Conserved gene regulatory module specifies lateral neural borders across bilaterians

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The lateral neural plate border (NPB), the neural part of the vertebrate neural border, is composed of central nervous system (CNS) progenitors and peripheral nervous system (PNS) progenitors. In invertebrates, PNS progenitors are also juxtaposed to the lateral boundary of the CNS. Whether there are conserved molecular mechanisms determining vertebrate and invertebrate lateral neural borders remains unclear. Using single-cell-resolution gene-expression profiling and genetic analysis, we present evidence that orthologs of the NPB specification module specify the invertebrate lateral neural border, which is composed of CNS and PNS progenitors. First, like in vertebrates, the conserved neuroectodermal lateral border specifier Msx/vab-15 specifies lateral neuroblasts in Caenorhabditis elegans. Second, orthologs of the vertebrate NPB specification module (Msx/vab-15, Pax3/7/pax-3, and Zicref-2) are significantly enriched in worm lateral neuroblasts. In addition, like in other bilaterians, the expression domain of Msx/vab-15 is more lateral than those of Pax3/7/pax-3 and Zicref-2 in C. elegans. Third, we show that Msx/vab-15 regulates the development of mechanosensory neurons derived from lateral neural progenitors in multiple invertebrate species, including C. elegans, Drosophila melanogaster, and Ciona intestinalis. We also identify a novel lateral neural border specifier, ZNF703/tp1-1, which functions synergistically with Msx/vab-15 in both C. elegans and Xenopus laevis. These data suggest a common origin of the molecular mechanism specifying lateral neural borders across bilaterians.

C. elegans | neural plate border | neural border | Msx/vab-15 | ZNF703/tp1-1

The vertebrate neural border is a transient embryonic domain located between the neural plate and nonneurogenic ectoderm from late gastrulation to early neurulation. The neural border is composed of the lateral neural plate border (NPB) and preplacode ectoderm (PPE) subdomains (1, 2). The NPB and PPE give rise to the neural crest and placode, respectively, both of which undergo epithelial-to-mesenchymal transition/delamination, migrate in prototypical paths, and give rise to the peripheral nervous system (PNS) and many other cell types (3, 4). However, the NPB and PPE also have many different features (5). For example, the PPE is confined to the anterior half of embryos and does not contribute to the central nervous system (CNS), whereas the NPB is the lateral border of the whole neural plate and consists not only of progenitors for the PNS but also those for the CNS in the dorsal neural tube. The juxtaposed localization of the CNS neuroectoderm and PNS progenitors also occurs in the trunk of invertebrate embryos such as nematodes, arthropods, annelids, and urochordates (6–9), reminiscent of vertebrate NPB. In Caenorhabditis elegans, lateral neuroblasts (P, Q, and V5 cells) are located between the embryonic CNS and skin from the birth of these cells (10). In addition, worm lateral neuroblasts possess several key cellular and developmental features of vertebrate NPB. For example, the medially located P neuroblasts display nuclear migration and give rise to multiple cell types, including CNS motor neurons (10). The laterally located V5 and Q neuroblasts give rise to PNS neurons; that is, V5 neuroblasts give rise to the mechanosensory neuron PVD, whereas QL and QR neuroblasts give rise to the migratory mechanosensory neurons AVM and PVM, respectively (10). Furthermore, Q neuroblasts undergo delamination and migrate along prototypical paths while dividing and differentiating (10), similar to the neural crest in vertebrates and mechanosensory bipolar tail neurons (BTNs) in tunicate (3, 9). At the molecular level, Msx/vab-15 establishes the identity of the lateral boundary of the neuroectoderm in fly, annelids, amphioxus, lamprey, and higher vertebrates. Also, their Msx/vab-15–expressing domains include both CNS and PNS progenitors (8, 11–14). Worm Msx/vab-15 expression was observed in lateral P neuroblasts, progenitors of larval CNS motor neurons. Furthermore, development of mechanosensory neurons is defective in Msx/vab-15 mutants (15). These evolutionary echoes suggest that studies in C. elegans may reveal new clues regarding whether there is a conserved molecular mechanism underlying development of worm lateral neuroblasts and vertebrate NPB. Using genetic analysis, we show here that worm Msx/vab-15 expresses in and specifies all lateral neuroblasts. We also did comparative analysis in multiple species, including C. elegans, Drosophila melanogaster, and Ciona intestinalis, to show that Msx/vab-15 regulates the development of mechanosensory neurons derived from their lateral neural borders, similar to its role in the vertebrate NPB. We profiled the expression of 90% conserved transcription factors in worm trunk ectoderm at the resolution of single cells and found that orthologs of the vertebrate NPB specification module are significantly enriched in worm lateral

Significance

The lateral neural plate border (NPB) gives rise to the neural crest, one of the precursors of the peripheral nervous system (PNS) and generally considered an evolutionary innovation of the vertebrate lineage. Recently, it has been reported that a rudimentary neural crest exists in protvertebrate Ciona, but whether this is true in other invertebrates and there is conserved molecular machinery specifying the NPB lineage are unknown. We present evidence that orthologs of the NPB specification module specify lateral neural progenitor cells in several invertebrates, including worm, fly, and tunicate. We propose that an ancient lateral neuroblast gene regulatory module was coopted by chordates during the evolution of PNS progenitors.

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neuroblasts. More interestingly, a novel NB specifier, ZNF703/tp1-1, was identified and shown to be synergistic with Msx/vab-15 in regulating lateral neural progenitors in both C. elegans and Xenopus laevis, providing another molecular clue to trace the origin and evolution of the lateral neural border.

Results

Msx/vab-15 Specifies Lateral Neuroblasts in C. elegans. A previous study in C. elegans did not detect Msx/vab-15 reporter expression in V5 and Q neuroblasts (15). To reveal the expression pattern of endogenous Msx/vab-15, we generated a knockin worm strain in which GFP was integrated into the 3′ end of the Msx/vab-15-coding sequence in the genome. Worms of the knockin strain do not show any detectable phenotype, suggesting the GFP knockin does not disturb the expression of vab-15. The knockin strain shows significant Msx/vab-15 expression in embryonic AB.p(lr)/apapa, which is the mother cell of Q and V5 neuroblasts (Fig. 1 A). After the division of this mother cell, Msx/vab-15 expression turns off in V5 neuroblasts but is maintained in Q cells until they divide (Fig. 1 B and C). P-neuroblast expression of Msx/vab-15 starts before hatching, is maintained while P nuclei migrate into the ventral midline in early L1-stage larvae, and shuts down after P neuroblasts divide (Fig. 1 B and D).

To investigate the function of Msx/vab-15 during worm development, we phenotyped Msx/vab-15 mutants. These mutants appear to have wild-type embryonic cell lineage and produce lateral neuroblasts that can even divide in larvae (Figs. 2 and 3B and Fig. S1), but their P neuroblasts are significantly defective in nuclear migration, and those that migrate into the midline frequently fail to give rise to hypodermal daughter cells or neurons (Fig. 2 and Table 1). Q neuroblasts in mutants show a defect in migration too (Fig. 3B), and fail to give rise to mechanosensory neurons with nearly full penetrance (Fig. 3D and F and Table 1). Similarly, V5 neuroblasts in mutants also fail to give rise to neurons (Fig. 3H).

V5 neuroblasts show a strong phenotype in Msx/vab-15 mutants even though Msx/vab-15 expression shuts down right after the separation from its sister cell Q in late embryos, indicating Msx/vab-15 specifies the cell fate of these neuroblasts at the embryo stage, instead of functioning at the larval stage. To dissect the temporal role of Msx/vab-15, we generated an Msx/vab-15 somatic knockout strain in which CAS9 is driven by a heat-shock promoter. Disrupting Msx/vab-15 at an early stage by heat shocking embryos results in a significant migration and differentiation defect of the lateral neuroblasts. On the contrary, disrupting Msx/vab-15 in newly hatched L1 larvae does not lead to a significant differentiation phenotype in V5 and Q neuroblasts (Table 1). Similarly, knocking out Msx/vab-15 at the early L1 stage does not affect the development of P neuroblasts, either (Table 1). In summary, our endogenous gene-expression analysis and temporal knockout assay show that Msx/vab-15 specifies the lateral neuroblasts at their initial formation and regulates the development of both the CNS and PNS.

Msx/vab-15 Regulates Development of PNS Derived from Lateral Neuroblasts in Fly. As in nematodes and vertebrates, Msx/vab-15 in D. melanogaster is also expressed in the lateral neural border encompassing both CNS and PNS progenitors (7, 16). Furthermore, by Msx/vab-15 is a specifier of lateral CNS progenitors (7, 16). To test whether Msx/vab-15 also regulates the PNS in fly, we generated an Msx/vab-15 mutant, ms*24, in D. melanogaster and focused on the lateral clusters of the mechanosensors, the chordotonal organs. In wild-type Drosophila embryos, each chordotonal organ is made of four cells derived from a single sense organ precursor (SOP) cell, including a neuron, glial cell, and two supporting cells. During PNS development, five SOPs for the chordotonal organs are formed in the Msx/vab-15–expressing domain lateral to the neuroectoderm in each segment (7). The progeny cells of these five SOPs migrate dorsally and give rise to a stretch of five characteristically arranged chordotonal organs (17). However, in ms*44 mutants, the chordotonal organs contained fewer neurons and glial cells (Fig. 4A). Furthermore, the migration of the progeny cells derived from the SOPs in ms*44 embryos appeared to be significantly desynchronized among different segments, as indicated by the position of the glial cells, whereas in the wild-type embryos these cells migrated to a similar position in all segments (Fig. 4B). So, like its role in nematodes and vertebrates, Msx/vab-15 in fly regulates PNS development in the lateral neural border.

Systematic Prediction of Lateral Neuroblast Regulators in C. elegans. To comprehensively reveal regulators that distinguish lateral neuroblasts from trunk CNS and the skin cells flanking them, we generated reporter transgenic lines for 339 out of all 377 worm transcription factors that have human orthologs (18) (Dataset S1). Seventy-five percent of our cloned promoters are more than 2-kb-long upstream regions. Other promoters are less than 2 kb,
mostly because their upstream intergenic regions are short. It has been demonstrated that 2 kb upstream of a worm gene accurately drives gene expression in most cases (19, 20). Therefore, the majority of our reporters should largely recapitulate endogenous gene expression. We profiled their expression in trunk ectoderm of newly hatched L1 larvae at the resolution of single cells using an imaging analysis pipeline (19) (Fig. 5). The annotation of L1 trunk ectodermal cells is highly reliable because of their well-separated and invariable position and characteristic nuclear morphology (10). All cell annotations were manually verified to be precise.

To predict neuroblast regulators, we defined a neuroblast-enrichment score as the logarithmic ratio of expression in neuroblasts over expression in skin or ventral cord motor neurons (see Materials and Methods for details). To test the prediction power of our neuroblast-enrichment score, we functionally validated seven top-scored genes: ZNF703/tp-1, Msx/vab-15, Atonal/lin-32, Zic/ref-2, Znf/ztf-6, Ash/hlh-3, and Pax3/7/pax-3 (Fig. 5). In addition to Msx/vab-15 described in this study, Atonal/lin-32 is a well-known master regulator of Q and V5 neuroblast development (21, 22). Our genetic analysis showed that Pax3/7/pax-3 and Zic/ref-2 are required for both migration and differentiation of P neuroblasts.

Fig. 2. Msx/vab-15, Pax3/7/pax-3, and Zic/ref-2 are required for migration and differentiation of P neuroblasts. Somatic knockout was induced by heat shocking embryos carrying the Phsp::CAS9 transgene. The Pref-2::H1::mCherry reporter was used as a marker to label P-neuroblast progeny. The Punc-25::GFP reporter was used to label D-type ventral cord motor neurons. VDs are P-derived D-type neurons. (A, E, I, and M) Wild-type L2 larvae, when all P progenies arrive at the ventral midline (A) and give rise to hypodermal Pn.p (E) and neurons (I and M) distributed with stereotypical patterns. Arrowheads indicate progenies of P neuroblasts that are defective in migration, as shown retained in the lateral body. In mutant worms, some P neuroblasts cannot migrate to the ventral cord and retain in the middle body of worm (B–D). Among P neuroblasts that successfully migrated into the ventral cord, some failed to give rise to hypodermal Pn.p (F–H), neurons (I–L), such as VD neurons (K–P). Dotted circles indicate missing nuclei. Each worm was arranged such that its anterior end was to the right and its ventral midline was at the top. Green fluorescence in pharynx represents the Pmyo-2::GFP coinjection marker (K, L, O, and P). All of the worms detected were at L2 stage. (Scale bar, 10 μm.)
Their progeny (47). AVM/PVM and PVD (59). Arrowheads indicate the PLM neuron as a landmark. (A) Pmc-4::H1::GFP; (B) Pmc-10::H1::mCherry; (C)egl-17::myr:mCherry; (D) Pme-4::H1::GFP; (E) Pmc-4::H1::GFP; (F) Pme-4::H1::GFP; (G) Pmc-4::H1::GFP; (H) Pmc-4::H1::GFP.

Fig. 3. Mxs/vab-15 is required for the development of worm lateral PNS progenitors. The boundary of the worm body is delineated by dashed lines. (A and B) Migration of Q neuroblast progeny cells. Dotted circles indicate Q daughter or granddaughter cells. Examined worms were at late L1 stage. Q daughters were separating due to their different migration rate in wild type (A), whereas they failed in migration in the vab-15 mutant so that even their granddaughters are clustered together (B). (C-F) Q-derived mechanosensory neurons AVM (arrow in C) and PVM (arrow in E) were missed in the vab-15 mutant (D and F). Examined were L2 larvae. Arrowheads indicate ALM (C and D) or PLM (E and F) neurons as a landmark. (G and H) V5-derived mechanosensory neuron PVD (arrow). egl-17: markers of Q neuroblasts and their progeny (47); mec-4: markers of AVMPVM (58); mec-10: markers of AVMPVM and PVD (59). Arrowheads indicate the PLM neuron as a landmark. Examined were L3 larvae. (Scale bars, 10 μm.)

nuclei (Fig. 2 and Table 1). However, mutants of the other three candidates (ZN7F03/tlp-1, Ash/hlh-3, and Znt/zf-6) show a weak to marginal phenotype (Table 1). ZN7F03/tlp-1 is expressed both in P neuroblasts and the mother cells of V5 and Q, similar to Mxs/vab-15 (Figs. 5 and 6A), so we further investigated the genetic interaction between ZN7F03/tlp-1 and Mxs/vab-15. Although P neuroblasts appear, there are no phenotypes in ZN7F03/tlp-1 single mutants. But P neuroblasts in ZN7F03/tlp-1; Mxs/vab-15 double mutants show a stronger defect in migration and differentiation than those in Mxs/vab-15 single mutants (Fig. 6B and C). Q neuroblasts display little migration defect in ZN7F03/tlp-1 single mutants, whereas ZN7F03/tlp-1; Mxs/vab-15 double mutants showed a stronger phenotype than Mxs/vab-15 single mutants (Fig. 6D). Significant synergistic effect was also observed upon V5 differentiation (Fig. 6E). Therefore, ZN7F03/tlp-1 is a novel neuroblast regulator that functions as a modulator of Mxs/vab-15 activity in worms. These data demonstrated that the neuroblast-enrichment score is strongly related to the molecular mechanisms specifying worm lateral neural precursor cells. Then, examining the correlation between the worm neuroblast-enrichment score and orthologs of the NPB specification module can suggest the conservation between the worm lateral neuroblasts and the vertebrate NPB.

The NPB Specification Module Is Conserved Between Worm and Chordates. A conserved four-gene NPB specification module (Mxs/vab-15, Pax3/7/pax-3, Zic/ref-2, and AP2/apf-1) establishes the identity of the NPB from jawless fish lamprey to mouse (11, 23). This gene set has neuroblast-enrichment scores significantly higher than those of other genes (Mann–Whitney test based on neuroblast-enrichment score, P value < 1.8 × 10−4). Actually, three out of these four genes are among the top-seven neuroblast-enriched genes (Fig. 5). Although the expression patterns of these three NPB specifiers (Mxs/vab-15, Pax3/7/pax-3, and Zic/ref-2) are overlapping, the expression domain of Mxs/vab-15 in many bilaterians expands more laterally than those of Pax3/7/pax-3 and Zic/ref-2 (8, 24). Consistently, only Mxs/vab-15, but not Pax3/7/pax-3 and Zic/ref-2, is expressed and plays a critical role in the most lateral V5 and Q neuroblasts, which are PNS progenitors in worm (Fig. 1 and Table 1). Another example of more lateral Mxs/vab-15 expression exists in the urochordate C. intestinalis. Two NPB cells (b8.18 and b8.21) give rise to BTNs, which are the counterparts to vertebral dorsal root ganglion (DRG) mechanosensory neurons (9). These two NPB cells express Mxs/vab-15 and Pax3/7/pax-3 but not Zic/ref-2 (9, 25). Nevertheless, our gene-knockdown experiments showed that Mxs/vab-15 is required for BTN differentiation (Fig. 4C). Therefore, Mxs/vab-15 regulates the development of the PNS from the lateral neural border in both Protostomia and Deuterostomia, regardless of the coexistence of other NPB specifiers such as Zic/ref-2.

ZN7F03/tlp-1 Is a Novel NPB Specifier in X. laevis. We have identified ZN7F03/tlp-1 as a novel regulator of lateral neuroblasts cooperating with Mxs/vab-15 in C. elegans. To investigate whether ZN7F03/tlp-1 has conserved function in vertebrates, we examined its expression and function in X. laevis. In situ RNA hybridization showed that ZN7F03/tlp-1 is expressed at the posterior ectoderm except for the dorsal midline in frog gastrulae. During neurulation, the expression of ZN7F03/tlp-1 in nonneurogenic lateral ectoderm diminishes so as to become enriched in the NP and neural crest (Fig. 6F). As in C. elegans, disturbing ZN7F03/tlp-1 alone has no detectable phenotype. But moderate knockdown of both ZN7F03/tlp-1 and Mxs/vab-15 significantly down-regulates the trunk expression of the neural crest specifier FoxD3, migratory DRG progenitor marker id5, and nonmigratory Rohon–Beard mechanosensory neuron progenitor marker Runx1 (Fig. 6G), with no detectable effect on cell proliferation or apoptosis (Fig. S2). High dosage Mxs/vab-15 morpholino blocks the differentiation of NPB in Xenopus, while low dosage does not (Fig. 6G and Fig. S3). Hence, ZN7F03/tlp-1 acts as a modulator of Mxs/vab-15 in both worm lateral neuroblasts and the vertebrate NPB, supporting our hypothesis on the conservation of the gene regulatory module specifying the lateral neural border across bilaterians.

Discussion

An important feature of Bilateria is the centralized nervous system, in which the CNS in the head and trunk midline integrates and processes sensory information from the PNS. Developmental processes to generate central nervous systems differ dramatically among different bilaterian phyla. In nema- todes such as C. elegans, cell fate is coupled with cell lineage and neurogenesis is multiclonal; that is, many independent progenitor cells give rise to neurons (6, 10). However, in vertebrate gastrulae, accompanying the determination of the dorsoventral axis by BMP signaling, ectoderm in the dorsal midline thickens to form a neural plate, from which their CNS is derived. Between the neural plate and nonneurogenic ectoderm are the NPB and PPE, from which the majority of the vertebrate PNS is derived (24). How nervous system centralization patterns come into being is a long-standing question in neural development and evolution (3, 26).

Regarding the molecular mechanisms determining CNS development, comparative studies have provided evidence that the CNS of vertebrates, annelids, and arthropods originated from a
common bilaterian ancestor (8, 27). For example, both the neuroectoderm of annelids and neural plate of vertebrates are subdivided into a sim/ilh-34-expressing midline and longitudinal Nk2.2/celh-22-, Nkx6/cog-1-, Pax6/vab-3-, Pax3/7/pax-3-, and Msa/vab-15-expressing domains from medial to lateral, likely representing the molecular architecture of trunk CNS in their last common ancestor, Urbilateria (8). However, there has been debate on the common origin of CNS centralization because some sister or outgroup species of annelids, arthropods, and chordates lack a centralized ventral cord (26, 28). For example, hemichordates differ from major chordates by possessing a diffuse nervous system and do not have mediolateral neural patterning (29). Characterization of the molecular mechanisms of neural patterning in distantly related bilaterians should provide a broader view of the evolution of nervous system centralization (28).

Nematodes diverged from arthropods about 550 Mya, close to the origin of Ecdysozoa (30). Unlike annelids and arthropods, nematodes have an unsegmented body plan and no appendages. C. elegans burrows in rotten matter and has a feeding organ isolated from the rest of the animal (31, 32). Correspondingly, its nervous system is more modestly organized than those in annelids and arthropods. The complex mediolateral patterning of trunk CNS is largely absent in worm, where there are only motor neurons in its ventral cord. Nevertheless, Pax3/7/pax-3 and Msa/vab-15 still express in the lateral border of the CNS neuroectoderm in worm (15, 33), consistent with the conserved expression patterns of these two lateral genes in arthropods, annelids, and chordates (8). Our genetic analysis shows that both Msa/vab-15 and Pax3/7/pax-3 specify P neuroblasts, which are worm CNS progenitors, similar to their roles in the lateral CNS progenitors in fly and vertebrates (11, 16, 34–36). Combining our data with the existing evidence of the common origin of trunk CNS from fly to vertebrates (8, 27), it is more likely that nematodes inherited the Msa/vab-15 specification of P neuroblasts from Urbilateria than the nematode branch lost the conserved lateral domain after diverging from fly and then regenerated it independently.

In addition to the conserved CNS molecular module shown in C. elegans, our data suggest that the molecular mechanism defining the lateral neural border, from which the majority of the PNS is derived, is also conserved across bilaterians. First, the lateral neural borders in nematodes, fly, annelids, and urochordates show conserved neural patterning where the medial portion of the Msa/vab-15-expressing domain gives rise to the CNS whereas the lateral portion generates the PNS. Second, their lateral PNS progenitors exhibit several key features of vertebrate PNS progenitors, such as migration and differentiation into mechanosensory neurons (7, 8, 10, 13, 34). Our genetic analysis also shows that Msa/vab-15 specifies these PNS progenitors in nematodes, fly (Fig. S4), and urochordates, similar to its role in the vertebrate NPB and PPE. The PPE is the neurogenic ectoderm anterolateral to the NPB, and is considered the epidermal subdomain of the vertebrate neural border that mostly contributes to cranial sensory systems (1, 37). The PPE also gives rise to mechanosensory hair cells of lateral lines in the trunk of anamniote vertebrates and those in vertebrate inner ears (1). The development of the mechanosensory hair cells in vertebrates and that of the mechanosensory neurons in worm and fly both depend on Atoh1/lin-32 (22, 38). On the contrary, Ciona BTNs and vertebrate DRG neurons require Neurogenin/ingr-1 (9). Hence, more molecular and comparative studies are needed to understand the evolutionary relationship of these mechanosensory cell types. Nevertheless, our study on the neural borders suggests the conservation of the molecular mechanism underlying the specification of PNS progenitors across bilaterians (39).

In theory, the similarity between the invertebrate and vertebrate lateral neural border can be explained by either common origin or convergence of independent evolution. To distinguish between these two hypotheses, it is necessary to compare molecular mechanisms specifying lateral neural borders in various bilaterians. Unlike the vertebrate NPB and neural crest, the molecular mechanism underlying the specification of worm lateral neuroblasts still remains largely unknown after years of genetic study (33, 40, 41). Our systematic expression profiling of 90% conserved transcription factors in C. elegans revealed that three NPB specifiers (Msa/vab-15, Pax3/7/pax-3, and Zic/ref-2) are coexpressed in worm lateral neuroblasts and that Msa/vab-15 expression is more lateral than that of Pax3/7/pax-3 and Zic/ref-2, like their expression patterns in neural borders of vertebrates, urochordates, amphioxus, and likely annelids (7, 8, 11, 13, 24). Furthermore, we showed that Msa/vab-15 regulates the differentiation of PNS progenitors in worm and fly, similar
Msx neurons in array of chordotonal organs in wild type but disorganized with only four are revealed by 22C10 immunostaining (identified a novel lateral neural border specifier, in PNS progenitors across bilaterians (3, 43). Finally, we have morpholino-injected larva represent BTNs with the down-regulated progenitors in other invertebrates. ( Dataset 51. 45). Each vector was injected into worms mixed with the Pyro-2-GFP conjugation marker. Synchronized embryos of transgenic strain were transferred to OP50-seeded nematode growth medium (NGM) plates and heat shocked as described, at 33 °C for 1 h (47). The embryos then grew at 20 °C until L2 stage. Imaging protocol. Larvae that grew for no more than 4 h after hatching were considered to be at early L1 stage. Larvae whose neuronal lineage P neurons finished cell division and whose gonad was composed of about 50 cells were considered to be at L2 stage. For live imaging, worms were placed on a 2% agarose pad and immobilized using levamisole (2.5 mg/mL). Then, one- to four-cell-stage embryos were transferred onto an agarose pad and heat shocked as described, at 33 °C for 1 h (47). The embryos then grew at 20 °C until L2 stage. To become robust to background, gene expression was normalized by DAPI fluorescence to account for spherical aberration. A nucleus with a normalized mCherry fluorescence of 500 was measured as described (19). Briefly, background fluorescence was estimated using 10 pseudonuclei. After subtracting background fluorescence, mCherry fluorescence was normalized by DAPI fluorescence to account for spherical aberration. A nucleus with a normalized mCherry fluorescence of 500 was measured as described (19). Gene-expression measurement. The gene-expression level for each cell was measured as described (19). Briefly, background fluorescence was estimated using 10 pseudonuclei. 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**D. melanogaster-Related Procedures.**

*Generation of Drosophila mutant.* *Msh* mutant was generated using the CRISPR/Cas9 method (50) and guide sequence 5′-GGCTAGACTGCTGGTGTGTC-3′ targeting the N-terminal *msh* coding region. *Msh*<sup>ΔN</sup> contains a single-nucleotide insertion at position 185 of the ORF so that it is a frameshift allelic and produces a truncated MSH protein of only 67 amino acids.

**Immunocytochemistry and microscopy.** Embryos were fixed and stained as previously described (51). Rat anti-Repo was from A. Tomlinson (Columbia University, New York). Embryos were observed using differential interference contrast optics with a Nikon Eclipse 80i microscope.

**X. laevis-Related Procedures.** Frogs were used with ethical approval from the Animal Care and Use Committee of Tsinghua University. *Xenopus* whole-mount in situ hybridization. Whole-mount in situ hybridization was performed based on standard protocols (52, 53). cDNA sequences for *ZNF703, Runx1, Islet1*, and *Sox2* were cloned into the vector pCS107 (www.xenbase.org). Primer sequences were as follows: *ZNF703* was performed based on standard protocols (52, 53). cDNA sequences for *X. laevis* was performed based on standard protocols (52, 53). cDNA sequences for *X. laevis* whole-mount in situ hybridization.

**Immunocytochemistry and microscopy.** Embryos were fixed and stained as previously described (51). Rat anti-Repo was from A. Tomlinson (Columbia University, New York). Embryos were observed using differential interference contrast optics with a Nikon Eclipse 80i microscope.

**Xenopus embryo manipulation and microinjection.** *Xenopus* eggs and embryos were obtained through standard procedures (54). Briefly, eggs harvested from HCG-injected female frogs were fertilized through artificial insemination techniques. Fertilized eggs were dejellied using 2% cysteine prepared in 0.1x Marc’s modified Ringer (MMR) (pH 7.9), followed by a thorough wash with 0.1x MMR. The dejellied embryos were then transferred into 2% Ficoll in 0.5x MMR for morpholino (MO) and/or mRNA injection. The MO sequences were as follows: *Mxl1*: 5′-GCCATACAGAGAGATCGG-3′; *ZNF703a*: 5′-ACACGAGGCGCAAAGATTCCCCTTGTT-3′; and *ZNF703b*: 5′-ACACGAGGCGAGATTCCCCTTAT-3′.

**Microinjection of antisense morpholino oligonucleotide.** MOs were obtained from Gene Tools. *ZNF703 MO* was a mixture of 5 ng *ZNF703 MO* and 5 ng *ZNF703 MO* per embryo. *Mxl1 MO* and/or *ZNF703 MO* mixed with 2 ng RLDx as lineage tracer were injected into the animal-pole two left-side cells at the eight-cell stage. For rescue experiments, after MO injection, *ZNF703 mRNA* was immediately injected into the same two cells before the needle hole cured. Beta-gal mRNA was coinjected as a lineage tracer in the *ZNF703 mRNA*-alone group. The right-injected embryos were sorted out under a fluorescence dissecting microscope (Olympus; SZX16) and cultured to stages 16 to 17. Embryos were fixed in Mops EGTA MgSO4 formaldehyde (MEMFA) solution for 4 h at room temperature followed by whole-mount in situ hybridization. *ZNF703 mRNA* (mixed with beta-gal)-injected embryos were stained by red-gal before in situ hybridization.

**Western blotting.** Western blotting was performed as described previously (55). Briefly, *Xenopus* embryos were lysed in TNE buffer (10 mM NaCl, 10 mM Tris HCl, 1 mM EDTA, pH 7.4) plus protease inhibitors, and pipetted several times on ice. Lysates were then cleared by centrifugation at 1,000 × g for 5 min. Supernatant was mixed with loading buffer and boiled for 15 min followed by SDS/PAGE separation. Primary antibody of HA (Roche; 1:2,000 dilution) and primary antibody of β-actin (1:5,000 dilution) were used.

**C. intestinalis-Related Procedures.** *C. intestinalis* adults were obtained from M-Rep. Sperm and eggs were collected by dissecting the sperm and gonad ducts. *Construct.* The Asicb reporter construct was designed based on the previously published enhancer sequence (56). Gene Tools. The anti-sense oligonucleotide sequence of the Mxl1 MO is 5′-ATT CGTTTACTGTACTTTAATT-3′. The MO was dissolved at a concentration of 0.5 mM in 1 mM Tris HCl (pH 8.0), 0.1 mM EDTA, 1 mg/mL tetramethylrhodamine.
dextran (D-1817; Invitrogen). Microinjection of MO was performed as described previously (57).

Image acquisition. Images of larvae were captured using a Zeiss AX10 epifluorescence microscope.

Statistics. Wilcoxon and Mann–Whitney tests, robust statistics tests with no assumption on the distribution of samples, were used to examine whether a set of worm genes orthologous to a vertebrate gene module had significantly higher neuroblast-enrichment or mechano-enrichment scores. In genetics and gene-knockdown analyses, Fisher’s exact test was used under the assumption that the fraction of animals with a phenotype followed binomial distribution, and a t test was used when a quantitative phenotype was scored under the assumption that the quantitative phenotype followed normal distribution. Variation was calculated based on binomial or normal distribution, and the similarity of variations was not assumed to decrease the false positive rate. The minimum number of scored animals was 22 in one batch analyzed, whereas the maximum was 300.

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Fig. S1. Retention of Q cells in Msx/vab-15 mutants. (A) Intact embryonic cell lineage of the vab-15(u781) mutant up to the 398-cell stage, in which the mother cell of V5 and Q has been generated. (B and C) Retention of Q cells in vab-15 mutant larvae. Prnt-1::H1::cherry, marker of Q and seam precursor cells. Shown are the lateral view (B) and ventral view (C) of newly hatched L1 larvae. (Scale bars, 10 μm.)

Fig. S2. Little effect on cell proliferation (A) and apoptosis (B) of Xenopus embryos by the moderate double knockdown of Msx/vab-15 and ZNF703/tlp-1. pH3, phosphorylated histone H3Serine10 as a cell proliferation marker. TUNEL, apoptosis marker. Embryos are viewed from the dorsal side with anterior on top. Asterisks indicate the morpholino injection side. (Scale bar, 0.5 mm.)
Fig. S3. Assessment of effects of ZNF703 and Msx1 depletion in Xenopus. (A) Immunoblots (IBs) showing that ZNF703 MO efficiently blocks translation of an MO-complementary Znf703-HA mRNA but not an MO-resistant Znf703-HA* mRNA. (B) Immunoblots showing that Msx1 MO efficiently blocks translation of an MO-complementary Msx1-HA mRNA but not an MO-resistant Msx1-HA* mRNA. β-Actin serves as a loading control in A and B. (C) Depletion of ZNF703 alone through injecting a mix of MOs up to 40 ng has no discernable effects on the expression of foxd3, sox2, runx1, and islet. However, depletion of Msx1 using 10 to 15 ng MO discernably decreased the expression of foxd3, runx1, and islet1 and expanded the expression domain of sox2. Asterisks indicate the injected side of an embryo. (Scale bar, 0.5 mm.)

Fig. S4. Disrupted chordotonal organs in Msx/vab-15/msh mutant Drosophila embryos. Shown are the lateral view of wild-type and msh4-4 embryos at early stage 12 with anterior side on the left and ventral side at the bottom. Glial cells in the chordotonal organs are revealed by Repo staining. Arrows indicate glial cells in the chordotonal organs. (Scale bar, 100 μm.)

Dataset S1. Information on the promoters in reporter constructs

Dataset S1
Dataset S2. Expression profile of 339 transcription factors across 60 trunk ectoderm cells/nuclei in L1 larvae