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Supplemental Information

Combining Developmental and Perturbation-Seq Uncovers Transcriptional Modules Orchestrating Neuronal Remodeling

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Figure S1

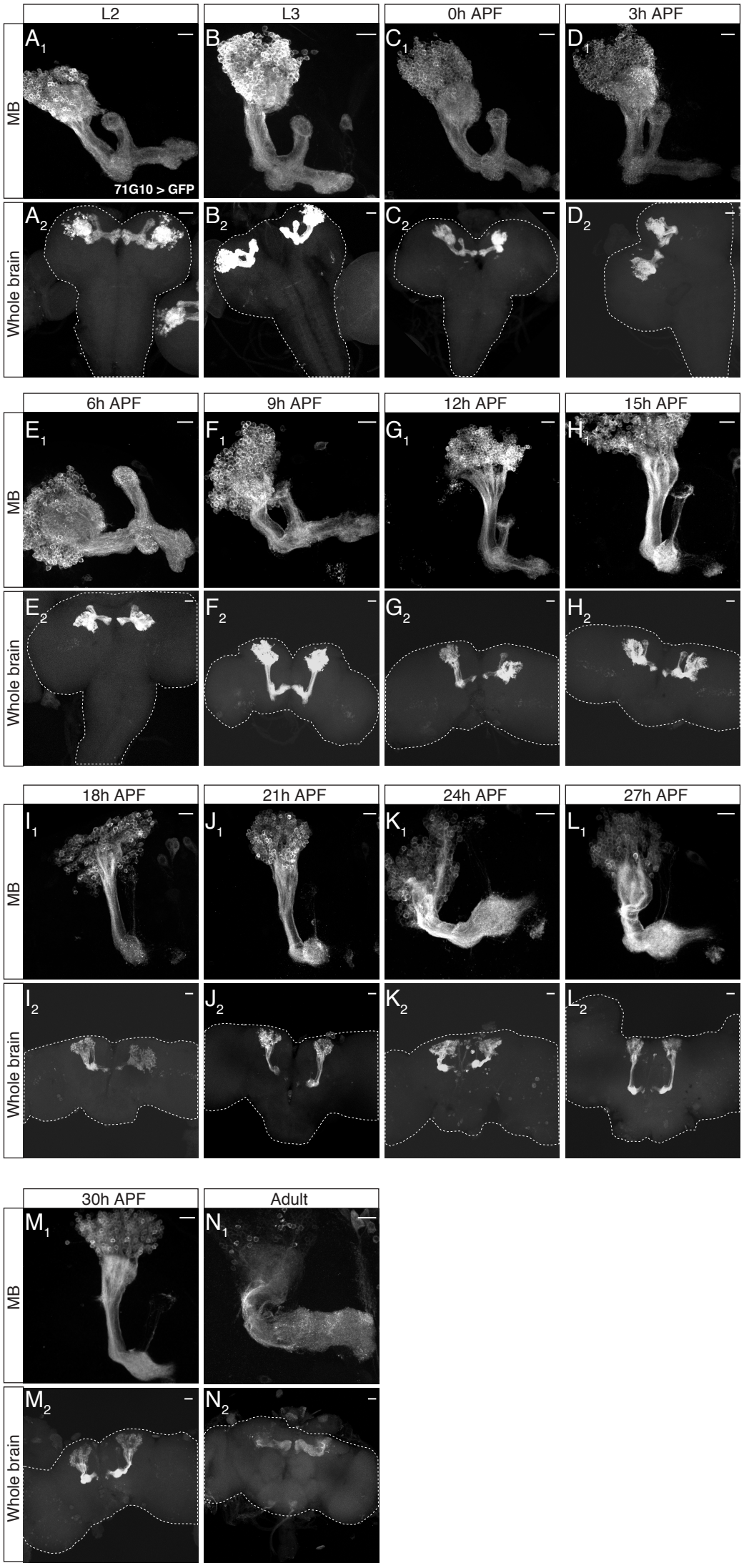

L2
L3
0h APF
3h APF
6h APF
9h APF
12h APF
15h APF
18h APF
21h APF
24h APF
27h APF
30h APF
Adult
MB
Whole brain
71G10 > GFP
A1
A2
B
B2
C1
C2
D1
D2
E1
E2
F1
F2
G1
G2
H1
H2
I1
I2
J1
J2
K1
K2
L1
L2
M1
M2
N1
N2

Figure S1 – **71G10-Gal4 is robustly and almost exclusively expressed in MB γ neurons throughout development, related to Figure 1**

Confocal Z-projections of MB lobe **(A_1-N_1)** and whole brains **(A_2-N_2)** where the γ neurons labeled with mCD8-GFP (GFP) driven by 71G10-Gal4 (71G10), at all the time points taken for the developmental RNA-seq. Whole brain projections are intentionally over exposed in order to emphasize the near absence of labeled neurons outside the MB expressing GFP. Scale bars represent 15 μm for MB-lobes and 30 μm for whole brains.

Figure S2

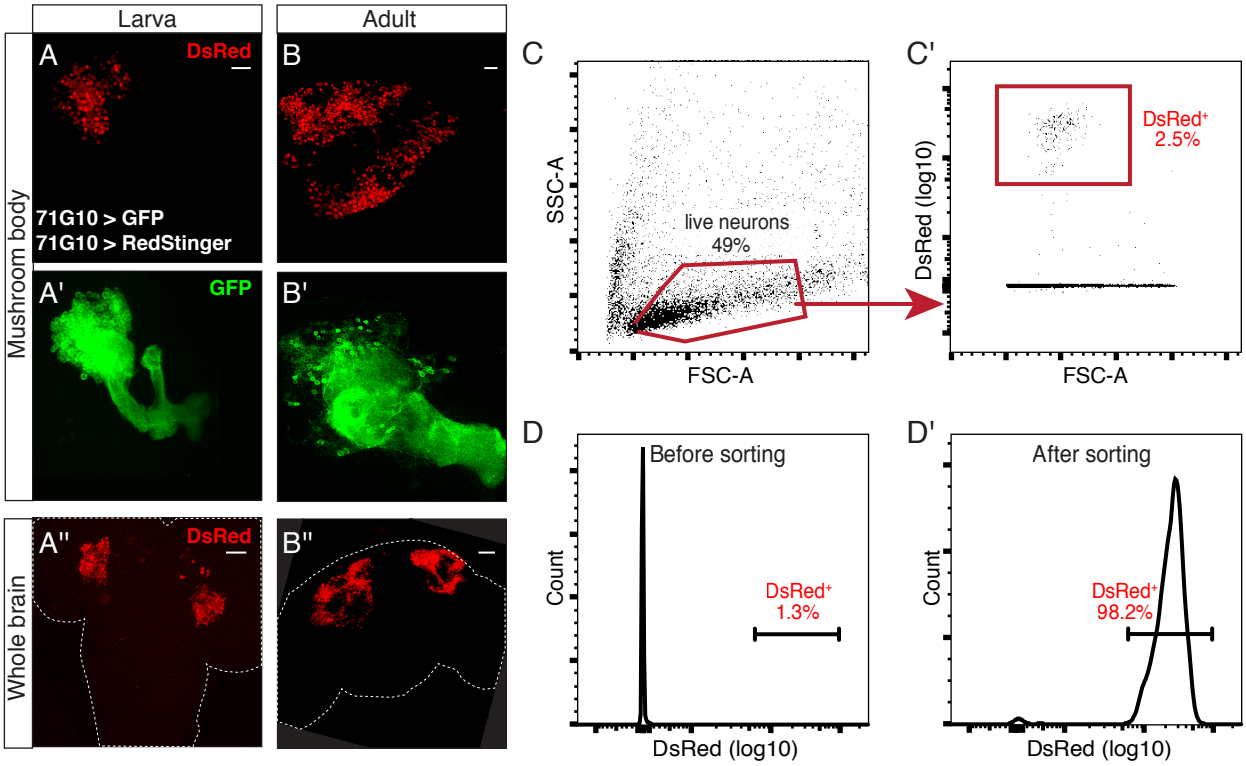

Larva
Adult
Mushroom body
Whole brain
A
DsRed
71G10 > GFP
71G10 > RedStinger
B
A'
GFP
B'
A''
DsRed
B''
C
SSC-A
live neurons
49%
FSC-A
C'
DsRed (log10)
DsRed+
2.5%
FSC-A
D
Before sorting
Count
DsRed+
1.3%
DsRed (log10)
D'
After sorting
Count
DsRed+
98.2%
DsRed (log10)

Figure S2 – **Quality control analyses of cell isolation, related to Figure 1.**

(A) Confocal Z-projections of larval **(A**-**A')** and adult **(B**-**B')** MB γ neurons labeled with nuclear DsRed (RedStinger) and mCD8-GFP (GFP) driven by 71G10-Gal4 (71G10). Low magnification projections that show whole brains (**A''**-**B''**) demonstrate the specificity of 71G10 within the entire brain. Scale bars represent 15 µm for lobes and 30 µm for whole brains.

(C) FACS gating strategy for isolating DsRed positive MB γ neurons from dissociated brains. Forward scatter – FSC is plotted against Side scatter – SSC (**C**) or DsRed expression (**C'**).

(D) FACS analysis of DsRed positive cells from dissociated brains before (**D**) and after (**D'**) positive sorting according to the gating strategy presented in **(C)**. Note that **D** represents the entire cell population resulting in lower $DsRed^+$ cell proportion (1.3%) compared to **C'**, where the analysis includes only live cells that passed a certain SSC and FSC threshold (2.5% $DsRed^+$ cells).

Figure S3

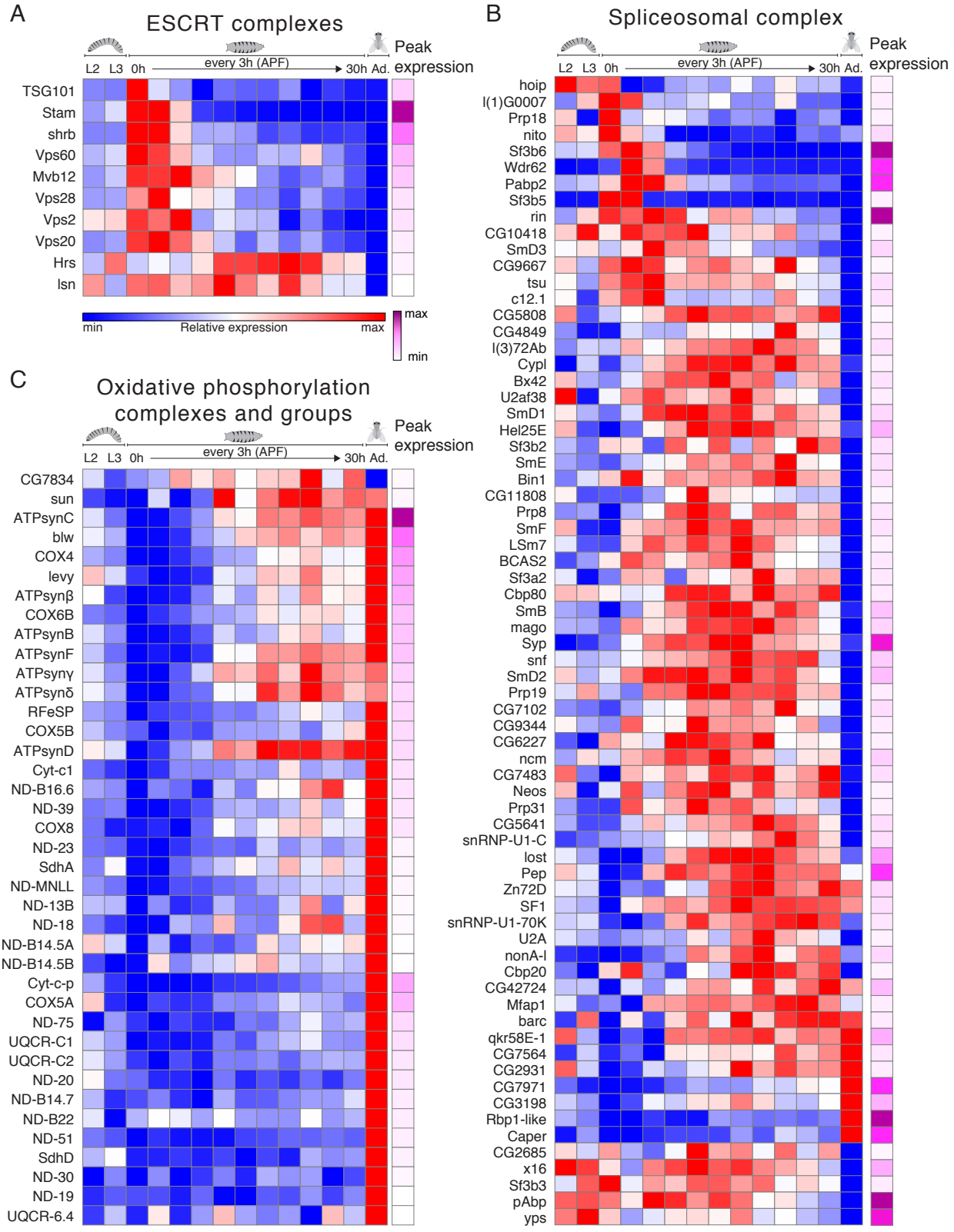

A
ESCRT complexes
L2 L3 0h
every 3h (APF)
30h Ad.
Peak expression
TSG101
Stam
shrb
Vps60
Mvb12
Vps28
Vps2
Vps20
Hrs
Isn
min
Relative expression
max
max
min
B
Spliceosomal complex
hoip
l(1)G0007
Prp18
nito
Sf3b6
Wdr62
Pabp2
Sf3b5
rin
CG10418
SmD3
CG9667
tsu
c12.1
CG5808
CG4849
l(3)72Ab
Cypl
Bx42
U2af38
SmD1
Hel25E
Sf3b2
SmE
Bin1
CG11808
Prp8
SmF
LSm7
BCAS2
Sf3a2
Cbp80
SmB
mago
Syp
snf
SmD2
Prp19
CG7102
CG9344
CG6227
ncm
CG7483
Neos
Prp31
CG5641
snRNP-U1-C
lost
Pep
Zn72D
SF1
snRNP-U1-70K
U2A
nonA-l
Cbp20
CG42724
Mfap1
barc
qkr58E-1
CG7564
CG2931
CG7971
CG3198
Rbp1-like
Caper
CG2685
x16
Sf3b3
pAbp
yps
C
Oxidative phosphorylation complexes and groups
CG7834
sun
ATPsynC
blw
COX4
levy
ATPsynβ
COX6B
ATPsynB
ATPsynF
ATPsynγ
ATPsynδ
RFeSP
COX5B
ATPsynD
Cyt-c1
ND-B16.6
ND-39
COX8
ND-23
SdhA
ND-MNLL
ND-13B
ND-18
ND-B14.5A
ND-B14.5B
Cyt-c-p
COX5A
ND-75
UQCR-C1
UQCR-C2
ND-20
ND-B14.7
ND-B22
ND-51
SdhD
ND-30
ND-19
UQCR-6.4

Figure S3 – **Gene expression profiles of dynamic genes from selected terms, related to Figure 2.**

(A-C) Heat map depicting the relative expression patterns of dynamic genes belonging to the described GO terms. The magenta scale depicts the intensity of the peak expression of each gene relative to that of other genes in the group. The genes are ordered according to their developmental cluster and then by their max expression peak.

Figure S4

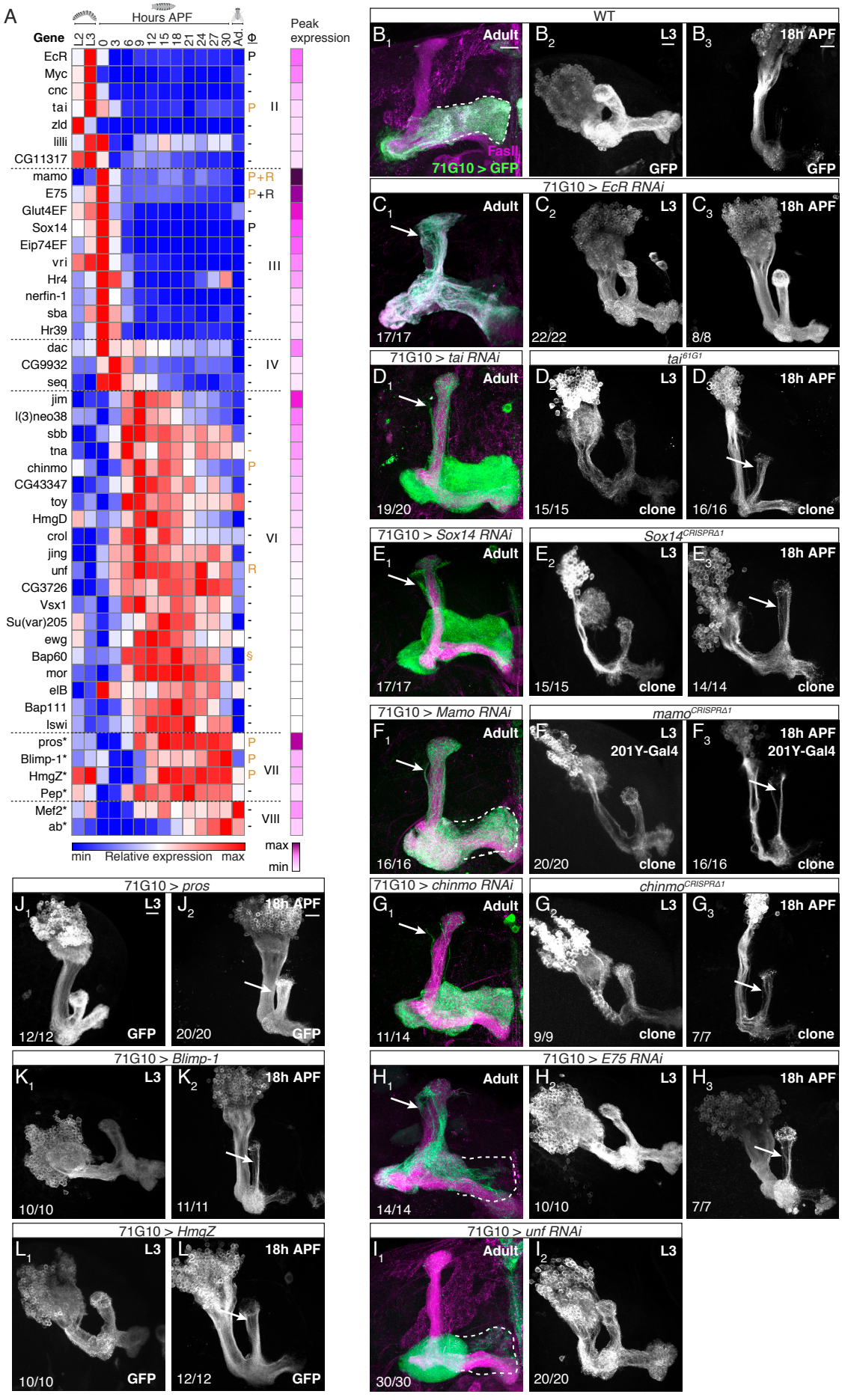

A
Hours APF
Gene
L2 L3 0 3 6 9 12 15 18 21 24 27 30 Ad.
Φ
Peak expression
EcR P
Myc -
cnc -
tai P II
zld -
lilli -
CG11317 -
mamo P+R
E75 P+R
Glut4EF -
Sox14 P
Eip74EF -
vri - III
Hr4 -
nerfin-1 -
sba -
Hr39 -
dac -
CG9932 - IV
seq -
jim -
l(3)neo38 -
sbb -
tna -
chinmo P
CG43347 -
toy -
HmgD -
crol - VI
jing -
unf R
CG3726 -
Vsx1 -
Su(var)205 -
ewg -
Bap60 §
mor -
elB -
Bap111 -
Iswi -
pros* P
Blimp-1* P
HmgZ* P VII
Pep* -
Mef2* - VIII
ab* -
min Relative expression max
max
min
WT
B1 Adult
FasII
71G10 > GFP
B2 L3
GFP
B3 18h APF
GFP
71G10 > EcR RNAi
C1 Adult
17/17
C2 L3
22/22
C3 18h APF
8/8
71G10 > tai RNAi
D1 Adult
19/20
tai61G1
D2 L3
15/15 clone
D3 18h APF
16/16 clone
71G10 > Sox14 RNAi
E1 Adult
17/17
Sox14CRISPRΔ1
E2 L3
15/15 clone
E3 18h APF
14/14 clone
71G10 > Mamo RNAi
F1 Adult
16/16
mamoCRISPRΔ1
F2 L3
201Y-Gal4
20/20 clone
F3 18h APF
201Y-Gal4
16/16 clone
71G10 > chinmo RNAi
G1 Adult
11/14
chinmoCRISPRΔ1
G2 L3
9/9 clone
G3 18h APF
7/7 clone
71G10 > pros
J1 L3
12/12 GFP
J2 18h APF
20/20 GFP
71G10 > Blimp-1
K1 L3
10/10
K2 18h APF
11/11
71G10 > HmgZ
L1 L3
10/10 GFP
L2 18h APF
12/12 GFP
71G10 > E75 RNAi
H1 Adult
14/14
H2 L3
10/10
H3 18h APF
7/7
71G10 > unf RNAi
I1 Adult
30/30
I2 L3
20/20

Figure S4 – **DNA-binding-protein screen uncovers new genes required for remodeling, related to Figure 3**

(A) Heat map of the relative expression patterns of the DNA binding proteins (DBPs) taken for the screen, sorted by cluster and peak expression levels (magenta). P, R and (-) stand for pruning, regrowth or WT phenotypes, respectively. While known roles of genes are labeled in black, new findings from this study are labeled in orange. Asterisks demarcate examples of genes whose expression is downregulated at the onset of pruning - which we therefore tested by overexpression. § demarcate the fact that while expression of Bap60 RNAi resulted in a pruning defect, we were not able to confirm this using mutant analysis.

(B-L) Confocal Z-projections of MBs of the indicated age in which 71G10-Gal4 drives the expression of mCD8-GFP as well as the indicated RNAi/overexpression transgenes (indicated by 71G10>transgene) OR, brains in which 71G10-Gal4 **(D_{2-3}**, **E_{2-3}**, **G_{2-3})** or 201Y-Gal4 **(F_{2-3})** drive the expression of mCD8-GFP within a MARCM clone of the indicated genotype.

Pruning defects are marked by arrows, and regrowth defects are evident when the adult γ lobe does not fully occupy the white dashed line. Scale bars represent 15 μm. The numbers (x/n) on the lower left corners depict the number of times the phenotype was observed out of the total hemispheres examined.

Figure S5

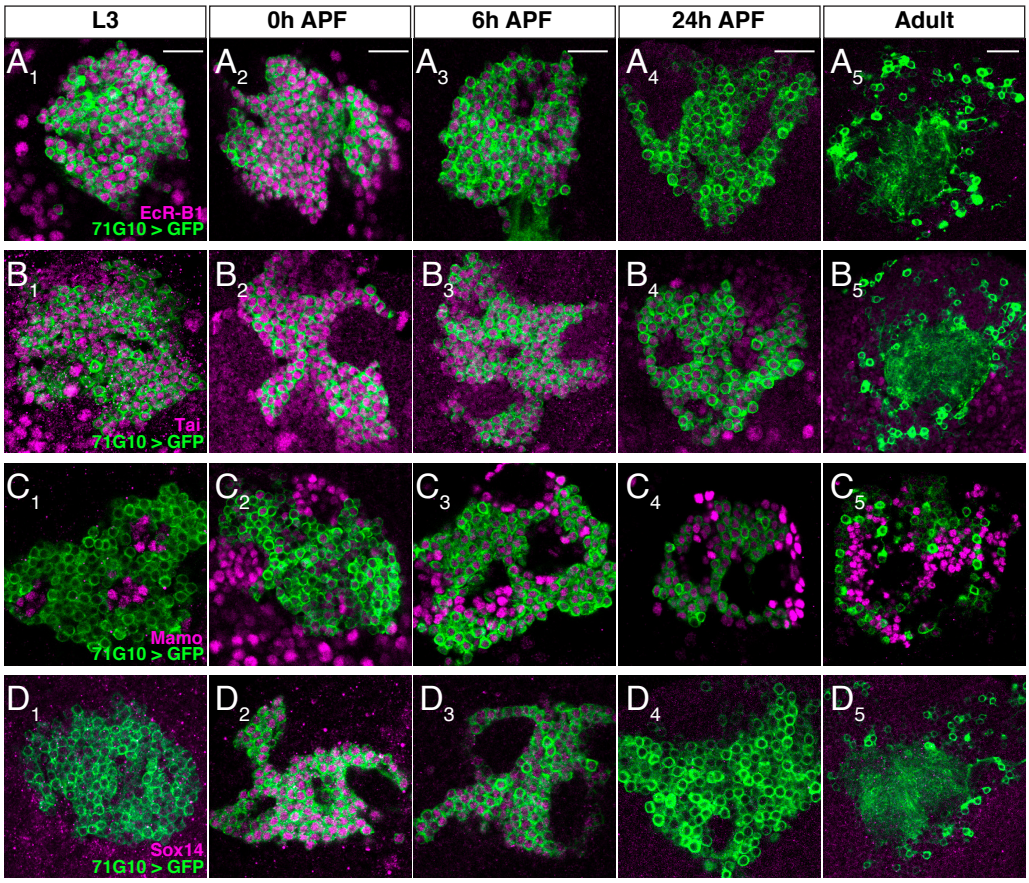
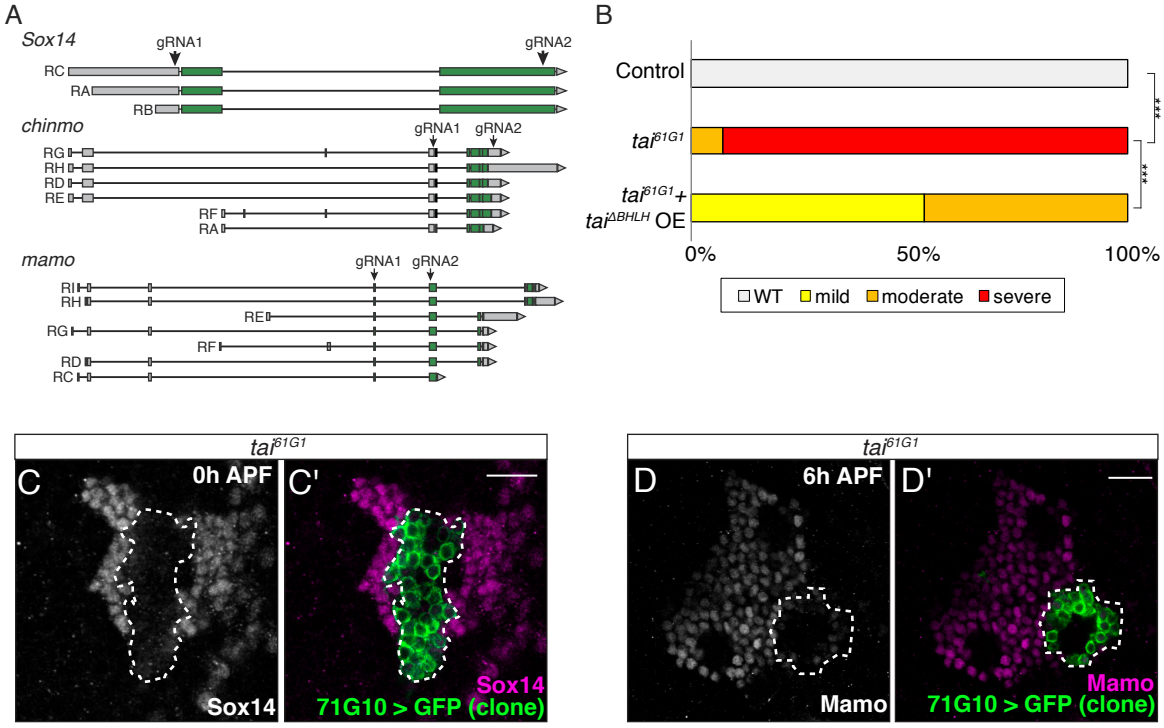

L3
0h APF
6h APF
24h APF
Adult
A1
A2
A3
A4
A5
EcR-B1
71G10 > GFP
B1
B2
B3
B4
B5
Tai
71G10 > GFP
C1
C2
C3
C4
C5
Mamo
71G10 > GFP
D1
D2
D3
D4
D5
Sox14
71G10 > GFP

Figure S5 – **Antibody staining of selected DBPs during development correlate to their RNA expression profile, related to Figure 3**

(A-D) Single confocal slices of the cell body region of MB neurons labeled with mCD8-GFP (GFP, green) driven by 71G10-Gal4 co-stained using the indicated antibodies (magenta) at the indicated developmental stages. Protein peak expression within γ neurons that are labeled in green, for each of the DBPs (0h APF for EcR-B1 and Tai, 6h APF for Mamo and 0h APF for Sox14) correlates to the RNA expression peak as referred from the RNA-seq. n>8 lobes for each experiment, scale bars represent 15 μm.

Figure S6


A
Sox14
gRNA1
gRNA2
RC
RA
RB
chinmo
gRNA1
gRNA2
RG
RH
RD
RE
RF
RA
mamo
gRNA1
gRNA2
RI
RH
RE
RG
RF
RD
RC
B
Control
tai61G1
tai61G1+
taiΔBHLH OE
0%
50%
100%
WT
mild
moderate
severe
***
***

tai61G1
C
0h APF
Sox14
C'
Sox14
71G10 > GFP (clone)

tai61G1
D
6h APF
Mamo
D'
Mamo
71G10 > GFP (clone)

Figure S6 – **CRISPR mediated deletion design, and *tai* function at the same epistatic level as EcR, related to Figure 3**

(A) A schematic representation of the gRNA design to induce CRISPR/Cas9-mediated deletions in *Sox14*, *chinmo* and *mamo*. Green bars represent coding exons, grey bars represent non-coding exonal sequences and lines represent introns. All annotated isoforms for each gene are displayed – while $Sox14^{CRISPR\Delta 1}$ and $chinmo^{CRISPR\Delta 1}$ alleles are deletions of most of the coding sequence, $mamo^{CRISPR\Delta 1}$ allele induces a frameshift 11 base pairs downstream of the annotated ATG (resulting in a premature stop codon). The gRNAs were designed to delete most of the coding sequence of the gene.

(B) Quantification of the pruning severity in Figure 3D, 3H and 3O using blind ranking analysis of the described genotypes. *** $p<0.001$, See experimental procedures for details.

(C-D) Single confocal sections of the cell body region of tai^{61G1} MARCM MB neuroblast clones stained for anti-Sox14 **(C,** n=6**)** or anti-Mamo **(D**, n=4**)** at 0h APF and 6h APF, respectively. Expression of Sox14 **(C)** and Mamo **(D)** is 4.2-fold ($p<0.001$) and 2.4-fold ($p<0.01$) decreased within the tai^{61G1} clone, respectively. Dashed lines demarcate the boundaries of the clone. Green represents mCD8-GFP driven by 71G10-Gal4, magenta represents Sox14 **(C)** or Mamo **(D)** antibody staining, which are also shown in grey. Scale bars represent 15 μm.

Figure S7

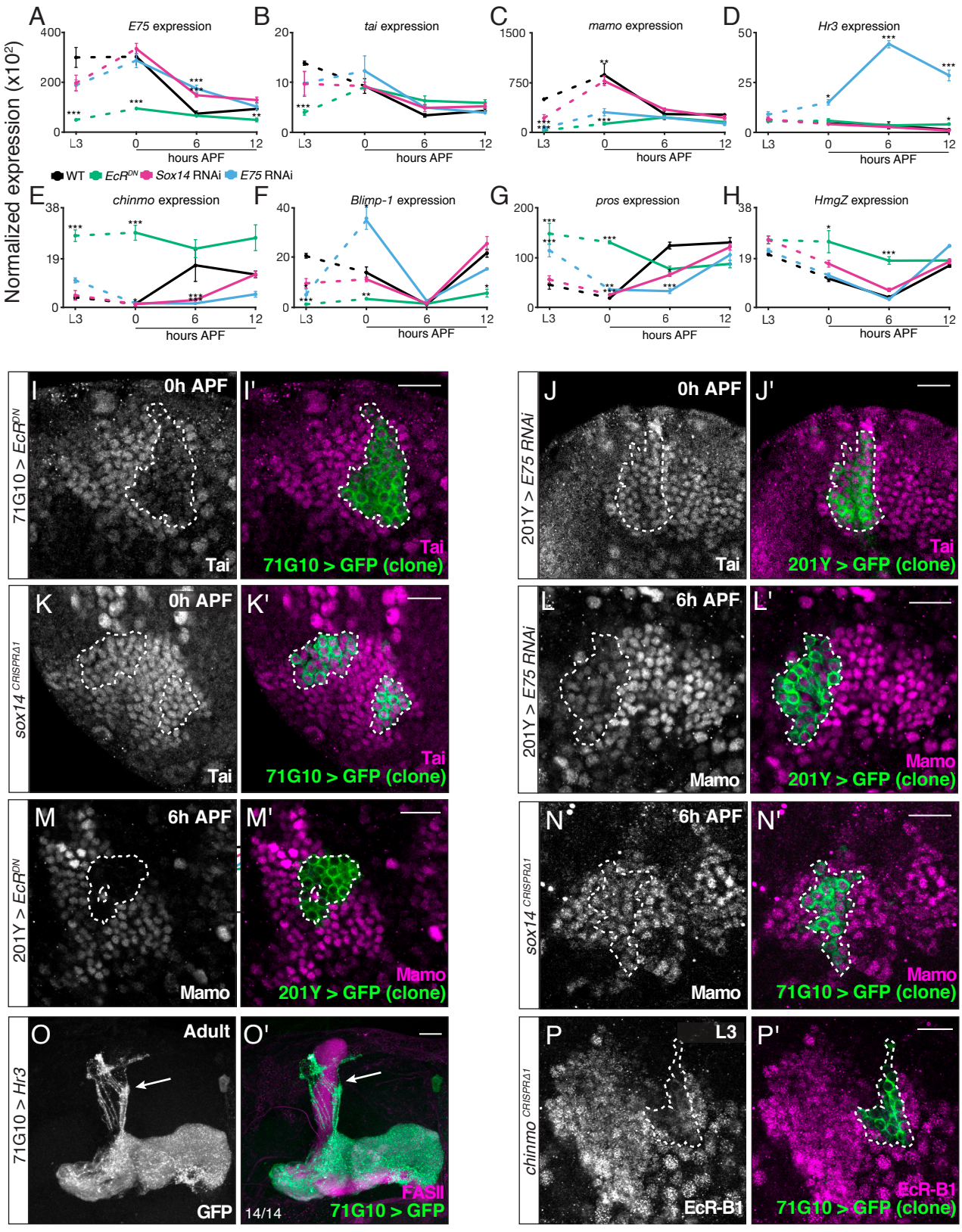

A
E75 expression
B
tai expression
C
mamo expression
D
Hr3 expression
Normalized expression (x10²)
hours APF
WT
EcR^DN
Sox14 RNAi
E75 RNAi
E
chinmo expression
F
Blimp-1 expression
G
pros expression
H
HmgZ expression
I
0h APF
Tai
I'
Tai
71G10 > GFP (clone)
71G10 > EcR^DN
J
0h APF
Tai
J'
Tai
201Y > GFP (clone)
201Y > E75 RNAi
K
0h APF
Tai
K'
Tai
71G10 > GFP (clone)
sox14 CRISPRΔ1
L
6h APF
Mamo
L'
Mamo
201Y > GFP (clone)
201Y > E75 RNAi
M
6h APF
Mamo
M'
Mamo
201Y > GFP (clone)
201Y > EcR^DN
N
6h APF
Mamo
N'
Mamo
71G10 > GFP (clone)
sox14 CRISPRΔ1
O
Adult
GFP
O'
FASII
14/14
71G10 > GFP
71G10 > Hr3
P
L3
EcR-B1
P'
EcR-B1
71G10 > GFP (clone)
chinmo CRISPRΔ1

Figure S7 – **Expression profiles of neurons perturbed for key transcription factors uncovers hierarchical regulation of axon pruning by regulatory factors, related to Figure 5**

(A-H) Normalized expression of *E75* **(A),** *tai* **(B)**, *mamo* **(C)**, *Hr3* **(D)**, *chinmo* **(E)**, *Blimp-1* **(F)**, *prospero* **(G)** or *HmgZ* **(H)** in WT MB γ neurons and in those expressing the indicated transgene (* p<0.05, ** p<0.01, *** p<0.001). Error bars indicate SEM, units on the y axis are arbitrary.

(I-N, P) Single confocal slices of MB cell bodies containing MARCM clones labeled with mCD8-GFP (GFP) driven by 71G10 **(I,K,N,P)** or 201Y **(J,L-M)** additionally expressing EcR^{DN} (**I**, n=6, **M**, n=4), *E75*-RNAi (**J**, n=8, **L**, n=8), homozygous for $Sox14^{CRISPR\Delta1}$ (**K**, n=9, **N**, n=6) or homozygous for $chinmo^{CRISPR\Delta1}$ (**P**, n=3) stained using the indicated antibody at the indicated developmental stage. The anti-Tai staining was decreased by 3.1-fold (p<0.001) within clones expressing EcR^{DN} **(I)**. The anti-Mamo staining was decreased by 2.2-fold (p<0.01) within clones expressing *E75*-RNAi **(L)** and by 7.1-fold within clones expressing EcR^{DN} **(M)**. The anti-EcR-B1 staining was decreased by 3.5-fold (p<0.001) within clones expressing $chinmo^{CRISPR\Delta1}$. The boundaries of the clones are represented by dashed lines.

(O) Confocal Z-projections of an adult MB labeled by 71G10-Gal4 driving the expression of mCD8-GFP and an *Hr3* transgene. Arrow indicates dorsally projecting unpruned γ axons. The numbers (x/n) on the lower left corners depict the number of times the phenotype was observed out of the total hemispheres examined.

Green represents mCD8-GFP expression driven by the indicated Gal4. Magenta and Gray represent the antibody indicated. Scale bars represent 15 μm.

Table S2 – Enrichment analysis of selected GO, KEGG and Reactome terms within developmental clusters

Cluster	Genes within cluster	Selected enriched terms	Term enrichment (see below)	Adjusted p value=
I	266	Synaptic transmission [GO:0007268]	35/184	5×10^{-13}
		Regulation of neurotransmitter levels [GO:0001505]	21/86	4×10^{-9}
		Voltage-gated cation channel activity [GO:0022843]	10/26	4×10^{-6}
II	188	Glycolytic process [GO:0006096]	10/16	7×10^{-7}
III	270	Proteasome-mediated ubiquitin-dependent protein catabolic process [GO:0043161]	38/123	4×10^{-22}
		Neuron projection morphogenesis [GO:0048812]	35/401	4×10^{-4}
IV	404	Programmed cell death [GO:0012501]	32/196	7×10^{-4}
		Autophagy [GO:0006914]	29/116	2×10^{-7}
		Endosome transport via multivesicular body sorting pathway [GO:0032509]	8/16	8×10^{-4}
		Endosomal transport [GO:0016197]	15/65	0.014
V	239	COP9 signalosome [GO:0008180]	6/9	1×10^{-5}
VI	309	Axon guidance [GO:0007411]	24/182	4×10^{-4}
		Axon development [GO:0061564]	28/242	3×10^{-4}
		Spliceosomal complex [GO:0005681] Spliceosome (map03040)	32/140 25/97	3×10^{-13} 2×10^{-10}
		Chromatin remodeling [GO:0006338]	19/78	2×10^{-7}
VII	247	Protein processing in endoplasmic reticulum [map04141]	13/96	0.002
VIII	316	Cellular respiration [GO:0045333]	21/79	1×10^{-7}
		Mitochondrial part [GO:0044429]	46/196	3×10^{-13}
IX	333	Synaptic transmission [GO:0007268]	34/184	7×10^{-10}
		Neurotransmitter secretion [GO:0007269]	18/80	1×10^{-5}
X	101	Ribosome [GO:0005840]	25/55	1×10^{-13}

Enrichment analysis of selected GO, KEGG and Reactome terms that are significantly enriched ($p<0.05$) after Holm–Bonferroni correction. Term enrichment refers to the number of term related genes within the cluster out of all of the *Drosophila* term related genes has significant expression in the γ neurons.

Table S6 – Enrichment analysis of selected GO, KEGG and Reactome terms within sub-clusters

Cluster	Genes within cluster	Selected enriched terms	Term enrichment	Adjusted p value=
Ia	72	Synaptic transmission [GO:0007268]	12/35	9×10^{-4}
Ib	75	Plasma membrane proton-transporting V-type ATPase complex [GO:0033181]	6/9	1×10^{-6}
Ic	43	Voltage-gated cation channel activity [GO:0022843]	5/10	1×10^{-4}
IIa	45	Glycolytic process [GO:0006096]	8/10	2×10^{-10}
IIb	38			
IIc	61			
IIIa	65	Proteasome complex [GO:0000502]	29/32	4×10^{-49}
IIIb	32			
IIIc	47	Neuron projection morphogenesis [GO:0048812]	13/35	0.003
IVa	125	Autophagy [GO:0006914]	14/29	1×10^{-4}
		ESCRT complex [GO:0036452]	4/7	0.035
		Endosome [GO:0005768]	10/26	0.01
IVb	101	programmed cell death involved in cell development [GO:0010623]	9/21	0.01
		Peptidase family C14A, cysteine active site [IPR033139]	3/3	0.01
IVc	33	Ferritin complex [GO:0070288]	2/2	0.009
Va	39			
Vb	63	RHO GTPases Activate Formins [R-DME-5663220]	3/3	0.048
VIa	36	Chromatin organization [GO:0006325]	10/41	0.01
VIb	35	Protein processing in endoplasmic reticulum [map04141]	7/13	3×10^{-4}
VIIa	47			
VIIc	40			
VIIIa	122	Mitochondrial part [GO:0044429]	23/46	6×10^{-7}
		Citrate cycle TCA cycle [map00020]	6/11	0.02
VIIIb	38			
IXa	135	Synaptic transmission [GO:0007268]	19/34	2×10^{-6}
IXb	57	Cation channel complex [GO:0034703]	4/8	0.049

Enrichment analysis of selected GO, KEGG and Reactome terms that are significantly enriched ($p<0.05$) after Holm–Bonferroni correction. Term enrichment refers to the number of term related genes within the cluster out of the number of genes from the functional group within the parent cluster.