            | ● 82.transmedullary Y neuron TmY8            |
| ● 21.centrifugal neuron C3                               | ● 52.neuron 12                            | ● 83.transmedullary neuron Tm1               |
| ● 22.columnar neuron T1                                  | ● 53.neuron 13                            | ● 84.transmedullary neuron Tm2               |
| ● 23.cone cell   | ● 54.neuron 14                            | ● 85.transmedullary neuron Tm20              |
| ● 24.distal medullary amacrine neuron Dm10               | ● 55.neuron 15                            | ● 86.transmedullary neuron Tm29              |
| ● 25.distal medullary amacrine neuron Dm12               | ● 56.neuron 16                            | ● 87.transmedullary neuron Tm3a              |
| ● 26.distal medullary amacrine neuron Dm3                | ● 57.neuron 17                            | ● 88.transmedullary neuron Tm4               |
| ● 27.distal medullary amacrine neuron Dm8                | ● 58.neuron 2                             | ● 89.transmedullary neuron Tm5c              |
| ● 28.distal medullary amacrine neuron Dm9                | ● 59.neuron 3                             | ● 90.transmedullary neuron Tm9               |
| ● 29.dopaminergic PAM neuron                             | ● 60.neuron 4                             | ● 91.unannotated                             |
| ● 30.ensheathing glial cell                              | ● 61.neuron 5                             | ● 92.uncharacterized CNS neuron              |
| ● 31.epithelial cell_head                                | ● 62.neuron 6                             |  |

**Fig. S8. Detailed cell types annotated in the head sample shown by tSNE.**  
91 head cell types are shown in the tSNE plot.

Figure S9

Body



- 01.16-cell germline cyst in germarium region 2a and 2b
- 02.CNS surface associated glial cell
- 03.adult Malpighian tubule principal cell
- 04.adult Malpighian tubule principal cell of initial segment
- 05.adult Malpighian tubule principal cell of lower segment
- 06.adult Malpighian tubule principal cell of lower ureter
- 07.adult Malpighian tubule stellate cell of main segment
- 08.adult alary muscle
- 09.adult differentiating enterocyte
- 10.adult fat body\_body
- 11.adult glial cell
- 12.adult heart ventral longitudinal muscle
- 13.adult hindgut
- 14.adult midgut enterocyte
- 15.adult midgut-hindgut hybrid zone
- 16.adult oenocyte
- 17.adult peripheral nervous system
- 18.adult reticular neuropil associated glial cell\_body
- 19.adult salivary gland
- 20.adult tracheal cell
- 21.adult ventral nervous system
- 22.anterior ejaculatory duct
- 23.antimicrobial peptide-producing cell
- 24.cardia (1)
- 25.cardia (2)
- 26.cardiomyocyte, working adult heart muscle (non-ostia)
- 27.cell body glial cell
- 28.copper cell
- 29.crop
- 30.cyst cell
- 31.ejaculatory bulb
- 32.ejaculatory bulb epithelium
- 33.enteroblast
- 34.enterocyte of anterior adult midgut epithelium
- 35.enterocyte of posterior adult midgut epithelium
- 36.enterocyte-like
- 37.enteroendocrine cell
- 38.eo support cell
- 39.epidermal cell that specialized in antimicrobial response
- 40.epithelial cell\_body
- 41.escort cell
- 42.female reproductive system
- 43.follicle cell
- 44.follicle stem cell and prefollicle cell
- 45.germline cell
- 46.gustatory receptor neuron
- 47.hemocyte\_body
- 48.indirect flight muscle
- 49.intestinal stem cell
- 50.leg muscle motor neuron
- 51.male accessory gland main cell
- 52.male accessory gland secondary cell
- 53.mechanosensory neuron of haltere
- 54.midgut large flat cell
- 55.multidendritic neuron
- 56.muscle cell
- 57.oviduct
- 58.perineurial glial sheath
- 59.pheromone-sensing neuron
- 60.polar follicle cell
- 61.posterior midgut\*
- 62.prefollicle cell/stalk follicle cell
- 63.principal cell\*
- 64.scolopidial neuron
- 65.seminal vesicle & testis epithelia
- 66.spermatid
- 67.spermatocyte
- 68.stalk follicle cell
- 69.subperineurial glial cell\_body
- 70.unannotated
- 71.visceral muscle of the crop
- 72.visceral muscle of the midgut
- 73.young germ cell

**Fig. S9. Detailed cell types annotated in the body sample shown by tSNE.**  
72 body cell types are shown in the tSNE plot.

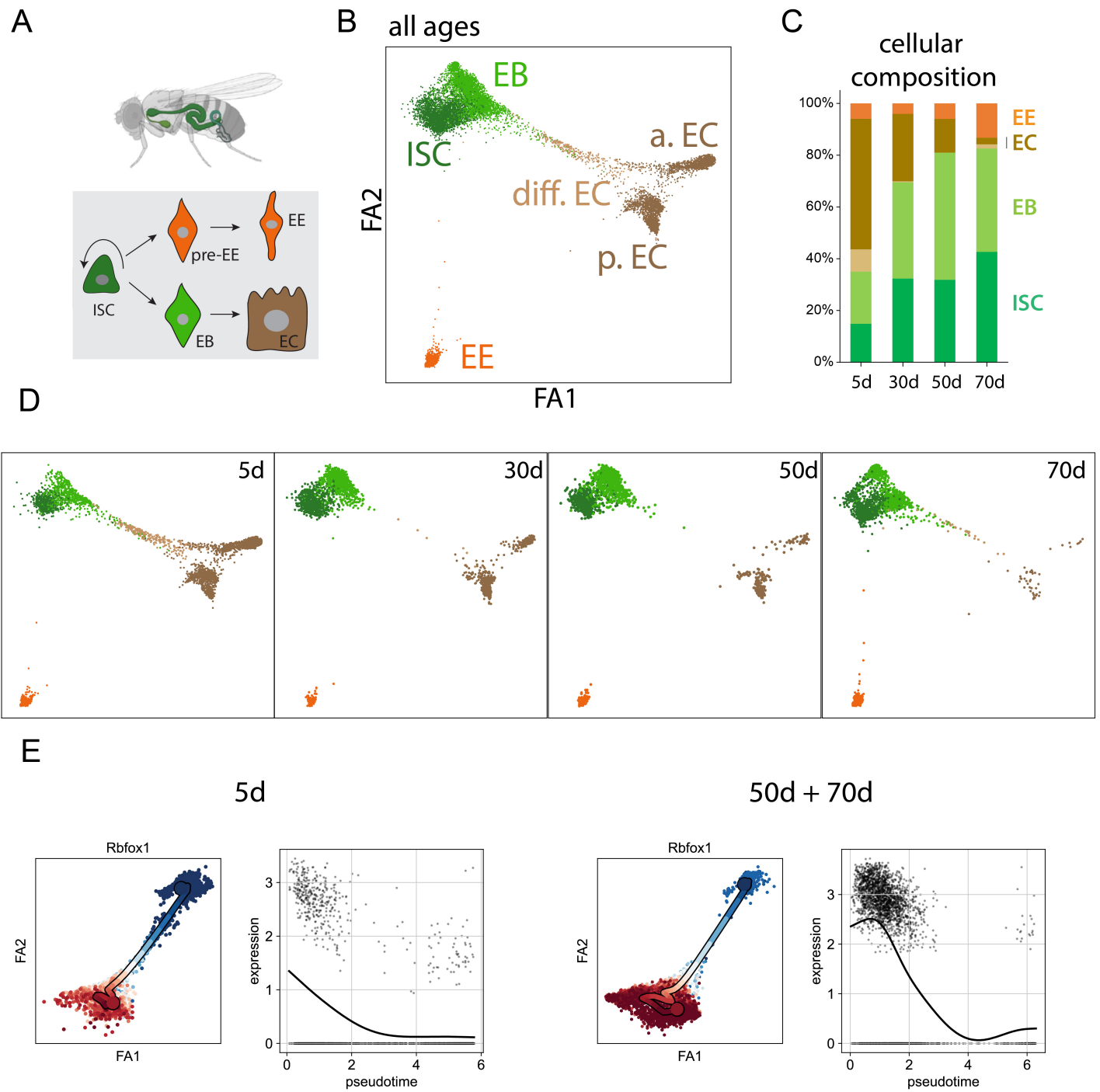




**Fig. S10. Marker genes identified from the newly identified neuron clusters.**

Markers of 17 newly identified neuron types are shown. Expression of general neuronal markers, Syt1, and para, indicates neuron specificity. Expression of VAcHT, Gad1, and VGlut indicates they are cholinergic, GABAergic, or Glutamatergic neurons.

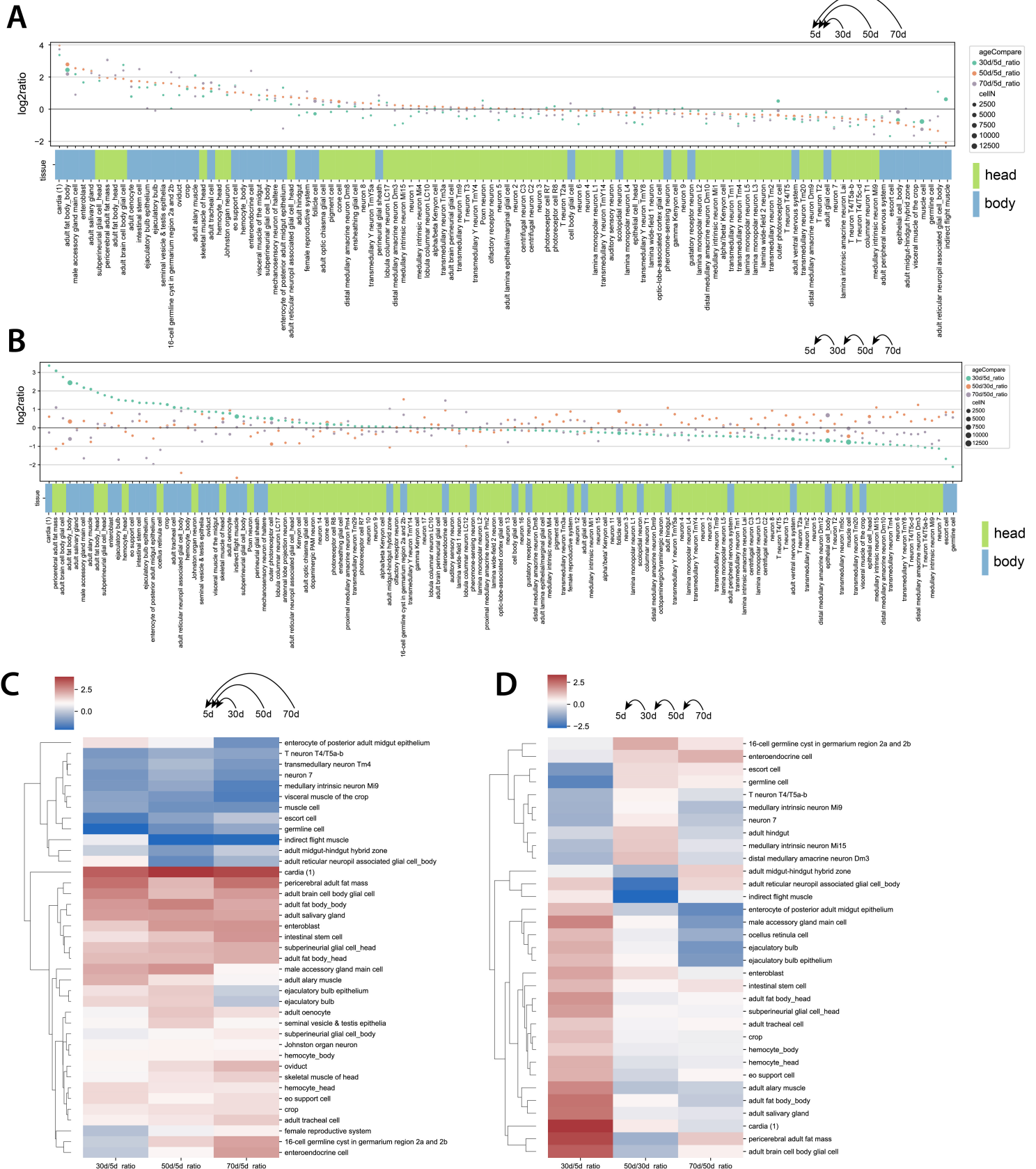
Figure S11



**Fig. S11 Pseudotime inference and cellular composition of ISC and ISC-differentiated cell types.**

A) Illustration of ISC differentiation process. B) Cell types shown in different colors. C) Changes of cellular composition in the ISC lineage. D) Age-specific profile of different cell types. E) Rbfox1 gene shows different patterns along the young and old ISC lineage. ISC, intestinal stem cell; EB, enteroblast; EC, enterocyte; EE, enteroendocrine cell; a. EC, anterior EC; p. EC, posterior EC; diff. EC, differentiating EC.

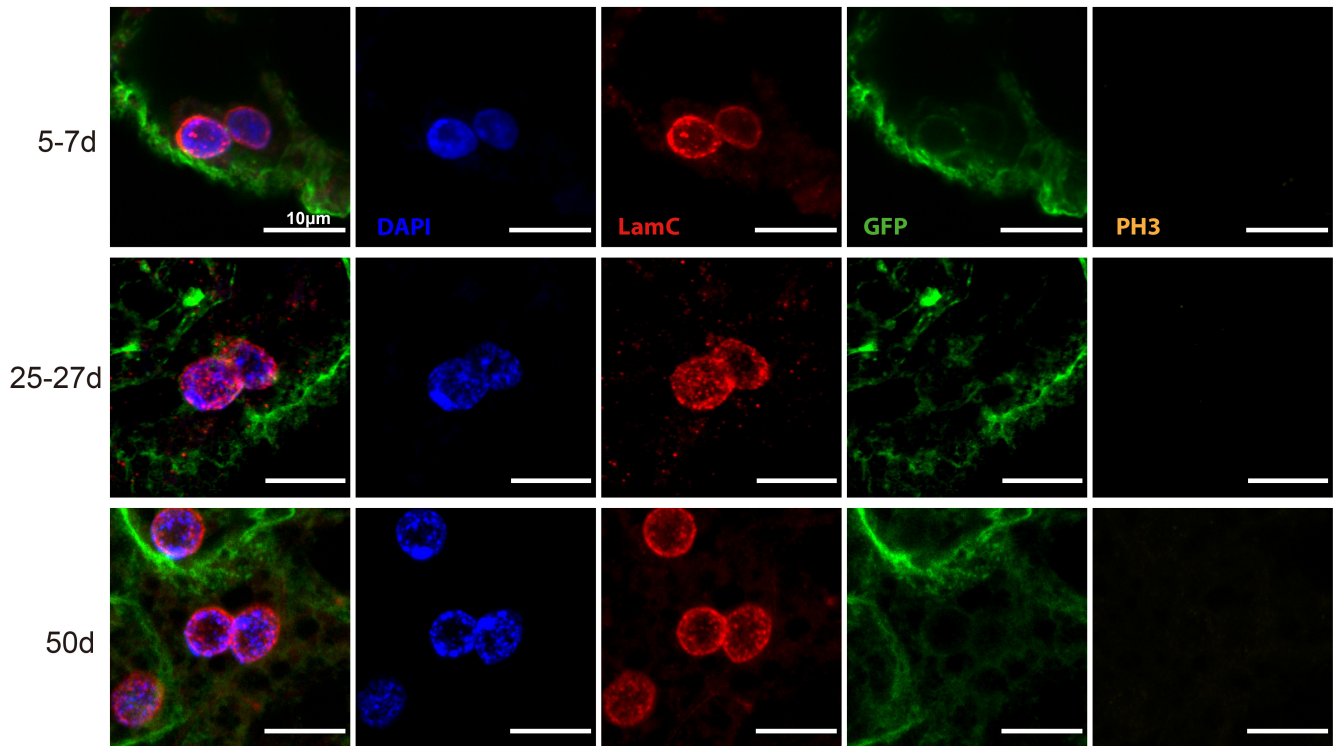
# Figure S12



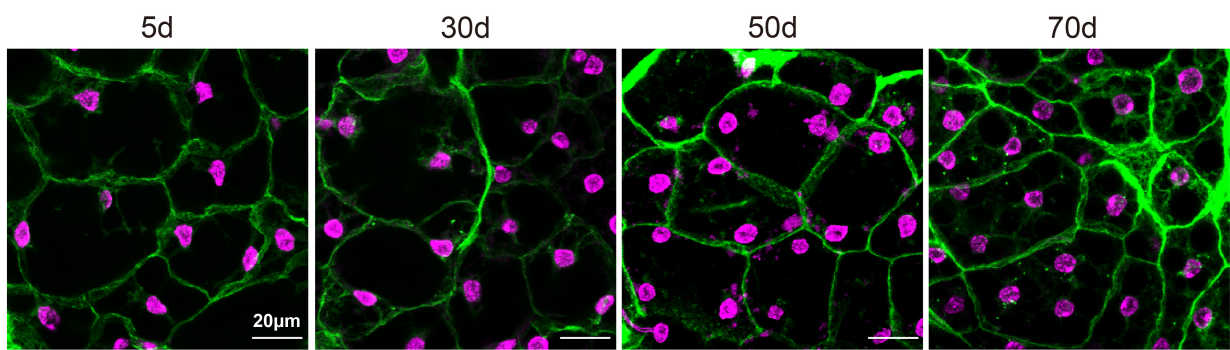
**Fig. S12 Changes of cell-type composition during aging.**

A) Changes in cellular composition by comparing the nuclear ratio of the old sample (30d, 50d, and 70d) to the ratio of the young one (5d). B) Changes of cellular composition by comparing the nuclear ratio between two consecutive ages. C) Cell types with more than 2-fold changes of cellular composition by comparing the old samples to the young ones. D) Cell types with more than 2-fold changes of cellular composition by comparing the sample to the previous age.

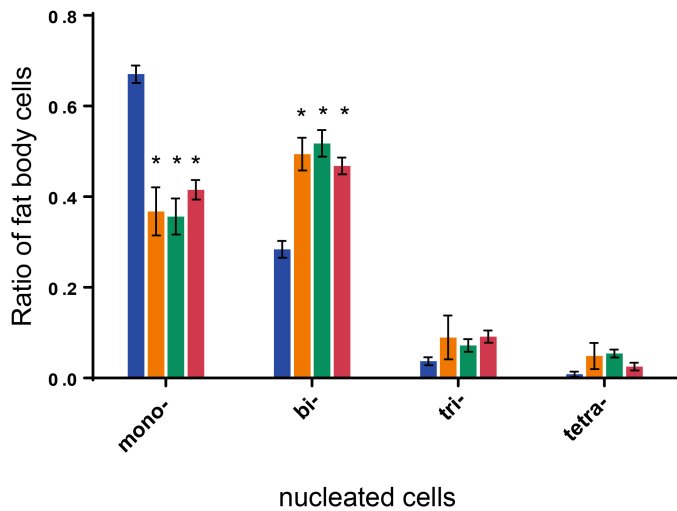
**A** fat body (*cg-GAL4>UAS-CD8GFP*)



**B**



Increase of nuclei per fat body cell upon aging



\*: significantly changed between 5d and the aged population.

**Fig. S13. Examples of dividing nuclei without the mitosis marker.**

A) Dividing nuclei from flies with different ages showing the absence of the mitosis marker (pH3). Fat body nuclei are collected from different ages, including 5-7d, 25-27d, and 50d. Dividing nuclei are stained by DAPI and LamC. The fat body membranes are labeled by *cg-GAL4 > UAS-CD8GFP*. B) The multinucleation of fat body cells in aged flies. The representative images of fat bodies of different ages are shown. The multinucleation of the fat body was examined for at least 324 cells (5d:359, 30d:324, 50d:369, and 70d:494 cells) in 7-10 animals of each age. The two-way ANOVA and Tukey's multiple comparisons test were performed. The asterisks show the  $P < 0.0001$ .

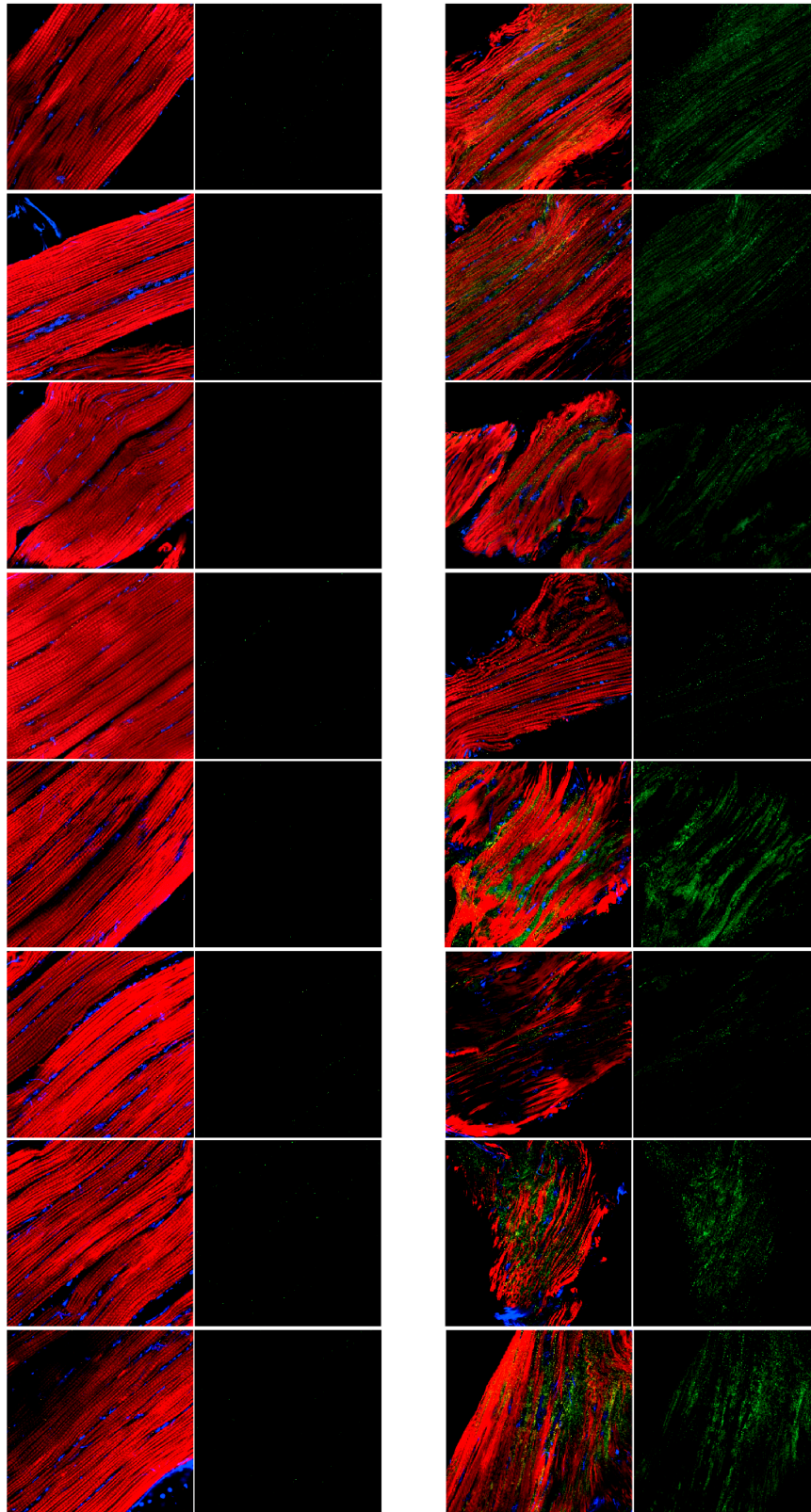


Figure S14

indirect flight muscle

young (5d)

old (50d)

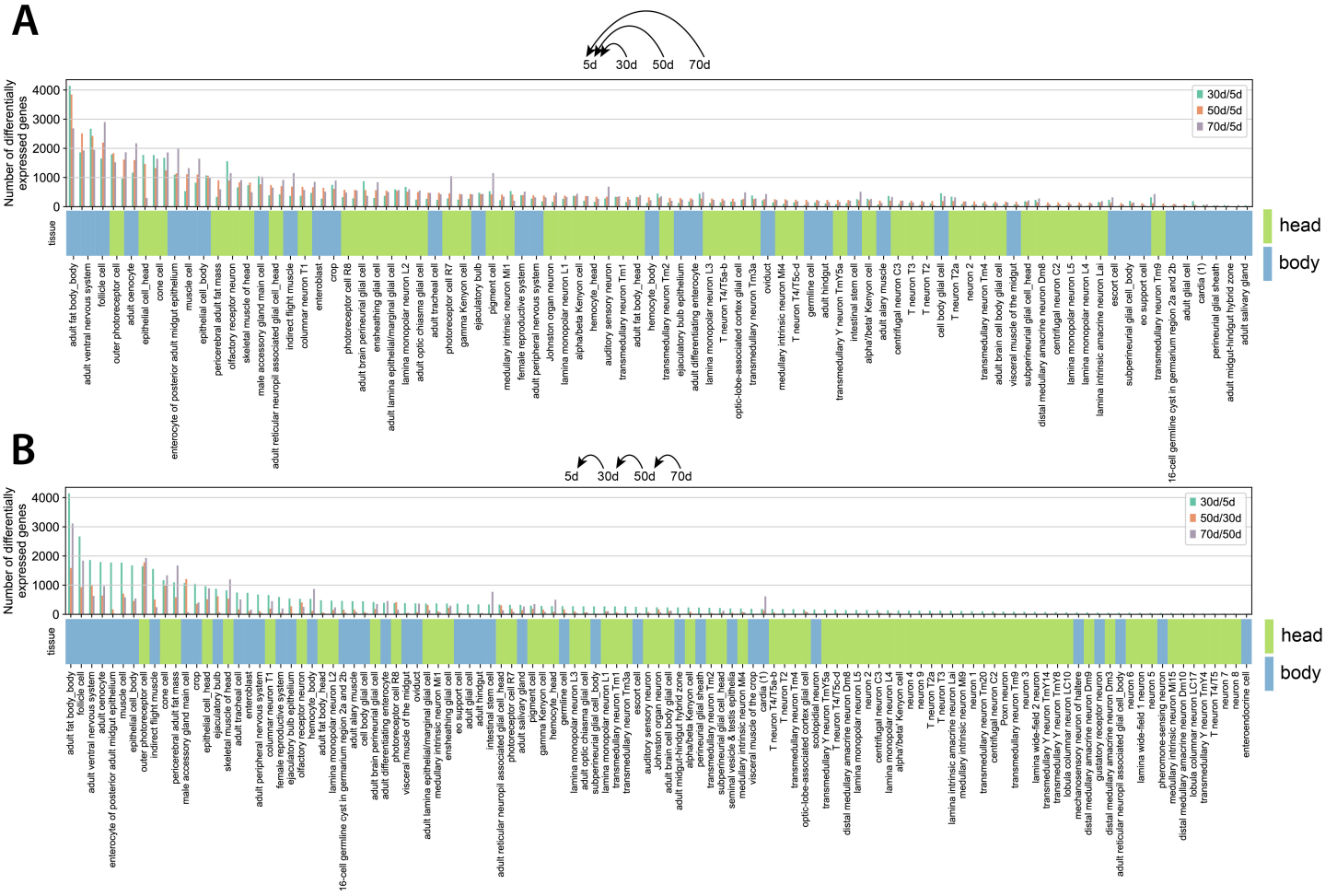


DAPI/Phalloidin/Caspase3

**Fig. S14 Apoptosis signals form the aged indirect flight muscle.**

Examples of indirect flight muscle stained by the apoptosis marker in the young and old flies. The indirect flight muscles are stained for the apoptosis events using a cleaved-Caspase3 antibody. Nuclei are stained by DAPI. Actin filaments in the muscle cells are labeled by Phalloidin.

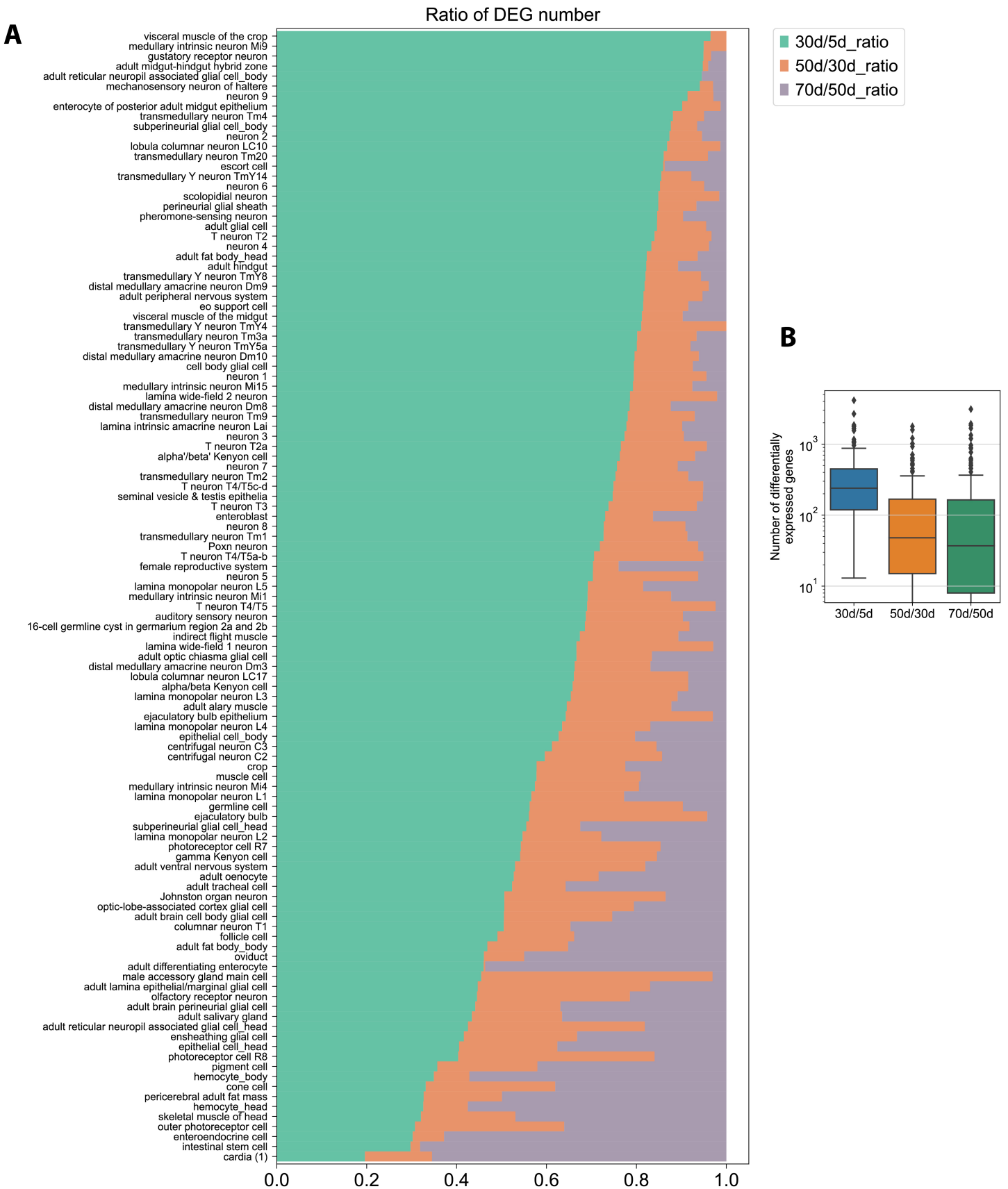
# Figure S15



**Fig. S15 DEG numbers detected in each cell type.**

A) DEG numbers from different cell types. The aged groups (30d, 50d, and 70d) are compared with the young population (5d). B) Two consecutive ages are compared to identify DEGs in each cell type.

Figure S16

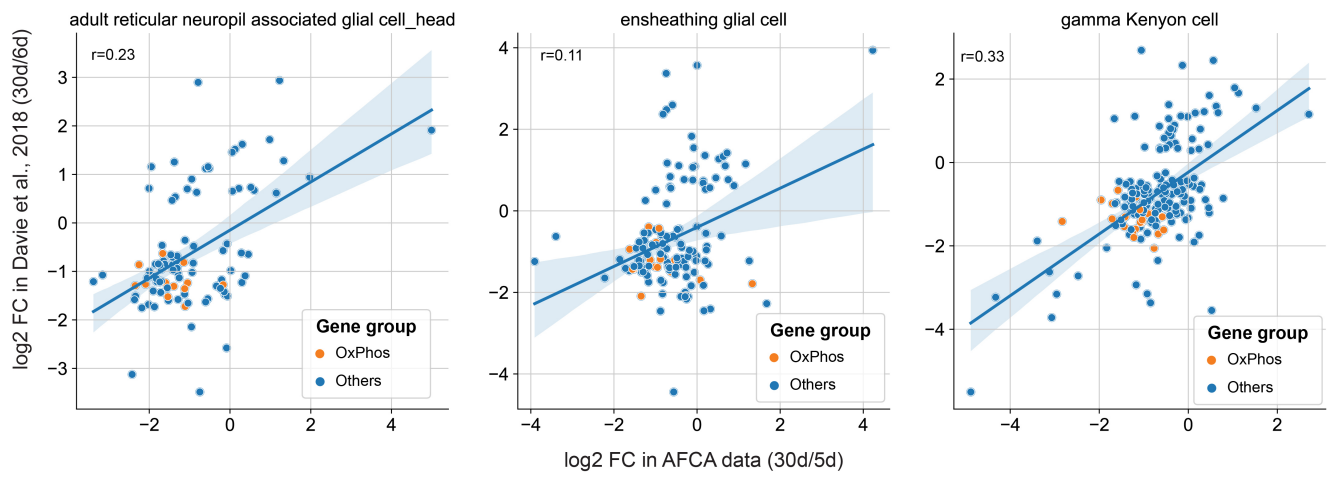


**Fig. S16 Detailed ratio of DEG number across different age periods.**

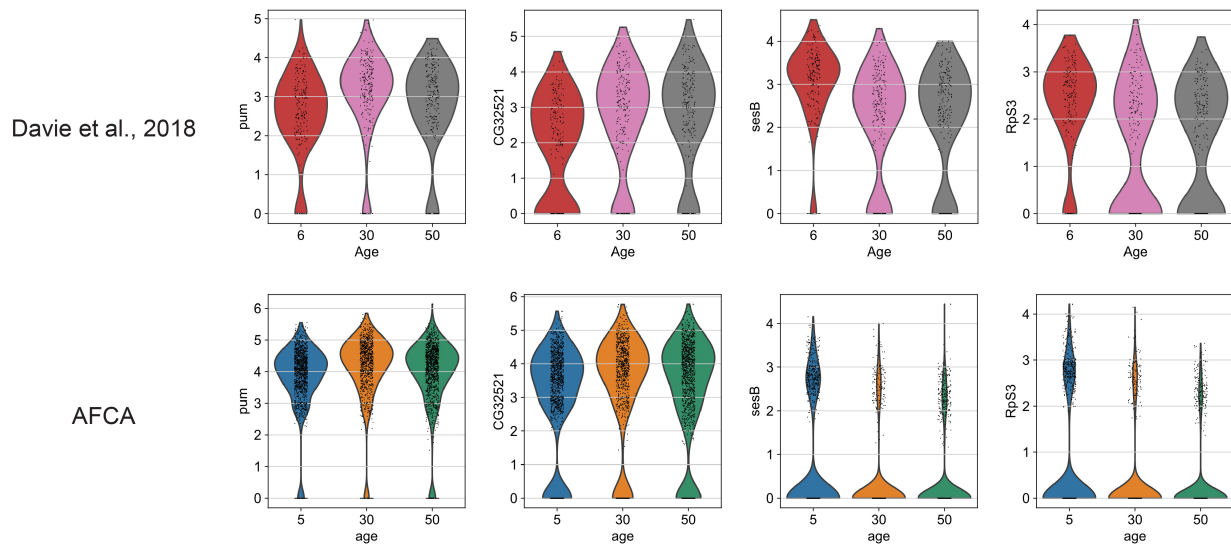
- A) The ratios of DEG numbers in different cell types. Each age period is shown in a different color.
- B) Number of differentially expressed genes detected in the different age periods.

Figure S17

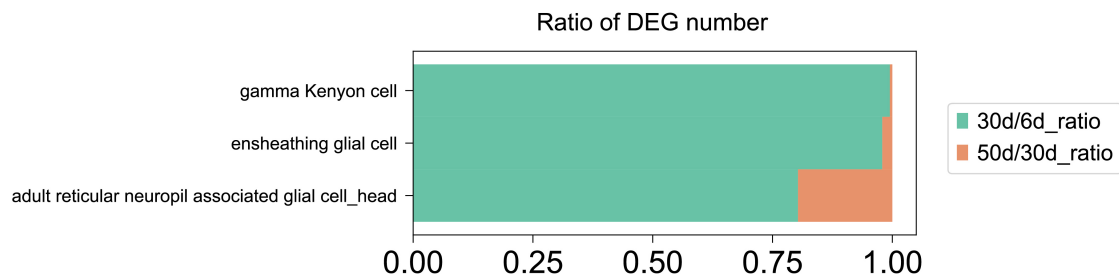
**A** DEG comparisons between AFCA and Davie et al., 2018



**B** ensheathing glial cell



**C** DEG ratio in Davie et al., 2018

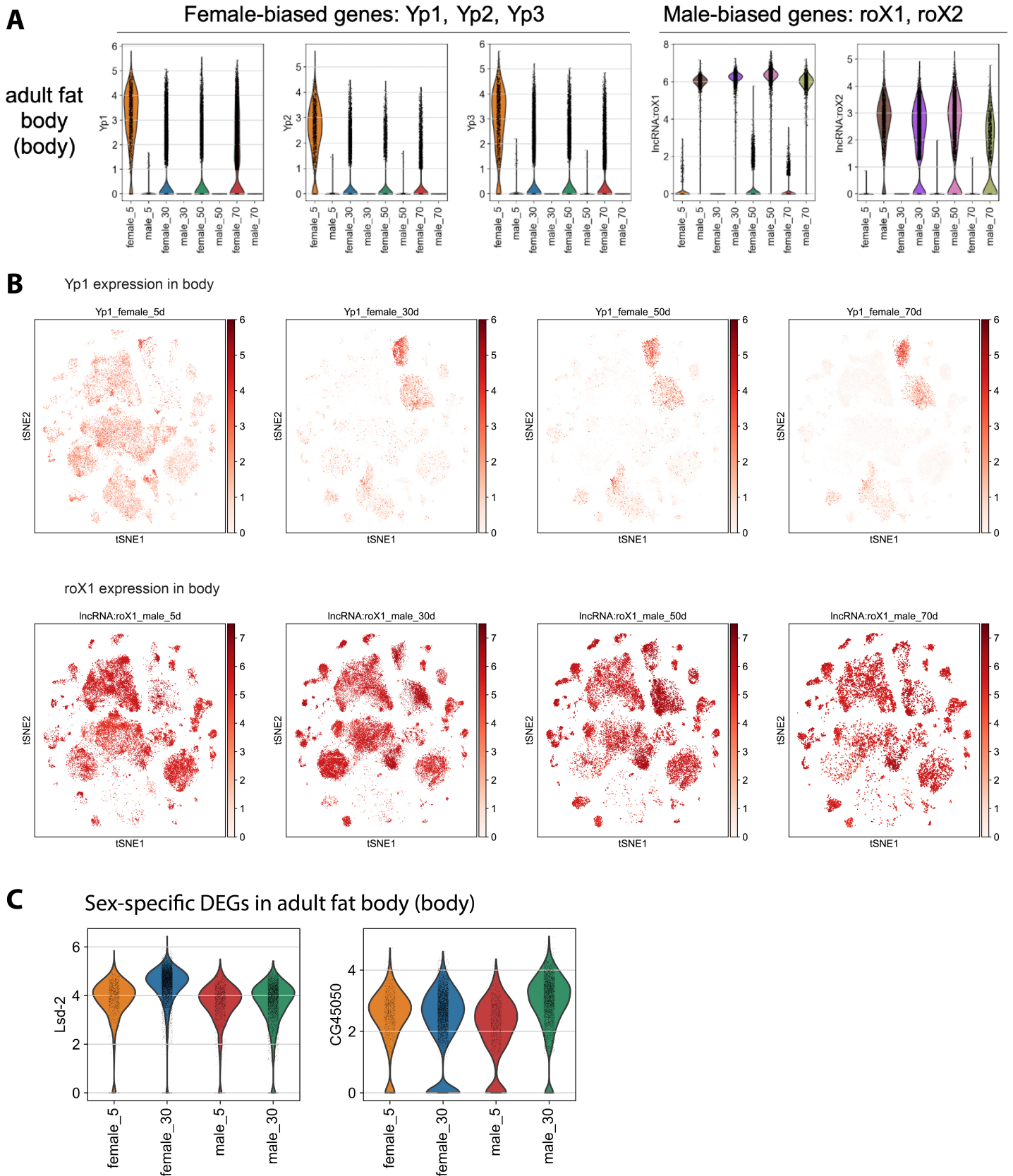


**Fig. S17. Comparisons between AFCA and Davie et al., 2018 data.**

A) Three cell types with the highest cell number from Davie et al., 2018 are compared to the corresponding cell types in the AFCA. X-axis indicates the log<sub>2</sub> fold changes of gene expression between 30d and 5d samples in AFCA, while Y-axis represents the log<sub>2</sub> changes between 30d and 6d samples in Davie et al., 2018. Orange dots are genes related to oxidative phosphorylation. Pearson correlations are performed to measure the correlation of log<sub>2</sub> fold changes between two datasets. B) Aging DEGs in ensheathing glial cells are similarly regulated in AFCA and Davie et al. 2018 samples. C) Three cell types show a higher DEG ratio in the 30d/6d age interval compared to the 50d/30d one.



Figure S18

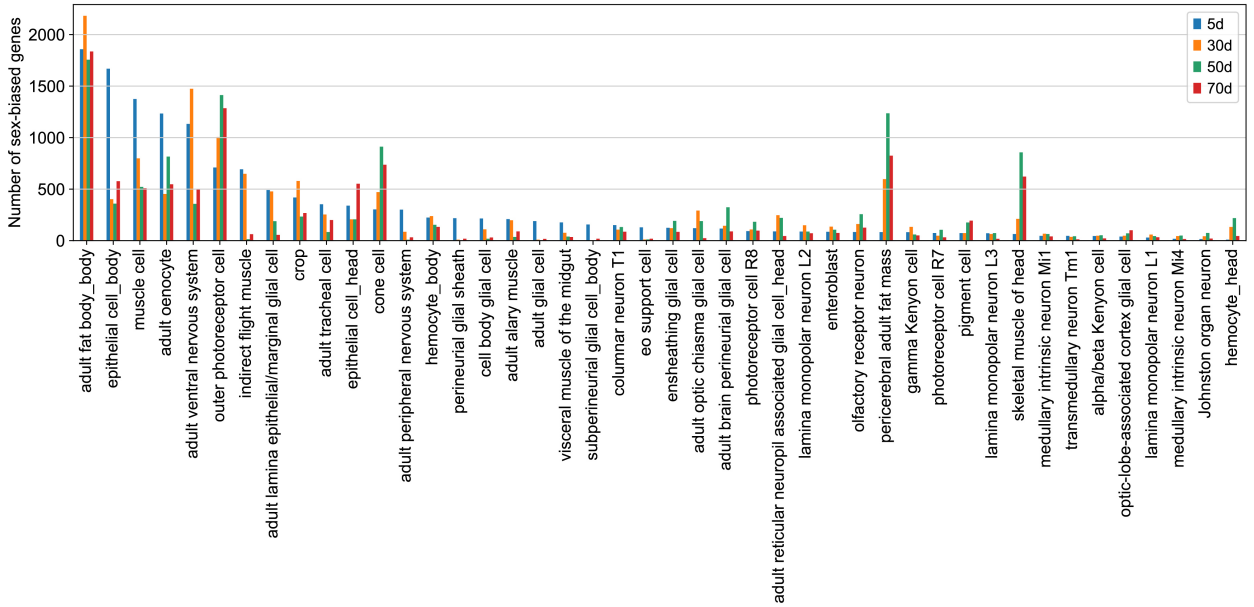


**Fig. S18 Sex-specific expression of genes.**

A) Expression of female- and male-biased genes from the fat body cells during aging. B) Expression of Yp1 and roX1 in body cells shown by tSNE. C) two example genes showing different change trends between males and females.

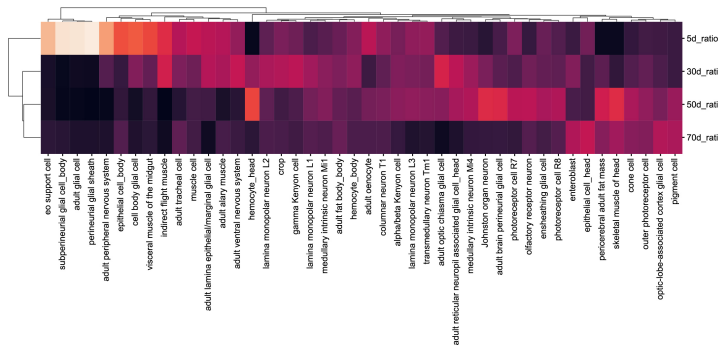
**A**

number of DEGs between male and female at different ages male female



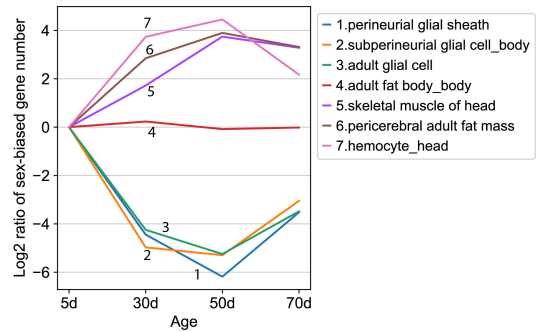
**B**

Sex-biased gene# from one age = Sex-biased ratio from one age  
Sex-biased gene# from all ages



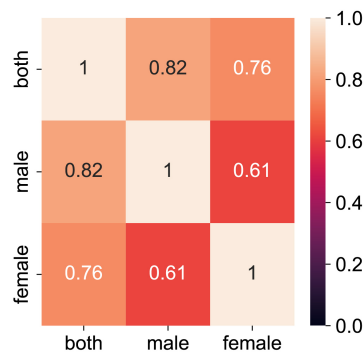
**C**

Sex-biased gene# from one age = Sex-biased ratio from one age  
Sex-biased gene# from 5d



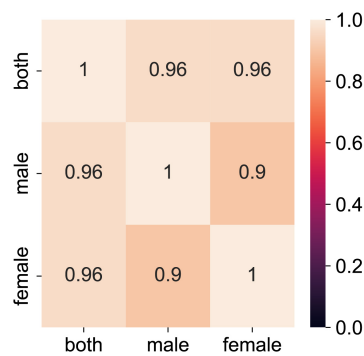
**D**

Cell-type composition separated by sex (50d/5d)



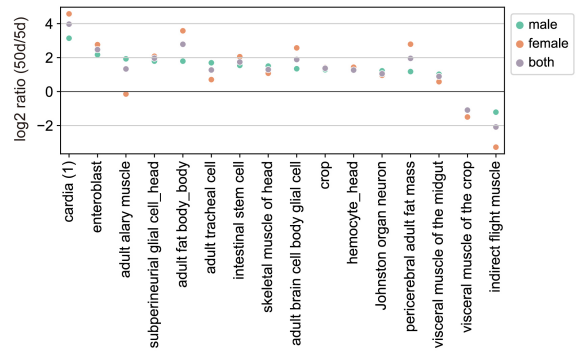
**F**

DEG# separated by sex (50d/5d)

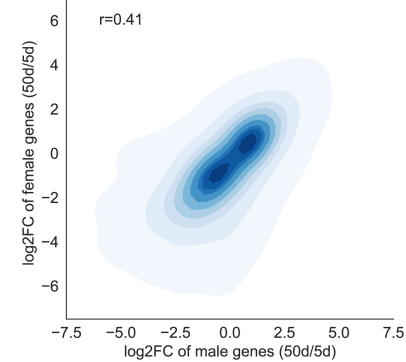


**E**

$|\log_2 \text{ratio}| \geq 1$  in male



**G**



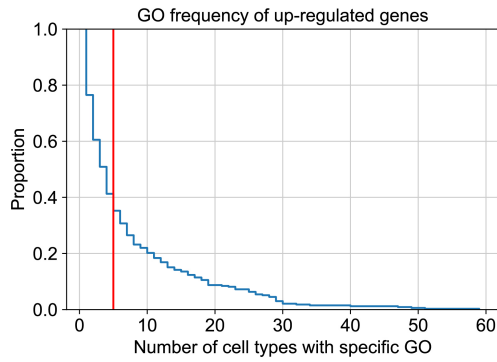
**Fig. S19 Sex-biased genes from each age.**

A) The number of sex-specific genes from each age. B) Sex-biased ratios from different ages. C) Illustration of sex-biased ratios from several cell types. D) Spearman's rank correlation of the change of cell-type composition between males and females. (E) Log<sub>2</sub> ratio of cell-type composition by comparing 50d to 5d samples in different sexes of flies. (F) Spearman's rank correlation of DEG number from each cell type by comparing 50d to 5d samples in male or female flies. (G) Comparison of log<sub>2</sub> fold changes of gene expression between male and female. The Pearson correlation coefficient of male and female log<sub>2</sub> fold changes is 0.41.

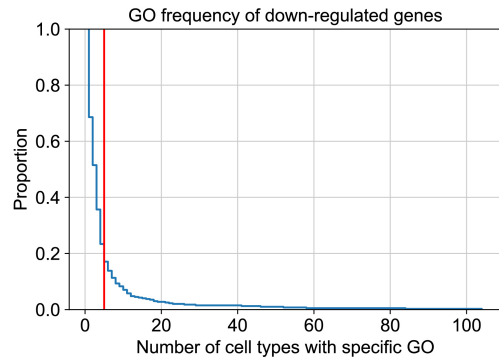
# Figure S20

**A**

Up-regulated



Down-regulated



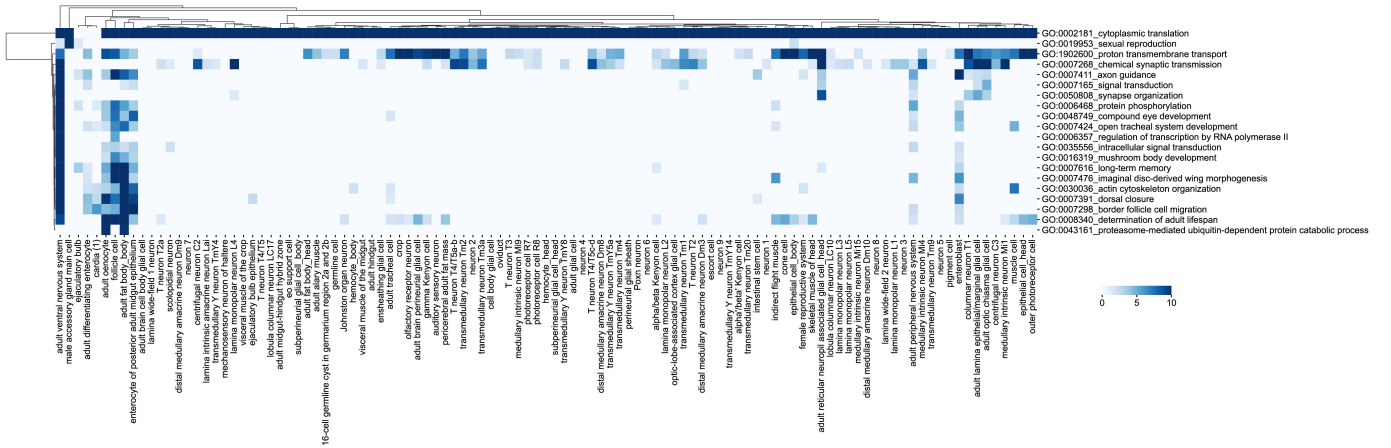
**B**

GOs with the lowest FDR using the up-regulated genes



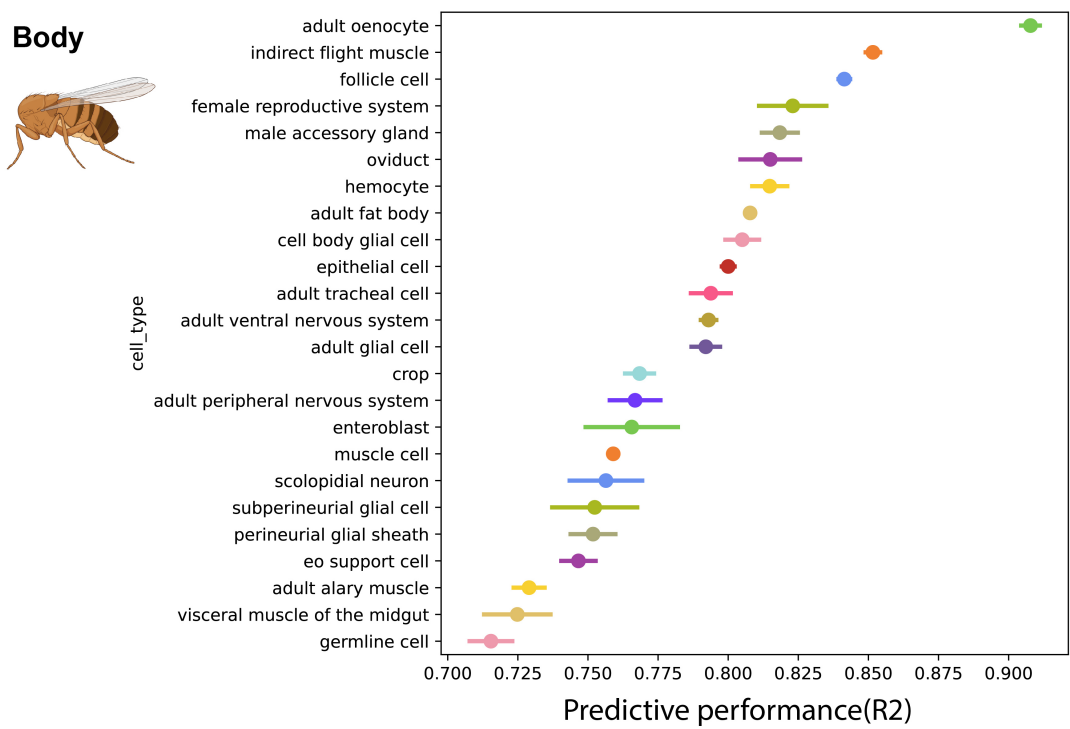
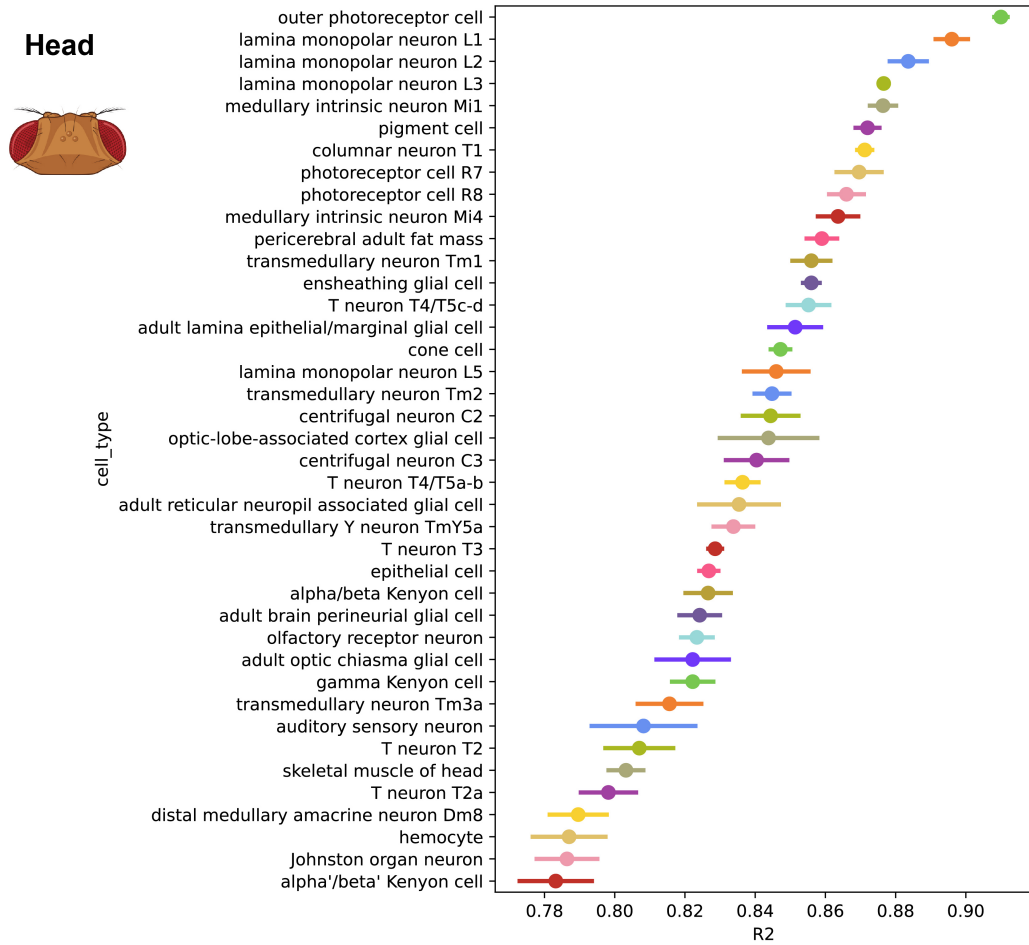
**C**

GOs with the lowest FDR using the down-regulated genes



**Fig. S20 GO frequency and the most significant GOs from the up- or down-regulated genes.**  
A-B) Up- and down-regulated DEGs are identified by comparing 50d flies to 5d flies. A) GO frequencies from up- or down-regulated genes. GO frequency indicates how many cell types have the corresponding GO. The red line indicates five cell types with the corresponding GO. B) GOs with the lowest FDR from the up-regulated genes. C) GOs with the lowest FDR from the down-regulated genes.

Figure S21

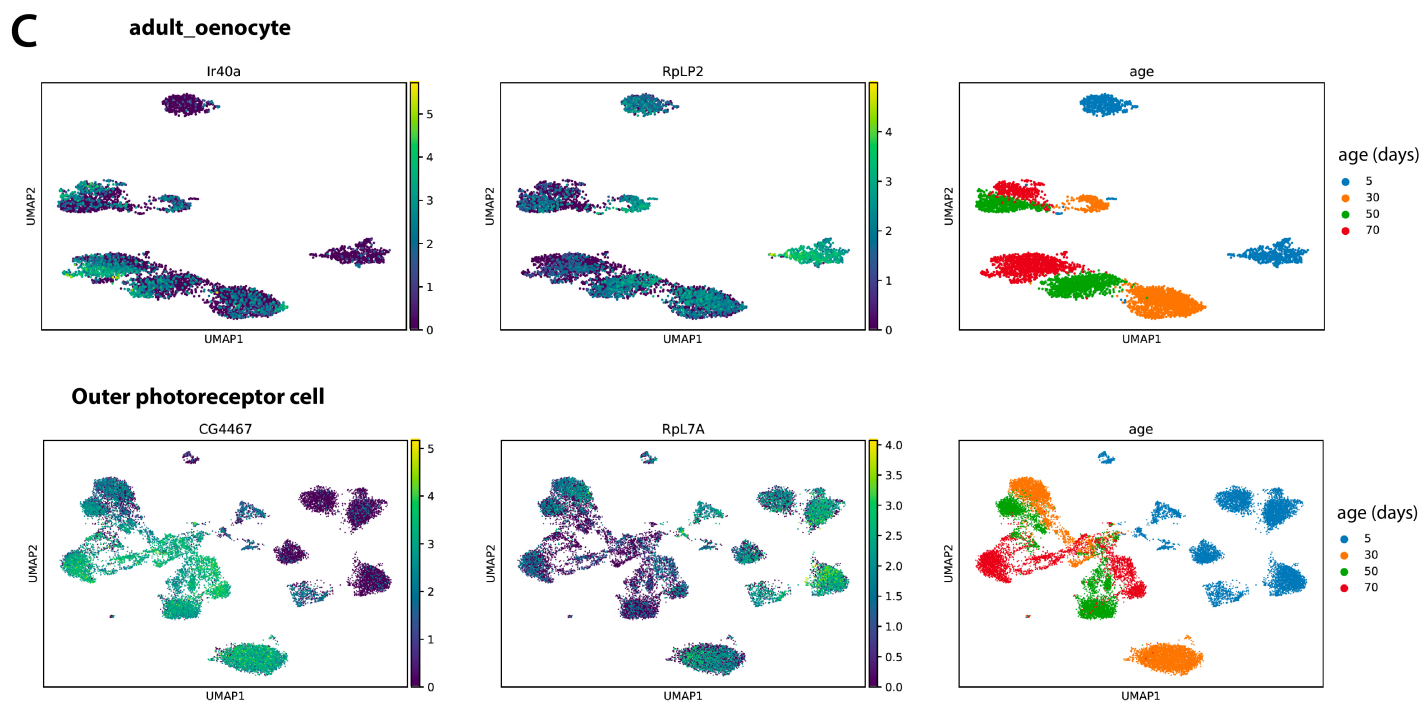
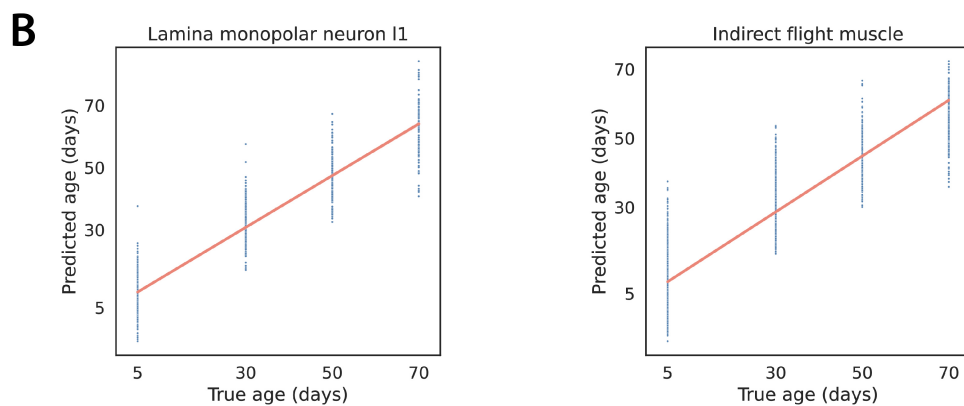
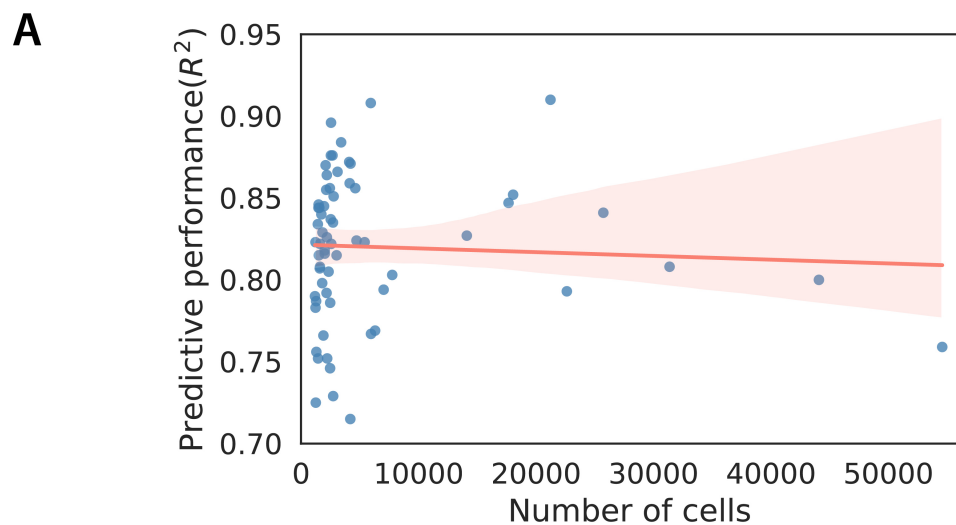


**Fig. S21 Predictive performance of aging clocks.**

Predictive performances of aging clocks from each cell type. Performances of head and body cell types are shown separately.



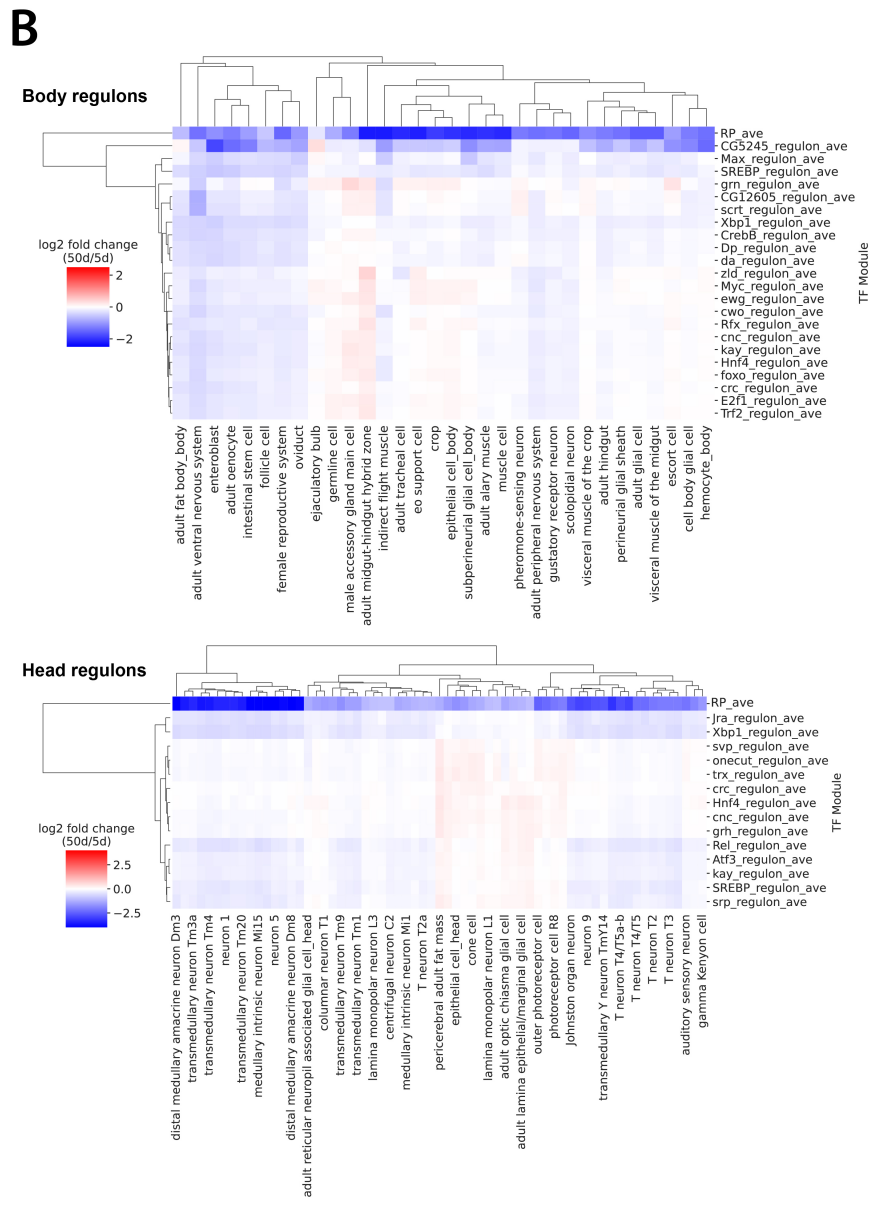
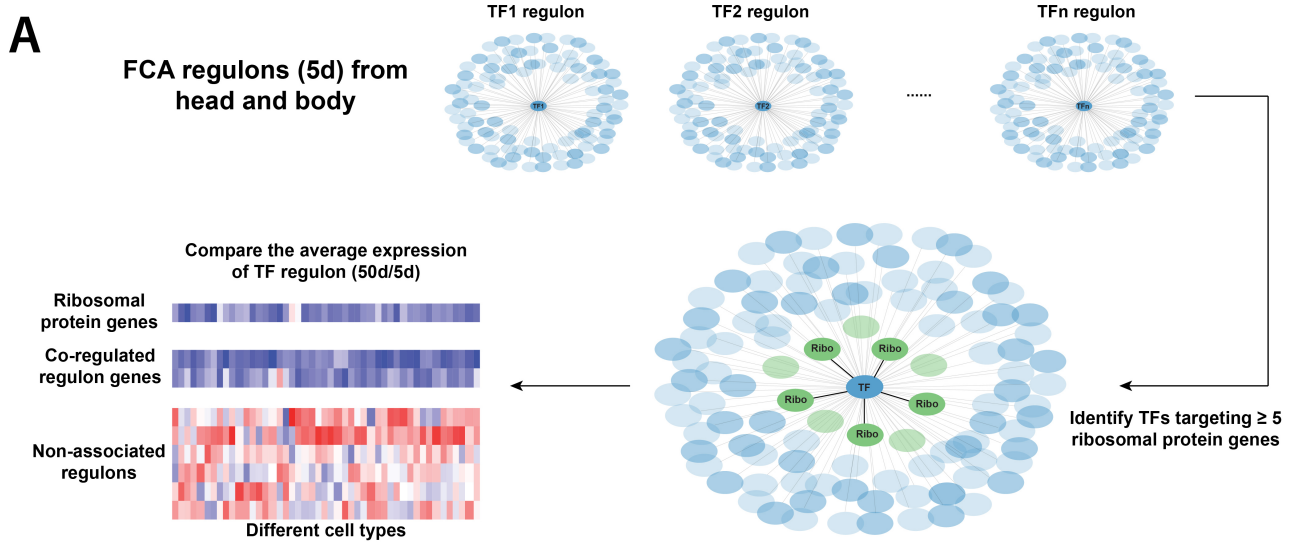
Figure S22



**Fig. S22 Aging clock profiles.**

A) The cell numbers from each cell type are not correlated to the predictive performance. B) Examples of two cell types with good prediction of true age. C) Expression of aging clock genes in two different cell types.

# Figure S23



**Fig. S23 Identification of possible TFs regulating the expression of ribosomal protein genes.**

A) The flowchart of identification of possible TFs of ribosomal protein genes. B) The averaged expressions of RP gene-related regulons.

# Figure S24

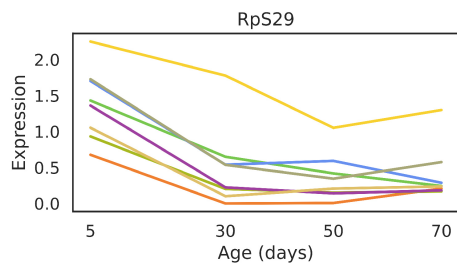
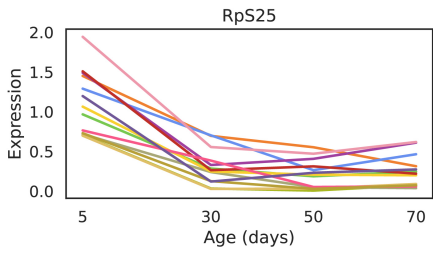
<b>fly_gene</b>	<b>mouse_ortholog</b>	<b># cell types in fly</b>	<b># cell types in mouse</b>
RpL13A	Rpl13a	8	24
RpS25	Rps25	13	18
RpS29	Rps29	5	24
RpS26	Rps26	10	10
RpS21	Rps21	3	15
RpS15	Rps15	15	2
RpS23	Rps23	6	11
RpL27A	Rpl27a	15	1
RpS7	Rps7	13	2
RpL21	Rpl21	11	2
RpL35A	Rpl35a	3	10
RpL14	Rpl14	11	1
RpS12	Rps12	7	5
RpS8	Rps8	11	1
RpL23	Rpl23	8	3
RpL38	Rpl38	1	10
RpL10Ab	Rpl10a	9	1
RpS16	Rps16	6	3
RpS3	Rps3	3	6
sta	Rpsa	7	1
RpL13	Rpl13	3	4
RpL31	Rpl31	4	3
RpS17	Rps17	2	5
RpLP0	Rplp0	4	2
RpS20	Rps20	5	1
RpS24	Rps24	4	1
RpS30	Fau	3	2
ATPsynbeta	Atp5b	2	2
RpL11	Rpl11	3	1
RpS11	Rps11	3	1
RpS6	Rps6	3	1
Hsc70-3	Hspa5	1	2
RpL24	Rpl24	1	1

**Fig. S24 Aging clock genes present in both fly and mouse.**

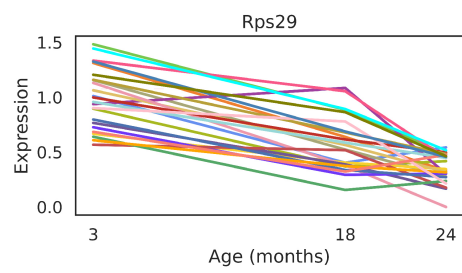
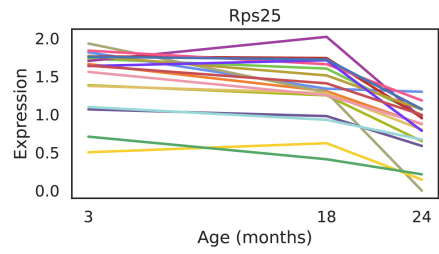
1-1 orthologs of 33 aging clock genes conserved between fly and mouse. The number of cell types shown in each species indicates that the aging clock genes are detected as aging clock genes in the corresponding number of cell types.

Figure S25

**Fly ribosomal protein gene**



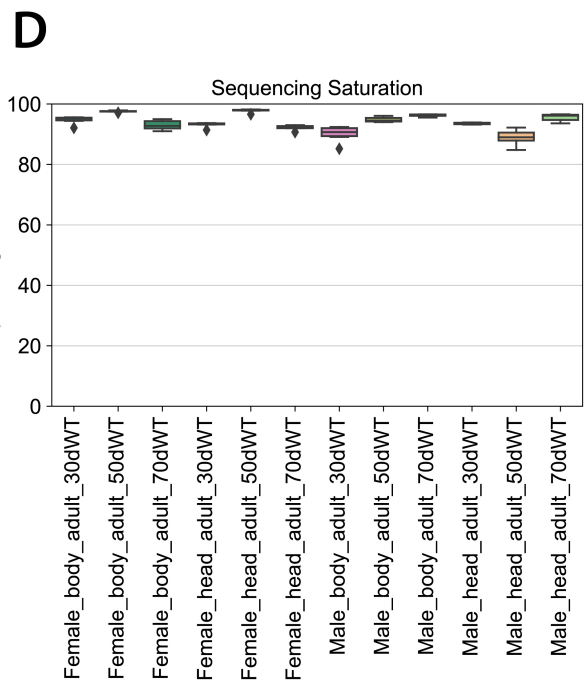
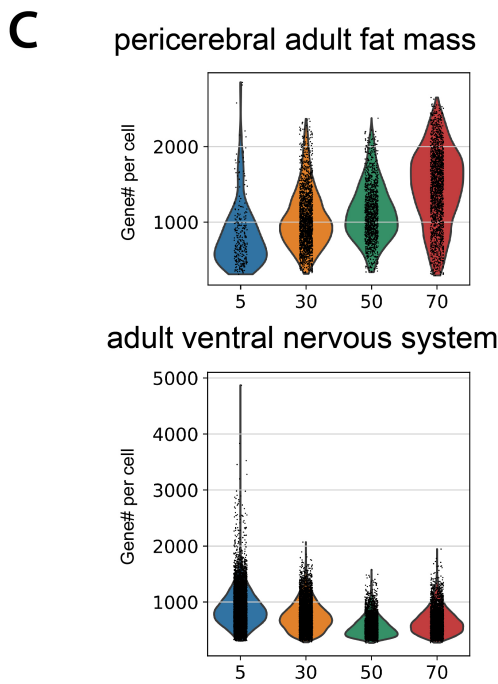
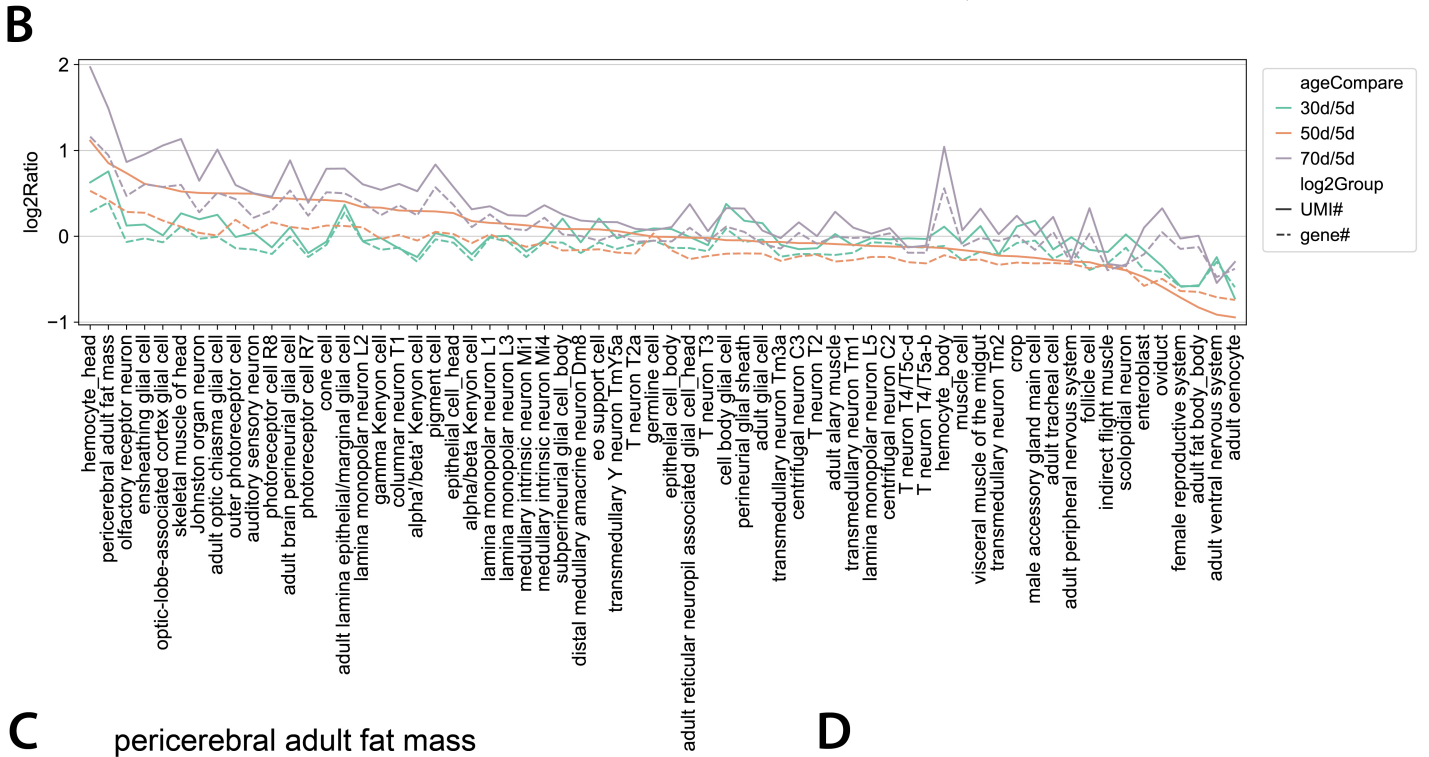
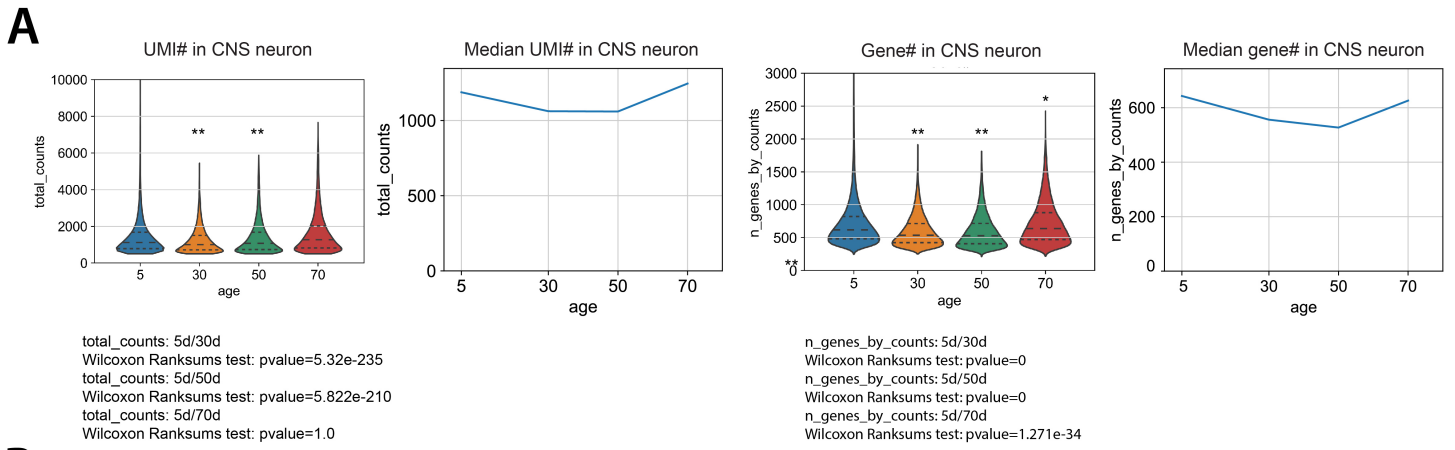
**Mouse ribosomal protein gene**



**Fig. S25 Examples of cross-species comparison of aging clock genes.**  
Expression of two orthologous aging clock genes.



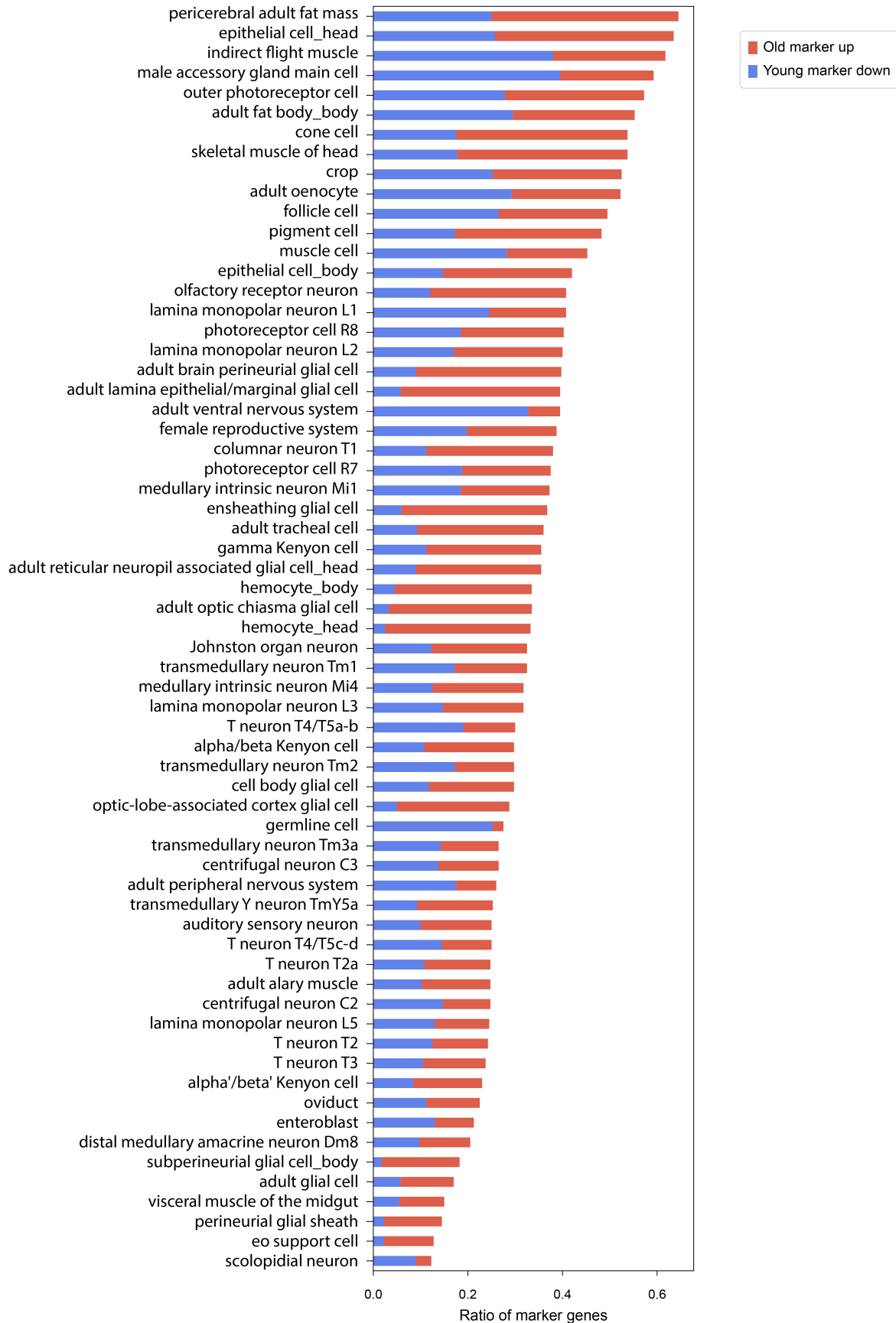
# Figure S26



**Fig. S26 Variance of UMI and expressed gene numbers during aging.**

A) Numbers of expressed genes and UMIs per cell are decreased in the aged CNS neurons (30d and 50d). B) Log<sub>2</sub> ratio of expressed gene or UMI number per cell during aging. C) Changes of expressed gene number in pericerebral adult fat mass and adult fat body from the body. D) Sequencing saturation of different batches of samples.

# Figure S27

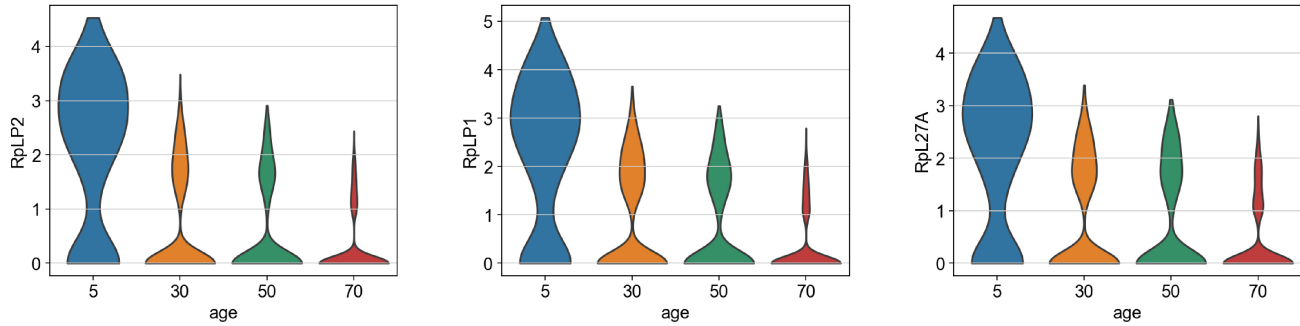


**Fig. S27 Changes of cell-type identity during aging.**

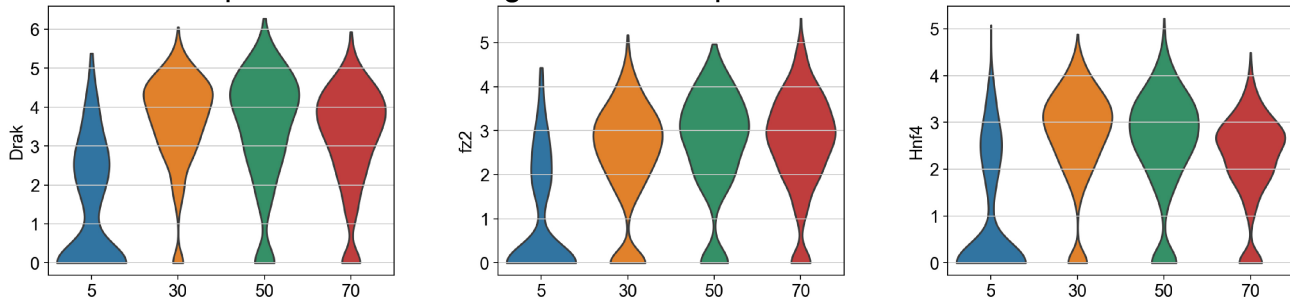
Cell types ranked by the decline of cell-type identity during aging. The decrease of young markers and the increase of old markers are shown separately in each cell type.

Figure S28

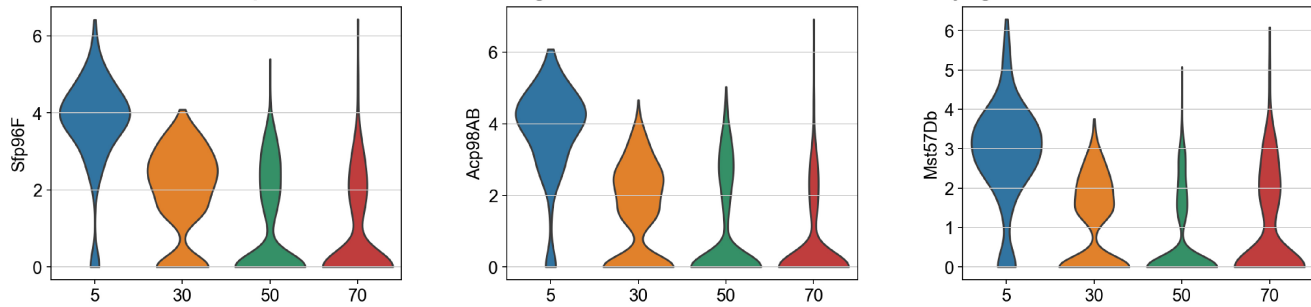
Decreased expression of marker gene from the pericerebral adult fat mass



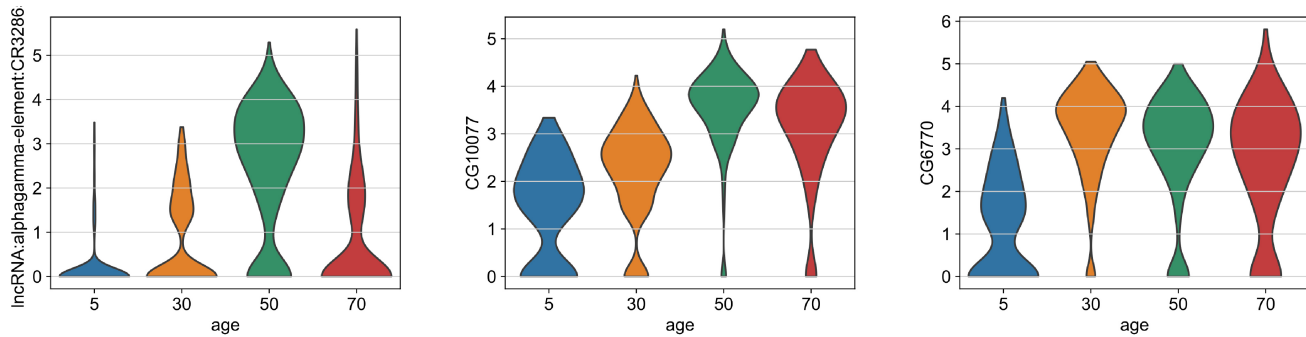
Increased expression of marker gene from the pericerebral adult fat mass



Decreased expression of marker gene from the male accessory gland main cell



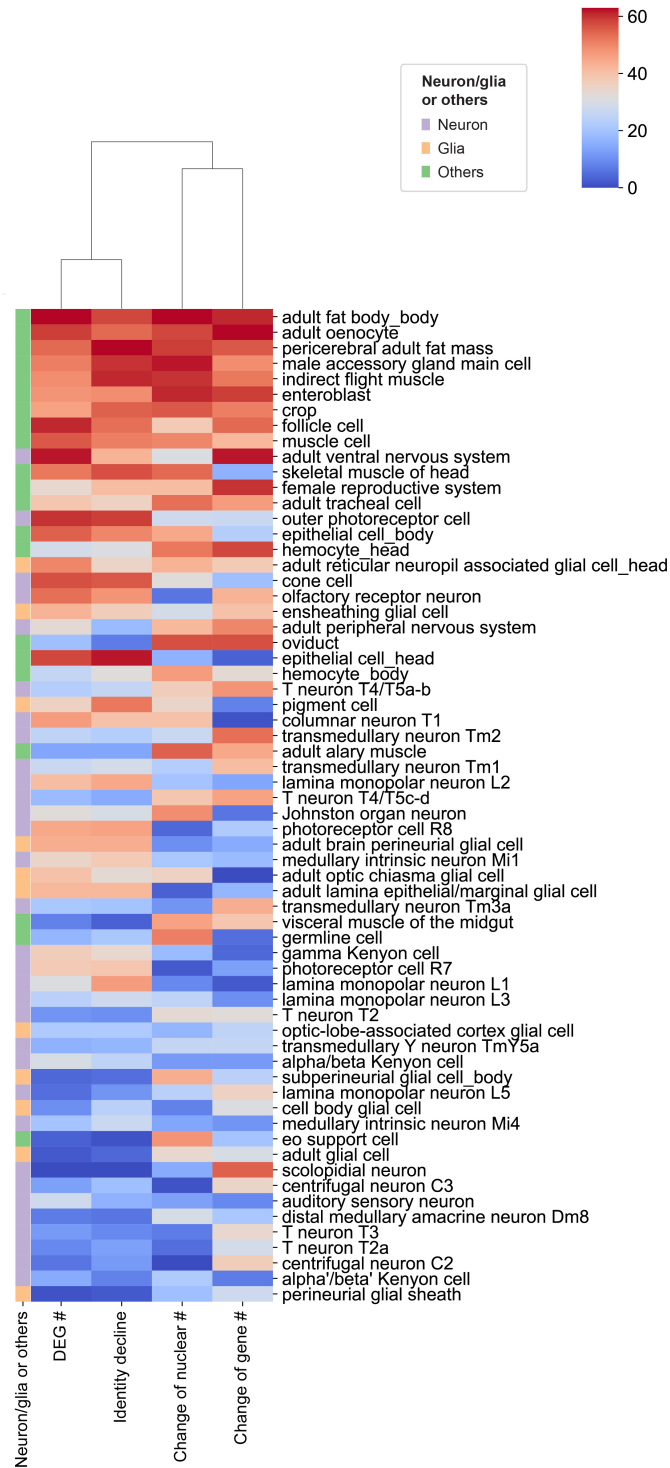
Increase expression of marker gene from the male accessory gland main cell



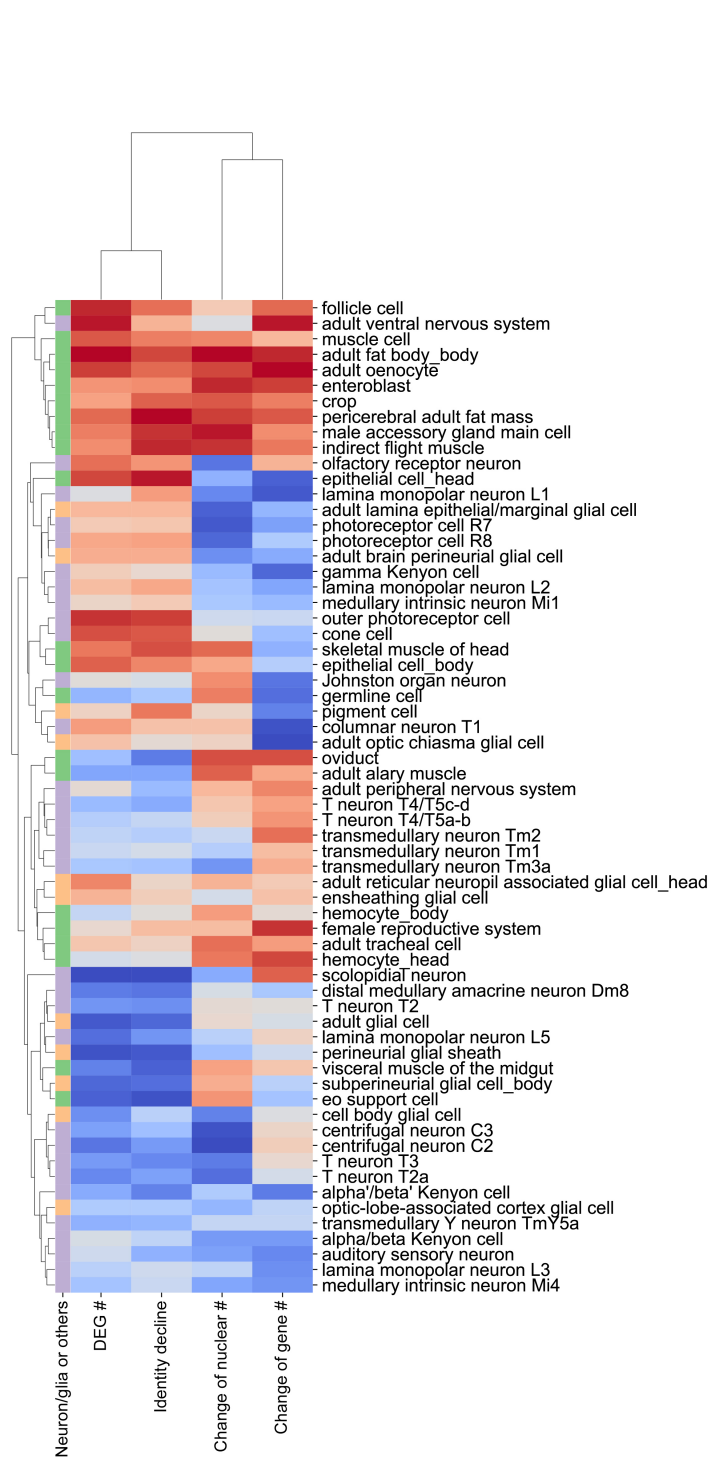
**Fig. S28 Marker genes that affect cell-type identities during aging.**

Examples of marker genes that caused the decline of cell-type identities.

## A Ranked by rank sum



## B Cluster different aging features

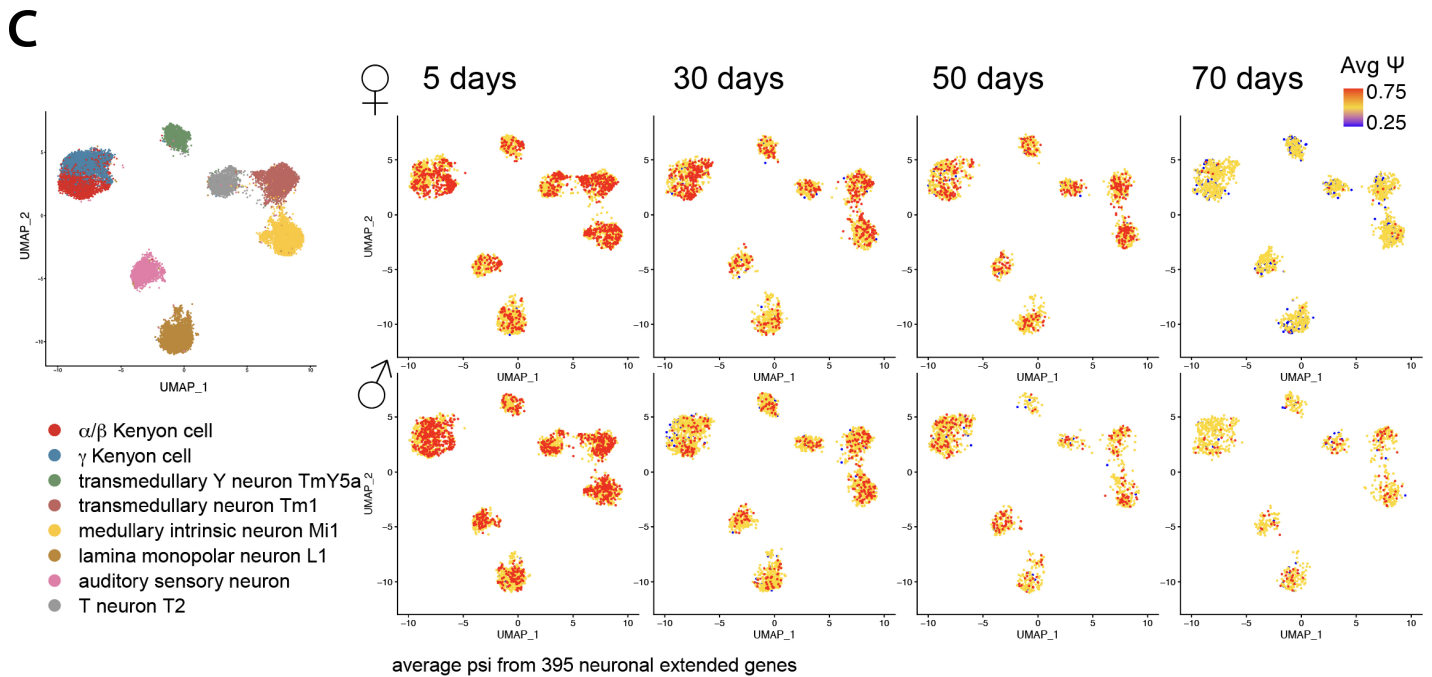
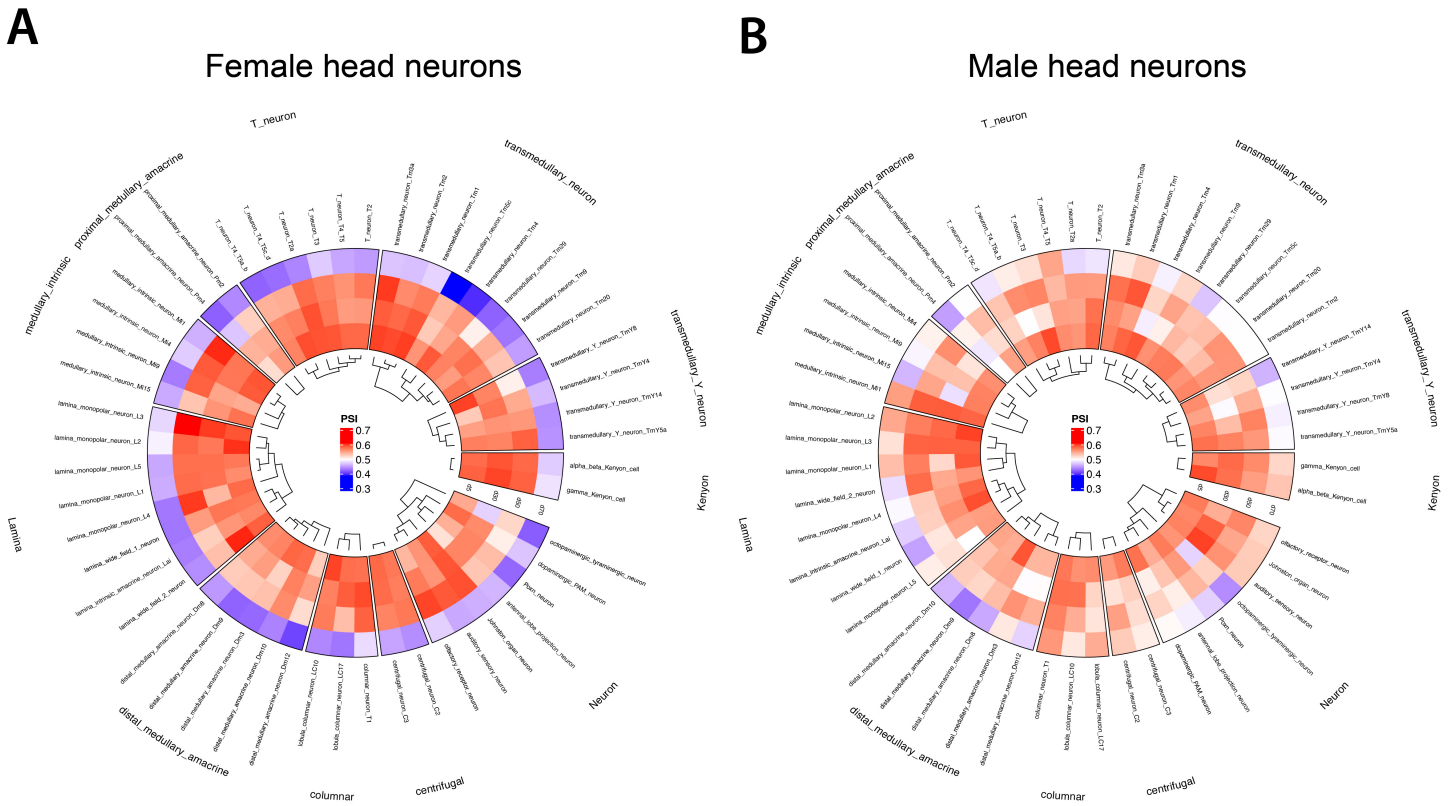


**Fig. S29 Rank sums of different aging features.**

A) Cell types ranked by the sum of different aging feature scores. B) Clustering of rank sum scores across different cell types.



Figure S30



**Fig. S30 Comparison of alternative polyadenylation (APA) scores across different ages.**

A-B) The circular heatmaps summarize APA trends from the annotated neuronal extended genes across different time points in females (A) and males (B). We use the LABRATsc package to calculate  $\psi$  and the average value for 391 neuronal extended genes, which are plotted in each of the neuronal types from the AFCA head data. C) UMAP of different neuronal types designated by the AFCA. Average 3' isoform usage of 391 neuronal extended genes is plotted in individual cells by different aging time points. Both analyses show that neuronal extended 3' isoforms are progressively depleted during aging. This phenotype is stronger in females at 70d.

**Movies S1-S3. Three examples of three-dimensional (3D) reconstructions of two attached nuclei in the same fat body cell.** The attached nuclei are stained by DAPI and LamC. The membranes of fat body cells are labeled by *cg-GAL4 > UAS-CD8GFP*.

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