

## RESEARCH ARTICLE

## STEM CELLS AND REGENERATION

# Transcription factor expression uniquely identifies most postembryonic neuronal lineages in the *Drosophila* thoracic central nervous system

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**ABSTRACT**

Most neurons of the adult *Drosophila* ventral nerve cord arise from a burst of neurogenesis during the third larval instar stage. Most of this growth occurs in thoracic neuromeres, which contain 25 individually identifiable postembryonic neuronal lineages. Initially, each lineage consists of two hemilineages – ‘A’ (Notch<sup>ON</sup>) and ‘B’ (Notch<sup>OFF</sup>) – that exhibit distinct axonal trajectories or fates. No reliable method presently exists to identify these lineages or hemilineages unambiguously other than labor-intensive lineage-tracing methods. By combining mosaic analysis with a repressible cell marker (MARCM) analysis with gene expression studies, we constructed a gene expression map that enables the rapid, unambiguous identification of 23 of the 25 postembryonic lineages based on the expression of 15 transcription factors. Pilot genetic studies reveal that these transcription factors regulate the specification and differentiation of postembryonic neurons: for example, Nkx6 is necessary and sufficient to direct axonal pathway selection in lineage 3. The gene expression map thus provides a descriptive foundation for the genetic and molecular dissection of adult-specific neurogenesis and identifies many transcription factors that are likely to regulate the development and differentiation of discrete subsets of postembryonic neurons.

**KEY WORDS:** *Drosophila*, Adult neurogenesis, Hb9, Gene expression map

**INTRODUCTION**

Understanding how cell-type diversity in nervous systems arises remains a key goal in developmental biology. Even simple nervous systems, such as those in insects, involve hundreds of different subtypes of cells. Over the last several decades, research using the *Drosophila* embryonic CNS as a model system has unveiled basic principles that underlie nervous system development in invertebrates and vertebrates (reviewed by Skeath and Thor, 2003; Lin and Lee, 2012). *Drosophila* and other holometabolous insects, however, undergo two distinct waves of neurogenesis: embryonic neurogenesis creates the larval nervous system; postembryonic neurogenesis creates the adult nervous system (Truman and Bate, 1988; Prokop and Technau, 1991). Relative to embryonic neurogenesis, we know little about the genetic and molecular control of postembryonic neurogenesis.

Within each hemisegment of the segmented embryonic nerve cord, 30 neuroblasts (NBs) divide in a stem cell manner to produce ~400 neurons and glia that interconnect to form a functional CNS (reviewed by Campos-Ortega and Hartenstein, 1997; Goodman and Doe, 1993). Towards the end of embryogenesis, NBs become quiescent or undergo apoptosis: in abdominal segments, most NBs die; in thoracic segments, 25 of the 30 NBs become quiescent and persist into larval stages (Truman and Bate, 1988; Britton and Edgar, 1998; Maurange and Gould, 2005). Here, we focus on the postembryonic neuronal lineages produced by these 25 NBs. During the second larval-instar stage, in response to glia-derived insulin signaling, thoracic NBs regain their proliferative activity (Chell and Brand, 2010; Sousa-Nunes et al., 2011). Initially, NBs divide slowly to produce a small number of large, Chinmo-positive (Chinmo<sup>+</sup>) neurons (termed early-born neurons). Shortly after larvae enter the third (last) instar stage, NBs divide more quickly and produce many, small Broad<sup>+</sup> neurons (termed late-born neurons), ceasing their proliferation in the early pupal stage (Truman et al., 2004; Truman et al., 2010; Zhu et al., 2006; Maurange et al., 2008).

Elegant mosaic analysis with a repressible cell marker (MARCM)-based lineage-tracing studies revealed that each neuronal lineage in the thoracic CNS is uniquely identifiable based on its relative position size and neuronal projection patterns (Truman et al., 2004). Each postembryonic NB, which resides in the ventral-most region of a lineage, divides in a stem cell manner to self-renew and produce a chain of secondary precursor cells, called ganglion mother cells (GMCs) (Betschinger and Knoblich, 2004; Wang and Chia, 2005; Yu et al., 2006). Typically, each GMC divides to produce sibling post-mitotic neurons that adopt distinct fates based on the state of Notch signaling – ‘A’ (Notch<sup>ON</sup>), ‘B’ (Notch<sup>OFF</sup>) (Truman et al., 2010). In contrast to the embryo, in which sequentially born ‘A’ (or ‘B’) daughter cells often adopt distinct identities (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999), most A (or B) cells within a given postembryonic lineage manifest the same cellular phenotype, extending projections along a common path to a similar target region (Truman et al., 2010). Thus, initially, each postembryonic lineage consists of a NB and some GMCs in the ventral region of the clone and two major subtypes of neurons (A and B) more dorsally. In some lineages, most or all cells of the A (or B) hemilineage undergo apoptosis, resulting in a monotypic lineage that consists largely, if not exclusively, of cells from the A or B hemilineage.

At present, the only reliable way to identify which lineage a group of postembryonic neurons belongs to is through labor-intensive MARCM-based lineage tracing methods (Lee and Luo, 1999; Truman et al., 2004; Truman et al., 2010). In the work reported here, by combining gene expression studies of 14 transcription factors with MARCM-based lineage tracing methods, we created a gene expression map that unambiguously identifies 23 of the 25

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postembryonic neuronal lineages and 29 of the 34 major neuronal hemilineages. Pilot functional studies reveal that the identified transcription factors direct the development and differentiation of the postembryonic neurons expressing them.

## RESULTS

### Characterization of postembryonic Hb9<sup>+</sup> neurons

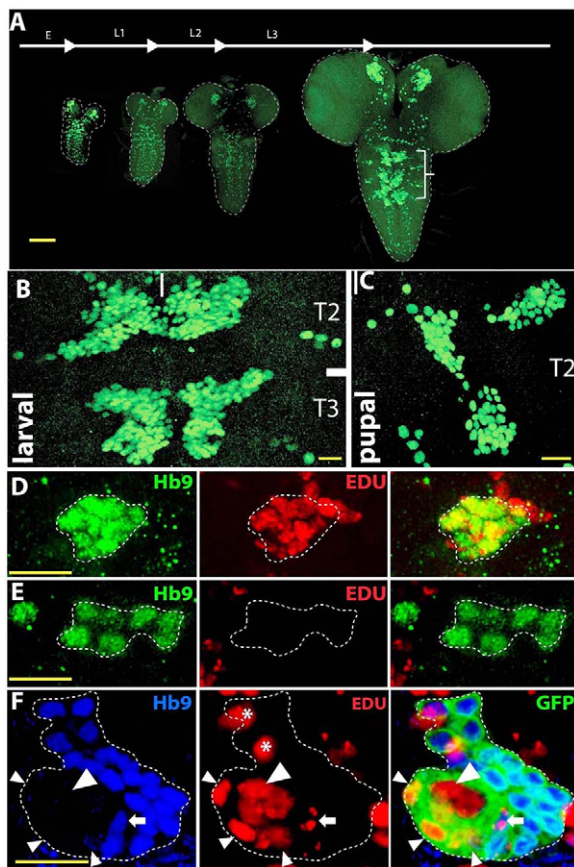
We previously determined that Hb9 (also known as Extra-extra; Exex – FlyBase) is expressed in and required for the differentiation of most embryonic motor neurons that innervate ventral body wall muscles (Broihier and Skeath, 2002). To assess whether additional Hb9<sup>+</sup> neurons develop postembryonically, we followed Hb9 expression in the ventral nerve cord during all larval stages and into the adult (Fig. 1). The pattern of Hb9<sup>+</sup> neurons in the nerve cord of first and early second instar larvae appeared similar to that observed in late stage embryos (Fig. 1A). In late third instar larvae, however, large clusters of Hb9<sup>+</sup> neurons formed in each thoracic segment (Fig. 1A,B). Visual inspection revealed that of the 120.6±7.5 neurons in these clusters, 101.8±5.1 (84.4%) were small, ventrally located

neurons and 18.8±2.1 (15.6%) were large, dorsally located neurons ( $n=5$  hemisegments). EdU birth-dating experiments confirmed that the small, ventral Hb9<sup>+</sup> neurons developed during the third larval instar stage. When larvae were fed EdU continuously from the start of the third larval stage and analyzed at the end of larval life, 96.4±1.0% of the small Hb9<sup>+</sup> neurons were labeled with EdU (Fig. 1D), but only 5±5% of the large Hb9<sup>+</sup> neurons were EdU positive (Fig. 1E;  $n=9$  hemisegments). Thus, most small Hb9<sup>+</sup> neurons arose during the third larval instar stage, and most large Hb9<sup>+</sup> neurons arose before this stage. Consistent with these data, visual inspection of five thoracic hemisegments revealed that 93.4±4.2% of small Hb9<sup>+</sup> neurons expressed Broad, a marker of late-born neurons, and 6.8±1.1% expressed Chinmo, a marker of early-born neurons; conversely, 92.0±2.3% of large Hb9<sup>+</sup> neurons expressed Chinmo and 9.1±2.6% expressed Broad (supplementary material Fig. S1). By the mid-pupal stage, these Hb9<sup>+</sup> neurons segregated into three clusters per hemineuromere (Fig. 1C), suggesting each cluster represents a distinct lineage. These Hb9<sup>+</sup> neurons persist into the adult, where they occupy stereotyped positions (supplementary material Fig. S2C). Thus, during postembryonic neurogenesis, three clusters of adult-specific Hb9<sup>+</sup> neurons develop in each thoracic neuromere.

To check whether Hb9 expression specifically labels post-mitotic neurons in the larval CNS, we marked lineages containing Hb9<sup>+</sup> neurons with the MARCM technique and visualized mitotic progenitor cells by short exposure to EdU. No Hb9<sup>+</sup> neurons labeled with EdU when CNS explants were treated with EdU for 6 hours prior to fixation ( $n=5$  clones; not shown), which is the time required for the birth of a GMC and its subsequent division to produce neurons (Truman and Bate, 1988). But, when explants were treated with EdU for 12 hours prior to fixation, on average 1.1±0.7 Hb9<sup>+</sup> neurons were labeled with EdU ( $n=5$  clones; Fig. 1F). In these clones, the NB, identified as the largest and most ventral cell in the clone, and adjacent GMCs strongly label for EdU, but lack Hb9 expression (Fig. 1F, arrowheads). Thus, Hb9 expression is restricted to post-mitotic neurons in the thoracic postembryonic CNS. We note that in all analyses of MARCM clones detailed below, we identified the NB on the basis of its size and ventral location and GMCs by their proximity to the NB.

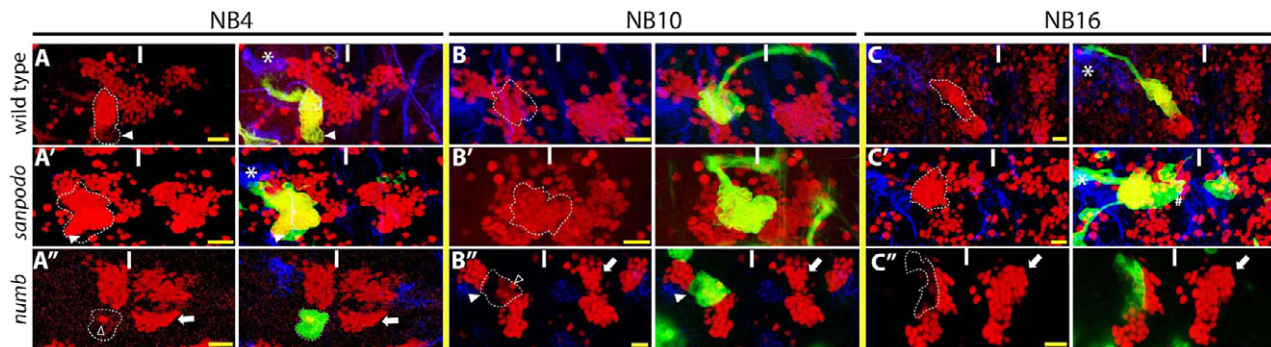
Only a few molecular markers have been identified that label distinct subsets of postembryonic neurons; the expression of even fewer of them has been mapped to defined postembryonic neuronal lineages. Thus, we used the MARCM system to map the clusters of Hb9<sup>+</sup> postembryonic neurons to individual lineages (Fig. 2), using FasII (also known as Fas2 – FlyBase) to label architectural features of the larval CNS (Landgraf et al., 2003). Each postembryonic lineage can be uniquely identified by its characteristic position in a neuromere, pattern of axonal projections, and ditopic (A- and B-type neurons) or monotopic (A- or B-type) nature. Thus, we compared these traits for each wild-type, *spdo* mutant or *numb* mutant MARCM clone that contained a defined set of Hb9<sup>+</sup> neurons to those previously determined for all 25 postembryonic lineages (Truman et al., 2004; Truman et al., 2010).

MARCM clones that contained the most posterior group of Hb9<sup>+</sup> neurons (Fig. 2A) as well as those that contained the most lateral group of Hb9<sup>+</sup> neurons (Fig. 2C) each extended a single neurite bundle laterally to the leg neuropil, with that of the posterior group ending posterior to the neuropil and that of the lateral group ending anterior to the leg neuropil (Fig. 2A-C, asterisks). Visual inspection of five clones of the posterior group revealed they contained 7±1 large, dorsal Hb9<sup>+</sup> neurons, 38.8±1.5 small, ventral Hb9<sup>+</sup> neurons, and 10.8±1.5 Hb9<sup>+</sup> cells per clone. Similar analysis of the lateral group revealed they contained 6±1 large, dorsal Hb9<sup>+</sup> neurons,



**Fig. 1. Postembryonic expression of Hb9 marks three clusters of neurons in the wild-type thoracic CNS.** (A) Hb9 expression in (left to right) the dissected CNS of a first instar, second instar, third instar and late third instar larva. Brackets identify thoracic segments. (B,C) Hb9 expression in two thoracic segments (T2, T3) of a late third instar larval CNS (B) and a single hemisegment (T2) of a mid-pupal CNS (48 hours after pupariation; C). (D,E) Hb9 (green) and EdU (red) labeling in the ventral (D) and dorsal (E) region of a thoracic neuromere of a late third instar larva. (F) Hb9 (blue) and EdU (red) labeling in a MARCM clone in an explant of a late third instar larval CNS treated with EdU for 12 hours immediately before fixation. The large arrowhead points to the NB, small arrowheads to GMCs; arrow points to the Hb9<sup>+</sup> and EdU<sup>+</sup> neuron. Anterior, up; white bar, midline. Scale bars: 100  $\mu$ m in A; 10  $\mu$ m in B-F.





**Fig. 2. Hb9 expression labels three postembryonic lineages.** (A–C) High magnification views of a wild-type thoracic neuromere of late third instar larvae labeled for Hb9 (red), GFP (green) and FasII (blue) expression. Wild-type MARCM clones are shown in A–C; *spdo* mutant MARCM clones are shown in A'–C'; *numb* mutant MARCM clones are shown in A''–C''. In A–C, asterisks denote the leg neuropil. In A, A' and B'', arrowheads point to Hb9<sup>+</sup> NB and GMCs. In C', # denotes a second, unrelated GFP<sup>+</sup> MARCM clone. In A'' and B'', open arrowheads point to Hb9<sup>+</sup> neurons located in different focal planes than the *numb* mutant MARCM clones. In A''–C'', arrows point to the Hb9<sup>+</sup> neurons of relevant lineages in the unaffected contralateral hemisegment. Dashed lines, lineage boundaries. Anterior, up; white bar, midline. Scale bars: 10  $\mu$ m.

31.0 $\pm$ 1.6 small, ventral Hb9<sup>+</sup> neurons, and 8.2 $\pm$ 1.2 Hb9<sup>−</sup> neurons per clone ( $n=5$ ). The Hb9<sup>−</sup> cells always resided in the ventral-most region of the clone and consisted of the NB, the largest cell in the clone, adjacent GMCs and probably some newly born neurons (Fig. 2, arrowheads). *spdo* mutant MARCM clones of each lineage, which duplicate the B hemilineage at the expense of the A hemilineage, were larger than wild-type clones and consisted mostly of Hb9<sup>+</sup> neurons that extended axons to the leg neuropil (Fig. 2A',C'). Conversely, *numb* mutant clones, which duplicate the A hemilineage, consisted of relatively few cells that did not extend axon projections; some of the cells expressed Hb9 transiently at lower levels (Fig. 2A'',C''). Thus, both lineages appear to be primarily monotypic B-type lineages. Lineages 4 and 16 are the only monotypic B-type lineages that extend neurite bundles laterally to the leg neuropil (Truman et al., 2004; Truman et al., 2010), and lineage 16 resides anterior and lateral to lineage 4. Thus, we conclude that the posterior group of Hb9<sup>+</sup> neurons identifies the B-hemilineage of lineage 4 (lineage 4b) and the lateral group of Hb9<sup>+</sup> neurons identifies lineage 16b.

MARCM clones that contained the remaining Hb9<sup>+</sup> neurons in the clusters resided anterior to lineage 4 and medial to lineage 16, and extended a single neurite bundle contralaterally (Fig. 2B). This lineage consisted of 5.6 $\pm$ 0.5 large, dorsal Hb9<sup>+</sup> neurons, 32.0 $\pm$ 2.4 small, ventral Hb9<sup>+</sup> neurons, and 5.8 $\pm$ 0.7 Hb9<sup>−</sup> neurons ( $n=5$ ); similar to the two other lineages, the Hb9<sup>−</sup> cells resided in the ventral-most region of the clone and included the NB, GMCs and probably some newly born neurons (not shown). *spdo* mutant MARCM clones were larger than wild-type clones and consisted mostly of Hb9<sup>+</sup> cells that extended axons contralaterally (Fig. 2B'); *numb* mutant MARCM clones consisted of relatively few neurons that did not extend axons; some of these cells transiently expressed Hb9 (Fig. 2B''). Based solely on location, this lineage could be lineage 2, 10 or 14 (Truman et al., 2004), but only lineage 10 is a monotypic B-type lineage that extends axons contralaterally (Truman et al., 2010). Thus, the Hb9<sup>+</sup> clusters of neurons appear to identify lineages 4b, 10b and 16b.

#### The expression patterns of Lim3, Islet, Nkx6 and Hb9 overlap in adult-specific lineages

The paucity of molecular markers known to label defined postembryonic neurons led us to investigate whether Nkx6 (also known as HGTX – FlyBase), Lim3 and Islet (also known as Tailup; Tup – FlyBase) – three transcription factors that act and are partially

co-expressed with Hb9 in the embryonic CNS (Thor et al., 1999; Broihier et al., 2004; Cheesman et al., 2004) – also label defined sets of postembryonic neurons. We found that they are partially co-expressed with each other in sets of postembryonic neurons (Fig. 3). Nkx6 was co-expressed with Hb9 in all neurons of lineage 4 and half the neurons of lineage 10 (Fig. 3A). Based on MARCM studies detailed below, Nkx6 expression also labeled four other neuronal clusters per thoracic neuromere: lineages 3a (Fig. 4A) and 11a (supplementary material Fig. S7A–C) and lineages 12b (Fig. 6D) and 15b (supplementary material Fig. S10B).

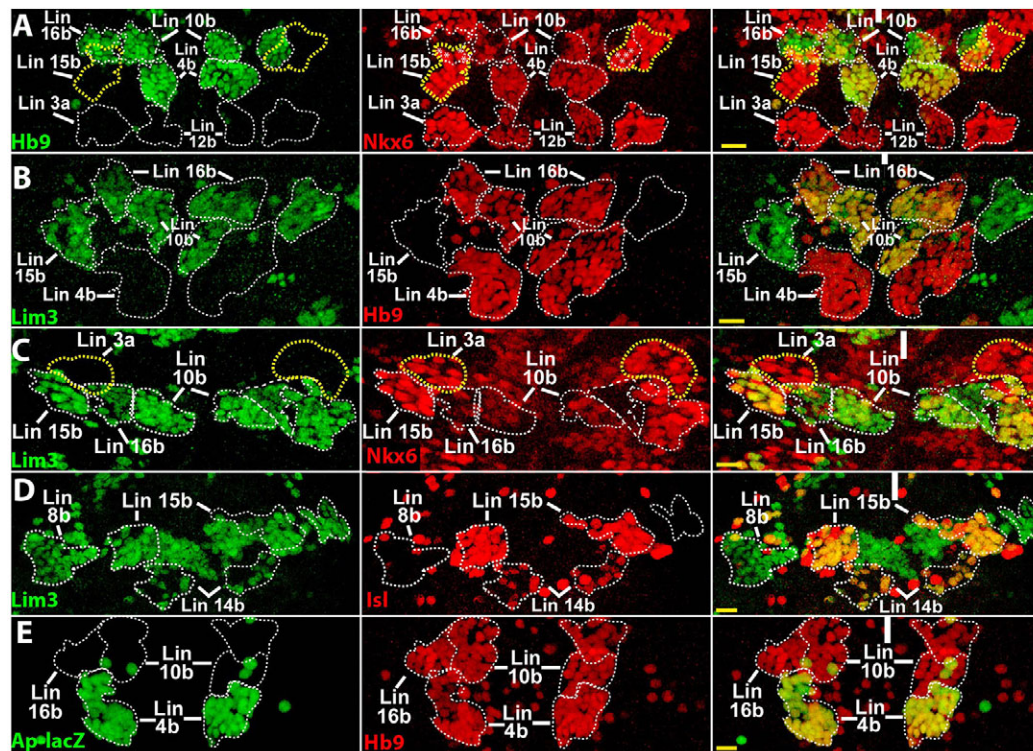
Lim3 was expressed in six neuronal clusters per thoracic hemineuromere. Lim3 was co-expressed with Hb9, Nkx6 or both in lineages 10b, 15b and 16b (Fig. 3B,C). As detailed below, MARCM analysis revealed that Lim3 labeled lineages 8b (supplementary material Fig. S6C), 9b (Fig. 5B) and 14b (supplementary material Fig. S9B) within three ditopic lineages. Thus, Lim3 expression, like that of Hb9, appears restricted to B-type hemilineages.

Islet was co-expressed with Lim3 in lineages 9b (Fig. 5E), 14b (Fig. 3D) and 15b (Fig. 3D). In addition, MARCM analysis mapped the remaining Islet<sup>+</sup> cluster to lineage 17a (supplementary material Fig. S10E). Thus, the overlapping expression patterns of Hb9, Nkx6, Lim3 and Islet label nine of the 25 postembryonic neuronal lineages per thoracic neuromere.

#### Construction of a gene expression map of the adult-specific neuronal lineages

To create a gene expression map of thoracic postembryonic neuronal lineages, we tested 26 other transcription factors that are expressed, identifying 11 that were expressed in discrete clusters of postembryonic neurons – Msh (also known as Drop; Dr – FlyBase), Vestigial (Vg), Cut (Ct), Dichaete (D), Unc4, Dbx, Engrailed (En), BarH (also known as MBH1 – FlyBase), Nmr-1 (also known as H15 – FlyBase), Dachshund (Dac) and Apterous (Ap) (supplementary material Table S3). In addition, Castor (Cas), a member of the temporal gene cascade (Kambadur et al., 1998; Ishiki et al., 2001), was expressed in a subset of post-mitotic neurons in all lineages (supplementary material Fig. S2E). We then took two complementary approaches to map each cluster of neurons that expresses one or more of these transcription factors to a characterized postembryonic neuronal lineage. First, we determined the expression of each transcription factor relative to that of Hb9, Lim3 and Nkx6 in the thoracic CNS of late third instar larvae. Second, we generated wild-type, *spdo* mutant and *numb* mutant MARCM clones for essentially





**Fig. 3. Overlapping expression of Hb9, Nkx6, Lim3, Islet and Apterous in thoracic CNS of late third instar larvae.** (A-E) High magnification ventral views of single wild-type neuromeres labeled for the indicated proteins. Dashed lines identify predicted boundaries for each lineage; lineage designations are provided for the left hemisegment. The boundaries of some lineages appear to overlap, but are in different focal planes. In C, Lim3 expression in lineage 8 is not in the focal plane of the image. Scale bars: 10  $\mu$ m. Anterior, up; white bar, midline.

every cluster of neurons marked by the expression of one or more of these transcription factors, analyzing over 400 MARCM clones in total (supplementary material Table S4). We analyzed all clones at the end of the third larval instar stage, using FasII to highlight landmarks of the larval CNS (Landgraf et al., 2003). As detailed below, this analysis gave rise to a gene expression map that can unambiguously identify 23 of the 25 postembryonic lineages and 29 of the 34 major hemilineages in each thoracic neuromere as *Drosophila* larvae enter metamorphosis.

#### Lineage 0

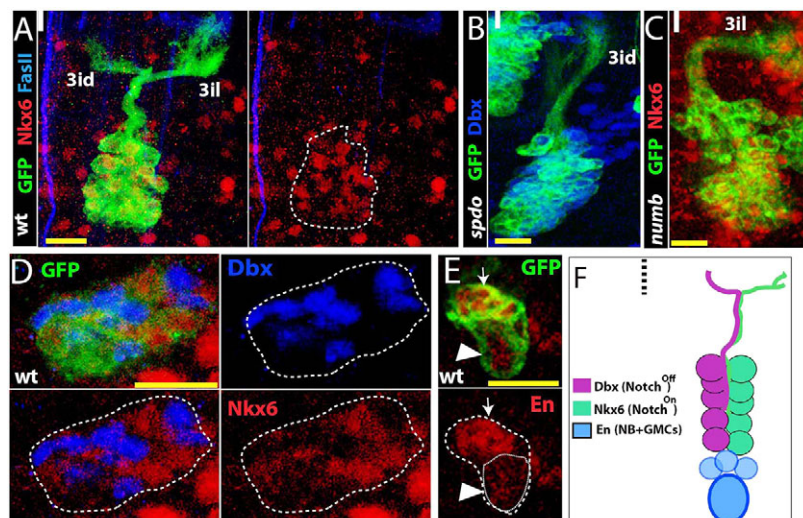
NB 0 generates a largely monotypic A-type lineage that resides at the midline at the posterior of each neuromere (Truman et al., 2004; Truman et al., 2010). We found that this lineage generates ~40  $En^+$  neurons that extended axons dorsally and anteriorly along the midline (supplementary material Fig. S3A).

#### Lineage 1

NB 1 generates a ditypic lineage that resides at the extreme anterior of each hemineuromere (Truman et al., 2004; Truman et al., 2010). This lineage generated ~70 neurons: Msh expression labeled most A-type neurons, which extended axons to the contralateral leg neuropil; Nmr1 expression labeled a subset of B-type neurons, which extended axons ipsilaterally to the leg neuropil in the next segment (supplementary material Fig. S3B,D). Consistent with these designations, *numb* mutant MARCM clones consisted mostly of Msh<sup>+</sup> neurons and extended axons contralaterally (supplementary material Fig. S3C); *spdo* mutant clones consisted of many Nmr1<sup>+</sup> cells and extended axons ipsilaterally (supplementary material Fig. S3E).

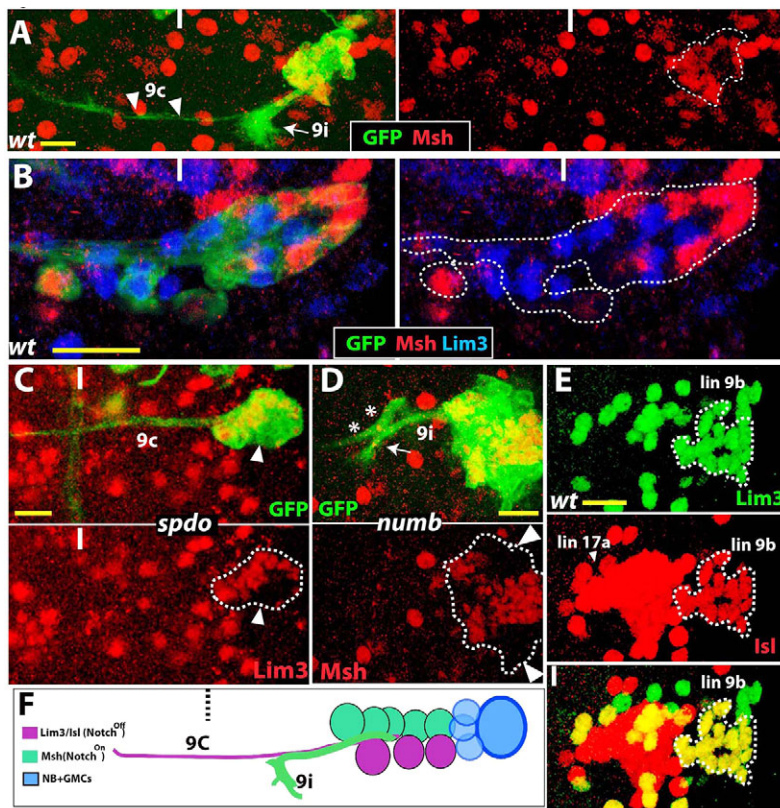
#### Lineage 2

Our expression studies failed to identify any molecular markers that labeled neurons of lineage 2.



**Fig. 4. Nkx6 and Dbx label the A and B hemilineages of lineage 3.** (A-C) Lineage 3 wild-type (A), *spdo* mutant (B) and *numb* mutant (C) MARCM clones in the second thoracic segment of late third instar larvae labeled for the indicated proteins. (A) Lineage 3 neurons extend two ipsilateral axonal bundles: 3id and 3il. (B) *spdo* mutant MARCM clone labeled for Dbx. (C) *numb* mutant MARCM clones labeled for Nkx6. (D) Wild-type MARCM labeled for Nkx6 (red) and Dbx (blue). (E) Ventral portion of a wild-type lineage 3 clone in a late third instar larva labeled for En (red): the NB (encircled, arrowhead), identified by its size, and adjacent GMCs (arrow) express En. (F) Schematic of transcription factor expression in lineage 3. Dashed lines, lineage boundaries. Anterior, up; white bar, midline. Scale bars: 10  $\mu$ m.





**Fig. 5. Msh and Lim3 label the A and B hemilineages of lineage 9.** (A–D) Lineage 9 wild-type (A,B), *spdo* mutant (C) and *numb* mutant (D) MARCM clones labeled for the indicated proteins in late third instar larva. (A) Most lineage 9 neurons project axons ipsilaterally (arrow, 9i), but a few extend axons contralaterally (arrowheads, 9c). (B) Msh (red) and Lim3 (blue) are expressed in distinct lineage 9 neurons. (C) *spdo* mutant MARCM clone labeled for Lim3; arrowhead indicates Lim3<sup>+</sup> NB and GMCs. (D) *numb* mutant MARCM clone labeled for Msh; arrows point to ipsilaterally projecting axons. Asterisks denote GFP<sup>+</sup> axons from cells not located in relevant clone. Arrowheads point to large NB-like cells, which are often duplicated in *numb* clones (Truman et al., 2010). (E) Portion of thoracic hemineuromere of late third instar larva labeled for Lim3 (green) and Islet (red). Lineage 9 neurons are the lateral-most Lim3<sup>+</sup> neurons in a hemineuromere and co-express Islet; Islet expression in lineage 17a neurons is also shown. (F) Schematic of transcription factor expression in lineage 9. Dashed lines, lineage boundaries. White bar, midline. Scale bars: 10  $\mu$ m.

### Lineage 3

NB 3 generates a large, ditopic lineage that resides at the posterior border of each hemineuromere (Truman et al., 2004; Truman et al., 2010). Neurons of this lineage extend a single axon bundle dorsally that bifurcates into a lateral (3il; A sibs) and dorsal (3id; B sibs) branch. The axonal arborization pattern of 3id neurons appears more diffuse in the first and second thoracic segments relative to the third thoracic segment (Truman et al., 2004; Truman et al., 2010). This lineage consists of >100 neurons: Nkx6 and Nmr1 expression labeled most A-type neurons (Fig. 4A,D; supplementary material Fig. S4C); Dbx and Dachshund expression together labeled most B-type neurons, but there were segment-specific differences in their expression (Fig. 4D; supplementary material Fig. S4A,B). In the first thoracic segment (T1), most B-type neurons expressed Dachshund but not Dbx; in T2, most B-type neurons co-expressed Dbx and Dachshund; but in T3, only a subset of B-type neurons appeared to express Dbx and none expressed Dachshund. The segment-specific differences in Dbx and Dachshund expression do not explain the segment-specific arborization pattern of the 3id branch: MARCM clones mutant for Dachshund displayed wild-type axonal arborization patterns of 3id (and 3il) in all thoracic segments (not shown). We also found that NB 3 and its adjacent GMCs expressed En (Fig. 4E). Truman et al. (Truman et al., 2004) identified lineage 3 as the postembryonic progeny of embryonic NB 7-1, which produces Dbx<sup>+</sup> B-type embryonic neurons (Lacin et al., 2009). Thus, this NB produces Dbx<sup>+</sup> B-type neurons during embryonic and postembryonic neurogenesis.

### Lineage 4

As shown in Figs 2 and 3, NB 4 generates a largely monotypic B-type lineage of Hb9<sup>+</sup> and Nkx6<sup>+</sup> neurons that extend a neurite bundle ipsilaterally to the leg neuropil (Fig. 2) (Truman et al., 2004;

Truman et al., 2010). Apterous- $\beta$ -gal expression also labeled most of these neurons; nitric oxide synthase (NOS) expression labeled half of them (Fig. 3E; supplementary material Fig. S2B). Nkx6 expression also labeled the lineage 4 NB (supplementary material Fig. S2A, arrowhead). Hb9 function is necessary to promote NOS expression in these neurons (H.L. and J.B.S., unpublished), and NOS expression is maintained into the adult in the Hb9<sup>+</sup> neurons of the first thoracic neuromere (supplementary material Fig. S2C). Neither *nkx6* nor *hb9* mutant lineage MARCM clones disrupted the axonal phenotype of this lineage (not shown).

### Lineage 5

NB 5 generates a monotypic B-type lineage that resides anterior-lateral to lineage 3 in the posterior of each hemineuromere (Truman et al., 2004; Truman et al., 2010). This lineage consists of ~50 neurons: Cut and Vestigial expression labeled most of these neurons, which extend axons dorsally and medially across the midline (supplementary material Fig. S5A,F). In most postembryonic neuronal lineages, Cut, like Chinmo, labeled early-born neurons (not shown), but in lineages 5 and 6, Cut also labeled most B-type neurons (supplementary material Fig. S5A,D,F).

### Lineage 6

NB 6 generates a ditopic lineage that resides medially near the midpoint of each neuromere (Truman et al., 2004; Truman et al., 2010). This lineage consists of ~90 neurons: we failed to identify markers of the A hemilineage, but En, Cut and Vestigial expression labeled most B-type neurons, which extend axons anteriorly and medially and then across the midline (supplementary material Fig. S5B–G). In *spdo* mutant MARCM clones, most lineage 6 neurons expressed Vestigial (supplementary material Fig. S5E), confirming that Vestigial and by extension En and Cut mark lineage 6b.

### Lineage 7

NB 7 generates a monotypic B-type lineage that resides near the midpoint of the hemineuromere (Truman et al., 2004; Truman et al., 2010). This lineage consists of ~50 neurons: Unc4 labeled most of these neurons, which extend axons contralaterally (supplementary material Fig. S6A), but did not label the NB or GMCs (supplementary material Fig. S6A, arrowhead). In *spdo* mutant MARCM clones, most lineage 7 neurons expressed Unc4 (supplementary material Fig. S6B), confirming Unc4 marks lineage 7b.

### Lineage 8

NB 8 generates a ditypic lineage that resides at the anterior-lateral edge of each hemineuromere (Truman et al., 2004; Truman et al., 2010). This lineage consists of ~55 neurons: none of the factors we assayed labeled the A hemilineage, which extends axons ipsilaterally, but Lim3 labeled B-type neurons, which project axons contralaterally (supplementary material Fig. S6C). In *spdo* mutant MARCM clones, most lineage 8 neurons expressed Lim3 and extended axons contralaterally (supplementary material Fig. S6D), confirming Lim3 marks lineage 8b.

### Lineage 9

NB 9 generates a ditypic lineage that resides in the anterior half of each neuromere and is composed mostly of A-type neurons and a few B-type neurons. Msh expression labeled most A-type neurons, which extend a large neurite bundle to the ipsilateral leg neuropil (Fig. 5A,B); Lim3 and Islet expression labeled most, but not all, B-type neurons, which extend a small neurite bundle contralaterally (Fig. 5B,E). In *spdo* mutant MARCM clones, most neurons express

Lim3 and extend a contralateral axon projection (Fig. 5C). In *numb* mutant MARCM clones, most neurons express Msh and extend short ipsilateral projections (Fig. 5D, arrow). Based on size, multiple NB-like cells reside within this clone (Fig. 5D, arrowheads). Truman et al. previously observed that *numb* mutant clones often contain multiple NB-like progenitor cells (Truman et al., 2010).

### Lineage 10

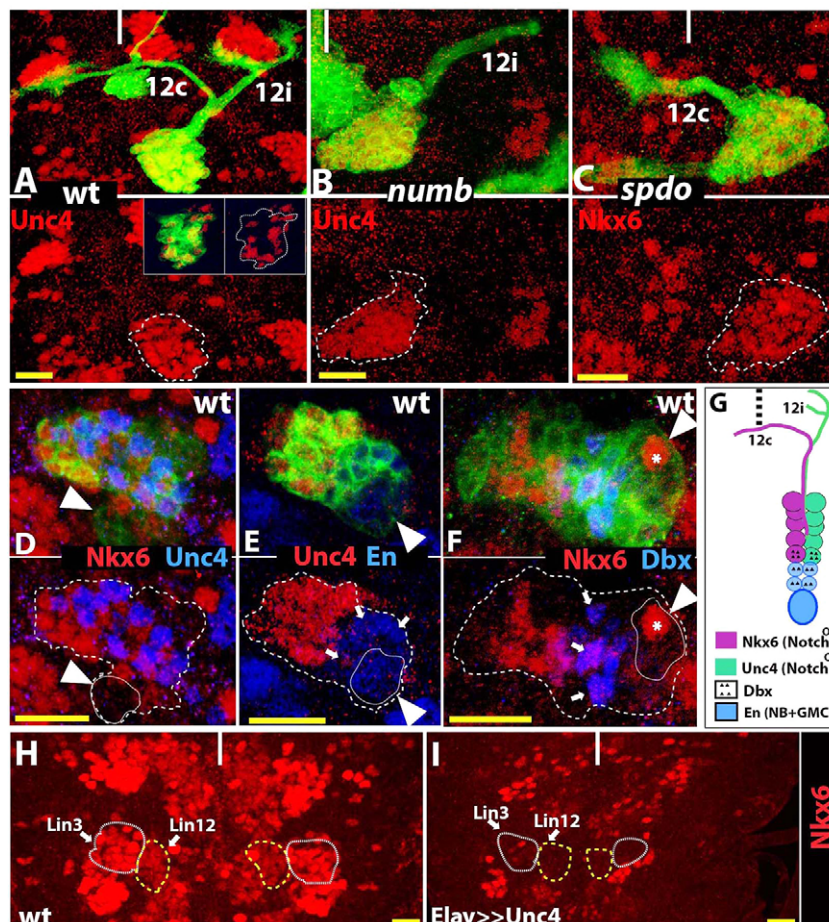
As shown in Figs 2 and 3, lineage 10 is a monotypic B-type lineage that extends axons contralaterally (Truman et al., 2004). Most neurons in this lineage express Hb9 and Lim3 and about half of them express Nkx6.

### Lineage 11

NB 11 generates a ditypic lineage that resides at the posterior-lateral region of each hemineuromere (Truman et al., 2004; Truman et al., 2010). Unc4 expression labeled most A-type neurons; Nkx6 labeled a subset of Unc4<sup>+</sup> neurons (supplementary material Fig. S7A,B); none of our markers labeled the B hemilineage. In *numb* mutant MARCM clones most neurons expressed Unc4 (supplementary material Fig. S7D), confirming Unc4 marks lineage 11a. NB 11 appeared to express En and its GMCs appeared to express En and Dbx (supplementary material Fig. S7C; not shown for En).

### Lineage 12

NB 12 generates a ditypic lineage that resides at the posterior-medial boundary of each hemineuromere (Truman et al., 2004; Truman et al., 2010). Unc4 expression labeled most A-type neurons (Fig. 6A,D,E), which extend neurites anteriorly and laterally (12i in



**Fig. 6. Unc4 and Nkx6 label the A and B hemilineages of lineage 12.** (A–C) Lineage 12 wild-type (A), *numb* mutant (B) or *spdo* mutant (C) MARCM clones labeled for the indicated proteins in late third instar larvae. (A) A wild-type lineage 12 clone contains Unc4<sup>+</sup> neurons and extends a contralateral projection (12c) and forked ipsilateral projections (12i). Some neurons in the clone do not express Unc4 as shown in a single z-section (inset). (B) *numb* mutant MARCM clone labeled for Unc4. (C) *spdo* mutant MARCM clone labeled for Nkx6. (D–F) Wild-type lineage 12 MARCM clones labeled for the indicated proteins; arrowheads point to NBs, which are encircled by solid lines. The Nkx6<sup>+</sup> cell (asterisk, F) does not reside in the clone. (G) Schematic of transcription factor expression in lineage 12. (H,I) Nkx6 expression (red) in a thoracic neuromere of a wild-type late third instar larva (H) and a late third instar larva in which Unc4 was expressed in all neurons (I): the location or expected location of Nkx6<sup>+</sup> neurons of lineage 3 and 12 are circled. Dashed lines, lineage boundaries (A–F) or predicted boundaries (H,I). Anterior, up; white bar, midline. Scale bars: 10  $\mu$ m.



Fig. 6A); *Nkx6* expression labeled most B-type neurons, which extend neurites medially to and across the midline (Fig. 6A,D,F). In *numb* mutant MARCM clones, most neurons expressed *Unc4* (Fig. 6B); in *spdo* mutant MARCM clones, most neurons expressed *Nkx6*<sup>+</sup> (Fig. 6C). We also found that pan-neuronal expression of *Unc4* repressed *Nkx6* expression in most *Nkx6*<sup>+</sup> postembryonic neurons, including those in lineages 12 and 3 (Fig. 6H,I). As per lineage 11, NB12 expressed *En* and its GMCs expressed *En* and *Dbx* (Fig. 6E,F).

#### Lineage 13

NB 13 generates a ditopic lineage that resides near the midpoint of each hemineuromere (Truman et al., 2004; Truman et al., 2010). *Dbx* expression labeled most A-type neurons, which extend axons ipsilaterally to the adjacent leg neuropil (supplementary material Fig. S8A,C); *Dichaete* and *Vestigial* expression labeled most B-type neurons, which extend axons to the contralateral leg neuropil (supplementary material Fig. S8B,C). In *spdo* mutant MARCM clones, most neurons expressed *Vestigial* (supplementary material Fig. S8D); in *numb* mutant MARCM clones, most neurons expressed *Dbx* (supplementary material Fig. S8E). Thus, *Vestigial* and *Dichaete* label lineage 13b; *Dbx* labels lineage 13a.

#### Lineage 14

NB14 generates a largely monotypic A-type lineage that resides in the anterior and medial first third of each hemineuromere (Truman et al., 2004; Truman et al., 2010). *Msh* expression labeled most A-type neurons, which extend axons to the contralateral leg neuropil (supplementary material Fig. S9A,B); *Lim3* and *Islet* expression labeled the few remaining B-type neurons (supplementary material Fig. S9B; see Fig. 3D for *Islet*), which extend axons anteriorly and across the midline (supplementary material Fig. S9A). In support of these assignments, *spdo* mutant MARCM clones were small and consisted largely of *Lim3*<sup>+</sup> neurons (supplementary material Fig. S9C); *numb* mutant MARCM clones were large and consisted mostly of *Msh*<sup>+</sup> neurons (supplementary material Fig. S9D).

#### Lineage 15

NB 15 produces a monotypic B-type hemilineage that resides in the anterior of each hemineuromere and consists of ~30 motor neurons (Truman et al., 2004; Truman et al., 2010). *Lim3*, *Nkx6* and *Islet* expression labeled most of these motor neurons (Fig. 3D; supplementary material Fig. S10A,B). These transcription factors are co-expressed with *Hb9* in many embryonic motor neurons (Broihier and Skeath, 2002; Broihier et al., 2004), but *Hb9* was not expressed in these postembryonic motor neurons. In *spdo* mutant MARCM clones, most neurons expressed *Lim3* (supplementary material Fig. S10C), confirming that *Lim3*, *Nkx6* and *Islet* mark lineage 15b. These neurons maintained *Lim3* and *Nkx6* expression into the adult (supplementary material Fig. S2D).

#### Lineage 16

As noted in Figs 2 and 3, lineage 16 is a monotypic B-type lineage composed of *Hb9*<sup>+</sup> and *Lim3*<sup>+</sup> neurons that extend axons anteriorly and laterally to the adjacent leg neuropil (Truman et al., 2004; Truman et al., 2010).

#### Lineage 17

NB17 generates a monotypic A-type lineage that resides at the anterior and lateral edge of each hemineuromere. *Unc4* and *Islet* expression labeled most neurons in this lineage, which extend axons medially towards the midline (supplementary material Fig. S10D,E).

We observed *Unc4* and *Islet* co-expression in the second and third, but not first, thoracic segment (supplementary material Fig. S10F). Truman et al. also failed to identify MARCM clones of lineage 17 in the first thoracic segment. Thus, lineage 17 may not form, or adopts a divergent morphology, in the first thoracic segment (Truman et al., 2004).

#### Lineage 18

NB 18 generates a monotypic B-type lineage that resides at the lateral edge of the CNS in the anterior half of each hemineuromere (Truman et al., 2004; Truman et al., 2010). *Unc4* expression labeled most of these neurons, which extend axons contralaterally (supplementary material Fig. S11A).

#### Lineage 19

NB 19 generates a ditopic lineage that resides at the extreme posterior-lateral edge of each neuromere (Truman et al., 2004; Truman et al., 2010). *Dbx* expression labeled most A-type neurons, which extend axons posteriorly to the leg neuropil (supplementary material Fig. S11C,E); *Unc4* expression labeled most B-type neurons, which extend axons across the midline to the contralateral hemineuromere (supplementary material Fig. S11B,E). In *numb* mutant clones, most neurons expressed *Dbx* but not *Unc4* (supplementary material Fig. S11D), confirming that *Dbx* and *Unc4* label lineage 19a and 19b, respectively. NB 19 and its GMCs also expressed *En* (supplementary material Fig. S11F).

#### Lineages 20 and 22

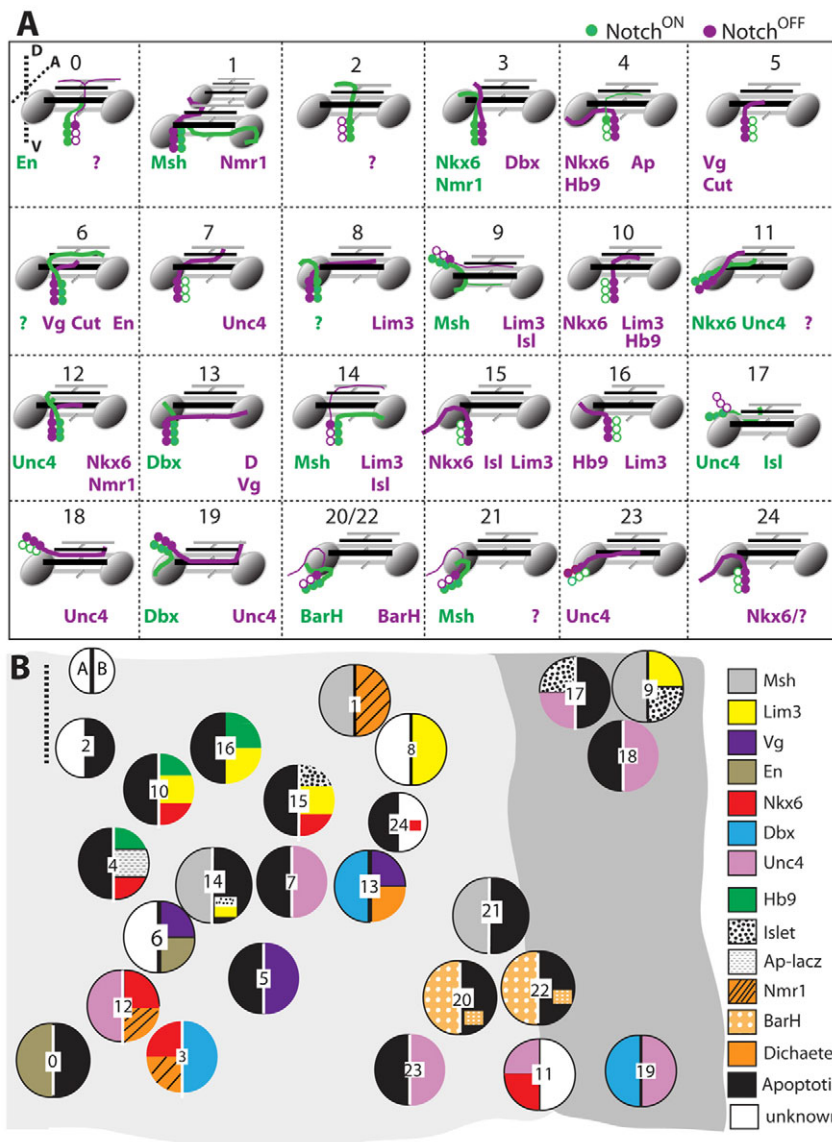
NBs 20 and 22 are adjacent NBs that produce identical, largely monotypic A-type lineages that reside in the posterior-lateral region of each neuromere (Truman et al., 2004; Truman et al., 2010). *BarH1/2* expression labeled most neurons in these lineages (supplementary material Fig. S12A): A-type neurons extend axons laterally into the adjacent leg neuropil; the surviving B-type neurons are motor neurons that extend axons to the ipsilateral muscle field (arrowheads, supplementary material Fig. S12A) (Truman et al., 2004). NBs 20 and 22 also expressed *Msh* (not shown). Finally, we determined that a regulatory region of the *FoxD* or *Fd59a* gene drives reporter gene expression in these neurons as well as in the *BarH1/2* motor neurons in the embryonic CNS (supplementary material Fig. S12B,C). In the embryo, these neurons derive from NB 5-4 (He and Noll, 2013). Thus, we speculate that embryonic NB 5-4 produces either postembryonic lineage 20 or 22.

#### Lineage 21

NB21, like NBs 20 and 22, produces a largely monotypic A-type lineage that resides next to lineages 20 and 22 (Truman et al., 2004; Truman et al., 2010). *Msh* expression labeled most A-type neurons, which extend short axons to the adjacent leg neuropil (supplementary material Fig. S13A); we failed to find a marker of lineage 21b. In *numb* mutant MARCM clones, most neurons expressed *Msh*, confirming *Msh* marks lineage 21a (supplementary material Fig. S13B).

#### Lineage 23

NB 23 generates a purely B-type monotypic lineage that resides in the posterior lateral region of each hemineuromere (Truman et al., 2004; Truman et al., 2010). *Unc4* expression labeled most neurons in this lineage, which project axons into the contralateral hemineuromere. NB 23 and its GMCs also appeared to express *En* (not shown).



**Fig. 7. Schematic models of transcription factor expression in postembryonic neuronal lineages.** (A) Transcription factor expression in each postembryonic lineage. Large gray ovals denote leg neuropils; black and gray lines denote commissures. Green circles represent A hemilineage neurons (Notch<sup>ON</sup>); purple circles represent B hemilineage neurons (Notch<sup>OFF</sup>). Empty circles represent hemilineages that die. Schematic reproduced with permission (Truman et al., 2010). (B) Schematic of transcription factor expression in postembryonic lineages in one thoracic neuromere. Left half of circles represents the 'A' hemilineage; right half represents the B hemilineage. Anterior, up; dotted line, midline. Dark gray shading represents the dorsal region of hemisegment.

#### Lineage 24

Lineage 24, which produces only motor neurons, was the second lineage for which we failed to identify molecular markers that reproducibly labeled its neurons. We did, however, observe Nkx6 expression in a subset of these motor neurons in two of four clones (supplementary material Fig. S13D,E). We presently do not understand why some clones contain Nkx6<sup>+</sup> neurons and others do not.

The results of the studies described above are summarized in Fig. 7 and supplementary material Table S4.

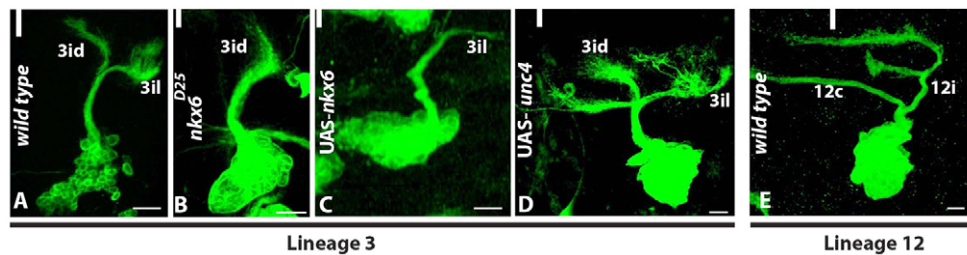
#### Nkx6 is necessary and sufficient to regulate axonal pathway selection in lineage 3

Our creation of a transcription factor expression map of postembryonic neuronal lineages should facilitate the dissection of postembryonic neurogenesis in two ways: it will increase the cellular resolution with which phenotypic studies can be conducted and it identifies transcription factors that are likely to regulate the development and differentiation of postembryonic neurons. To test these suppositions, we focused on lineage 3, a ditopic lineage, in which Nkx6 and Dbx are expressed in mutually exclusive subsets

of neurons (Fig. 4). A-type neurons extend an axon bundle dorsally and then laterally (3il); B-type neurons extend an axon bundle just dorsally (3id; Fig. 4A) (Truman et al., 2004; Truman et al., 2010). In *spdo* mutant MARCM clones, most neurons expressed Dbx and projected axons within 3id (Fig. 4B); thus, Dbx expression marks lineage 3b. In *numb* mutant MARCM clones, most neurons expressed Nkx6 and projected axons within 3il (Fig. 4C); thus, Nkx6 expression marks lineage 3a.

To test whether Dbx or Nkx6 regulate the differentiation of lineage 3 neurons, we used MARCM analysis to remove the function of or to overexpress each gene in cells of this lineage. Loss or ubiquitous expression of *dbx* function had no gross effect on Nkx6 expression or pathway selection of either axon bundle in lineage 3 (not shown), but loss or overexpression of *nkx6* function was necessary and sufficient to direct axonal pathway selection in lineage 3 neurons (Fig. 8A-C). In *nkx6* mutant MARCM clones ( $n=7$ ), the 3id bundle formed normally, but the 3il bundle failed to form (Fig. 8B). Conversely, in MARCM clones that expressed Nkx6 in all neurons ( $n=10$ ), the 3il bundle formed normally, but the 3id bundle failed to form 40% of the time and was reduced in size in the remainder (Fig. 8C). Thus, *nkx6* is necessary to direct proper axonal





**Fig. 8. *Nkx6* is necessary and sufficient to direct axonal pathway selection in lineage 3.** (A) A lineage 3 wild-type MARCM clone extends two axon bundles: 3id and 3il. (B) A lineage 3 *nkx6* mutant MARCM clone contains the 3id bundle, but lacks the 3il bundle. (C) A lineage 3 MARCM clone that overexpresses *nkx6* contains the 3il bundle, but lacks the 3id bundle. (D) A lineage 3 MARCM clone that overexpresses *Unc4* extends three axon bundles. (E) A lineage 12 wild-type MARCM clone (E). Scale bars: 10  $\mu$ m. Anterior, up; white bar, midline.

pathway selection of lineage 3a neurons in which it is normally expressed, and its ectopic expression in lineage 3b neurons is sufficient to disrupt their axonal pathway selection.

As shown in Fig. 6, generalized *Unc4* expression was sufficient to repress *Nkx6* expression in most postembryonic neuronal lineages, including lineage 3 (Fig. 6H,I). *Nkx6* is expressed in lineage 3a neurons, which extend axon bundles (3il) away from the midline (Fig. 4, Fig. 8A). *Unc4* is expressed in lineage 12a neurons, which reside next to lineage 3 and extend axon bundles toward the midline (Fig. 4, Fig. 8E). Thus, we asked whether forced *Unc4* expression in lineage 3 redirected its constituent neurons to project axons toward the midline. We found that lineage 3 neurons that expressed *Unc4* extended three, rather than the normal two, neurite bundles; many axons in all three bundles extended towards the midline (compare Fig. 8D with 8A,E). Thus, forced *Unc4* expression appeared to partially transform lineage 3 toward that of lineage 12. Together with the functional studies on *Nkx6*, this result indicates that many of the identified transcription factors help direct the development and differentiation of their respective postembryonic neurons.

## DISCUSSION

By a ten-to-one ratio, postembryonic neurons outnumber embryonic neurons, and the adult fly CNS is composed almost entirely of postembryonic neurons. Yet we know much less about postembryonic neurogenesis than embryonic neurogenesis. Our molecular marker map of postembryonic thoracic neuronal lineages helps bridge this gap by extending the work of Truman et al. that characterized these lineages on the basis of morphology (Truman et al., 2004; Truman et al., 2010). The map enables the identification of 23 of 25 postembryonic neuronal lineages based on gene expression alone, buttressing the descriptive foundation of postembryonic neurogenesis and illustrating that the combinatorial code of neuronal specification (Thor et al., 1999) extends to the postembryonic thoracic CNS. Below we discuss the apparent lack of cell-type diversity in postembryonic neuronal lineages, the utility of the gene expression map in matching postembryonic lineages to their cognate embryonic lineages, and the similarity of hemilineages in flies to pools of neurons in vertebrates.

Despite their larger size, postembryonic lineages appear less complex than their embryonic counterparts. For example, embryonic NB 3-1 produces four motor neurons and a variable number of intersegmental and local interneurons (Bossing et al., 1996; Schmid et al., 1999). Postembryonic lineage 4, which appears to derive from NB 3-1, generates almost 50 cells, but based on morphology and gene expression, neurons in this lineage can be grouped into at most two subtypes of neurons (supplementary material Fig. S2). Are thoracic postembryonic neuronal lineages less complex than their

embryonic counterparts? The jury remains out. Studies of postembryonic neurogenesis have not reached the resolution of those in the embryo, and most have assessed postembryonic neuronal lineages at the end of larval life, when the vast majority of neurons have arisen, but still days away from their final differentiation. Thus, even though neurons in a given postembryonic lineage display simple gene expression profiles and extend axons along only one or two paths before metamorphosis, they may manifest complex patterns of target innervation and gene expression after metamorphosis. In this context, our molecular marker map is a key antecedent for studies that dissect the cellular and molecular complexity of postembryonic lineages at single-cell resolution at later stages of development.

Within the thoracic nerve cord, postembryonic neurons derive from the same NBs that generate embryonic neurons. With few exceptions, it has proved difficult to pair postembryonic neuronal lineages with their cognate embryonic lineages and NBs (Truman et al., 2004; Truman et al., 2010). To do so would provide a continuum of knowledge from the genetic mechanisms that drive the NB formation and specification in the embryo to those that govern the development and differentiation of postembryonic neurons.

In those cases in which the common ancestry of embryonic and postembryonic neuronal lineages is known, the two lineages share similar gene expression profiles. For example, Truman et al. found that postembryonic lineages 3 and 4 derive from embryonic NBs 7-1 and 3-1, respectively (Truman et al., 2004). In embryos, NB 7-1 expresses *Nkx6* and generates *Eve*<sup>+</sup> A-type motor neurons and *Dbx*<sup>+</sup> B-type interneurons. In larvae, NB 7-1 continues to produce *Dbx*<sup>+</sup> B-type interneurons and generates *Nkx6*<sup>+</sup>, rather than *Eve*<sup>+</sup>, A-type neurons. In embryos, NB 3-1 produces B-type Hb9<sup>+</sup>, *Nkx6*<sup>+</sup>, *Lim3*<sup>+</sup> and *Islet*<sup>+</sup> RP1, three to five motor neurons. In larvae, NB 3-1 produces B-type Hb9<sup>+</sup> and *Nkx6*<sup>+</sup>, but *Lim3*<sup>-</sup> and *Islet*<sup>-</sup>, interneurons. Thus, lineally related embryonic and postembryonic neurons share similar gene expression profiles and are likely to share functional attributes.

The shared gene expression profiles of lineally related postembryonic and embryonic neurons suggest our gene expression map will help match postembryonic lineages to their cognate embryonic lineages. For example, in embryos NB 2-2 generates six B-type neurons that express Hb9, *Nkx6* and *Lim3* (Lacin et al., 2009). In larvae, lineage 10, a monotypic B-type lineage, is the only postembryonic lineage that expresses this combination of transcription factors, and similar to the embryonic neurons produced by NB 2-2, lineage 10 neurons extend axons across the midline as part of the anterior commissure. A systematic pairing of embryonic and postembryonic lineages will still require sophisticated lineage tracing methods that induce clones in the early embryo and analyze the embryonic and postembryonic lineages of single NBs in the

CNS of late third instar larvae. Here, the simultaneous use of molecular markers that identify defined embryonic and/or postembryonic neuronal lineages will enable the matching of individual embryonic and postembryonic lineages. Only through such studies will we be able to follow CNS development uninterrupted from the embryo to the adult.

Truman et al. identified the hemilineage as the developmental unit of the postembryonic CNS: most neurons within an individual hemilineage project axons within the same bundle to similar targets (Truman et al., 2010). We extend these findings by showing that within a given lineage, most transcription factors are expressed in A- or B-type neurons, but not both. Thus, hemilineages are composed of tightly clustered groups of neurons that share common transcription factor expression profiles and extend axons in the same bundle to innervate similar targets.

At the morphological and molecular level, neuronal hemilineages in flies resemble pools of neurons in vertebrates. Individual motor or inter-neuron pools are composed of clustered groups of neurons that share common transcription factor expression profiles and extend axons in the same bundle to innervate similar targets. For example, motor neurons with cell bodies located medially within the lateral motor column (LMC) express *Islet* and project axons to ventrally derived limb muscle; motor neurons with cell bodies located laterally in the LMC express *Lim1* and project axons to dorsally derived limb muscles (Landmesser, 1978a; Landmesser, 1978b; Kania et al., 2000; Kania and Jessell, 2003). These parallels between neuronal hemilineages in flies and pools of neurons in vertebrates suggest that individual pools of vertebrate neurons share a common lineage and state of Notch activation.

## MATERIALS AND METHODS

### MARCM clone generation

The MARCM technique was used to generate wild-type MARCM clones and MARCM clones mutant for *spdo*, *numb*, *nkx6*, *hb9* or *islet* (Lee and Luo, 1999). To generate MARCM clones, 0- to 6-hour-old embryos of the appropriate genotype were grown at 25°C for 20-24 hours. Newly hatched larvae were heat shocked at 37°C for 1 hour and grown to the late third instar stage, at which time their nerve cords were dissected, fixed and stained. Fly strains used in these studies are given in supplementary material Table S1.

### Antibody production, gene expression studies and EdU labeling

DNA encoding amino acids 169-428 of *Unc4* was cloned in-frame into pET29a (Novagen), and standard methods were used to express and purify the resulting protein in bacteria (Wang et al., 1989). Purified *Unc4* protein was used as an immunogen to generate an *Unc4*-specific antibody response in rabbits (Pocono Rabbit Farm and Laboratory).

Immunofluorescence and immunohistochemical stainings were performed as described in Patel (Patel, 1994). Antibodies used in this study are given in supplementary material Table S2.

To label cells in S phase, recently molted third instar larvae were fed yeast paste containing 300  $\mu$ M EdU. At the late third instar larval stage, larvae were dissected, fixed and stained as described previously (Daul et al., 2010).

### Transgenic flies

To generate a full-length *UAS-unc4* transgene, we cloned the entire *unc4* coding region into the *NorI* and *XhoI* restriction sites of pUAST (Brand and Perrimon, 1993). We generated transgenic P[*UAS-unc4*] flies using P-element-based transformation methods and verified *Unc4* expression by staining *Elav-GAL4::UAS-unc4* embryos for *Unc4* protein (not shown).

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

H.L. and J.B.S. conceived the study, analyzed the data, and co-wrote the manuscript. H.L. performed the majority of experiments; B.A.W. helped H.L. with most experiments. Y.Z. made the *Unc4* antibody and the *UAS-Unc4* transgene.

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### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.102178/-/DC1>

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<b>Table S1: Fly strains</b>
<b>Wild type and mutant stocks</b>
w <sup>1118</sup>
Canton S
<i>Apterous-lacZ</i>
<i>numb</i> <sup>15</sup> , <i>P{neoFRT}40A/CyO</i>
<i>P{ry[+t7.2]=neoFRT}82B e<sup>s</sup>, spdo<sup>G104</sup>/TM3 Sb</i>
<i>nkx6</i> <sup>D25</sup> , <i>P{FRT(w[hs])}2A/TM6b Tb</i>
<i>hb9</i> <sup>ij154</sup> , <i>P{FRT(w[hs])}2A/TM6b Tb</i>
<i>tup</i> <sup>isl-1</sup> , <i>P{ry[+t7.2]=neoFRT}40A/CyO</i>
<i>dac</i> <sup>E462</sup> <i>P{neoFRT}40A/CyO</i>
<b>Fly lines used for the Gal4/UAS experiments</b>
<i>UAS-hb9</i>
<i>UAS-nkx6</i>
<i>UAS-unc4</i>
<i>Gene Switch-Elav-Gal4</i>
<b>Fly lines used for MARCM analysis</b>
<i>P{GawB}elav[C155] ; P{FRT(w[hs])}G13, P{tubP-GAL80}LL2</i>
<i>P{GawB}elav[C155], P{FRT(w[hs])}G13 P{UAS-mCD8::GFP.L}LL5</i>
<i>P{hsFLP}1 ; P{FRT(w[hs])}G13 P{tubP-GAL80}LL2/CyO,</i> <i>P{FRT(w[hs])}G13 P{tubP-GAL80}LL2</i>
<i>P{tubP-GAL80}LL10 P{neoFRT}40A/CyO</i>
<i>y[1] w<sup>1118</sup> ; P{tubP-GAL80}LL9 P{FRT(w[hs])}2A/TM3, Sb</i>

<b>Table S2: Antibodies used in gene expression studies</b>		
Antibody Specificity	Species	Reference
Hb9	Rabbit, guinea pig	Broihier and Skeath, 2002
Lim3	Guinea pig	Broihier et al., 2004
Islet	Rat	Broihier et al., 2004
Nkx6	Rat	Broihier et al., 2004
Dbx	Guinea pig	Lacin et al., 2009
Nmr1	Rabbit	Leal et al., 2009
Msh	Rabbit	McDonald et al., 1998
Bar-H1/2	Rabbit	Higashijima et al., 1992
Unc4	Rabbit	this paper
Dichaete	Rabbit	Nambu and Nambu, 1996
Vestigial	Rabbit	Guss et al., 2008
NOS	Guinea pig	Lacin et al., submitted
GFP	Rabbit	Torrey Pines and Roche
β-galactosidase	Mouse and Rabbit	Invitrogen
Invected/engrailed 4D9	Mouse	Patel et al., 1989
Cut 2B10	Mouse	Blochinger et al., 1993
FasII 1D4	Mouse	van Vactor et al., 1993
Chinmo	Rabbit	Zhu et al., 2006
Broad-Complex	Mouse	Emery, 1994



<b>Table S3: Genes assayed for expression in post-embryonic neurons</b>			
<b>Transcription factors expressed in discrete clusters of neurons in post-embryonic lineages</b>			
Vestigial	Msh	Cut	Dichaete
Unc4	Dbx	Nmr1	BarH1/2
Engrailed	Apterous	Hb9	Lim3
Islet	Nkx6	Dachshund	
<b>Transcription factors expressed in subsets of neurons in most or all post-embryonic lineages</b>			
Zfh-1	Lim1	Castor	Hunchback
Kruppel	Pdm2		
<b>Transcription factors not expressed in clusters of neurons in post-embryonic lineages</b>			
Pdm3	Dimmed	FoxD	Pointed
Eve (may label one lineage)	Eagle (expressed in progenitors)	Eyes Absent	

<b>Table S4: Transcription factor expression in post-embryonic lineages</b>		
<b>Lineage</b>	<b>A hemi-lineage</b>	<b>B hemi-lineage</b>
<b>Lineage 0</b>	En (n>10*)	PCD*
<b>Lineage 1</b>	Msh (n>10)	Nmr1 (n=4)
<b>Lineage 2</b>	?	PCD
<b>Lineage 3</b>	Nkx6 (n>10); Nmr1 (n=3)	Dbx (n>10); Dacs (n=4)
<b>Lineage 4</b>	PCD	Hb9 (n>10); Nkx6 (n=4); Ap-lacZ (n=0)**
<b>Lineage 5</b>	PCD	Vg (n=2); Cut (n=7)
<b>Lineage 6</b>	?	En (n=5); Vg (n=3); Cut (n=4)
<b>Lineage 7</b>	PCD	Unc4 (n>10)
<b>Lineage 8</b>	?	Lim3 (n=5)
<b>Lineage 9</b>	Msh	Lim3 (n=7); Islet (n=0)**
<b>Lineage 10</b>	PCD	Hb9 (n>10); Nkx6 (n=3); Lim3 (n=5)
<b>Lineage 11</b>	Nkx6 (n=4); Unc4 (n=6)	?
<b>Lineage 12</b>	Unc4 (n>10)	Nkx6 (n>10); Nmr1 (n=4)
<b>Lineage 13</b>	Dbx (n>10)	Dichaete (n=7); Vg (n=1)
<b>Lineage 14</b>	Msh (n>10)	PCD; Lim3 (n=4); Islet (n=0)**
<b>Lineage 15</b>	PCD	Lim3 (n>10); Nkx6 (n>10); Islet (n=0)**
<b>Lineage 16</b>	PCD	Hb9 (n=>10); Lim3 (n=7))
<b>Lineage 17</b>	Unc4 (n=4); Islet (n=2)	PCD
<b>Lineage18</b>	PCD	Unc4 (n=6)
<b>Lineage 19</b>	Dbx (n=6)	Unc4 (n=8)
<b>Lineage 20</b>	BarH (n=7) <sup>#</sup>	PCD*/BarH
<b>Lineage 21</b>	Msh (n>10)	PCD*
<b>Lineage 22</b>	BarH(n=7) <sup>#</sup>	PCD*/BarH
<b>Lineage 23</b>	PCD	Unc4 (n=5)
<b>Lineage 24</b>	PCD	Nkx6/? (n=2 of 4)

PCD: Indicates that most cells in hemilineage die

\*: denotes number of wild-type clones assayed for this lineage and transcription factor.

\*\*: Islet expression in lineages 9, 14, and 15 based on co-expression with Lim3; Ap-lacZ expression in lineage 4 based on co-expression with Hb9

#: 7 BarH<sup>+</sup> clones were generated for lineages 20 and 22.



**Figure S1**

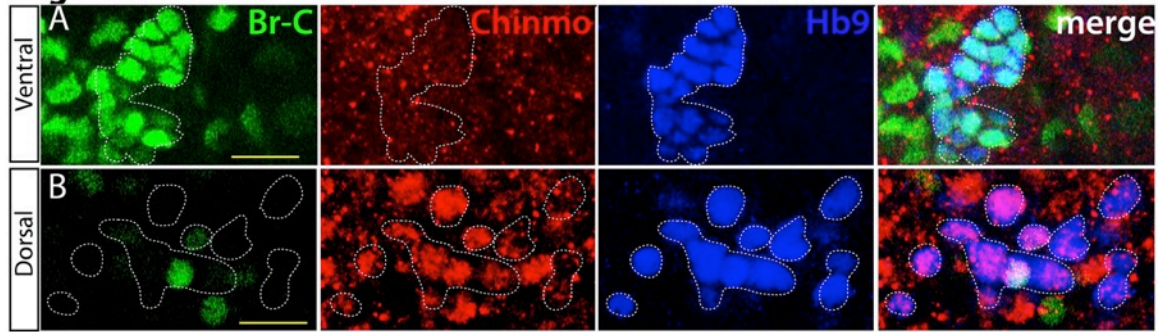


Figure S1) Hb9, Chinmo, and Broad expression in a thoracic neuromere of a late third instar larva.

(A, B) Ventral (A) and dorsal (B) views of a portion of a thoracic hemineuromere labeled for Broad (green), Chinmo (red), and Hb9 (blue). (A) Most small, ventral Hb9<sup>+</sup> neurons express Broad. (B) Most large, dorsal Hb9<sup>+</sup> neurons express Chinmo; a few express Broad. Anterior - up; scale bar: 10um.

**Figure S2**

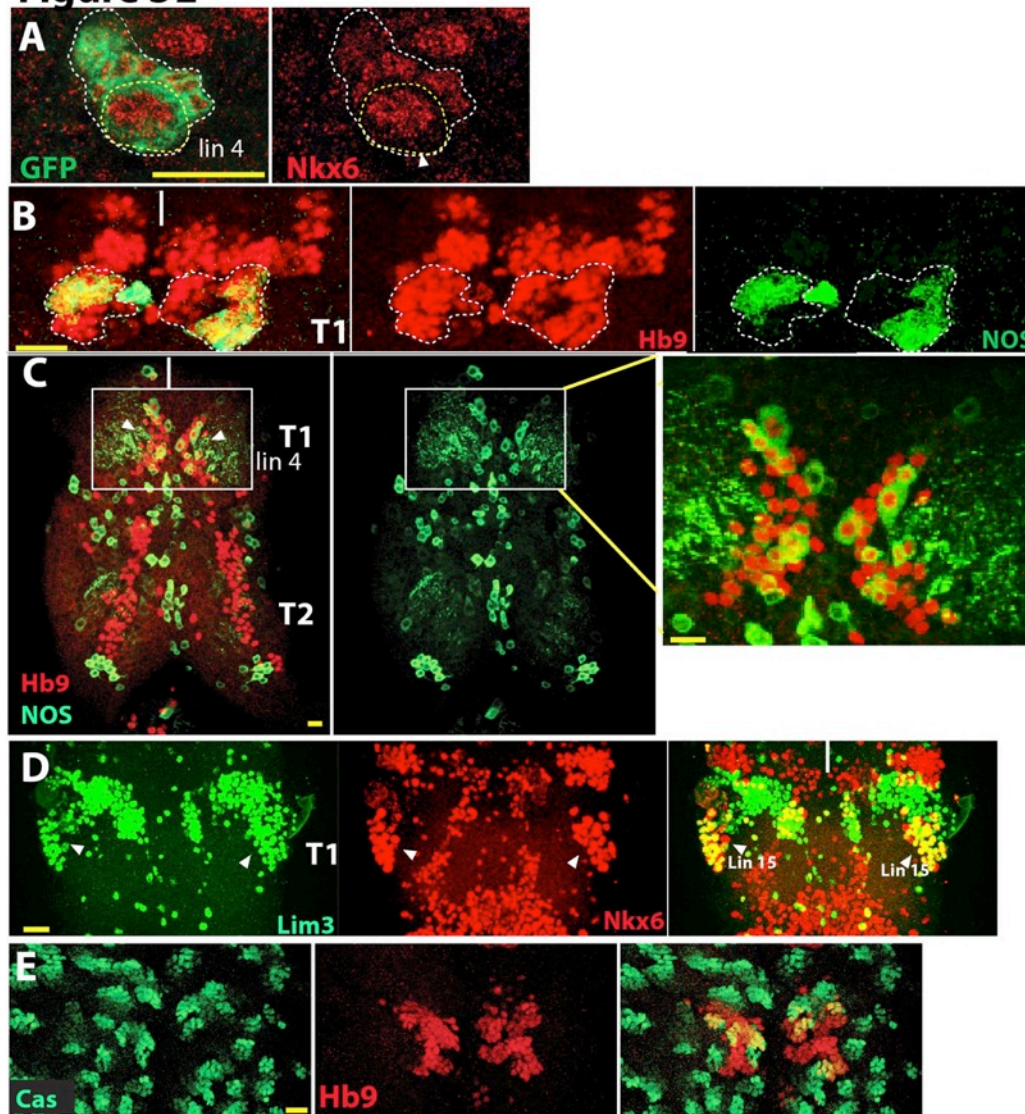


Figure S2) Hb9, Nkx6, Lim3, and Cas expression in thoracic post-embryonic lineages. (A) Wild-type lineage 4 MARCM clone in late-third instar larval CNS labeled for Nkx6 expression (red); arrowhead points to the NB, identified based on its large size and ventral position within clone. (B) Hb9 (red) and NOS (green) co-expression in lineage 4 neurons (dashed lines) of a late-third instar larva. (C) NOS expression (green) is maintained in Hb9<sup>+</sup> neurons (red; arrowheads) in the first thoracic segment of the adult nerve cord. (D) In the adult CNS, Lim3 and Nkx6 co-expression in lineage 15 neurons (arrowheads), all of which are motor neurons. For simplicity, only the first thoracic segment is shown. (E) In late-third instar larvae, Cas (green) and Hb9 (red) expression in post-embryonic neuronal lineages. In B-D T1, T2, and T3 refer to identity of thoracic segment. Anterior is up; white line marks ventral midline. Scale bar – 10μm.

**Figure S3**

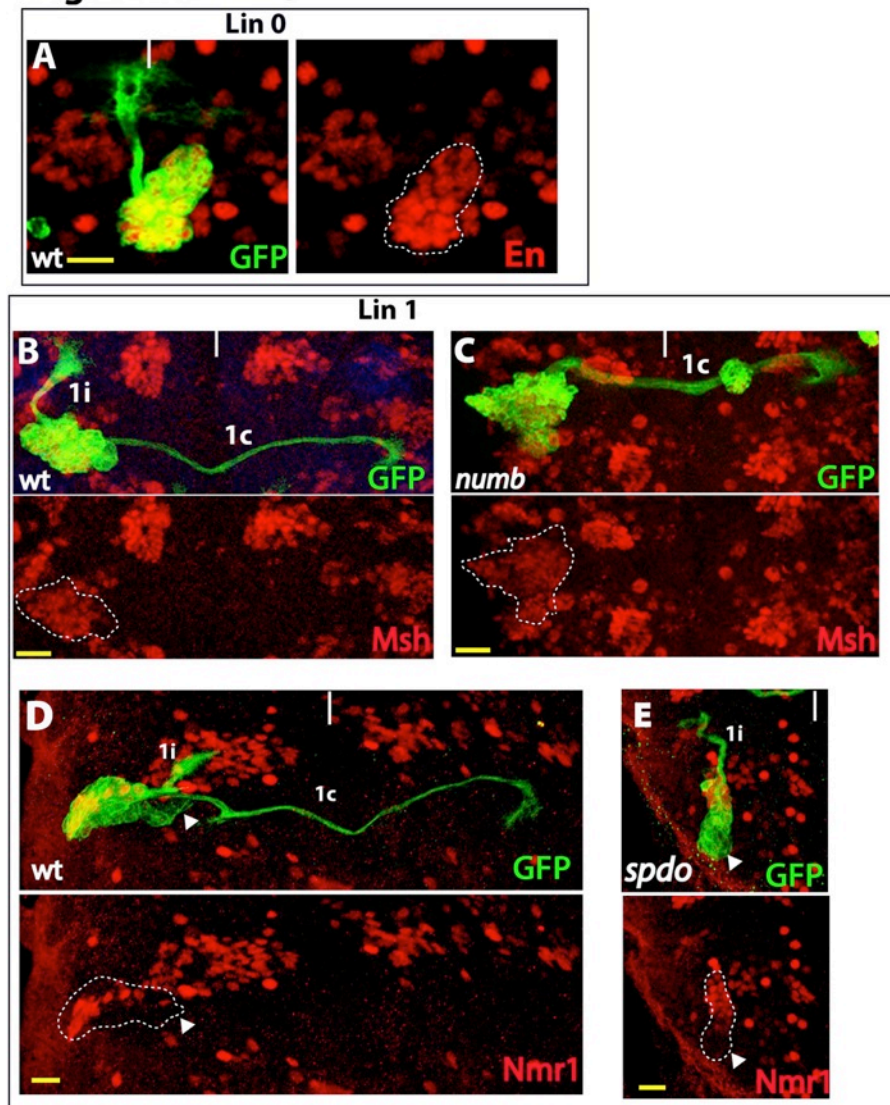


Figure S3) MARCM analysis of lineages 0 and 1 in late-third instar larvae.

(A) Wild-type lineage 0 clone labeled for Engrailed. (B, D) Wild type lineage 1 MARCM clones labeled for Msh (B) and Nmr1 (D) extend neurite bundles contralaterally (1c) and ipsilaterally (1i). (C) *numb* mutant lineage 1 MARCM clone labeled for Msh contains mostly Msh<sup>+</sup> neurons and extends a neurite bundle contralaterally (1c). (E) *spdo* mutant lineage 1 MARCM clone labeled for Nmr1 contains mostly Nmr1<sup>+</sup> neurons and extends a neurite bundle ipsilaterally (1i). Arrowheads in D, E point to the NB, identified based on size and ventral position within clone, which do not express Nmr1. Anterior is up; white bar indicates midline; dashed lines outline clone boundaries. Scale bar – 10µm.



**Figure S4**

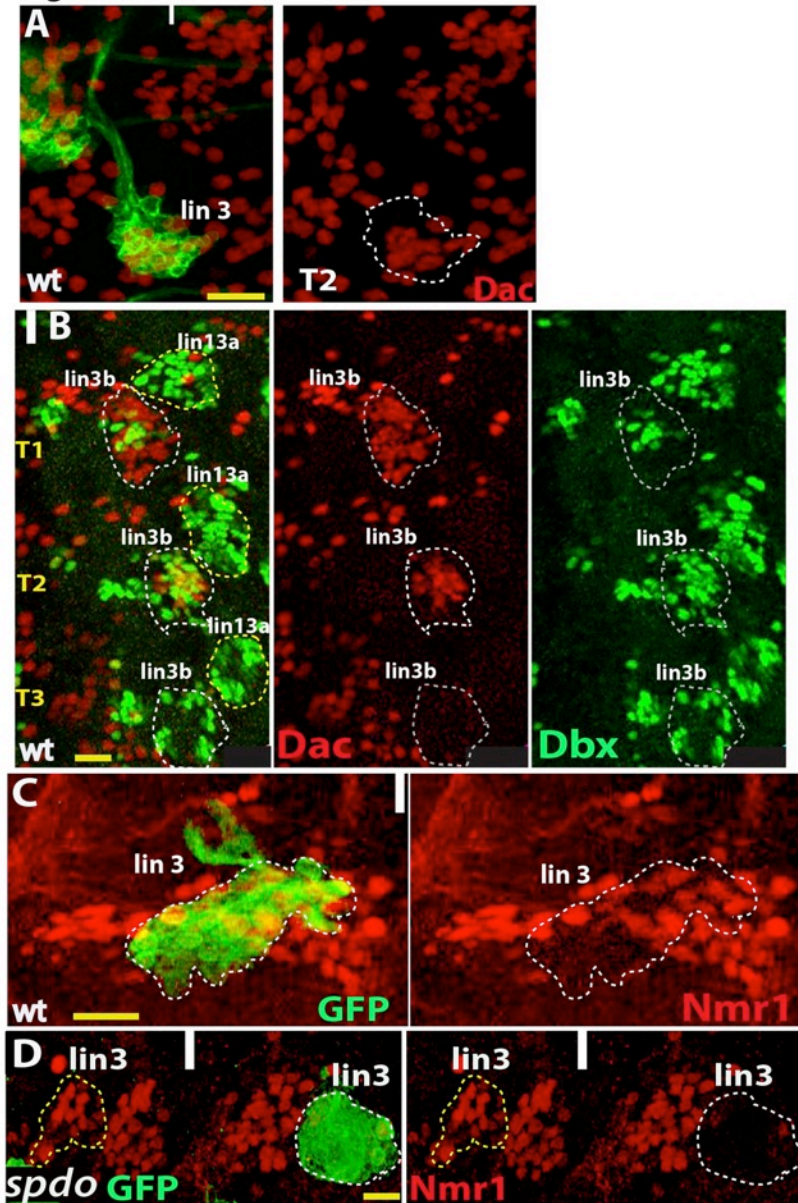


Figure S4) Dachshund, Dbx, Nkx6, and Nmr1 expression in lineage 3 in thoracic segments of late-third instar larvae.

(A) Wild-type lineage 3 MARCM clone labeled for Dachshund (Dac, red). (B) Dac and Dbx expression in lineage 3b neurons. Dashed lines outline lineage 3b and lineage 13a neurons. These neurons were mapped to the indicated lineages based on analysis of MARCM clones generated in other animals (e.g. see panel A). In A and B, T1, T2, and T3 refer to first, second, and third thoracic segments, the borders of which are identified by the  $Dbx^+$  lineage 13a, which only occurs in T1, T2, and T3. (C) Wild-type lineage 3 MARCM clones labeled for Nmr1 (red). (D) *spdo* mutant lineage 3 MARCM clone (green) lacks Nmr1 (red) expression; Nmr1 expression in lineage 3 neurons is shown in contralateral hemisegment. Anterior is up; white bar indicates midline. Scale bar = 10um.

**Figure S5**

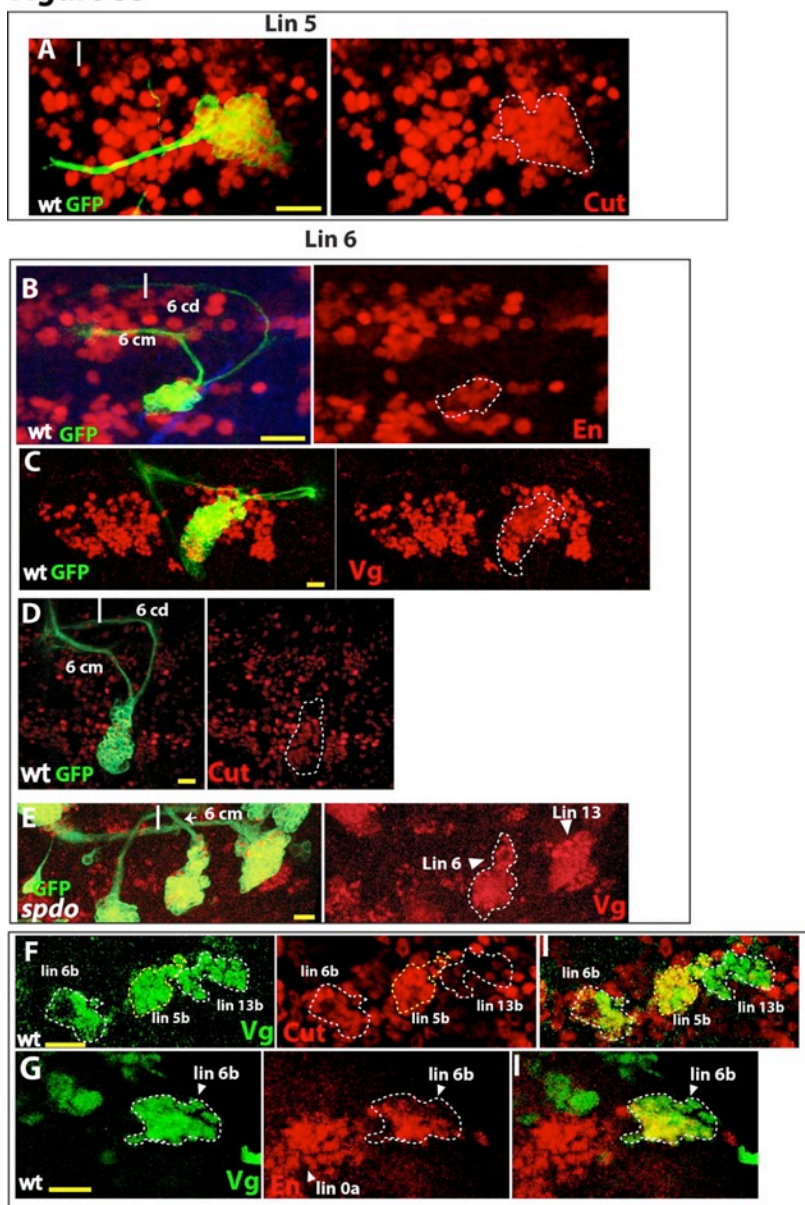


Figure S5) MARCM analysis of lineages 5 and 6 in late-third instar larvae.

(A) Wild-type lineage 5 MARCM clone labeled for Cut. (B-D) Wild-type lineage 6 MARCM clones labeled for Engrailed (En, B), Vestigial (Vg, C), and Cut (D) extend two neurite bundles contralaterally (6cm, 6cd). (E) In *spdo* mutant lineage 6 MARCM clones, most neurons express Vestigial; Vestigial expression in lineage 13 is also indicated. (F) Co-expression of Vg (green) and Cut (red) in lineage 5b and 6b neurons; Vestigial expression in lineage 13b neurons is also shown. (G) Co-expression of Vg (green) and Engrailed (red) in many lineage 6b neurons; Engrailed expression is also shown in lineage 0a neurons. Anterior is up; white bar indicates midline. Scale bar – 10µm.



**Figure S6**

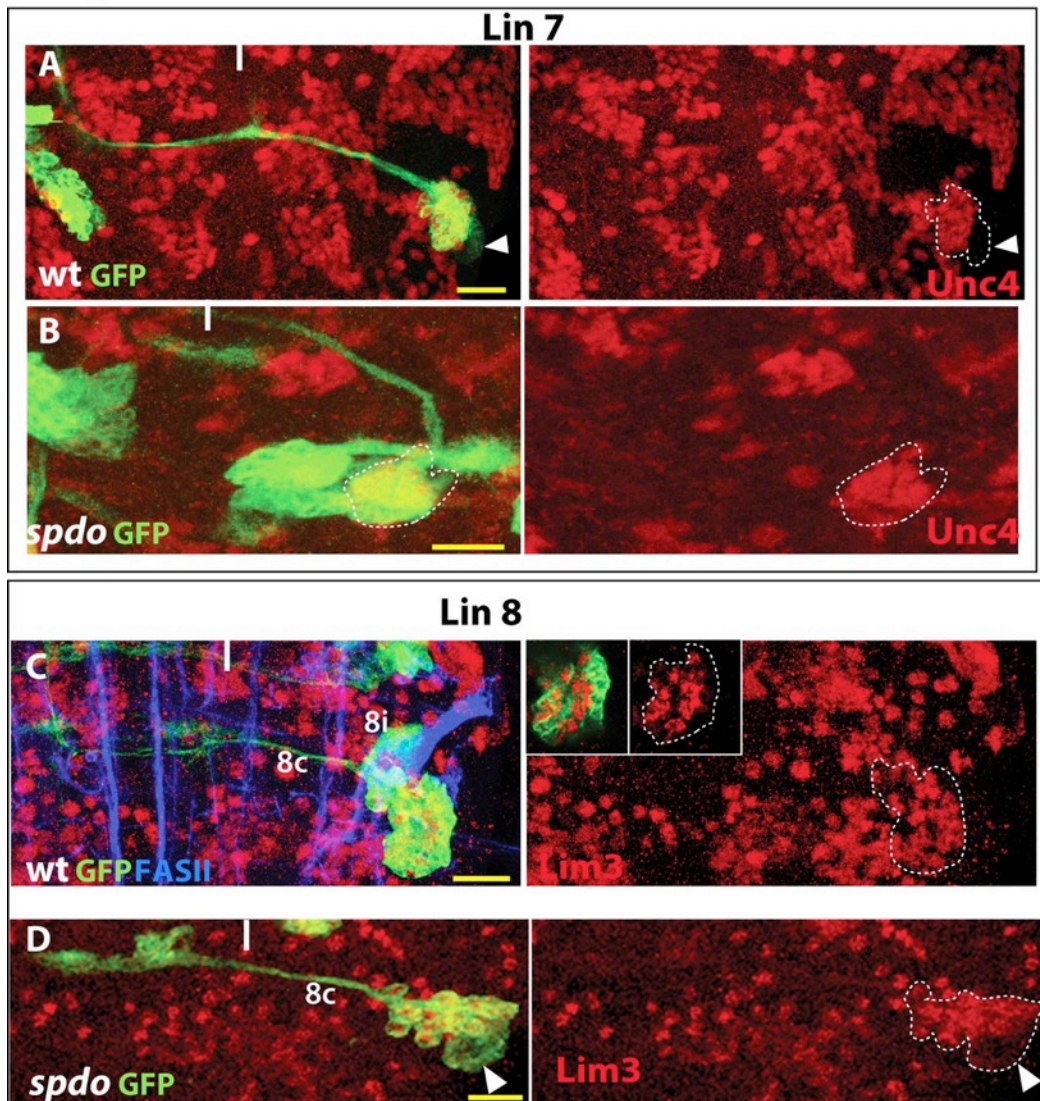


Figure S6) MARCM analysis of lineages 7 and 8 in late-third instar larvae.

(A) Wild-type lineage 7 MARCM clone labeled for Unc4 (red). Arrowhead points to NB, identified based on its large size and ventral position in clone, and adjacent GMCs that do not express Unc4. (B) *spdo* mutant lineage 7 MARCM clone labeled for Unc4 (red): the number of Unc4-expressing neurons increases. Note that two adjacent MARCM clones obscure boundary of lineage 7 clone. (C) Wild-type lineage 8 MARCM clone labeled for Lim3 (red) and Fas II (blue) extend neurite bundles ipsilaterally (8i) and contralaterally (8c). Inset shows a single Z-section of the clone revealing Lim3 expression in a subset of neurons in clone. (D) *spdo* mutant lineage 8 MARCM clone, most neurons express Lim3 and project axons contralaterally (8c). Arrowhead points to NB, identified based on size and ventral position, which lacks Lim3 expression. Anterior is up; white bar indicates midline; dashed lines outline clone boundaries. Scale bar – 10um.



Figure S7

# lin 11

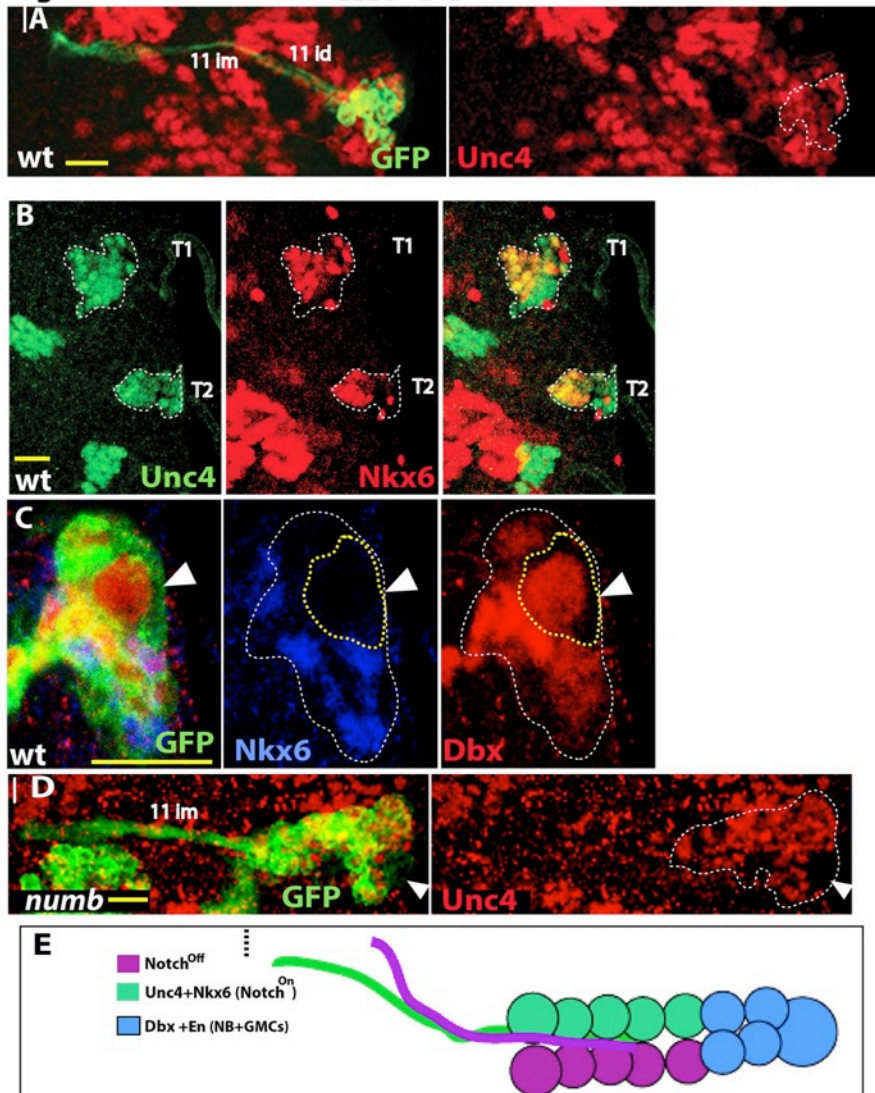


Figure S7) MARCM analysis of lineage 11 in late-third instar larvae.

(A) Wild-type lineage 11 MARCM clone labeled for Unc4 (red) extends a neurite bundle that forks as it approaches the midline (11im, 11id). (B) Unc4 (green) and Nkx6 (red) co-expression in lineage 11 neurons (arrowheads; dashed lines). Predicted boundaries of lineage 11 neurons based on analysis of Unc4 and Nkx6 expression in lineage 11 MARCM clones generated in other animals. T1 and T2 refer to first and second thoracic segments. (C) Dbx (red) and Nkx6 (blue) expression in a wild-type lineage 11 MARCM clone. Dbx expressed in the NB (arrowhead), identified based on its size, and adjacent GMCs. (D) *numb* mutant lineage 11 MARCM clone labeled for Unc4 (red): the clone is larger than in wild-type and contains mostly Unc4<sup>+</sup> neurons. Arrowhead points to NB, identified by its size and ventral position in clone, which does not express Unc4. (E) Schematic of gene expression in lineage 11. Anterior is up; white bar indicates midline; dashed lines outline clone boundaries. Scale bar – 10 μm.

**Figure S8** **lin 13**

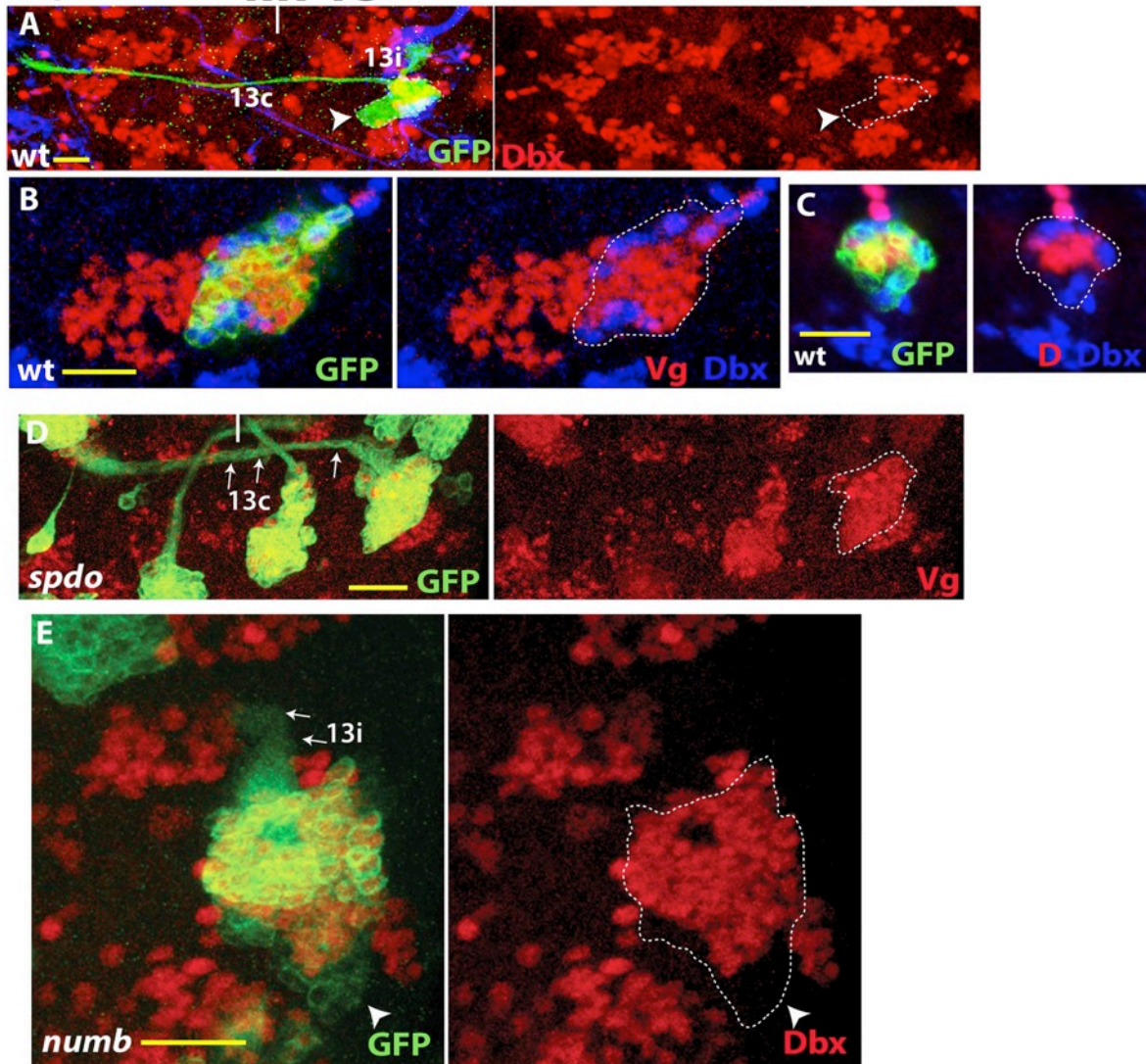


Figure S8) MARCM analysis of lineage 13 in late-third instar larvae.

(A) Wild-type lineage 13 MARCM clone labeled for Dbx (red) extends a short neurite bundle ipsilaterally (13i) and a long neurite bundle contralaterally (13c). Arrowhead points to Dbx<sup>+</sup> NB, identified based on size and position within clone. (B) Wild-type lineage 13 MARCM clone labeled for Vestigial (red) and Dbx (blue): the two proteins are expressed in mutually exclusive sets of neurons. (C) Wild-type lineage 13 MARCM clone labeled for Dichaete (red) and Dbx (blue): the two proteins are expressed in mutually exclusive sets of neurons. (D) *spdo* mutant lineage 13 MARCM clone labeled for Vestigial (red): most neurons in clone express Vg and extend the 13c neurite bundle contralaterally (arrows). (E) *numb* mutant lineage 13 MARCM clone: most neurons express Dbx and extend the short 13i neurite bundle ipsilaterally (arrows). Arrowhead points to the NB and adjacent GMCs identified based on size and ventral location in the clone, which lack Dbx expression. Anterior is up; white bar indicates midline. Scale bar: 10um.



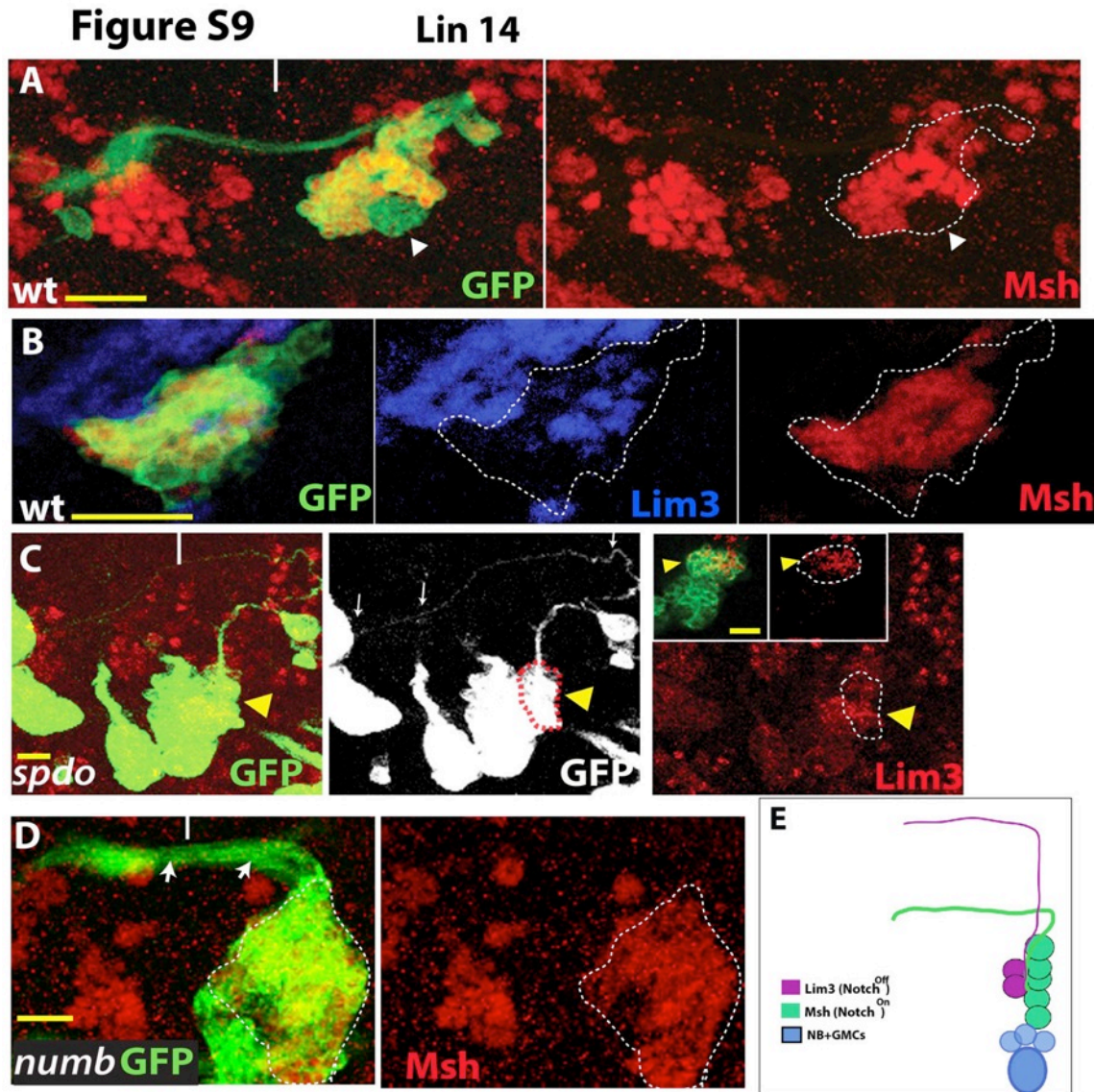


Figure S9) MARCM analysis of lineage 14 in late-third instar larvae

(A) Wild-type lineage 14 MARCM clone labeled for Msh (red) extends a thick ventral neurite bundle contralaterally and a thin dorsal neurite bundle contralaterally, the latter being too faint to be visible in the image. Arrowhead points to the Msh<sup>+</sup> NB, identified by its size and position in clone, and adjacent GMCs. (B) Wild-type lineage 14 MARCM clone labeled for Lim3 (blue) and Msh (red): the two proteins are not co-expressed in neurons of this lineage. (C) *spdo* mutant lineage 14 MARCM clone (dotted lines) labeled for Lim3 expression: most neurons in the clone express Lim3 and extend a thin dorsal neurite bundle (arrows). Boundaries of lineage 14 mutant clone were determined by analysis of individual Z-sections as shown in inset. (D) *numb* mutant lineage 14 MARCM clone labeled for Msh expression: most neurons express Msh and extend a thick ventral neurite bundle (arrows). (E) Schematic of gene expression in lineage 14. Anterior is up; white bar indicates midline; dashed lines outline clone boundaries. Scale bar – 10µm.



**Figure S10**

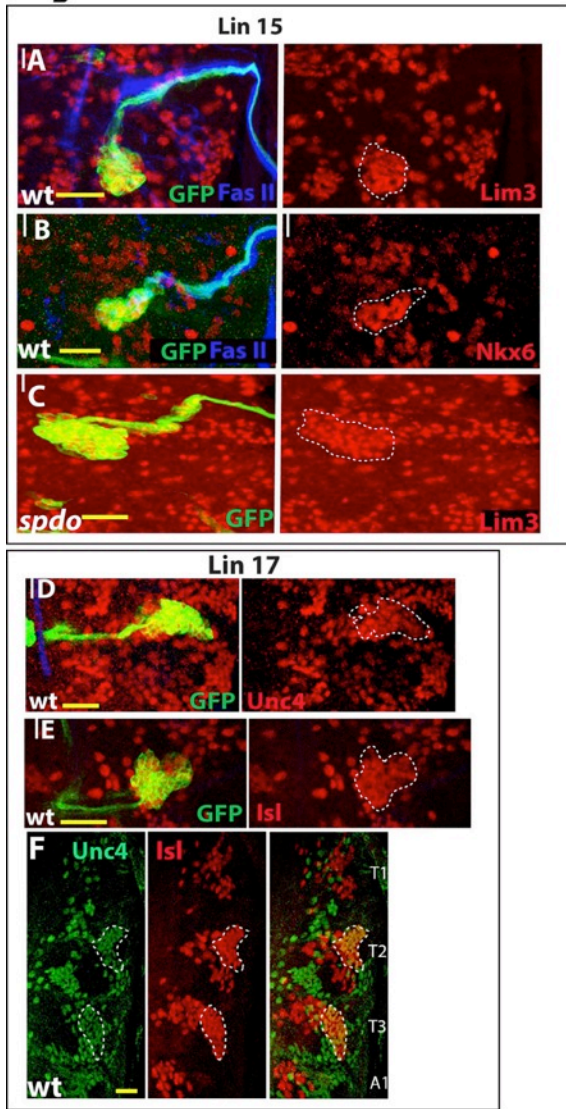


Figure S10) MARCM analysis of lineages 15 and 17 in late-third instar larvae (A, B) Wild-type lineage 15 MARCM clone labeled for Lim3 (red in A), Nkx6 (red in B), and Fas II (blue in A, B). (C) *spdo* mutant lineage 15 MARCM clone labeled for Lim3 (red): the clone is larger and most neurons express Lim3. (D, E) Wild-type lineage 17 MARCM clone labeled for Unc4 (D) and Isl (E) extends an ipsilateral neurite bundle towards the midline. (F) Co-expression of Unc4 (green) and Isl (red) in lineage 17 occurs in the second and third, but not first, thoracic segments. Anterior is up; white bar indicates midline. Scale bar: 10um.

**Figure S11**

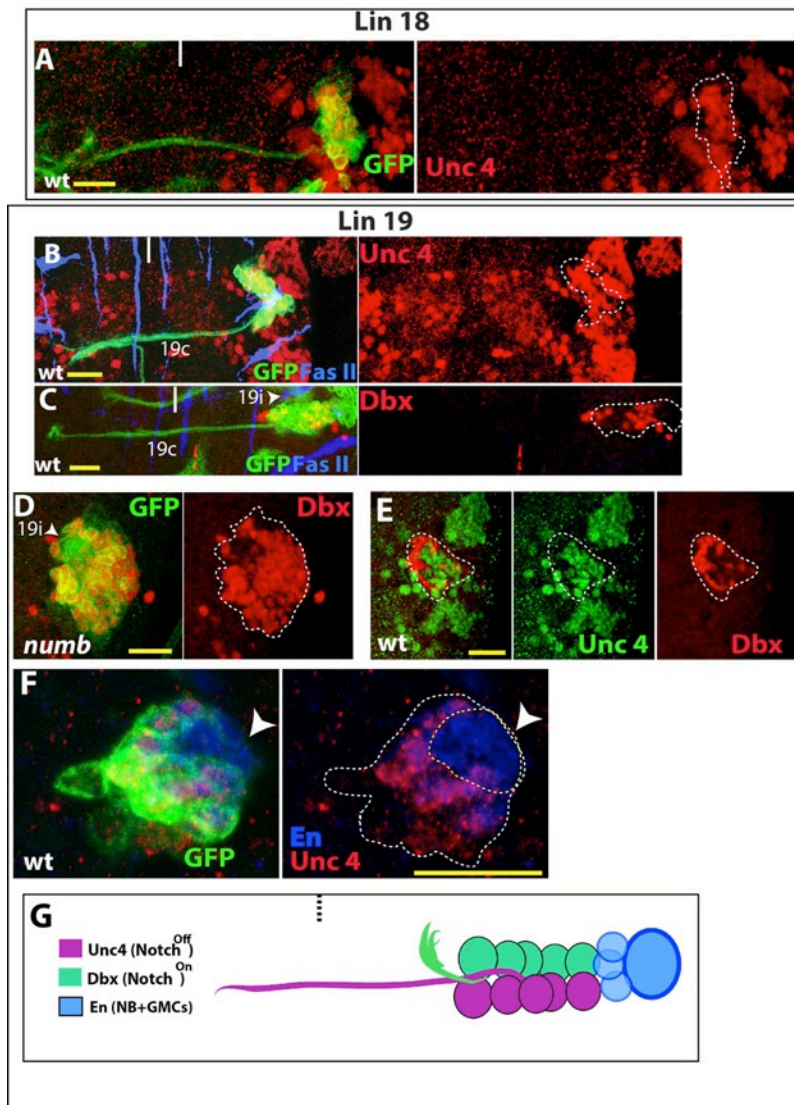


Figure S11) MARCM analysis of lineages 18 and 19 in late-third instar larvae  
 (A) Wild-type lineage 18 MARCM clone labeled for Unc4 (red) extends a contralateral axon projection. (B, C) Wild-type lineage 19 MARCM clone labeled for Unc4 (red, B) and Dbx (red, C) extends a short ipsilateral neurite bundle (19i, C) and a long contralateral neurite bundle (19c). (D) *numb* mutant lineage 19 MARCM clone labeled for Dbx: most neurons express Dbx. (E) Dbx (red) and Unc4 (green) expression in lineage 19: the two proteins are not co-expressed in neurons. (F) Wild-type lineage 19 MARCM clone labeled for Engrailed (blue) and Unc4 (red): arrowhead points to Engrailed<sup>+</sup> NB, identified based on its size and location, and adjacent GMCs. (G) Schematic of gene expression in lineage 19. Anterior is up; white bar indicates midline; dashed lines outline clone boundaries. Scale bar: 10µm.

## Figure S12

### Lin 20-22

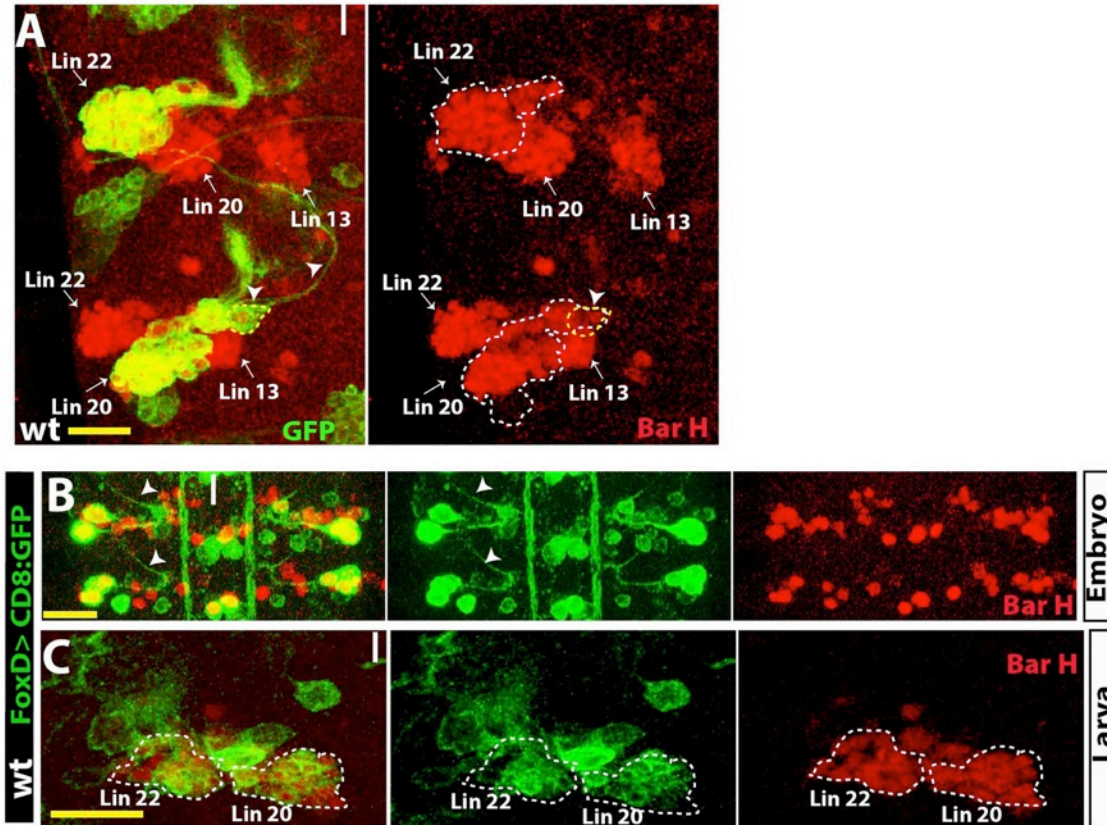


Figure S12) MARCM analysis of lineages 20 and 22

(A) Wild-type MARCM clones of lineages 20 and 22 in a late-third instar larva labeled for BarH1/2 (red). These lineages are identical in morphology; thus, the labeling is arbitrary. BarH1/2 is expressed in all neurons of lineage 20 and 22, including motor neurons. Arrowheads point to motor neuron and motor axon. (B, C) BarH1/2 (red) and GFP reporter gene expression driven by a FoxD enhancer (green) shown in the CNS of a late stage embryo (B) and late-third instar larva (C). (B) BarH1/2 is expressed in motor neurons that derive from NB 5-4 in embryo (arrowheads indicate motor neuron axons). (C) The FoxD enhancer drives reporter expression in BarH1/2-expressing neurons of post-embryonic lineages 20 and 22. Anterior is up; white bar indicates midline. Scale bar: 10um.



**Figure S13**

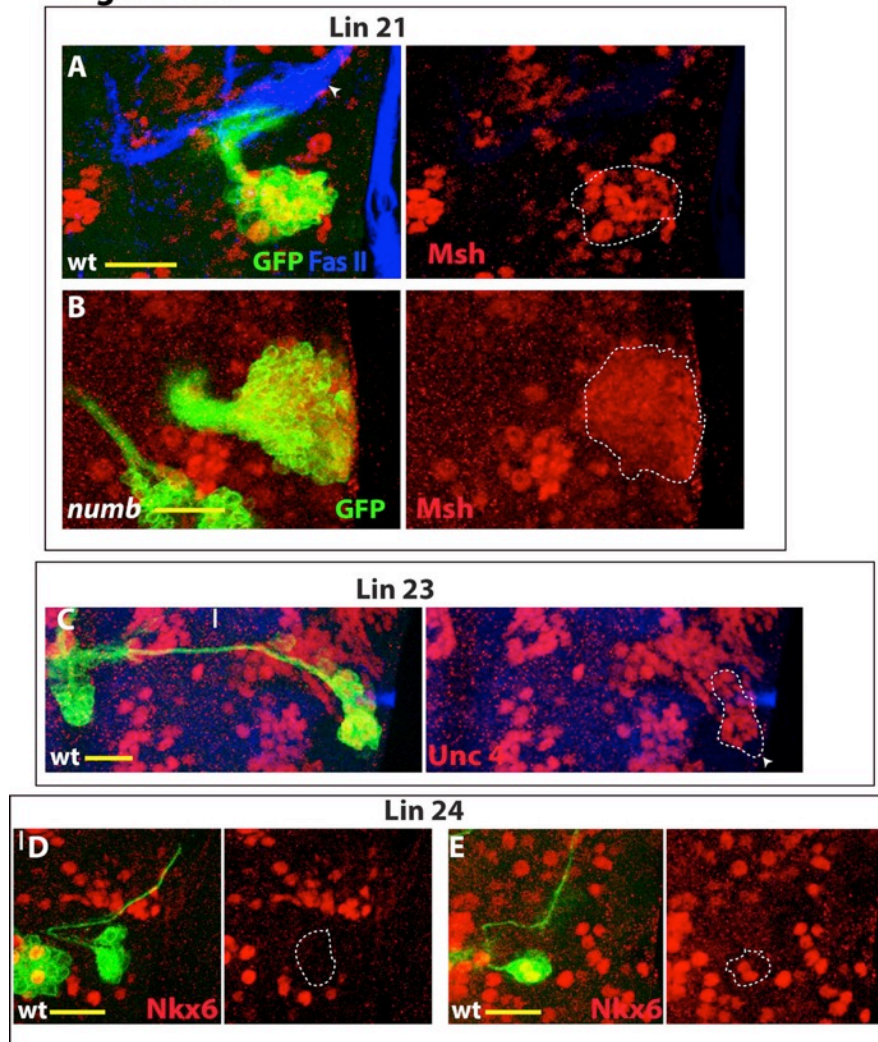


Figure S13) MARCM Analysis of lineages 21, 23 and 24 in late-third instar larvae  
 (A) Wild-type MARCM clone of lineage 21 labeled for Msh (red) and FasII (blue) extends an ipsilateral neurite, which terminates posterior to the lateral cylinder (arrowhead). (B) *numb* mutant lineage 21 MARCM clones labeled for Msh expression: the clones are bigger than wild-type; most neurons in them express Msh. (C) Wild-type MARCM clone of lineage 23 labeled for Unc4 (red) extends a contralateral neurite bundle. (D, E) Wild-type MARCM clones of lineage 24 labeled for Nkx6 (red): some clones lack Nkx6<sup>+</sup> neurons (D), and other clones contain Nkx6<sup>+</sup> neurons (F).

Anterior is up; white bar indicates midline; dashed lines outline clone boundaries. Scale bar: 10µm.

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