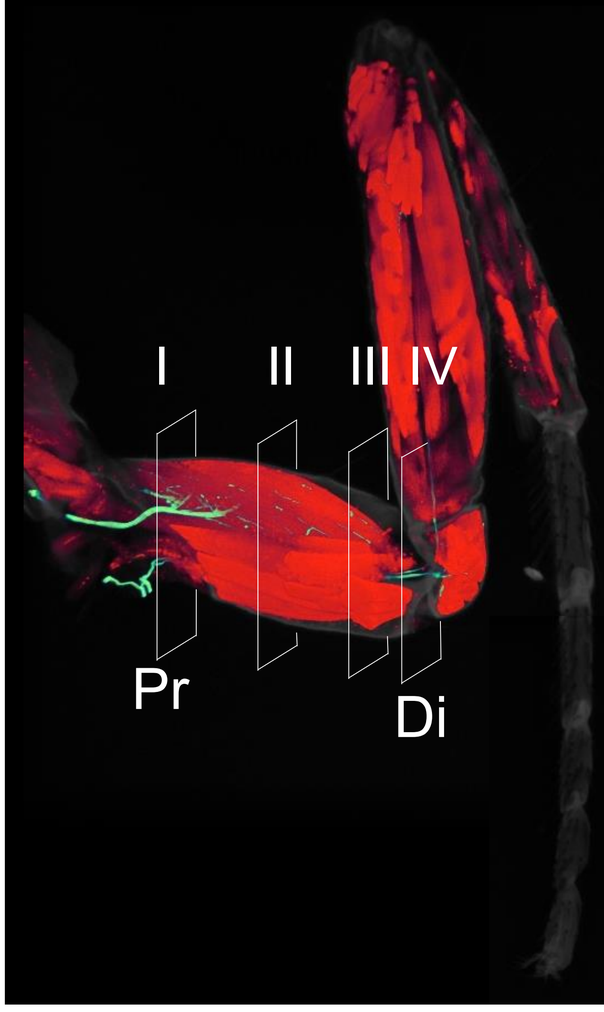
Figure S1. *Enriquez et al.*

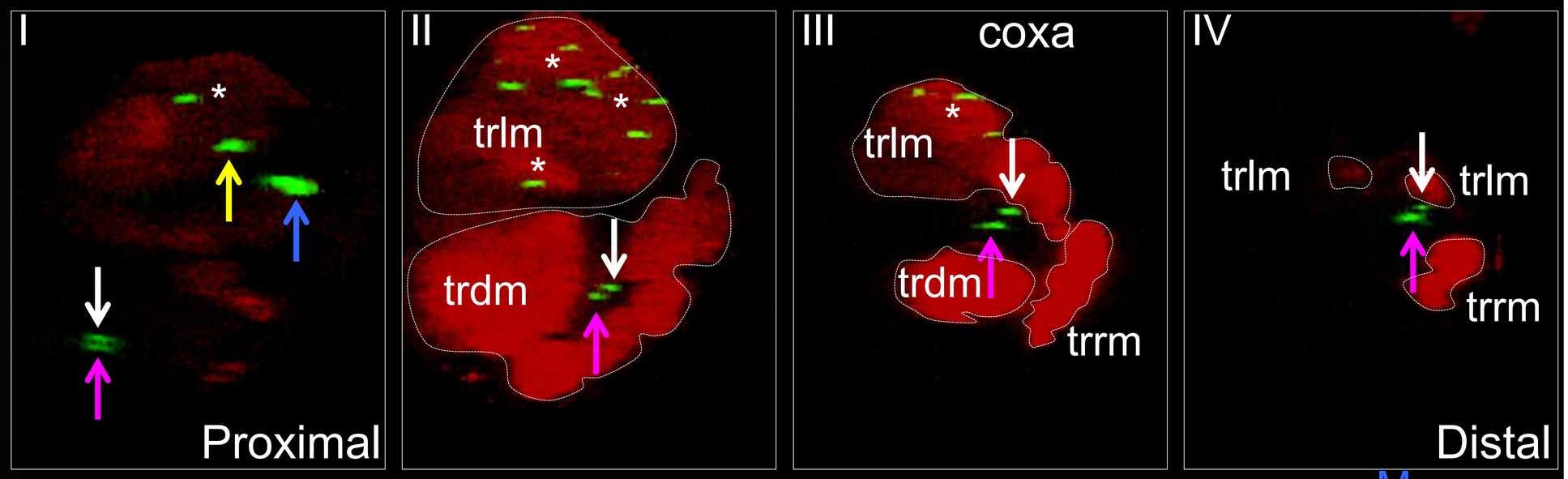
Lin B MARCM*: VGlut>mCD8::GFP, Mhc-RFP*

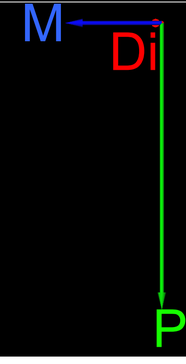
A Cuticle MNs Muscles



MNs Muscles

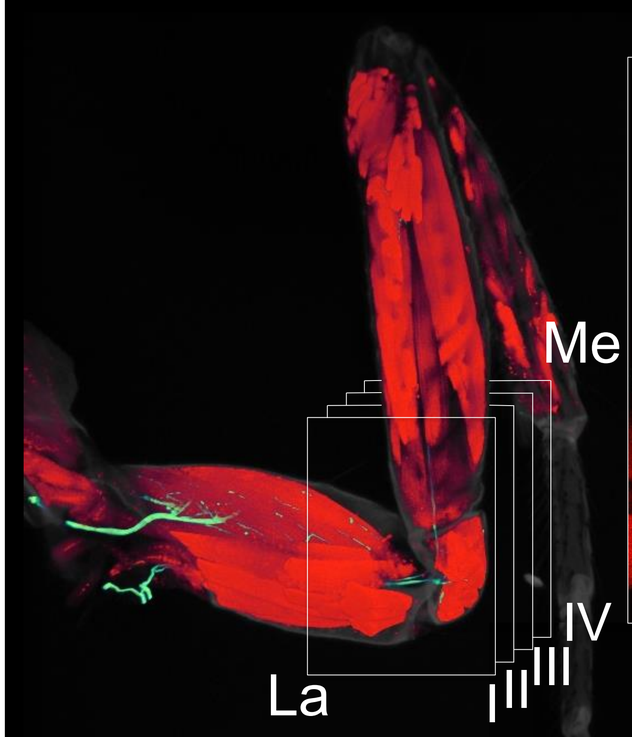
Transverse sections of the coxa





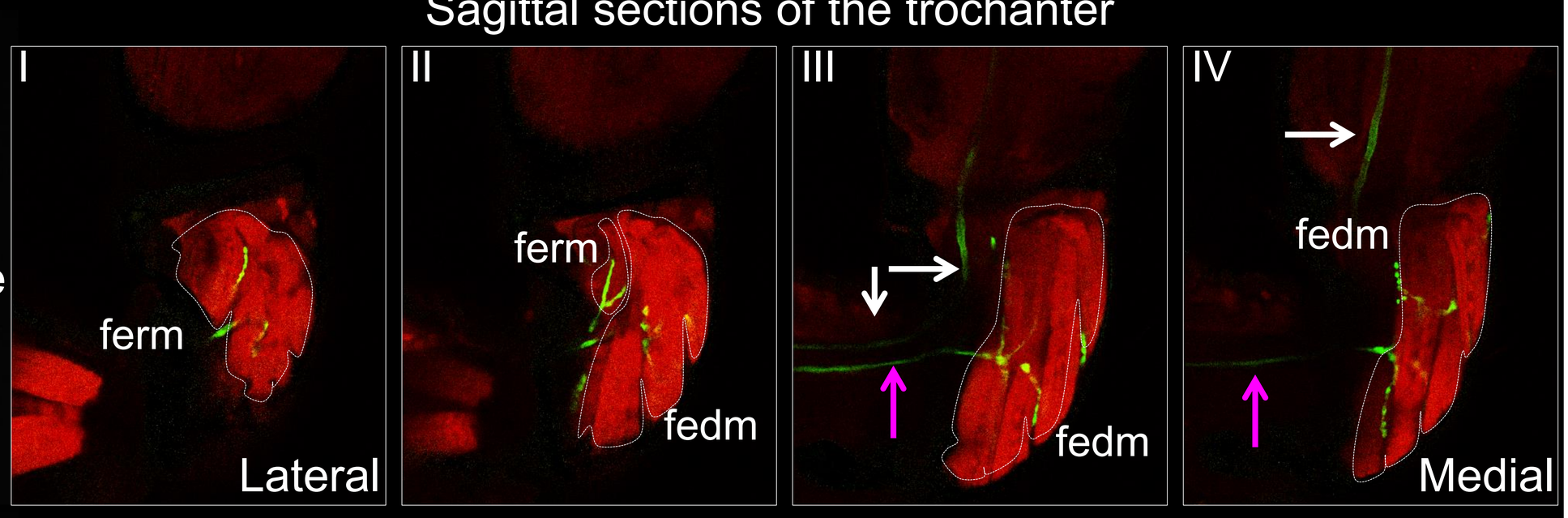
→ Coxa 1
→ Coxa 2/4 axons
→ Femoral 1 axon
→ Trochanter 1/2 axons

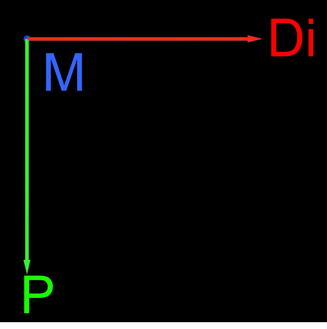
B Cuticle MNs Muscles



MNs Muscles

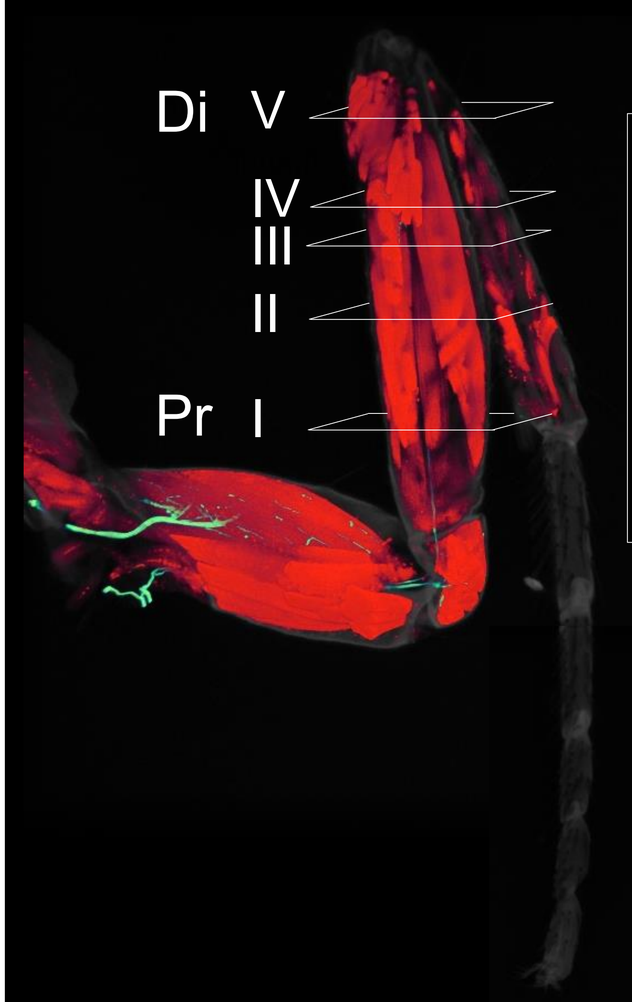
Sagittal sections of the trochanter





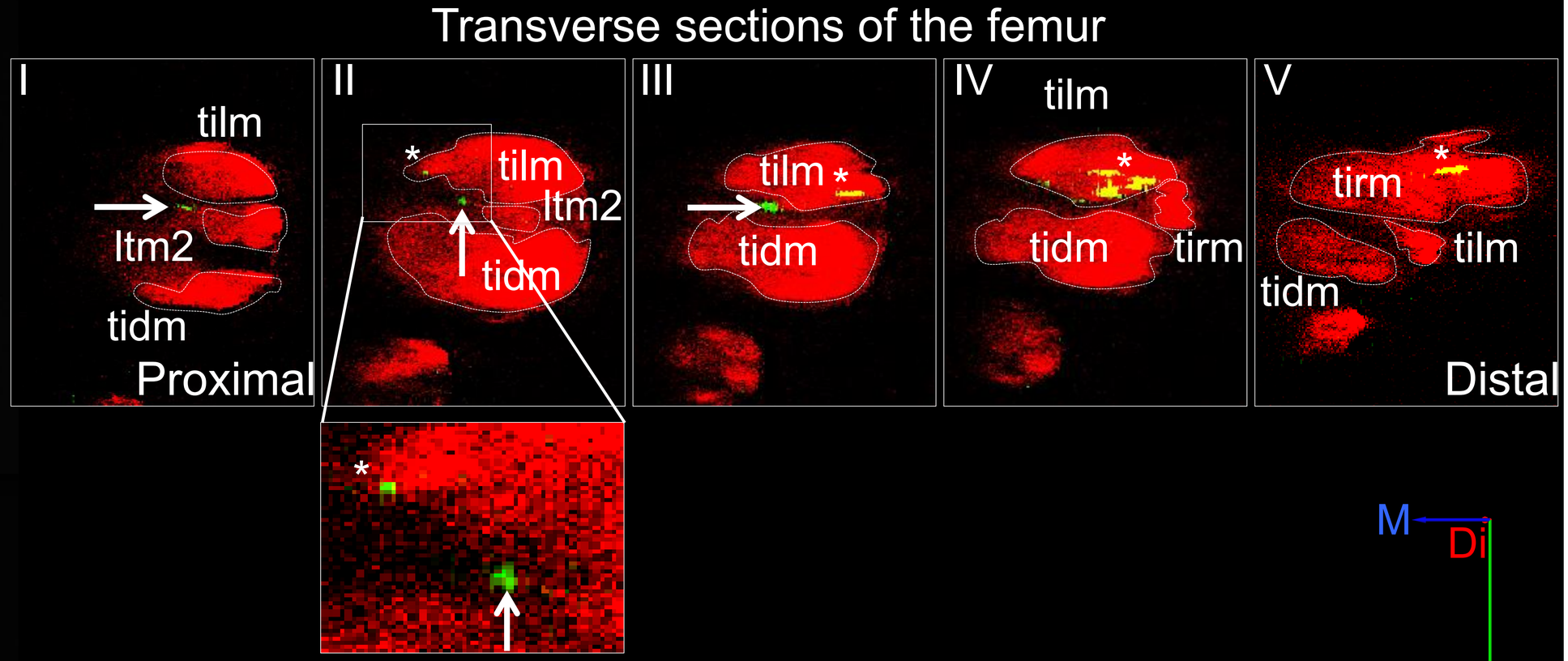
→ Femoral 1 axon
→ Trochanter 1/2 axons

C Cuticle MNs Muscles



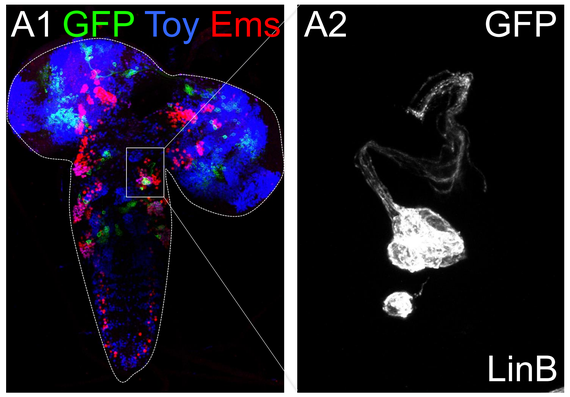
MNs Muscles

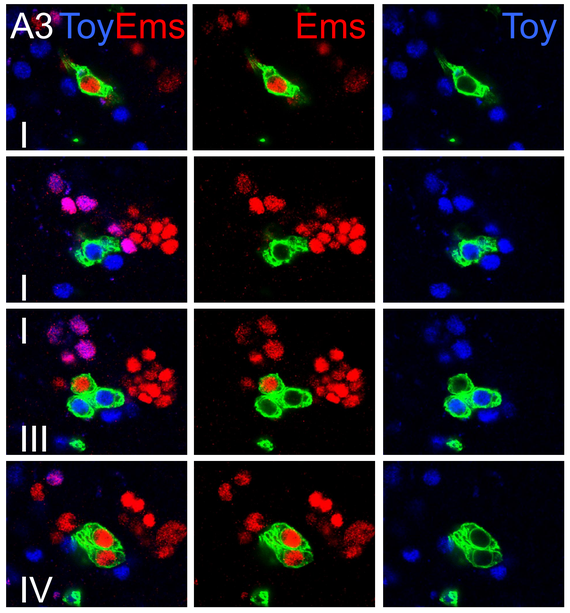
Transverse sections of the femur

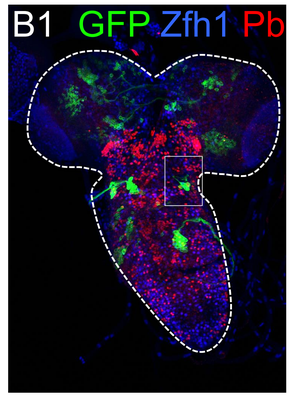


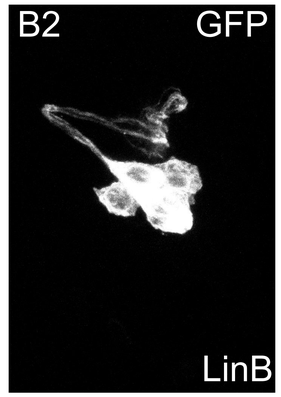
→ Femoral 1 axon

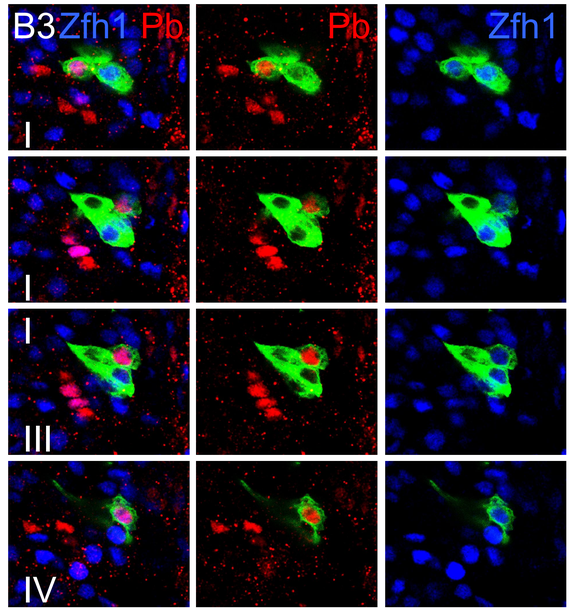
Figure S2. *Enriquez et al.*

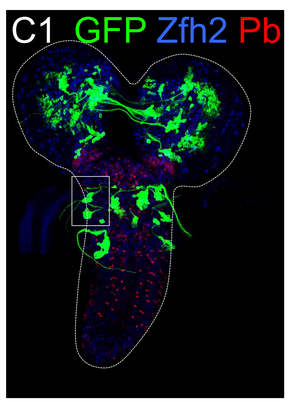


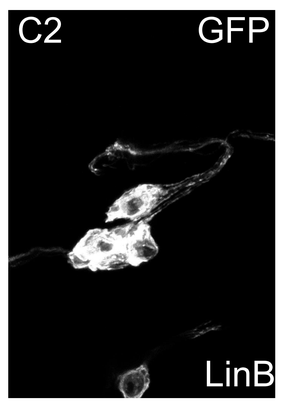


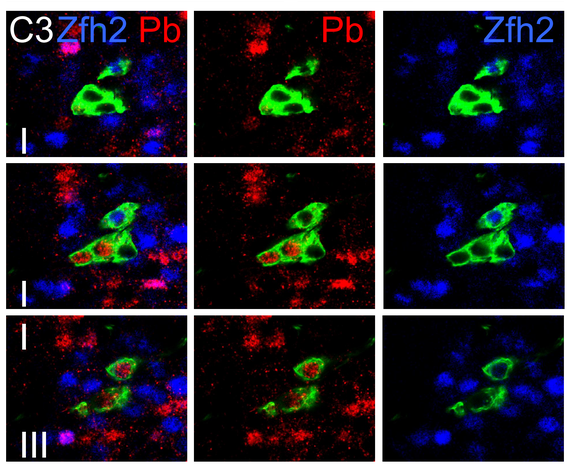


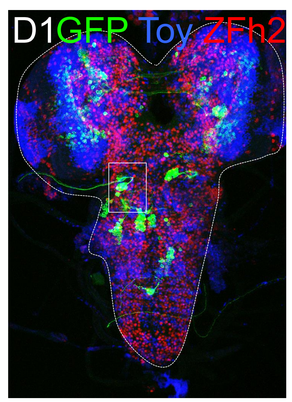


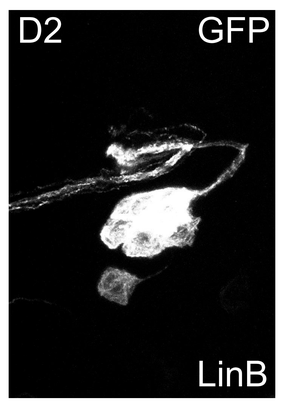


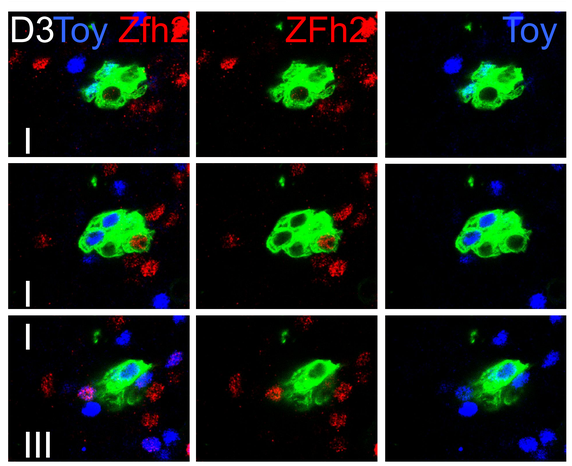


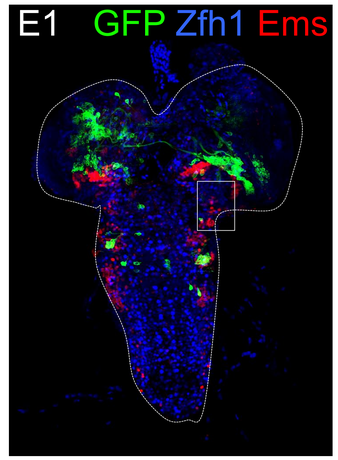


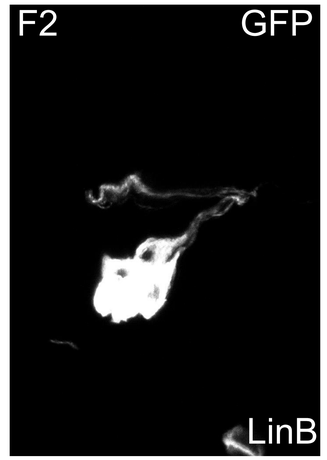


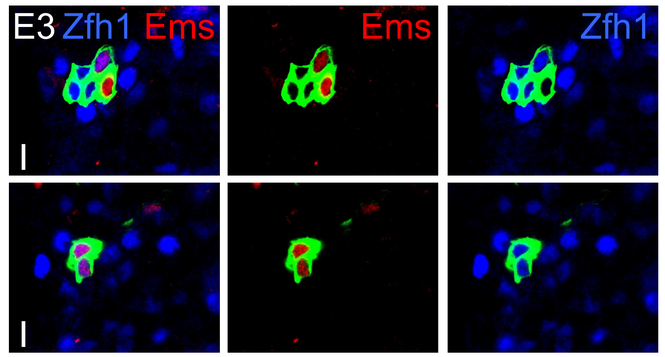


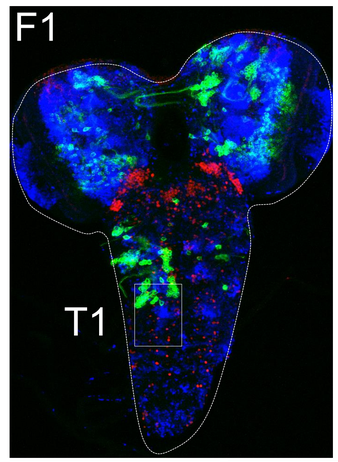


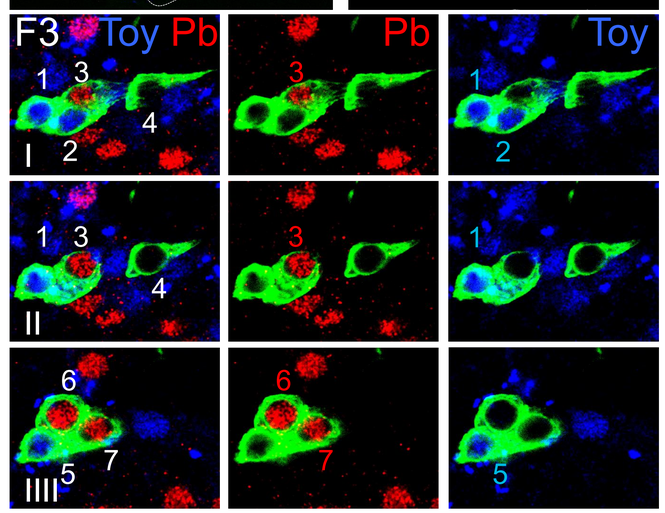


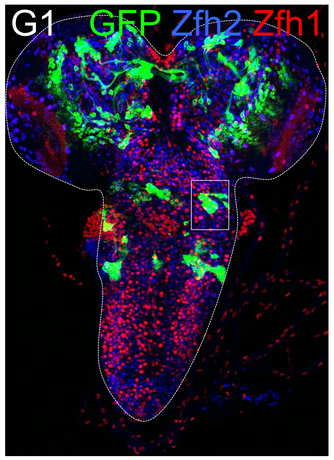


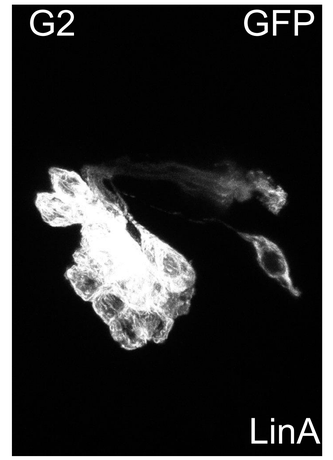












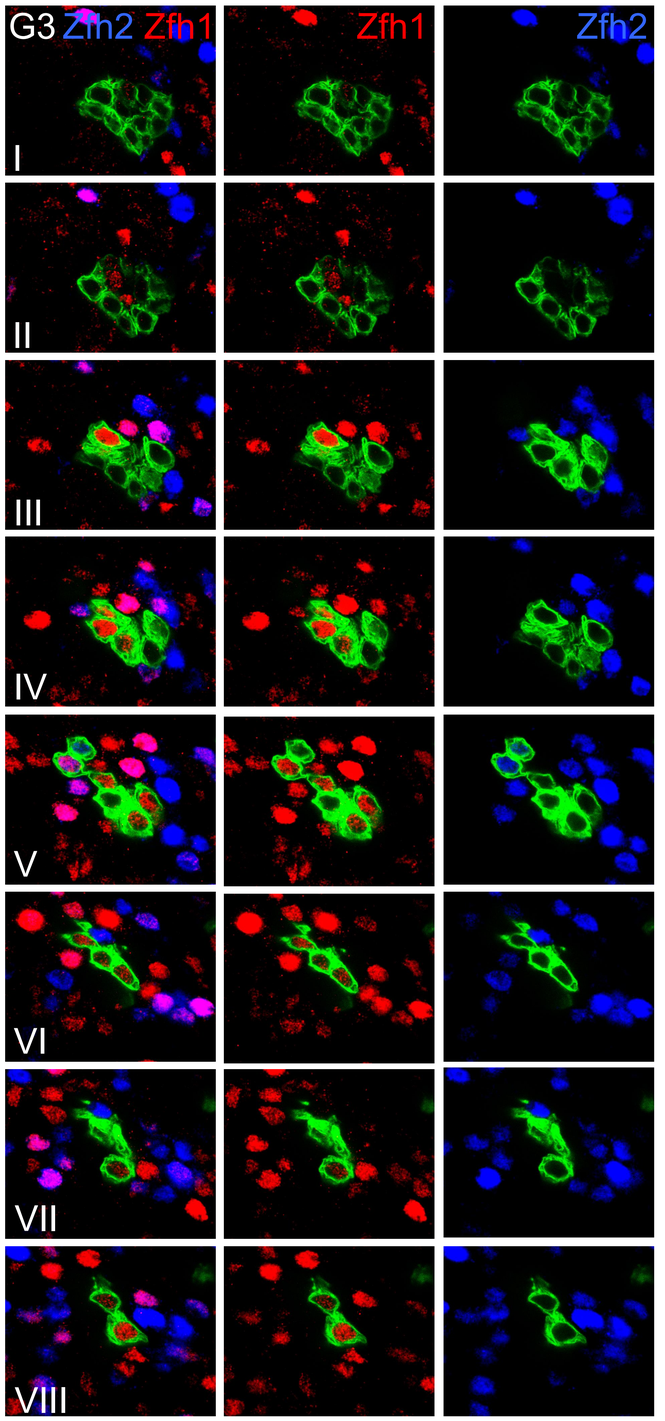
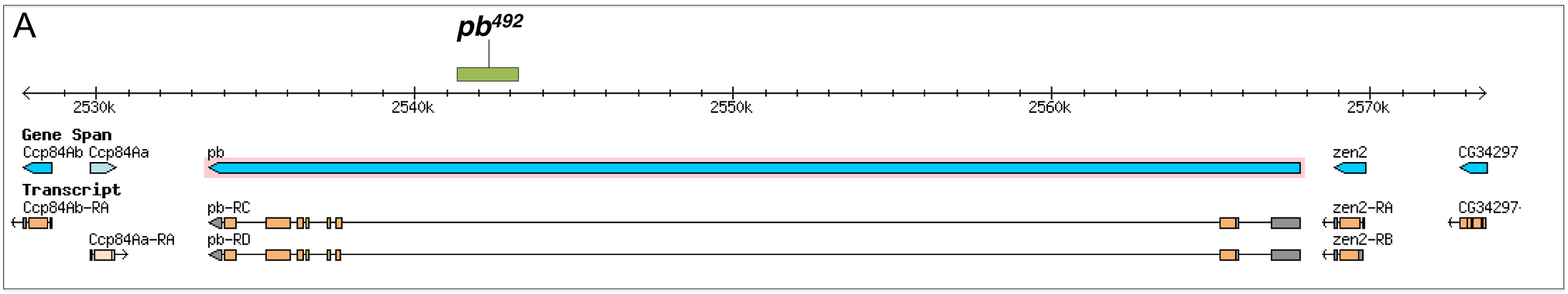
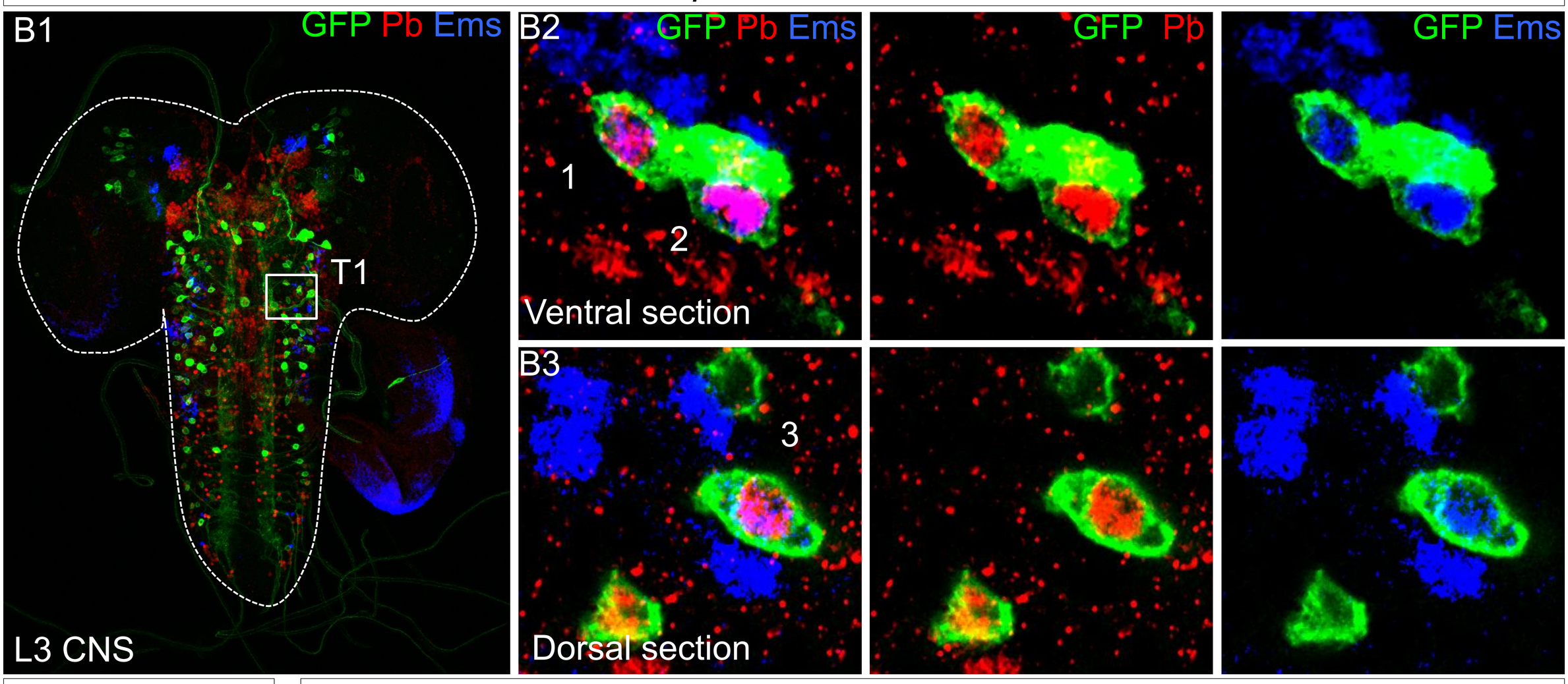


Figure S3. *Enriquez et al.*

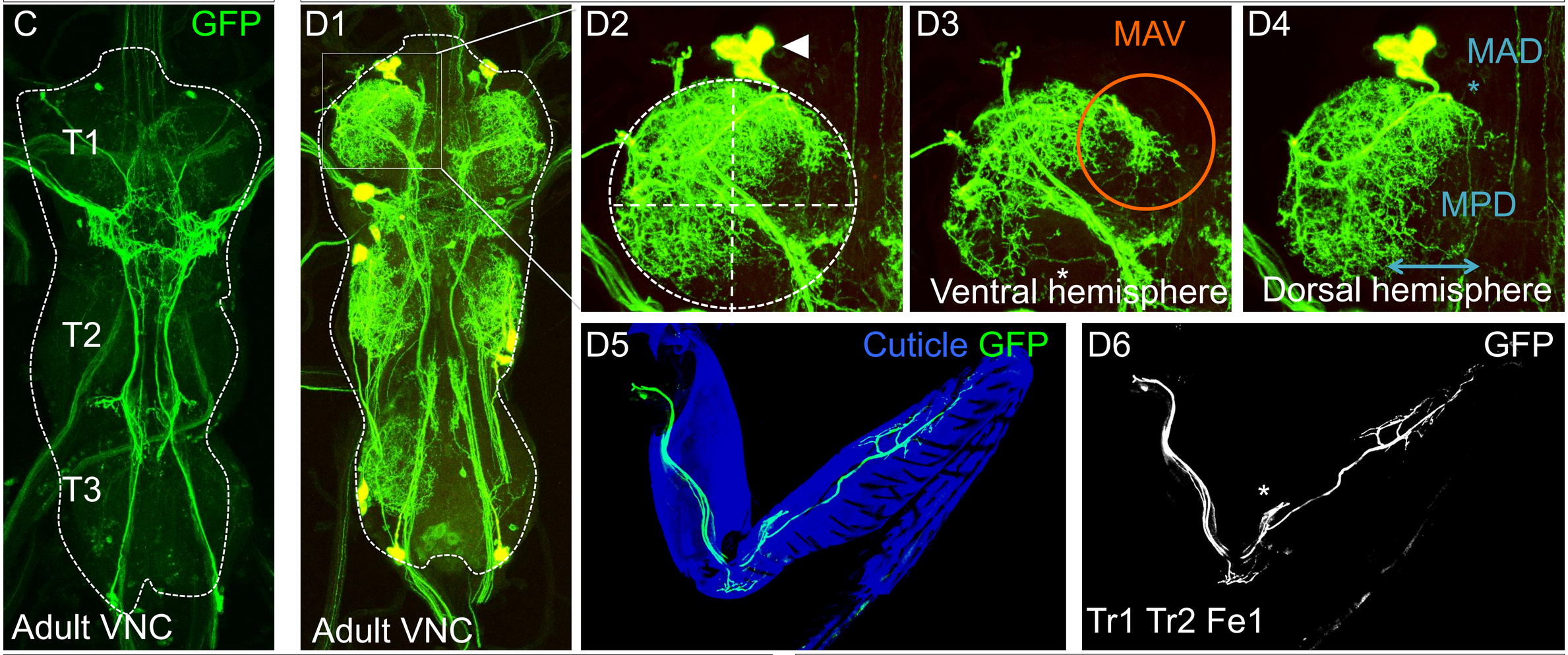


pb492>GFP



pb492>GFP

pb492>Flp, VGlut>>GFP



zfh2LP30>GFP

Lin B MARCM

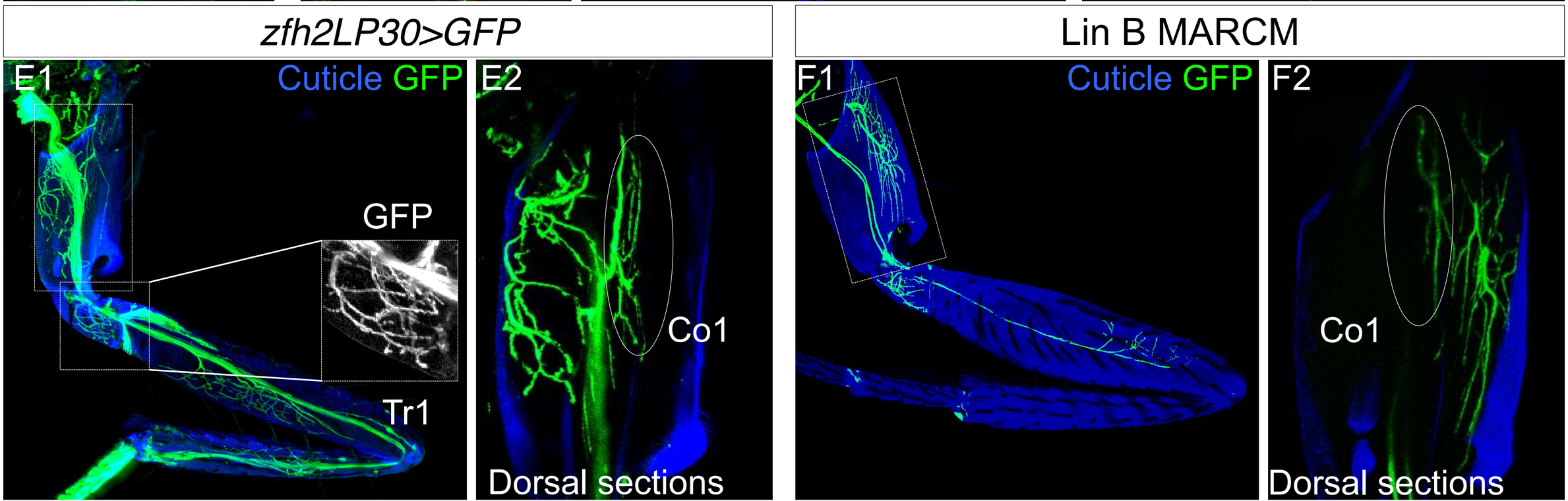


Figure S4. *Enriquez et al.*

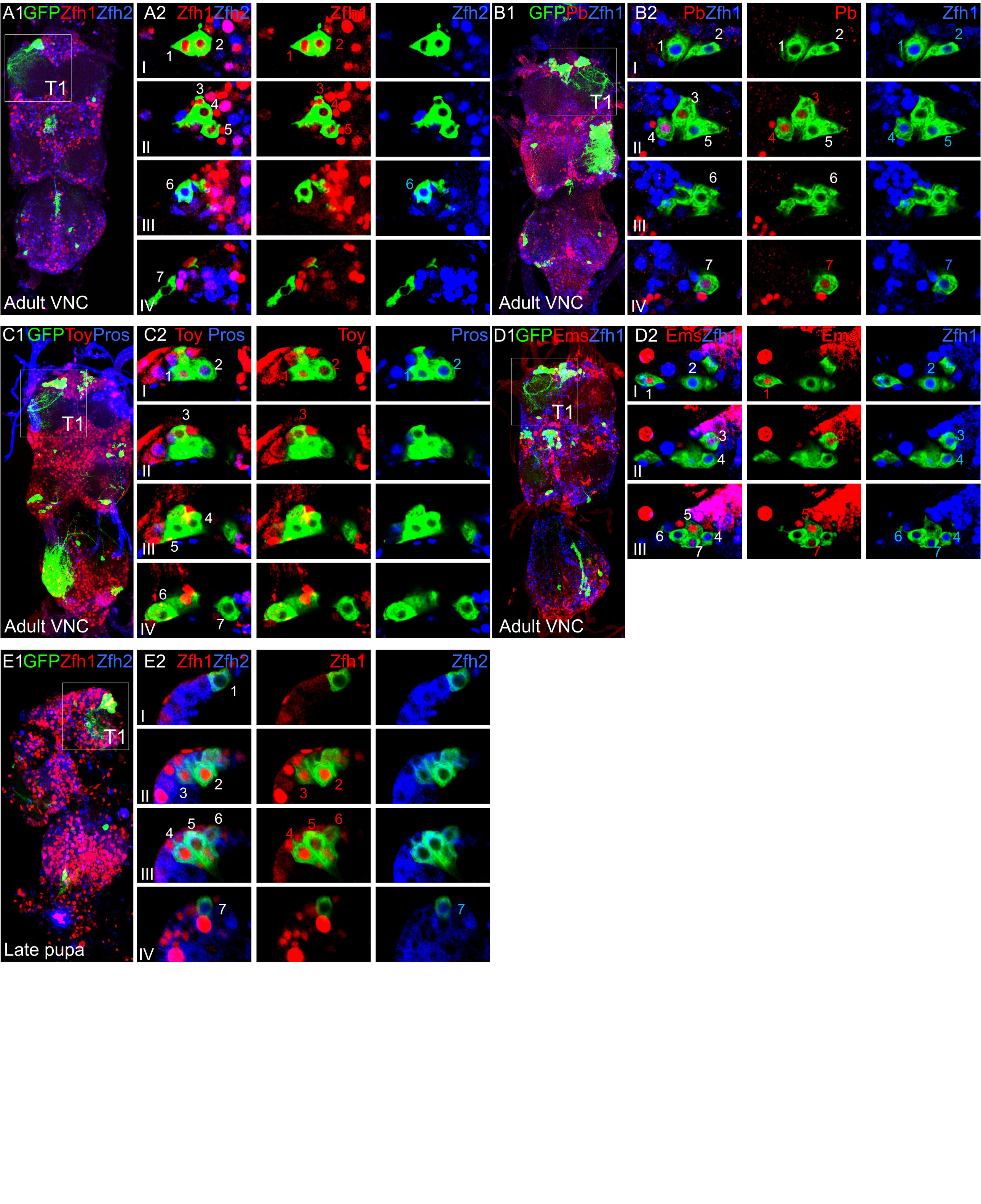


Figure S5. *Enriquez et al.*

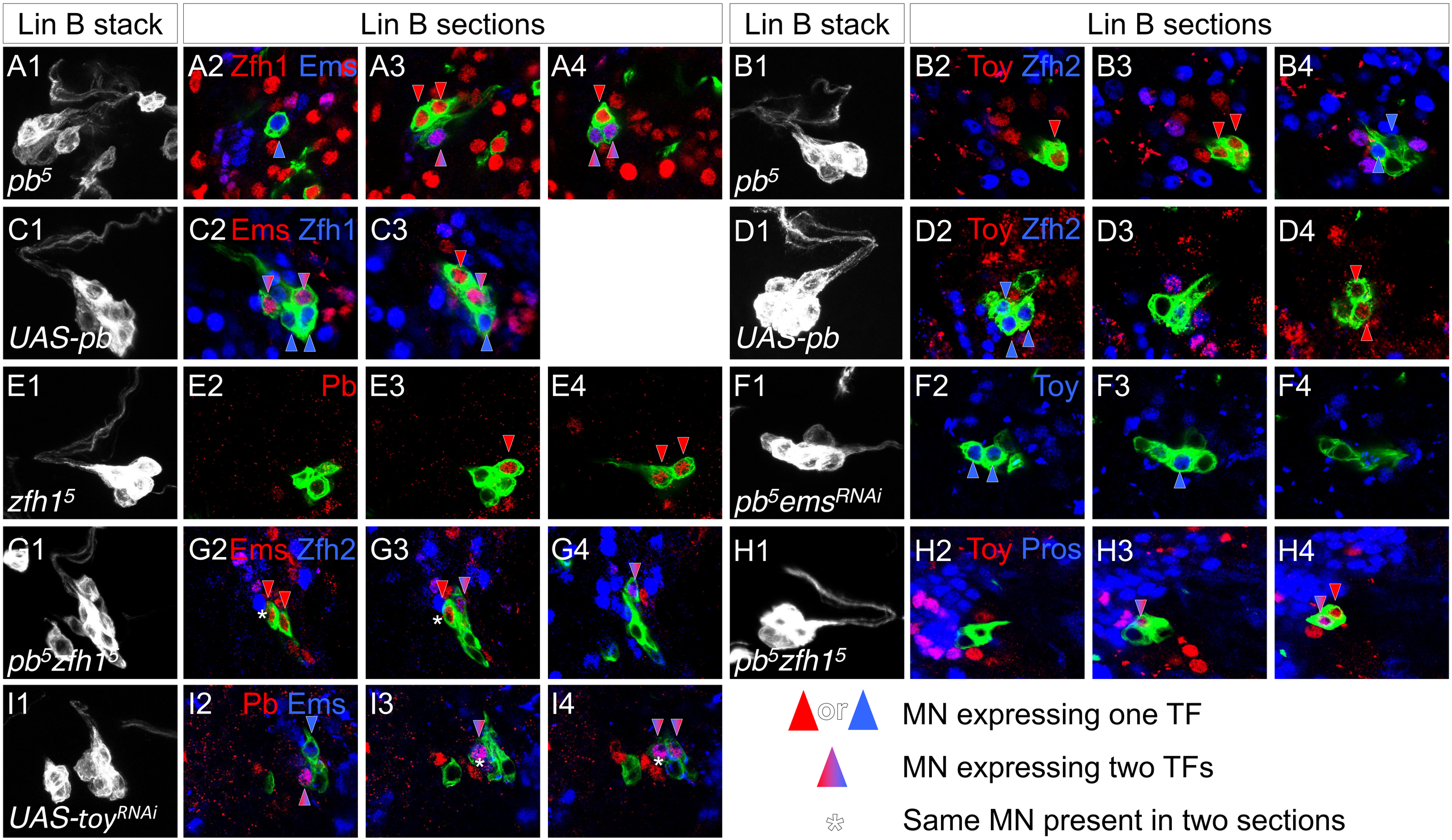


Figure S6. *Enriquez et al.*

A

WT Lin B

A1

A2

A3

A4

A5

A6

A7

T1

T2

T3

A

P

D

V

MAV

MPD

Ventral view

Anterior view

Ventral

Dorsal

N=10

Full stacks

Ventral

Dorsal

1

10

B

pb^{5} Lin B

B1

B2

B3

B4

B5

B6

B7

T1

T2

T3

Ventral view

Anterior view

Ventral

Dorsal

N=10

Full stacks

Ventral

Dorsal

1

10

C

C1

mcd8::GFP Cuticle

Coxa(4)

Tibia (1)

Trochanter (2)

C2

mcd8::GFP

Tr

Fe

D

D1

mcd8::GFP Cuticle

D2

mcd8::GFP

Tr

Fe

E

pb^{27} Lin B

E1

E2

E3

E4

T1

T2

T3

*

Ventral view

Anterior view

Ventral

dorsal

F

WT Lin B

F1

F2

F3

pb^{27}*-/-* Lin B

F4

F5

F6

N=5

Full stacks

Ventral

Dorsal

G

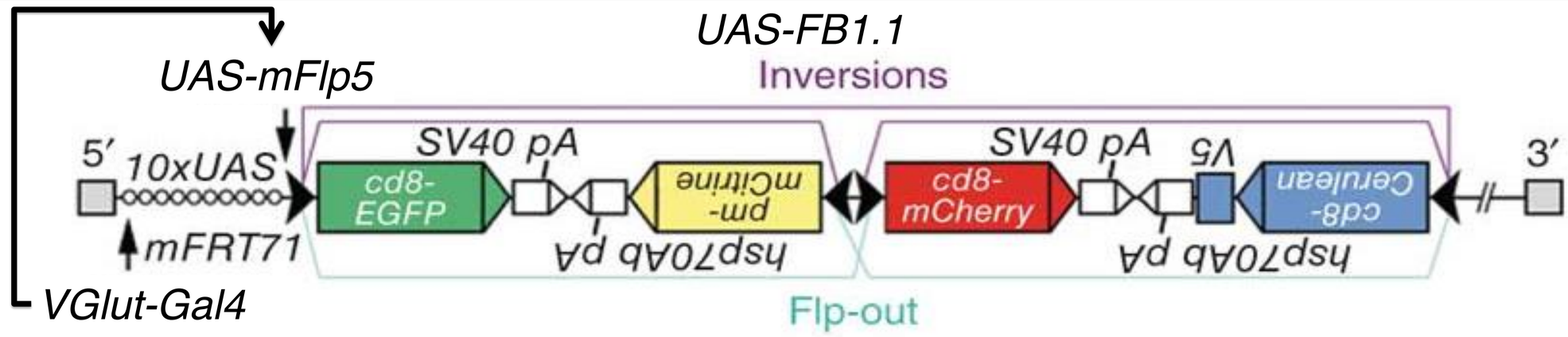
pb^{27}

Tr

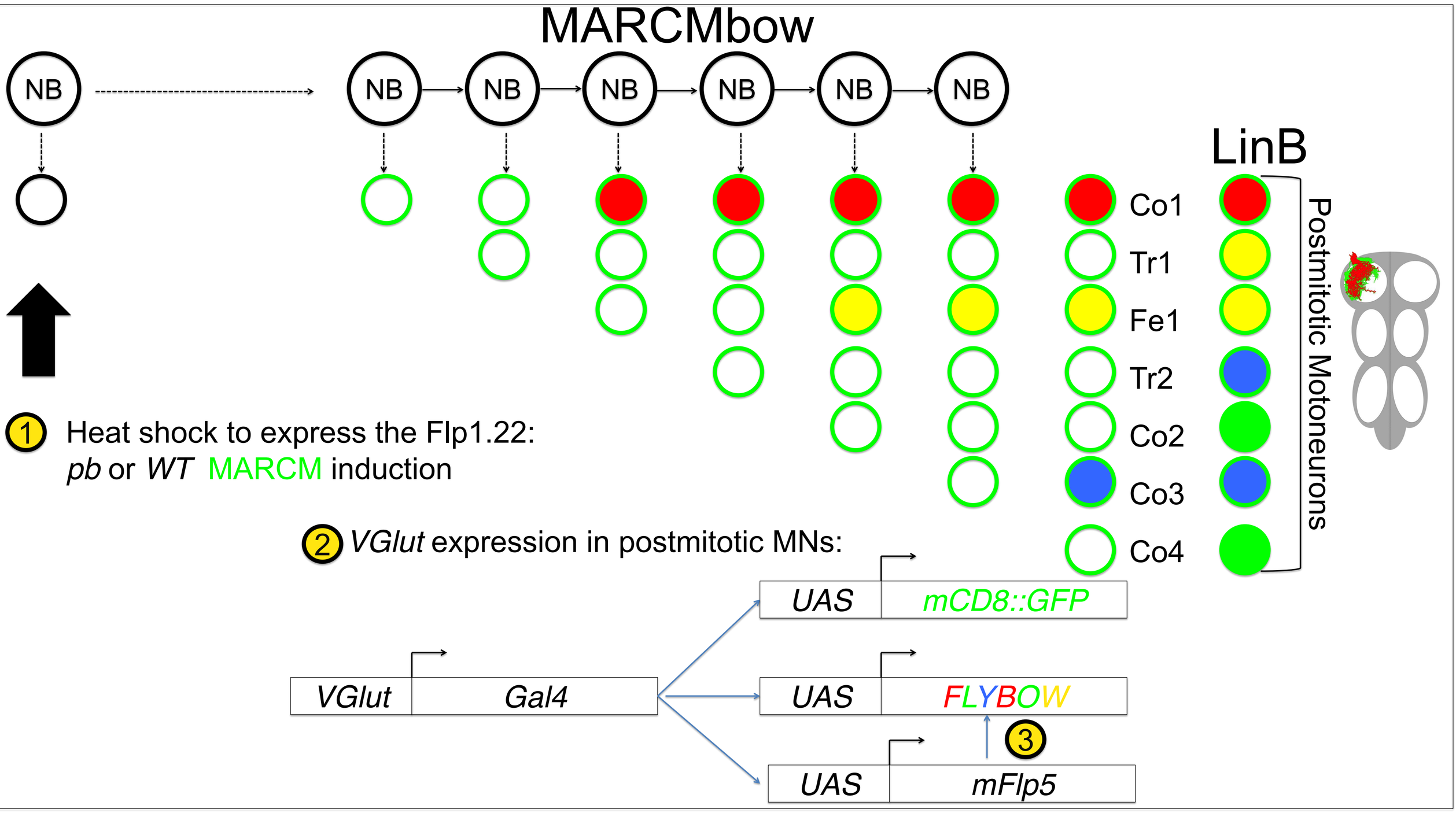
Fe

Figure S7 . *Enriquez et al.*

A



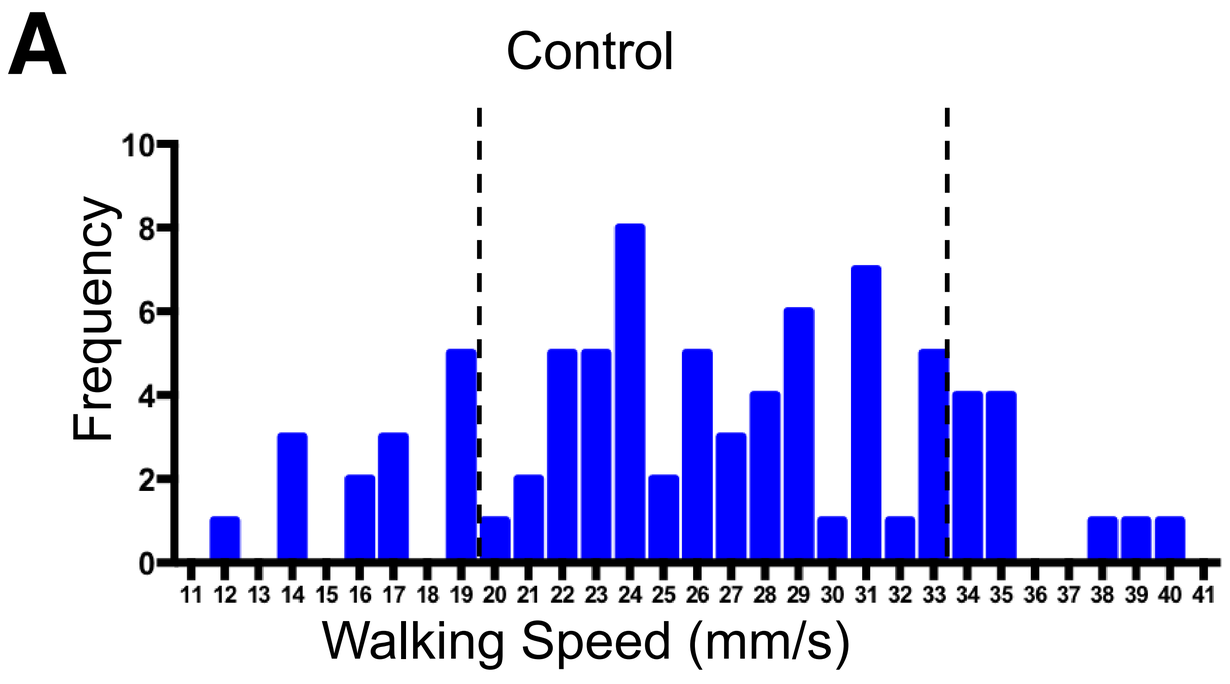
B

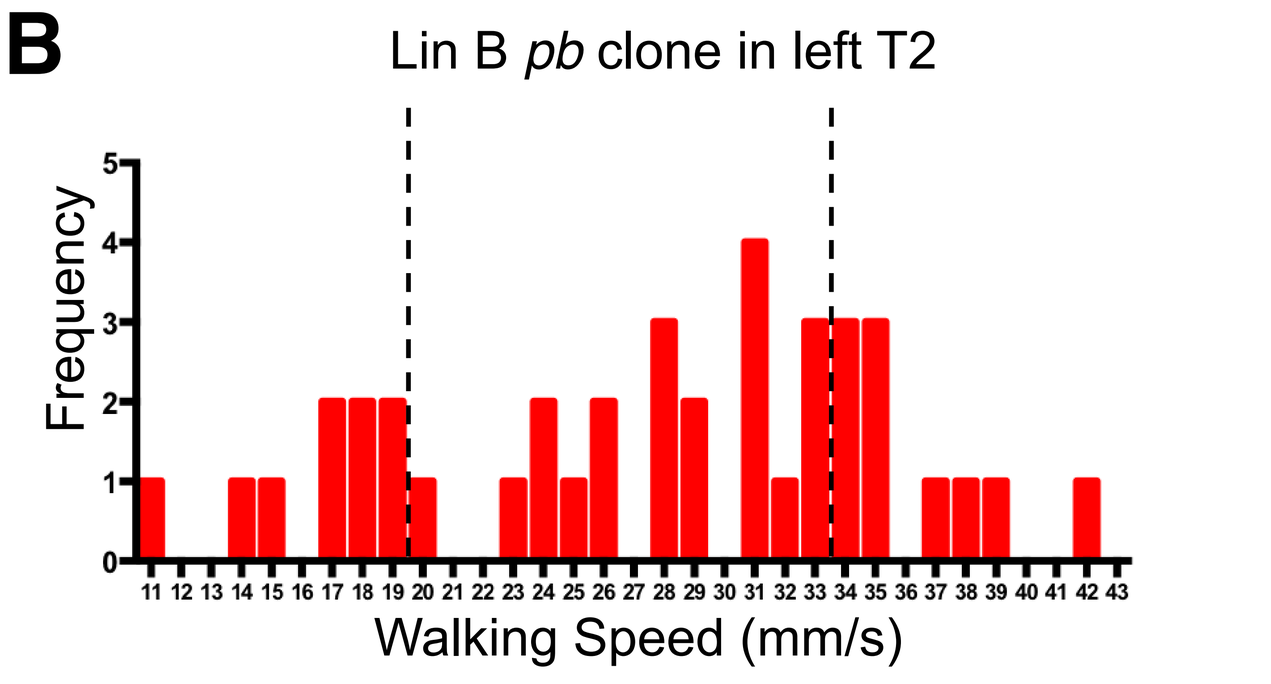


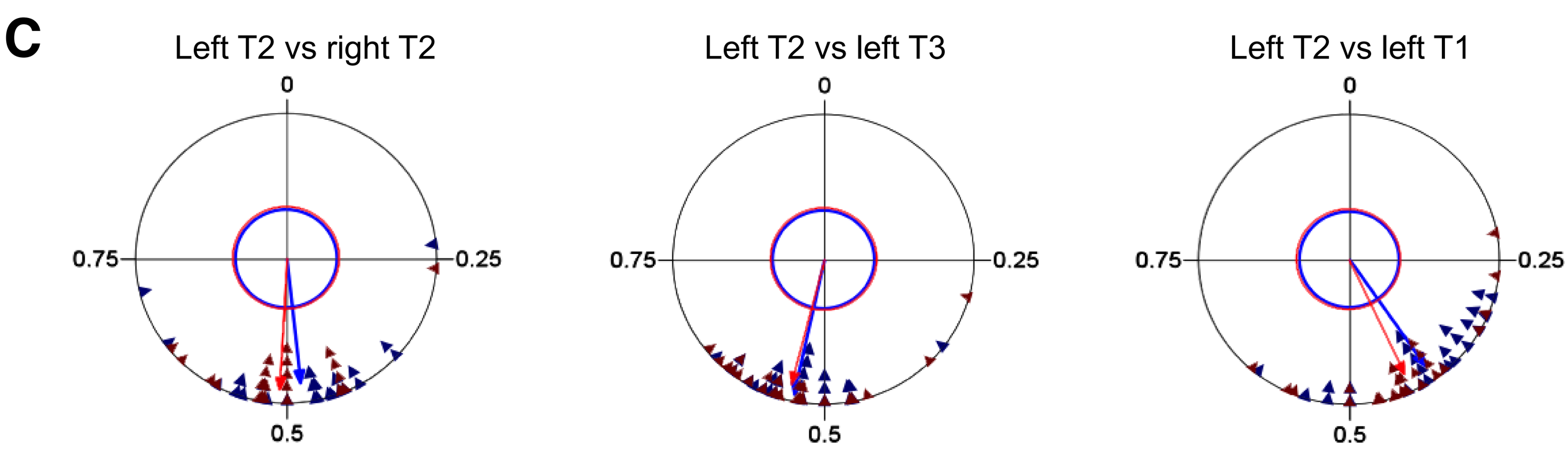
C

Wild type		*pb-/-*	
	Number	No phenotype	**Number**
Cox4	1	Cox3/4	2
Fe1	5	Fe1	2
Tr1	7	Tr1	4
Tr2	7	Tr2	3
Multiple MNs	11	Class I	14
		Class II	7
		Class III	5
Total	**31**	**Total**	**37**

Figure S8. *Enriquez et al.*







Supplemental Information.

Supplemental Figure Legends

Figure S1. Lin B muscle targeting. Relates to Figure 1.
(A-C) Transverse **(A, C)** and sagittal **(B)** sections of coxa, trochanter and femur of a T1 leg, Lin B GFP+ axons (green) and RFP+ muscle (red). Asterisks indicate terminal branches and arrows indicate axons. Reference axes are red (Di, Distal), green (P, Posterior), and blue (M, Medial). La, lateral; Pr, proximal **See also movies 1-3.**

Figure S2. TF co-staining in Lin B and Lin A MARCM clones. Relates to Figure 2.
(A-G) L3 CNSs with a Lin B **(A1-F3)** or Lin A **(G1-G3)** MARCM clones expressing mCD8::GFP under the control of *VGlut-Gal4* in the T1 segment. **(A1, B1, C1, D1, E1, F1, G1)** Ventral view of maximal confocal projections of L3 CNSs stained, with anti-Toy (blue) and anti-Ems (red) **(A1)**, anti-Zfh1 (blue) and anti-Pb (red) **(B1)**, anti-Zfh2 (blue) and anti-Pb (red) **(C1)**, anti-Toy (blue) and anti-Zfh2 (red) **(D1)**, anti-Zfh1 (blue) and anti-Ems (red) **(E1)**, anti-Toy (blue) and anti-Pb (red) **(F1),** anti-Zfh2 (blue) and anti-Zfh1 (red) **(G1)**. **A2, B2, C2, D2, E2, F2, G2** magnify the GFP+ MARCM clones in **A1, B1, C1, D1, E1, F1**, **G1** respectively**. A3, B3, C3, D3, E3, F3, G3** show individual confocal sections of Lin B **(A3-F3)** or Lin A **(G3)** clones from ventral (top) to dorsal (bottom).

Figure S3. Pb is expressed in Fe1, Tr1 and Tr2; Zfh2 is expressed in Co1 and Tr1.
(A): The *pb* locus; the green box marks the position of fragment pb^{492} used to drive the expression of Gal4 in **(B-C)**.
(B-C): Expression of mCD8::GFP under the control of pb^{492}-*Gal4* in the L3 CNS **(B1-3)** and in the adult VNC **(C). (B1)** Ventral view of a maximal confocal projection stained for Pb (blue), Ems (red), and GFP (green). **(B2)** ventral and **(B3)** dorsal confocal sections of the inset in **(B1)**; pb^{492} is expressed in the three Lin B MNs expressing Ems and Pb. **(C)** Although the expression of pb^{492} is not maintained in the adult VNC, it is active in some sensory neurons (green axons).
(D): Example of lineage traces in the adult using pb^{492}-*Gal4*. GFP expression is observed in Fe1, Tr1 and Tr2. **(D1)** ventral view of the VNC; **(D2-D4)** enlarges the region boxed in **D1**. **(D5-6),** in the ipsilateral T1 leg GFP+ axons target the femur and the trochanter and do not target the coxa; the asterisk indicates GFP expression in sensory

neurons in the femur chordotonal organ. The circles and double arrows indicate medial dendrites characteristic of Lin B (see figure 4); MAV, medial-anterior-ventral; MAD, medial-anterior-dorsal; MPB, medial-posterior-dorsal.

(E) When used to drive expression of mcd8::GFP, the *zfh2^{LP30}-Gal4* enhancer trap labels Tr1 and Co1 (F2).

(**F**) For comparison, Co1 is labeled in a Lin B MARCM clone.

Figure S4. Combinatorial expression of TFs in Lin B at pupal and adult stages. Relates to Figure 2.

(A-E): Adult VNCs **(A-D)** and late pupa **(E)** with Lin B MARCM clones in T1 expressing mCD8::GFP under the control of *VGlut-Gal4*. **(A1, B1, C1, D1, E1)** Ventral views of maximal confocal projections immunostained with anti-Zfh2 (blue) and anti-Zfh1 (red) **(A1)**, anti-Zfh1 (blue) and anti-Pb (red) **(B1)**, anti-Pros (blue) and anti-Toy (red) **(C1)** and anti-Zfh1 (blue), anti-Ems (red) **(D1)** and anti-Zfh2 (blue) and anti-Zfh1 (red) **(E1)**. The squares in **A1, B1, C1, D1 and E1** surround Lin B GFP+ clones in the T1 segment**. (A2, B2, C2, D2, E2)** show confocal sections of each Lin B clone in **(A1-E1)** from ventral (I) to dorsal (IV).

Figure S5. Additional data supporting the mTF code. Relates to Figures 2, 3, 6 and 7.

(A-F) No changes in mTF expression were observed in *pb^5* MARCM clones (**A, B**; Zfh1, Zhf2, Ems, and Toy), in ectopic Pb MARCM clones (**C, D;** Ems, Zfh1, Zfh2, Toy), in *zfh1^5* MARCM clones (**E**; Pb), in *pb^5, ems^{RNAi}* MARCM clones (**F**; Toy), in *pb^5, zfh1^5* MARCM clones (**G, H**; Ems, Pros, Zfh2, Toy) or in *toy^{RNAi}* MARCM clones (**I;** Pb, Ems). Note that Toy was undetectable in *toy^{RNAi}* MARCM clones, and Ems levels were strongly reduced in *ems^{RNAi}* MARCM clones **(data not shown)**.

Figure S6. Dendritic and axonal phenotypes of *pb* mutant Lin B MARCM clones in T2 and using a second null allele of *pb*. Relates to Figure 4.

(A-D) *WT* **(A, C)** and *pb^5* mutant (**B, D)** Lin B MARCM clones expressing mCD8::GFP under the control of *VGlut-Gal4* in the right T2 hemisegment. **(A, B)** On the left are schematics of the VNCs showing the positions of the clones. **(A1-A4, B1-B4)** Lin B clones are labeled in orange for the ventral hemisphere and in blue for the dorsal hemisphere. **(A5-A7, B5-B7)** Heat maps quantifying the extent of overlap among the

dendrites of *WT* **(A5-A7)** and *pb* mutant **(B5-B7)** Lin B clones; N, number of Lin B samples. **(C, D)** axonal targeting of *WT* (**C)** and *pb* mutant (**D)** Lin B clones, GFP+ axons are green **(C1, D1)** or white **(C2, D2)**.

(E) pb^{27} mutant Lin B MARCM clones expressing mCD8::GFP under the control of *vGlut-Gal4* in the right T1 hemisegment, note: this *pb* allele is different from the one used in Figure 3 but the phenotype is the same; on the left a schematic of the VNC showing the position of the clone. **(E1-E5)** *pb* Lin B clone labeled in orange for the ventral hemisphere and in blue for the dorsal hemisphere. **(F)** Heat maps quantifying the extent of overlap among the dendrites of *WT* **(F1-F3)** and pb^{27} mutant **(F4-F6)** Lin B MARCM clones; N, number of Lin B samples used for these analyses. **(G)** GFP+ axons (white) of a *pb* mutant Lin B MARCM clone in trochanter (Tr) and femur (Fe) segments.

Figure S7. MARCMbow technique. Relates to Figures 4 and 5.

(A) In the Flybow 1.1 (FB1.1) construct (Hadjieconomou et al., 2011), mFlp5 induces Flp-out or inversion of DNA sequences by acting on FRT elements (black arrowheads). In the set up used here, mFlp5 is under the control of Gal4/UAS binding sites. Consequently, in *VGlut-Gal4* MARCM clones, FB1.1 recombination will be induced only in postmitotic MNs.

(B) MARCMbow schematic.

(1) *WT* and *pb* mutant Lin B MARCM clones expressing mCD8::GFP under the control of *VGlut-Gal4* are induced in L1 larva;

(2) *VGlut-Gal4* activates the expression of the UAS-mFlp5 in Lin B postmitotic MNs,

(3) mFlp5 randomly induces recombination in the flybow system in postmitotic MNs, the cassette selected is only expressed in Lin B postmitotic neurons.

(C) Summary of MARCMbow samples. Each sample (WT=31 total; *pb*-/-=37 total) had at least one MN labeled with a flybow color within a Lin B MARCM clone. See text for details regarding the definitions of Class I, II, and III.

Figure S8. Flies with Lin B *pb* mutant MARCM clones in the T2 leg exhibit no defects in average speed, interleg coordination or stepping parameters. Relates to Figure 5.

(**A-B**) Distributions of average walking speeds for control (blue, n = 80) and $T2_L$ Lin B *pb*-/- (red, n = 39) animals. Orange dotted lines delineate the three occurring speed cohorts (slow, intermediate, and fast). No significant difference between overall average

speeds of $T2_L$ Lin B *pb*-/- (**A**) and control (**B**) animals (p = 0.303, unpaired t test with Welch's correction), or between average speeds within each two of three cohorts (p = 0.817, p = 0.0375, and p = 0.457, unpaired t test with Welch's correction for slow, intermediate, and fast cohorts, respectively). To control for the difference in speed distribution within the intermediate cohort, a subset of speed-matched control data points were selected for all subsequent analyses. (**C**) Radial coordination plots of leg phase values for contralateral (left) and ipsilateral (middle, right) leg pairs containing the affected T2 leg. Triangles indicate multiple individual phase values from videos of fast-walking control (blue, n = 10) and $T2_L$ Lin B *pb*-/- (red, n = 10) animals; blue and red arrows are respective vector sums with the arrow direction indicating the average phase value. Inner red and blue circles represent a Rayleigh p value = 0.05 for the respective data set; if an arrow falls within its respective significance threshold, the data are distributed randomly and the leg pair is considered uncoordinated. For all three phase plots, vector sums of control and $T2_L$ Lin B *pb*-/- data fall well outside of the significance threshold (leg pairs are coordinated) and directions of vectors are comparable (no difference in interleg coordination parameters between control and $T2_L$ Lin B *pb*-/- animals). (**D-G**) Box plots of four step parameters for the $T2_L$ leg only. No significant differences in step length (**D**), swing speed (**E**), swing duration (**F**), or stance duration (**G**) were observed in any speed cohort.

Supplemental Experimental Procedures

Immunostaining of L3 Larva

Inverted L3 larvae were fixed in 4% formaldehyde with PBS for 20 minutes. L3 larval CNS were dissected and incubated with primary antibodies for two days and secondary antibodies for one day at 4°C. Fresh PBT (PBS with 0.1% Triton X-100, 1% BSA) was used for the blocking step, incubation and washing steps: five times for 20 minutes at room temperature after fixation and after primary/secondary antibodies.

Adult leg and VNC preparation

After removal of abdominal and head segments, adult legs attached to thoracic segments were fixed overnight at 4°C followed by five washes in PBT for 20 minutes at room temperature. VNC and legs were dissected and mounted onto glass slides using Vectashield mounting medium (Vector Labs).

3D dendrite analysis

To image MN dendrites in the neuropil, ventral and dorsal Z-stacks were generated using NIH Image J. Branches from lineages other than Lin A or Lin B and not in direct contact were removed on individual stacks of some samples. Z-stacks were used to generate 3D reconstructions of Lin A or Lin B clones in the VNC by using the Volren rendering in Amira 3D software. The color codes used for MARCM and MARCMbow clones were Volrengreen for the dorsal hemispheres and Volrenred for the ventral hemispheres. The color codes used for MARCMbow clones were white constant color for Lin B and red constant color for single MN labeling. BRP (Bruchpilot) labeled neuropils in Figure 1 were visualized using the Volren rendering with a grey color code, the neuropil was rendered transparent by using the isosurface rendering.

3D Leg analysis

Z-stacks were generated using NIH Image J. A specific cuticle background was generated using the Argon laser 488 and by placing the confocal detector out of the range of the GFP emission wavelength. This cuticle background of the leg was used to remove the nonspecific signal from the GFP, YFP, Citrine and mCherry signal using ImageJ. Z-stacks were used to generate 3D reconstructions of legs with Amira 3D software. The color codes used were Volrengreen to visualize mCD8::GFP or Volrenred to visualize rab3::YFP (**Figures 1, 4**) and white constant color, yellow constant color and red constant color to visualize GFP, Citrine and mCherry respectively **(Figure 3)**. The color used for cuticles was grey (**Figures 1, 4**).

Plasmid constructions and transgenic lines

UAS-mFlp5: A *KpnI-mFp5-XbaI* fragment from *hs-mFlp5* (REF) was inserted into pRVV70 (*pUAS-attB* vector). pRVV70 is a 5xUAS-containing vector created by modification of pUASTattB (Bischof et al., 2007). pUASTattB contains ~500bp intervening sequence between XbaI and the beginning of the early SV40 polyA transcriptional terminator sequence. This feature was undesirable to us and the intervening sequence was removed and the late SV40 was placed as a transcriptional terminator immediately following XbaI. In addition, an NheI site was added to the MCS preceding the 5xUAS sequences. The resulting *pUAS-mFlp5-attB* construct was inserted in position 3B on the X chromosome by injection into embryos carrying PBac{yellow[+]-attP-3B}.

Casper-VGlut->y+stop>-LexAVP16: The 5.9 kb sequence upstream from the *VGlut* translation start site was PCR amplified from genomic DNA using the following primers, VGlut-F (BglII): GATC AGA TCT TAG ATG CTA CTA CTT TGG AGA T and VGlut-R (KpnI): GATC GGT ACC CTT GCT GCT CAG CTA GTA GT. This *VGlut* fragment was digested with BglII and KpnI and cloned into a pStinger vector digested with BglII and KpnI. A NotI-*LexAVP16-XbaI* fragment from a *pBS-LexAVP16* vector (Lai and Lee, 2006) was cloned into *pCasper-nucLacZ* vector digested with XbaI from which nucLacZ was removed. In a second step, *VGlut* enhancer region was amplified from *pStinger-VGlut* using VGlut-F (KpnI): GAT CGG TAC CTA GAT GCT ACT ACT TTG GAG AT and VGlut-R (KpnI): GAT CGG TAC CCT TGC TGC TCA GCT AGT AGT primers, digested with KpnI and cloned into *Casper-LexAVP16* using the KpnI site. In a third step *>y+stop>* fragment was digested from *pFC17* (Gary Struhl) using KpnI and was inserted into *Casper-VGlut-LexAVP16* vector which was partially digested with Kpn1 to restrict only one KpnI site.

plexAop-mCD8::GFP: The *mCD8::GFP* fragment from *pBS-mCD8GFP* (Liqun Luo) was inserted into *pLOT* containing *lexAop* (Lai and Lee, 2006) using XhoI and XbaI. P element transformant flies carrying *plexAop-mCD8::GFP* and *VGlut->y+stop>-LexAVP16* transgenes on chromosomes X, II and III has been used for the lineage tracing experiment.

Supplemental movies

Supplementary Movie 1. Relates to Figure 1. Adult VNC expressing cd8::GFP under the control of *vGlut-Gal4,* and stained for BRP (Bruchpilot).

Supplementary Movie 2. Relates to Figure 1. Axon and muscle innervation patterns of Lin B MNs in the coxa.

Supplementary Movie 3. Relates to Figure 1.: Terminal axon branches of Lin B MNs in the trochanter. RFP: *Mhc-RFP*; GFP: *mcd8::GFP*; YFP: *rab3::YFP*.

Supplementary Movie 4. Relates to Figure 1.: Terminal axon branches of Lin B MNs in the trochanter. The left panel shows sagittal sections of a plane moving through the image on the right along the lateral to medial axis.

Supplementary Movie 5. Relates to Figure 1.: Terminal axon branches of a Lin B MARCM clone in the femur.

Supplementary Movie 6. Relates to Figure 3.: WT and *pb-/-* Lin B MARCMbow clones in the adult VNC, with Fe1 labeled in red and all MNs labeled in white.

Supplementary Movie 7. Relates to Figure 3. WT and *pb-/-* Lin B MARCMbow clones in the adult VNC, with Tr1 labeled in red and all MNs labeled in white.

Supplementary Movie 8. Relates to Figure 4 . WT Lin B MARCMbow clone in the adult leg, with Tr1 labeled in red, Tr2 labeled in yellow, and all MNs labeled in white.