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Developmental Biology 291 (2006) 253 – 263
DEVELOPMENTAL BIOLOGY
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Zfh1, a somatic motor neuron transcription factor, regulates axon exit from the CNS

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Received for publication 7 September 2005; revised 1 December 2005; accepted 5 December 2005
Available online 3 February 2006

Abstract

Motor neurons are defined by their axon projections, which exit the CNS to innervate somatic or visceral musculature, yet remarkably little is known about how motor axons are programmed to exit the CNS. Here, we describe the role of the *Drosophila* Zfh1 transcription factor in promoting axon exit from the CNS. Zfh1 is detected in all embryonic somatic motor neurons, glia associated with the CNS surface and motor axons, and one identified interneuron. In *zfh1* mutants, ventral projecting motor axons often stall at the edge of the CNS, failing to enter the muscle field, despite having normal motor neuron identity. Conversely, ectopic Zfh1 induces a subset of interneurons—all normally expressing two or more "ventral motor neuron transcription factors" (e.g. Islet, Hb9, Nkx6, Lim3)—to project laterally and exit the CNS. We conclude that Zfh1 is required for ventral motor axon exit from the CNS.

Keywords: Motoneuron; Kheper; mnr2; Interneuron; zfh1; hb9; lim3; Islet; Neuronal identity; Axon projection; Axon outgrowth; unc-5; Axon guidance

Introduction

Motor neurons are defined by their axon projection out of the CNS to a muscle target, whereas interneuron axons remain within the CNS to synapse with other neurons. Although these differences are essential for the development of neuronal circuitry controlling movement, remarkably little is known about the mechanisms that promote motor axon exit from the CNS and keep interneuron axons from leaving the CNS.

In vertebrates, most motor neurons develop in the ventral region of the spinal cord and project laterally out of the CNS. Motor neuron-specific transcription factors such as MNR2 and HB9 (Arber et al., 1999; Thaler et al., 1999) are excellent candidates for regulating motor neuron-specific features such as axon exit from the CNS. Ectopic expression of Hb9 in chick interneurons can induce expression of motor neuron markers and axon projection out of the CNS (Tanabe et al., 1998), yet mice lacking HB9/MNR2 form normal numbers of motor neurons and their axons project out of the CNS (Arber et al., 1999; Thaler et al., 1999). Many axon guidance molecules have been discovered over the last decade (Tessier-Lavigne and Goodman, 1996), and some are known to regulate motor axon outgrowth in vitro (Varela-Echavarria et al., 1997); however, none appear to be required for directing motor axons out of the CNS (reviewed in Schneider and Granato, 2003).

In *Drosophila*, motor neurons project ipsilaterally or contralaterally to innervate body wall musculature (Bossing et al., 1996; Schmidt et al., 1999; Schmidt et al., 1997). They exit the CNS at two stereotyped positions to pioneer the intersegmental nerve (which has ISN, ISNb, and ISNd branches), the segmental nerve (which has SNa and SNc branches), and the transverse nerve. Motor axons will exit the CNS in the absence of muscles (Prokop et al., 1996) or in the absence of the glia that prefigure the nerve root exit sites (Sepp

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doi:10.1016/j.ydbio.2005.12.009

et al., 2001). Thus, motor axon that exits from the CNS appears to be primarily regulated by factors acting within the CNS or within the motor neurons.

There are no known pan-motor neuron transcription factors that regulate common aspects of motor neuron development in *Drosophila*. Instead, the combinatorial expression of several homeodomain transcription factors regulates development of different motor neuron subtypes (Shirasaki and Pfaff, 2002). Dorsal-projecting motor neurons exit the CNS via the ISN, innervate dorsal body wall muscles, and express the transcriptional repressor Even-skipped (Eve) (Fujioka et al., 2003; Landgraf et al., 1999). In the absence of Eve, these motor neurons are correctly specified but most (~80%) fail to exit the CNS, and all fail to reach their normal dorsal muscle field (Fujioka et al., 2003). Ventral-projecting motor neurons exit via the ISNb/d or SNa/c and many express the homeodomain proteins Islet, Hb9, Lim3, and/or Nkx6 (Broihier and Skeath, 2002; Broihier et al., 2004; Cheesman et al., 2004; Odden et al., 2002; Thor and Thomas, 1997; Thor et al., 1999). Motor neurons that lack expression of any one of these transcription factors show defects in axon guidance within the muscle field (Broihier and Skeath, 2002; Broihier et al., 2004; Cheesman et al., 2004; Landgraf et al., 1999; Odden et al., 2002; Thor and Thomas, 1997; Thor et al., 1999), but only loss of Nkx6 results in failure of some axons to exit the CNS (Broihier et al., 2004). Mutations in axon guidance molecules that regulate interneuronal projections towards and away from the midline of the CNS have been identified (Dickson, 2002), but none have significant effects on motor axon projection out of the CNS (Schneider and Granato, 2003). Thus, in both vertebrates and *Drosophila*, it remains unclear how motor neuron axons are induced to exit the CNS while interneuron axons are retained within the CNS.

Here, we describe the role of Zfh1 in motor neuron development. Zfh1 has nine zinc finger domains and one homeodomain, and it functions as a repressor during mesoderm development (Lai et al., 1993; Postigo et al., 1999; Su et al., 1999). *zfh1* mutants show defects in somatic and cardiac muscle development (Lai et al., 1993; Postigo et al., 1999; Su et al., 1999) and in gonadal mesoderm development (Broihier et al., 1998). Zfh1 is expressed in complex pattern within the CNS, but is reported to have no striking CNS phenotype (Lai et al., 1991, 1993). Here, we show that Zfh1 is found in all somatic motor neurons, in glia that associate with the CNS surface and that enwrap motor axon projections, and one identified interneuron. *zfh1* mutants show impaired ventral motor axon exit from the CNS, and ectopic Zfh1 can induce a small pool of interneurons to send their axons out of the CNS.

Results

Zfh1 is expressed in all embryonic somatic motor neurons

We began a detailed analysis of Zfh1 because it was known to be expressed in many motor neurons and no identified interneurons (Isshiki et al., 2001; Lundell et al., 2003; Novotny et al., 2002; Skeath and Doe, 1998), and thus was a candidate for regulating motor neuron versus interneuron specification. Using markers for identified motor neurons, we confirm and extend previous results, showing that Zfh1 is detected in the following identified motor neurons: the Eve$^+$aCC, RP2 and U1–U5 (Figs. 1A, B), the Islet$^+$Hb9$^+$Nkx6$^+$RP1/3/4/5 cluster (Fig. 1C), the Islet$^+$Hb9$^+$Eagle$^+$GW (Fig. 1D), the Islet$^+$Hb9$^+$Nkx6^{+-} lateral cluster (Fig. 1D), and the 22C10$^+$VUMs (data not shown). In addition, we performed double-labels with Zfh1 and the putative motor neuron marker phospho-Mad (pMad) (Marques et al., 2002). We detect nuclear pMad in ~35 cells per abdominal hemisegment at stage 16, which is consistent with the number of motor neurons (Landgraf et al., 1997; Schmid et al., 1999), and virtually all are Zfh1$^+$ (96%, n = 875; Fig. 2). The few pMad$^+$Zfh1$^-$ cells were found in random positions, and we

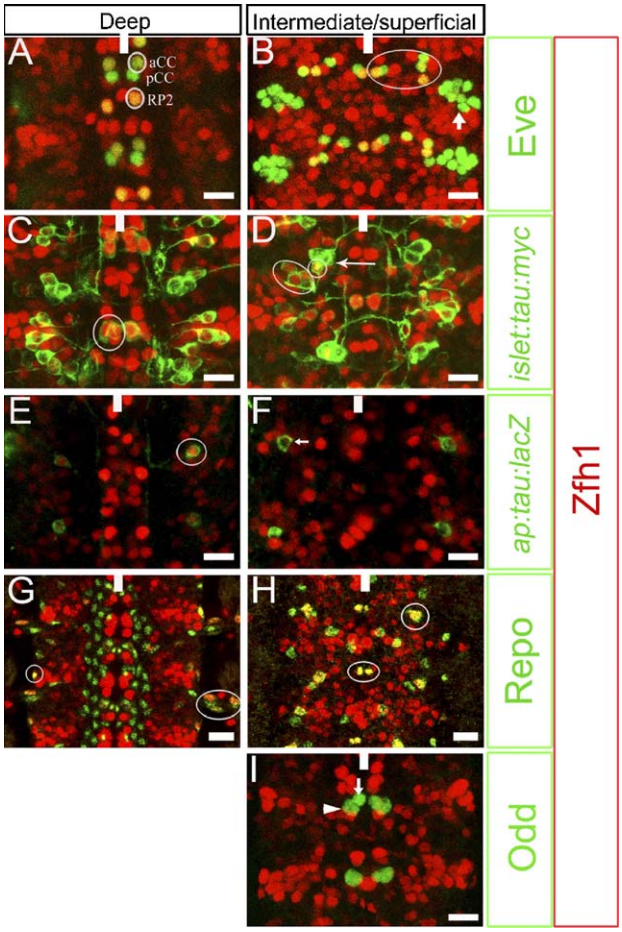
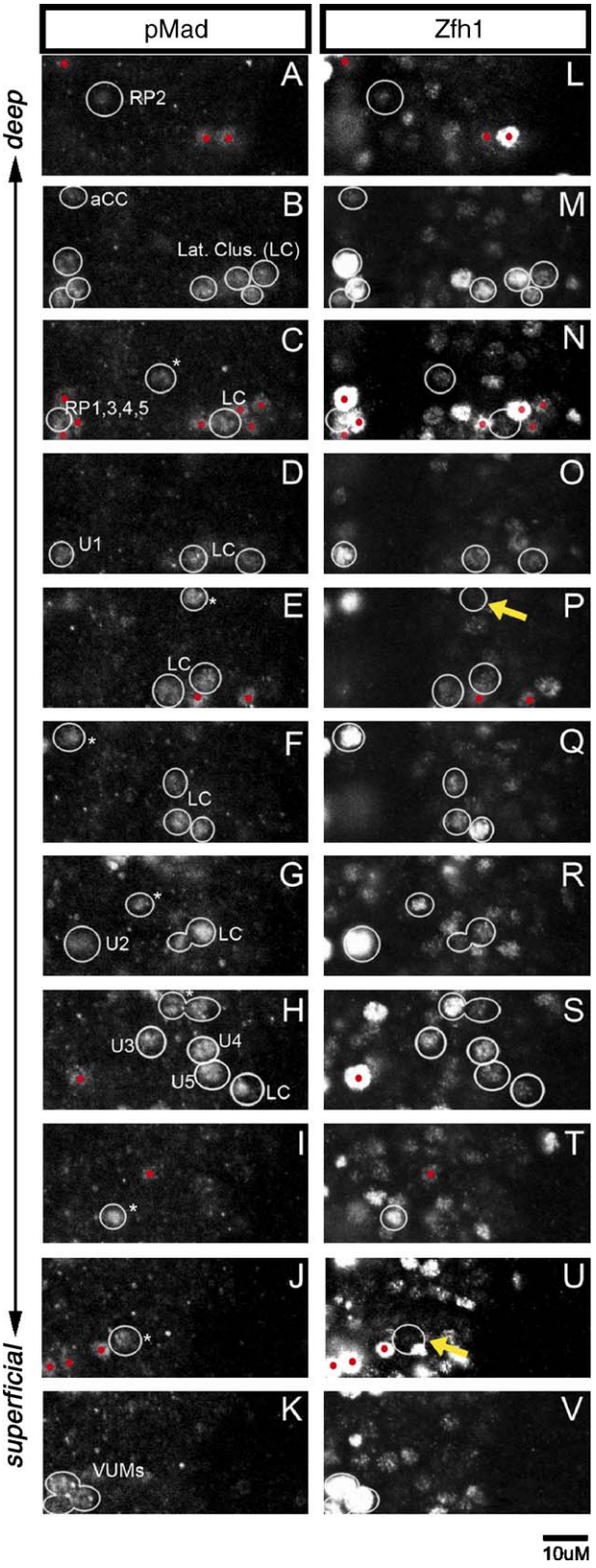


Fig. 1. Zfh1 is expressed in all somatic motor neurons, one interneuron, and a subset of glia. Wild-type stage 16 embryos stained for Zfh1 protein (red) and markers for identified motor neurons, interneurons, and glia (green). Confocal optical sections are shown of deep layer neurons (deep) and more superficial layer neurons (intermediate/superficial). Zfh1 is present in following motor neurons: the Eve$^+$aCC, RP2 (A, circled) and U1–U5 (B, circled); the *islet-tau:myc* RP1,3,4,5 (C, circled) and GW (D, small circle), and the lateral cluster (D, large circle). Zfh1 is absent from the following interneurons: the Eve$^+$pCC (A) and ELs (B, arrow); the *islet-tau:myc* EW1–EW3 (D, arrow), and the *apterous-tau:lacZ* ventral interneuron (F, arrow). Zfh1 is present in the *apterous-tau:lacZ* AD interneuron (E, circled), and in the Repo$^+$ peripheral/exit glia that enwrap the motor nerves (G, oval), Channel glia derived from NB7-4 (H, oval), and the surface-associated glia (G, H, circles). Midline is indicated by white line, and anterior is up in this and all subsequent figures. Scale bar = 10 μm.

suspect that this may reflect developmental or transient modulation of Zfh1 levels, rather than the presence of specific pMad$^+$Zfh1$^-$ neurons. We also find that Zfh1 is not expressed in dMP2 (Fig. 1I), a visceral motor neuron that projects within the CNS longitudinal axon tracts until exiting the posterior of the CNS to innervate the hindgut (Miguel-Aliaga and Thor, 2004). We conclude that Zfh1 is expressed in all embryonic somatic motor neurons.



To determine if Zfh1 is expressed in interneurons, we assayed all molecularly identifiable interneurons. Zfh1 is not detected in the *apterous-tau:lacZ* positive AV interneuron (Fig. 1F); the Eve$^+$pCC or 10 EL interneurons (Figs. 1A, B); the three Islet$^+$Hb9$^+$EW1–EW3 interneurons (Fig. 1D); the Odd-skipped$^+$vMP2 interneuron (Fig. 1I); the six Hb9$^+$pMad$^-$ ventral cluster interneurons (data not shown); or the ~50 Dachshund$^+$pMad$^-$ interneurons (data not shown). Within this pool of over 70 interneurons, Zfh1 is detected in just one interneuron, the *apterous-tau:lacZ* expressing AD interneuron (Fig. 1E) (Lundgren et al., 1995). In addition, there are about 20 other Zfh1$^+$Elav$^+$ neurons in each hemisegment; these cells are not mature motor neurons or glia because they do not express pMad or Repo, but we cannot determine if these cells are interneurons or late differentiating motor neurons (see Discussion). We conclude that Zfh1 is expressed in at least one interneuron.

Zfh1 is also expressed in a subset of Repo$^+$glia, including glia that enwrap motor neuron axons and most or all CNS surface-associated glia; it is not detected in the longitudinal glia or the cell body glia within the CNS (Figs. 1G, H).

zfh1 is required for a subset of motor axons to exit the CNS

To determine if Zfh1 regulates motor neuron specification, we scored *zfh1* mutants for altered expression of motor neuron identity markers. We assayed embryos heterozygous for two independently isolated, protein-negative *zfh1* mutant alleles $zfh1^5$ and $zfh1^{75.26}$ (Broihier et al., 1998; Lai et al., 1993) or homozygous for $zfh1^5$. We found no difference in phenotype between $zfh1^5/zfh1^{75.26}$ and $zfh1^5/zfh1^5$ and thus we will refer to both as "*zfh1* mutants" below. We find that *zfh1* mutants have no striking change in the number of neurons expressing the motor neuron subtype identity genes *eve*, *isl*, *hb9*, *nkx6*, and *lim3*; nor are there major changes in the number of neurons expressing the pan-motor neuronal markers pMad (Supplemental Figs. 1A–M) or *late bloomer* (data not shown). Conversely, there is no significant change in the number of Zfh1$^+$ neurons in *hb9*, *islet*, *lim3*, and *nkx6* mutant backgrounds (Supplemental Fig. 1N). Thus, Zfh1 is not required to specify motor neuron identity.

Fig. 2. Zfh1 is expressed in all embryonic pMad$^+$ somatic motor neurons. Wild-type stage 16 embryo double-labeled for the motor neuron marker pMad (left column) and Zfh1 protein (right column); one hemisegment is shown with each panel representing a single confocal optical section at 3 μm intervals progressing from the deepest layer (top) to the most superficial layer of the CNS (bottom). The midline is to the left. pMad$^+$ neurons are circled and the circles are superimposed over the Zfh1 panel to allow precise alignment of pMad/Zfh1 nuclei. Each red dot indicates a nucleus that has been circled in an adjacent optical section. Virtually all pMad$^+$ neurons are also Zfh1$^+$ (96%; n = 875); there is an average of 1–2 pMad$^+$Zfh1$^-$ nuclei in each hemisegment (two shown in this hemisegment, yellow arrows) but their position varies from segment to segment. Neuronal identity is labeled where known, and was determined based on cell position, size, and pMad double-labels with motor neuron molecular markers (data not shown). Asterisks indicate unidentified cells.

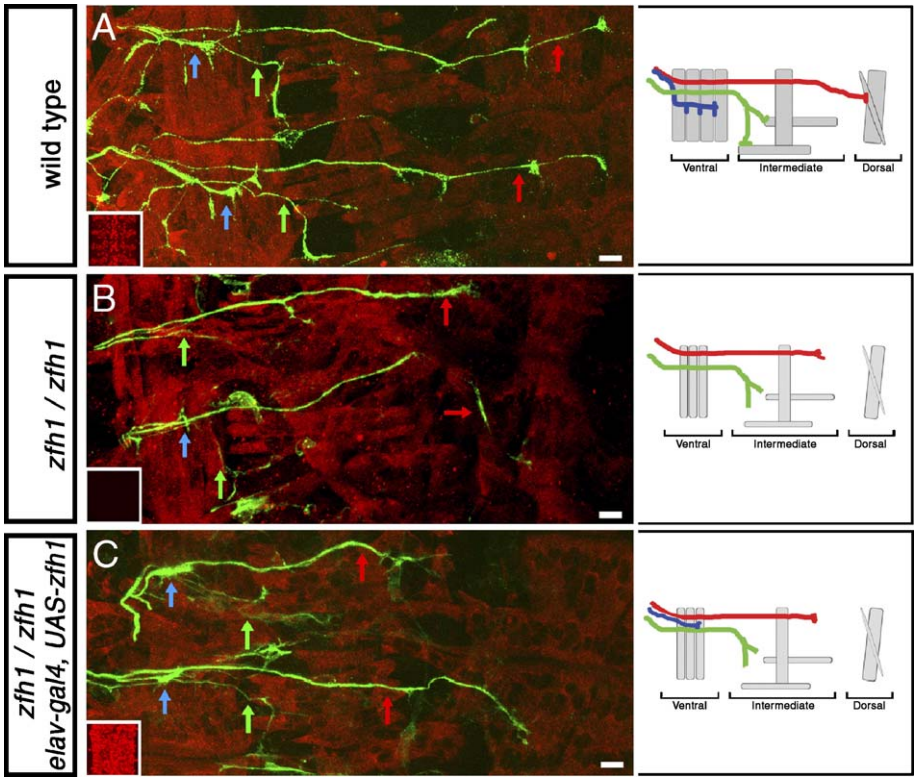


Fig. 3. *zfh1* mutants show defects in the formation of ventral motor nerves. Stage 16 flat mounted embryos stained for the motor axons (FasII, green) and body wall muscles (muscle myosin, red). CNS to left, muscle field to right, two hemisegments shown. ISN, red arrow; ISNb, blue arrow; SNa, green arrow. To the right are schematics showing the ISN (red), ISNb (blue), and SNa (green) nerves relative to a subset of muscles (grey). zfh1 mutants have variable reduction in muscle number and size. Insets show Zfh1 protein expression. (A) Wild type has normal Zfh1 protein levels (inset) and normal ISN, ISNb, and SNa projections. (B) *zfh1* mutants have no detectable Zfh1 protein (inset) and show a loss or truncated ISNb and SNa nerves; the ISN nerve is always present but has projection defects that are not rescued by Zfh1 neuronal expression (see panel C). (C) *zfh1* mutants with *elav-gal4 UAS-zfh1* rescue show ubiquitous Zfh1 protein in the CNS (inset) and a rescue of ISNb and SNa nerve projections into the muscle field. This genotype does not rescue the pathfinding or branching defects in ISNb, SNa, or ISN and thus they may arise from defects in the muscle field (Lai et al., 1993). For quantification, see Table 1.

The defining feature of a motor neuron is that it extends an axon out of the CNS to a muscle target. Here, we assay whether Zfh1 plays a role in promoting motor neuron axon exit from the CNS. In wild type, there are three major early-developing nerves (ISN, ISNb, SNa) and two minor late-developing nerves (SNc, ISNd) (Fig. 3A; Table 1) (Vactor et al., 1993). In *zfh1* mutants, we only scored the major early-developing ISN, SNa, ISNb nerves. We did not observe, or could not identify, the minor SNc/ISNd nerves in *zfh1* mutants. Because of embryonic defects, staging the embryos was difficult and we cannot rule out that the CNS stops developing before the formation of these minor nerves. Using the FasII antibody, which labels all motor axons, we find that the ISN exits the CNS, but the ISNb and SNa nerves are frequently absent (Fig. 3B; quantified in Table 1). The ISNb and SNa exit defects are substantially rescued by neuronal *zfh1* expression (Fig. 3C; Table 1), showing that the axon outgrowth phenotype is not due to muscle defects, which are present in *zfh1* mutants (Lai et al., 1993). In addition, *zfh1* mutant motor axon phenotypes are not rescued by *zfh1* expression in glia (Table 1), suggesting that they are not due to loss of *zfh1* glial expression.

Table 1
Zfh1 is required for motor axon exit from the CNS

	Nerve extends out of CNS			
	ISN (FasII)	ISNb (FasII)	ISNb (FasIII)	SNa (FasII)
Wild type	100% (n = 42)	95% (n = 42)	95% (n = 37)	100% (n = 42)
zfh1/zfh1	100% (n = 46)	35% (n = 46)	29% (n = 31)	80% (n = 46)
zfh1/zfh1, elav-gal4 uas-zfh1	100% (n = 45)	71% (n = 45)	n.a.	93% (n = 45)
zfh1/zfh1, gcm-gal4 uas-zfh1	100% (n = 24)	25% (n = 24)	n.a.	75% (n = 24)

To determine if absence of the ISNb nerve is due to failure to extend an axon out of the CNS or abnormal fasciculation with the ISN, we scored more specific axon markers. In wild-type embryos, the FasIII antibody and the *isl-tau:myc* transgene label the ISNb axons but not the ISN or SNa axons, and the ISNb observed exiting the CNS is over 95% of the hemisegments (Figs. 4A, C; quantified in Table 1). In *zfh1* mutants, the FasIII$^+$ or isl-tau:myc$^+$ axons frequently fail to exit the CNS, typically stalling at the edge of the CNS (73%); in the minority of cases where they project into the muscle field, they are often truncated and always fail to branch normally (Figs. 4B, D; quantified in Table 1). These results show that loss of Zfh1 does not transform motor neurons into interneurons or induce motor neuron cell death; rather, loss of Zfh1 results in failure of motor axon exit from the CNS and reduced axonal branching.

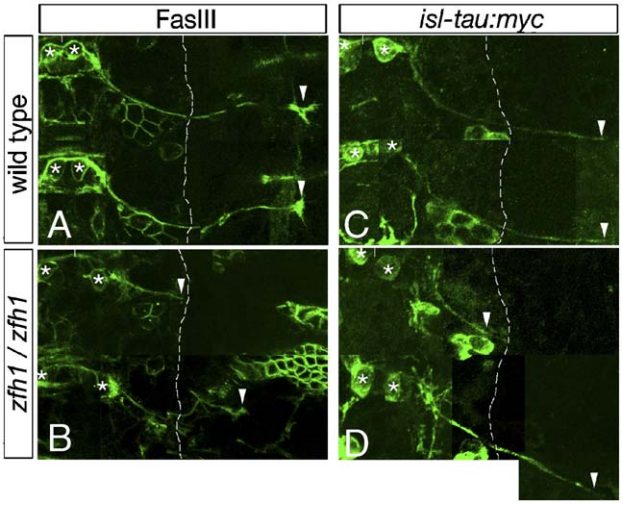


Fig. 4. *zfh1* is required for ISNb axons to exit the CNS. Dissected and flat-mounted embryos stained for the FasIII (A, B) or isl-tau:myc (C, D) to highlight ISNb axon projections; two segments shown. Montages of multiple focal planes were used to show the entire ISNb axon. RP motor neuron cell bodies, asterisk; CNS midline, white line; lateral edge of CNS, white dashed line; terminal end of ISNb axon, arrowhead. (A) Wild-type early stage 16 embryo showing the ISNb nerve. (B) *zfh1* mutant embryo showing the ISNb terminating within the CNS (top) or truncated without branching in the muscle field (bottom). (C) Wild-type stage 15 embryo showing the ISNb nerve. (D) *zfh1* mutant embryo showing the ISNb terminating within the CNS (top) or extending into the muscle field (bottom).

Ectopic Zfh1 induces a small number of interneurons to project out of the CNS

Because loss of *zfh1* resulted in reduced motor axon outgrowth from the CNS, we next tested whether ectopic *zfh1* could induce interneurons to project out of the CNS. We used three genotypes to drive pan-neuronal zfh1 expression: *hs-zfh1* (Lai et al., 1991, 1993; Postigo et al., 1999), *sca-gal4 UAS-zfh1*, and *elav-gal4 UAS-zfh1* (Fig. 5). We first tested whether motor neuron or interneuron identity is altered by *zfh1* misexpression. Consistent with the *zfh1* mutant analysis, misexpression of *zfh1* results in no change in the number or pattern of motor neuron and interneuron markers (Eve, Islet, Nkx6, Lim3, Hb9, En, Dachshund [Dac]; Supplemental Fig. 2 and data not shown), and only a small increase in the number of pMad + neurons (~3–6 extra cells per hemisegment). Thus, Zfh1 misexpression does not have a major impact on the specification of interneuron or motor neuron identity.

Next, we assayed embryos misexpressing Zfh1 for motor axon phenotypes. Ectopic Zfh1 results in a slight thickening and occasional misrouting of the ISNb nerve within the muscle field, as well as gaps in the longitudinal axon fascicles within the CNS (Figs. 5B, C, E). These results suggest a decrease in interneuronal projections within the longitudinal fascicles of the CNS and that there may be an increase in axon projections

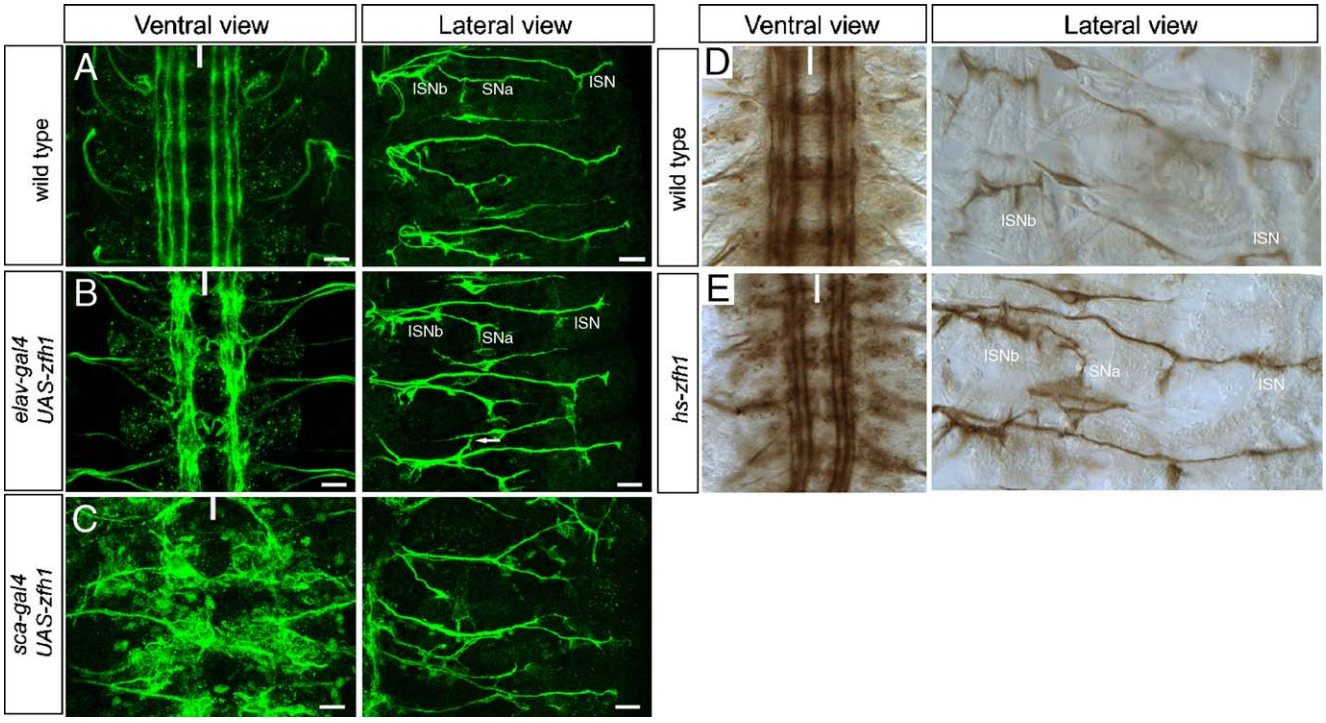


Fig. 5. Ectopic Zfh1 leads to a thickening of the ISNb motor nerve and thinning of interneuronal connectives. Stage 16 embryos stained for the FasII to highlight all motor axon projections and three interneuronal fascicles in the longitudinal connectives; anterior up, three segments shown. Left, ventral view of the CNS; white line marks ventral midline. Right, lateral view of the motor axon projections (CNS to left, muscle field to right). (A) Wild-type embryo showing the three early-developing ISN, ISNb, and SNa motor nerves and three FasII + interneuronal fascicles. (B) *elav-gal4 UAS-zfh1* drives Zfh1 expression weakly in neuroblasts and GMCs and strongly in postmitotic neurons (Pearson and Doe, 2003), resulting in ISNb ectopic branching into adjacent hemisegment (arrow) and disrupted interneuronal projections. (C) *sca-gal4 UAS-zfh1* drives Zfh1 expression strongly in neuroblasts and more weakly in GMCs and neurons (Pearson and Doe, 2003), resulting in thicker motor nerves, although the pattern is too disorganized to identify individual nerves; interneuronal projections are also severely perturbed. (D) Wild-type embryo showing the ISN and ISNb motor nerves (SNa is out of the plane of focus) and three FasII + interneuronal fascicles. (E) *hs-zfh1* embryo showing thickening of the ISNb nerve.

out the ISNb (the opposite of the *zfh1* mutant phenotype); however, we cannot rule out general axon misrouting of the interneuron projections that populate the longitudinal fascicles. They also show that, at best, only a small pool of interneuronal axons can be redirected out of the CNS, consistent with the observed small increase in pMad expression.

To determine the frequency that interneuron axons are redirected out of the CNS by ectopic Zfh1, if any, we DiI-labeled a random subset of neuroblast lineages in *elav-gal4 UAS-zfh1* embryos. The wild-type clones for all neuroblasts are known (Bossing et al., 1996; Schmidt et al., 1999; Schmidt et al., 1997), so we could readily identify ectopic motor projections. We observed ectopic motor neuron projections in at least 3 of the 12 neuroblast lineages that were scored multiple times. Neuroblast 1-1 normally generates a single Eve$^+$ aCC motor neuron in thoracic segments; following ectopic Zfh1, there are two motor projections (Figs. 6A, D). Neuroblast 5-2 normally produces a single motor neuron with a contralateral projection out the ISNb; after ectopic Zfh1, there is an additional ipsilateral axon projection out the ISNb (Figs. 6B, E). Neuroblast 7-3 normally produces a small clone containing three interneurons (EW1, EW2, EW3) and a single motor neuron (GW); following ectopic Zfh1, there is at least one ectopic motor projection out of the CNS (Figs. 6C, F). In addition, Zfh1 misexpression also resulted in the formation of ectopic filopodia emanating from axon shafts as well as pronounced defasciculation of normally tightly bundled axons (Fig. 7). We conclude that ectopic Zfh1 can induce ectopic axon projections out of the CNS in some but not all neuroblast lineages.

Ectopic Zfh1 can induce EW1–EW3 interneurons and dMP2 visceral motor neuron to project laterally out of the CNS

To extend our analysis of the ectopic Zfh1 phenotype, we focused on the NB7-3 lineage because it is small (three EW interneurons and one GW motor neuron in abdominal segments) and there are good genetic and molecular reagents available (Higashijima et al., 1996; Isshiki et al., 2001; Lundell et al., 2003; Novotny et al., 2002). We used *eagle-gal4* to misexpress Zfh1 throughout the 7-3 lineage, which results in ectopic Zfh1 in all three EW interneurons. Wild-type EW1–EW3 interneurons project medially across the posterior commissure before extending anteriorly and posteriorly in a contralateral connective (Higashijima et al., 1996); they never project out of the neuropil or extend ipsilaterally towards the edge of the CNS (0%, n = 46 hemisegments) and are typically not pMad$^+$ (5%, n = 155 cells; Fig. 7A). In contrast, ectopic Zfh1 in the EW1–EW3 interneurons results in their axons extending ipsilaterally, out of the neuropil, towards the edge of the CNS or into the somatic muscle field (63%; n = 82 hemisegments; Fig. 7B) and an upregulation of nuclear pMad staining (56%; n = 202 cells; Fig. 7D). Interestingly, axons of EW1–EW3 interneurons ectopically expressing Zfh1 always

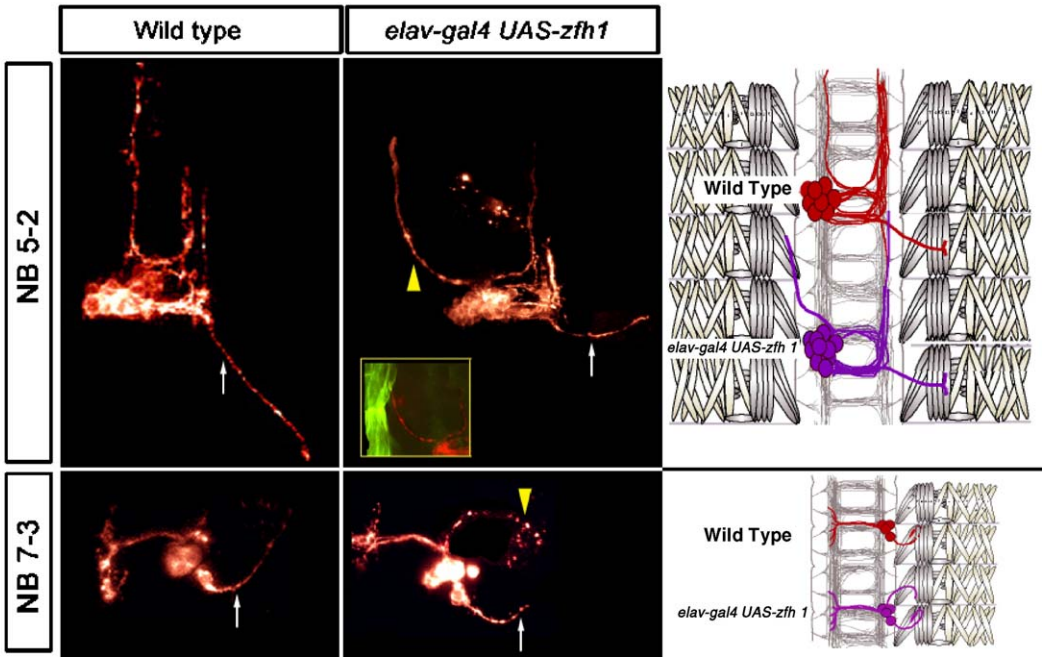


Fig. 6. Ectopic Zfh1 induces ectopic lateral axon projections in a small subset of neurons. DiI-labeled neuroblast clones in wild-type (A, C) and *elav-gal4 UAS-zfh1* (B, D). Right, schematics of the wild-type clone (top, red) and ectopic Zfh1 clone (bottom, purple). Ectopic lateral projections (yellow arrowhead), normal motor projections (white arrow), ventral midline (white triangle), anterior is up. (A, B) Abdominal NB5-2 clones. (A) Wild-type clone contains a motor neuron projecting ispilaterally, and interneurons projecting anteriorly in both ipsi- and contralateral fascicles. (B) Ectopic Zfh1 does not alter the ipsilateral interneuron or motor neuron projections, but the contralateral interneuron now extends laterally out of the CNS to terminate on a somatic muscle (inset: phalloidin stained muscles, green). The extralateral projection was observed in two of five clones analyzed. (C, D) Abdominal NB7-3 clones. (C) In wild type, NB7-3 generates three EW interneurons that project contralaterally and the GW motor neuron that projects out of the CNS ipsilaterally. (D) Ectopic Zfh1 results in one or more interneurons sending ipsilateral projections out of the CNS to somatic muscles (arrowhead). The extralateral projections were observed in two of six clones analyzed.

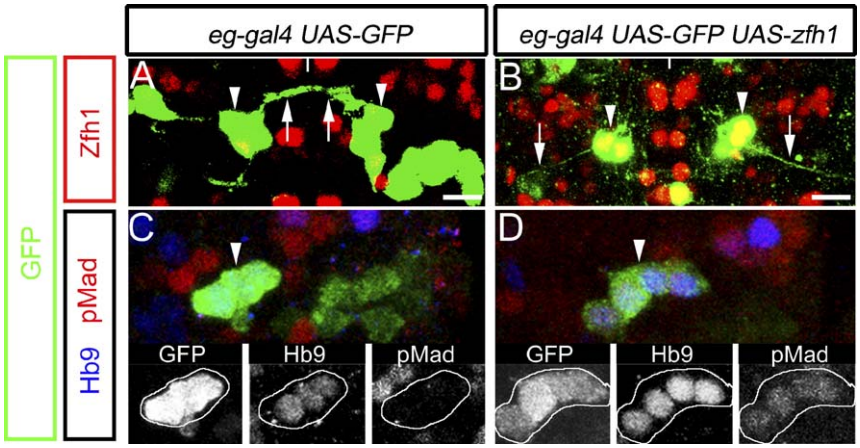


Fig. 7. Ectopic Zfh1 redirects EW1-3 interneuron axons out of the CNS. EW1–EW3 interneurons labeled with *eagle-gal4* (*eg-gal4*) *UAS-GFP* in stage 16 embryos. One segment is shown in panels A and B (midline, white line) and one hemisegment is shown in panels C and D (midline, left side of panels). Scale bar is 10 μm in panels A and B. Panels A/B and C/D were collected with identical confocal settings. (A, C) Wild-type EW1–EW3 interneurons (arrowheads) project medially across the posterior commissure (arrows) (A) and do not express pMad (C); insets show single label data. (B, D) Ectopic Zfh1 in the EW1–EW3 interneurons (arrowheads) redirects their axons laterally out the CNS (arrows) (B) and induces nuclear pMad staining (D).

project on the same trajectory out to the CNS and do not wander within the CNS indicating that rather than disrupting the axons ability to properly pathfind, ectopic Zfh1 switches the neuron's preferred trajectory from medial to lateral. We conclude that ectopic Zfh1 can promote EW interneurons to extend axons laterally out of the CNS and become nuclear pMad-positive.

The wild-type dMP2 axon initially extends posteriorly in a medial longitudinal fascicle within the CNS, similar to a projection interneuron (Schmid et al., 1999; Spana et al., 1995). In the three most posterior segments (A6–A8), however, the dMP2 differentiates into a visceral motor neuron, projecting out the posterior of the CNS to innervate the hindgut, becoming pMad$^+$, and expressing the neurotransmitter proctolin (Miguel-Aliaga and Thor, 2004). In anterior segments, the dMP2 undergoes apoptosis without exhibiting any motor neuron features (Miguel-Aliaga and Thor, 2004). Importantly for our analysis, the dMP2 axon never extends laterally out of the CNS in any segment (0%; n = 89) (Fig. 8A). In embryos where Zfh1 is expressed in the dMP2 neuron (using the dMP2-specific *22B-Gal4* driver; A.G. and S.T., unpublished), the dMP2 axon frequently extends laterally out of the CNS into the muscle field (45%; n = 96) or laterally to the edge of the CNS (5%; n = 96) (Fig. 8B). We conclude that ectopic Zfh1 in the dMP2 neuron can switch its axon projection from a longitudinal fascicle of the CNS to a lateral direction out of the CNS.

Dual misexpression of Zfh1/Nkx6 or Zfh1/Hb9

Only a small subset of neurons respond to Zfh1 by projecting axons out of the CNS, raising the question of what these neurons have in common that gives them the competence to respond to Zfh1. EW1–EW3 and dMP2 are among the only neurons to express two ventral motor neuron transcription factors (from the Hb9, Nkx6, Islet, Lim3 family) without projection out of the CNS, so we hypothesized that expression of three motor neuron factors (Zfh1 plus two others) may be sufficient to induce axon projection out of the CNS. To test this theory, we performed dual misexpression of Zfh1/Nkx6, Zfh1/Hb9, or Hb9/Nkx6, reasoning that the many interneurons that normally express just one motor neuron transcription factor might be induced to project out of the CNS upon addition of two additional factors. We found that dual misexpression experiments with *elav-gal4* driving *UAS-zfh1* and *UAS-hb9*, *UAS-zfh1* and *UAS-nkx6*, or *UAS-nkx6* and *UAS-hb9* did not result in a noticeable change in either the number of pMad positive cells or additional lateral projections detectable with anti-Fas2 (data not shown). We conclude that co-expression of Zfh1, Nkx6, and Hb9 is not sufficient to induce axon projection out of the CNS.

We next tested the relationship of Zfh1 and the dorsal motor neuron transcription factor, Eve. First, we attempted to drive

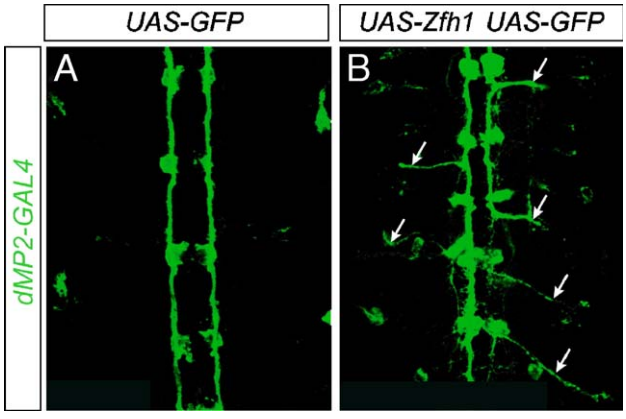


Fig. 8. Ectopic Zfh1 redirects dMP2 interneuron axons out of the CNS. (A) Control embryo expressing mycEGPF in the postmitotic dMP2; the dMP2 interneurons project posteriorly and remain tightly fasciculated in the longitudinal connective. Genotype: *22B dMP2-gal4/UAS-mycEGFP* at room temperature. (B) Ectopic Zfh1 in the postmitotic dMP2 redirects its axon laterally out of the connective or out of the CNS into the lateral muscle field (arrows). Genotype: *22B dMP2-gal4/UAS-mycEGFP; UAS-zfh1/*$^+$ at room temperature. For all experiments, we only assay thoracic and anterior abdominal segments where dMP2 has interneuronal morphology; in segments A6–A8, the dMP2 exits the CNS posteriorly to innervate the hindgut (Miguel-Aliaga and Thor, 2004).

ectopic Zfh1 in the Eve + neurons within the CNS. We used four different Gal4 lines to drive *UAS-zfh1* expression, but surprisingly, we could not generate detectable levels of Zfh1 protein in the Eve + EL interneurons, despite normal levels of the co-expressed axon reporter gene (Supplemental Fig. 3). We conclude that the Eve$^+$ interneurons have a mechanism for posttranscriptional repression of *zfh1* mRNA or proteins levels, and consequently we were unable to test whether Eve$^+$ interneurons would project out of the CNS in response to ectopic Zfh1.

Other interneurons, such as the ventral Apterous$^+$ interneurons, show no change in their axon projection when ectopically expressing *zfh1* (data not shown). It is not surprising that Zfh1 does not induce lateral axon exit of the ventral Apterous$^+$ interneuron, because the dorsal Apterous$^+$ interneuron normally expresses Zfh1. Furthermore, ectopic Zfh1 does not induce all neurons to project axons laterally out of the CNS (Fig. 6 and data not shown). These data suggest that Zfh1 is not sufficient to induce lateral axon exit (as in dMP2) or induce lateral axon exit and interneuron/motor neuron fate change (as in EW1–EW3) in all neurons. We conclude that Zfh1 controlled lateral axon projection and CNS exit requires additional unknown factors to enhance its ability to regulate these processes and that these factors are inherent in the EW1–EW3 interneurons and dMP2.

Discussion

Zfh1 is a transcriptional repressor required for mesoderm development and cell migration (Broihier et al., 1998; Lai et al., 1991, 1993; Postigo and Dean, 1997, 1999; Postigo et al., 1999; Su et al., 1999). Here, we show that Zfh1 regulates lateral axon growth and CNS exit in ventral-projection motor neurons, and that misexpression of Zfh1 can induce lateral axon projections and an interneuron to motor neuron fate transformation in cells competent to respond to ectopic Zfh1. In vertebrates, there are typically two Zfh1 homologs: ZFHX1A and ZFHX1B in humans (Sekido et al., 1996), deltaEF1 and Sip1 (also called ZEB1 and ZEB2, respectively) in mouse (Postigo, 2003), and Kheper in fish (Muraoka et al., 2000). These Zfh1 orthologs are required for proper muscle and CNS development (Bassez et al., 2004; Muraoka et al., 2000; Takagi et al., 1998). The worm homolog *zag-1* is expressed in muscle, interneurons, and motor neurons where it regulates axon outgrowth (Clark and Chiu, 2003; Wacker et al., 2003). Thus, the Zfh1 family members appear to have a conserved role regulating CNS and mesoderm development.

Zfh1 is expressed in somatic motor neurons, one interneuron, and a subset of glia

Drosophila Zfh1 is detected in all embryonic somatic motor neurons, but not in the dMP2 visceral motor neuron. Thus, its expression correlates better with lateral axon projection or innervation of body wall muscles rather than simply motor neuron identity. Zfh1 is detected in at least one interneuron that does not project laterally out of the CNS, the Apterous AD interneuron, and thus Zfh1 is not sufficient to induce lateral axon projections (except in certain contexts; see below). Zfh1 is also expressed in about 20 neurons that are negative for the motor neuron marker pMad and the glial marker Repo. These cells could be interneurons or late-differentiating motor neurons, but due to the lack of axon projection markers, it has not been possible to determine the identity of these cells. Zfh1 is expressed in a subset of glia, including the exit glia that enwrap motor nerves and the surface glia. The association of Zfh1$^+$ exit glia with Zfh1$^+$ motor neurons is intriguing, but we currently have no phenotype that suggests a role for Zfh1 glial function.

zfh1 mutants have normal motor neuron specification but reduced ventral motor axon projections

We initially investigated Zfh1 as a candidate for regulating the difference between motor neurons and interneurons. Analysis of the *zfh1* mutant phenotype clearly shows that it is not required to promote motor neuron identity or suppress interneuron identity. All known motor neuron markers (Eve, Islet, Hb9, Lim3, Nkx6, Late bloomer, and pMad) and interneuron markers (Apterous, Engrailed, and Dachshund) are normal in *zfh1* mutant and/or misexpression embryos.

Instead, we find that *zfh1* mutants show a severe defect in lateral axon projections of the ventral nerves ISNb/SNa; axons typically extend laterally to the edge of the CNS before arresting. This explains why there are missing nerves (the axons do not leave the CNS) but a normal pattern of nuclear pMad in motor neurons (they are at the edge of the CNS where they may be exposed to BMP signals from ventral muscles). In contrast, the dorsal projecting ISN appears to exit the CNS normally (100%), but is often truncated and has pathfinding defects. Although we cannot rule out mesodermal defects as the origin of these phenotypes, there are several reasons to think that the ISN defects are due to loss of neuronal Zfh1. First, *grain* mutant embryos show completely normal muscle development but fail to express Zfh1 specifically in the aCC motor neuron, and the aCC shows truncation and pathfinding defects that are qualitatively and quantitatively similar to the *zfh1* mutant ISN phenotype (A.G. and S.T., data not shown). Second, the *grain* mutant phenotype can be partially rescued by *zfh1* expression specifically in the aCC motor neuron (A.G. and S.T., data not shown). Thus, Zfh1 does not appear to be required for ISN motor axon exit from the CNS, but rather for later aspects of motor axon outgrowth and target recognition.

The fact that ISNb and SNa motor axons stall at the edge of the CNS raises the possibility that the phenotype is due to loss of Zfh1 in the exit glia. Exit glia express Zfh1, are intimately associated with motor axons as they exit the CNS, and regulate the site of motor axon exit from the CNS (Sepp et al., 2001). However, we observe no glial positioning or migration phenotypes, and we can rescue the motor axon exit phenotype with neuronal Zfh1 expression but not glial Zfh1 expression. Moreover, the lateral exit glia are not required for motor axons to exit the CNS (Sepp et al., 2001); the only motor neuron known to require a non-neuronal guidance cue to exit the CNS

is the transverse nerve motor neuron, which requires a specialized pair of midline mesodermal cells to exit the CNS (Gorczyca et al., 1994). We conclude that the *zfh1* mutant motor neuron exit phenotype is due to loss of *zfh1* in motor neurons.

In addition to Zfh1, two other *Drosophila* transcription factors regulate motor axon exit from the CNS. *nkx6* mutants show a 90% loss of ISNb projection out of the CNS (Broihier et al., 2004), despite normal Zfh1 levels in the affected neurons (Supplemental Fig. 1). Thus, both Nkx6 and Zfh1 are required for motor axon outgrowth via the ISNb nerve. Similarly, *eve* mutants show 98% and 80% loss of aCC and RP2 motor axon projections out the ISN nerve, respectively (Fujioka et al., 2003), but in these mutants, we find that aCC/RP2 neurons have little or no Zfh1 expression (Supplemental Fig. 4). Thus, it is unclear if Eve alone is required for ISN axon outgrowth, or if Eve and Zfh1 function redundantly to promote ISN axon outgrowth.

It has been reported that the expression of the *Drosophila* Eph transmembrane receptor is restricted to interneuronal axons, and that RNAi knockdown of Eph levels leads to redirection of some interneuron axons laterally out of the CNS (Bossing and Brand, 2002). It is tempting to speculate that Zfh1 keeps Eph levels low in motor neurons to allow them to exit the CNS. However, the situation is complicated by the recent finding that the *Eph* null mutant does not show abnormal interneuron projections out of the CNS (M. Boyle and J.B. Thomas, personal communication), and thus the role of Eph in restricting interneuron axons to the CNS remains unclear.

Zfh1 is likely to have additional later functions in motor neurons that we have not addressed. Vertebrate Zfh1 homologs ZEB-1 (Smad interacting protein 1/Sip1) and ZEB-2 (deltaEF1) are known to bind pSmad (Postigo, 2003), the nuclear effecter of the BMP signaling pathway. In flies, BMP signaling from the muscle field induces nuclear pMad in motor neurons (Aberle et al., 2002; Marques et al., 2002). This raises the possibility that Zfh1/pMad may collaborate to regulate later aspects of motor neuron differentiation. Analysis of later aspects of *zfh1* mutant phenotype in motor neurons—such as target recognition, synaptic branching, or formation of a functional synapse—is complicated by the fact that *zfh1* mutants have reduced muscle numbers and size (Lai et al., 1993). Analysis of potential later functions of Zfh1 awaits methods for removing *zfh1* expression from individual motor neurons, similar to recent elegant studies of Eve motor neuron function (Fujioka et al., 2003).

Misexpression of Zfh1 induces a subset of neurons to project out of the CNS

Ectopic Zfh1 does not induce all neurons to project axons laterally out of the CNS, but rather a modest thickening of the ISNb nerve and a slight increase in the number of $pMad^{+}$ neurons, due in part to the aberrant projection of dMP2 and the EW1–EW3 interneurons. Why do just these interneurons respond to ectopic Zfh1? The EW1–EW3 and dMP2 neurons are the only neurons we are aware of that express two or more "motor neuron transcription factors" (Hb9, Islet, Nkx6, or Lim3) but do not project to the lateral body wall muscles (Broihier and Skeath, 2002; Broihier et al., 2004; Novotny et al., 2002; Odden et al., 2002). However, the fact that dual misexpression of Zfh1/Hb9, Zfh1/Nkx6, or Hb9/Nkx6 does not induce a detectable increase in pMad staining or lateral projections above that observed in any of the single misexpression experiments suggests that the combined activity of these motor neuron factors is not sufficient to drive axon exit from the CNS. There may be an additional, unknown positive factor present in the EW1–EW3 and dMP2 neurons that gives them competence to exit the CNS in response to Zfh1 misexpression; alternatively, most interneurons could express a negative factor preventing lateral axon projection that cannot be overcome by Zfh1 expression.

How does Zfh1 induce nuclear pMad expression and lateral axon outgrowth in the EW1–EW3 and dMP2 neurons? In the EW1–EW3 neurons, expression of a constitutively-activated Tkv type I BMP receptor does not induce nuclear pMad (data not shown); thus, misexpression of Zfh1 in EW1–EW3 must induce competence to respond to the BMP ligand as well as promote lateral axon outgrowth. In contrast, the dMP2 visceral motor neuron is competent to respond to BMP signaling even without endogenous Zfh1 expression (Miguel-Aliaga and Thor, 2004). Thus, misexpression of Zfh1 may only promote lateral axon outgrowth in dMP2.

Model for Zfh1 function in ventral-projecting motor neurons

Our results support a role for Zfh1 in regulating lateral exit of the ISNb motor neurons from the CNS. Both *zfh1* and *nkx6* mutants show a loss of ISNb motor axons in the muscle field (Figs. 3 and 4; Broihier et al., 2004), suggesting that both genes are required to promote proper lateral CNS exit of ISNb motor neurons (Fig. 9A). Because Zfh1 and Nkx6 do not require each other for expression in ventral projecting motor neurons (e.g. RP1, 3, 4, 5), these two genes must be independently activated but are likely to have common downstream genes to induce lateral axon outgrowth (Fig. 9). It will be interesting to determine if common targets of Zfh1 and Nkx6 are involved in promoting lateral CNS exit and if they are required for lateral CNS exit in other motor neuron subtypes. One might predict that a gene that regulates lateral CNS exit in all motor neurons would be a target of Zfh1, Nkx6, and Eve. One potential candidate is *unc5*, which encodes a repulsive Netrin receptor that promotes cell/growth cone movement away from Netrin on the midline (Keleman and Dickson, 2001). Misexpression of Unc5 within the *Drosophila* CNS results in a

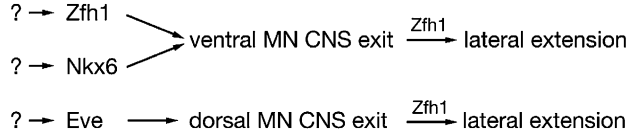


Fig. 9. Model for Zfh1 function in motor neuron exit from the CNS and lateral extension in the muscle field. Ventral motor neuron (MN) exit includes ISNB and ISNd only; dorsal MN exit includes the ISN. See text for details.

subset of interneurons redirecting their axon projections laterally out of the CNS (Keleman and Dickson, 2001) (data not shown). However, misexpression of Unc-5, but not Zfh1, results in lateral displacement of the EW1–EW3 cell bodies (data not shown), so it is unlikely that the Zfh1 EW1–EW3 phenotype is solely due to upregulation of Unc-5 expression. In addition, *zfh1* mutant ISNb motor neurons initially project away from the midline, only stalling at the edge of the CNS, which is inconsistent with the phenotype being due to failure to express the Unc-5 receptor. In the future, it will be important to test directly whether Zfh1 regulates unc-5 directly or indirectly, as well as identify novel direct targets regulated by Zfh1. Because eve and nkx6 mutants also show motor neuron axon exit defects, it will be especially interesting to compare direct targets of Eve, Nkx6, and Zfh1; common targets may help identify genes important for promoting motor neuron guidance out of the CNS in both flies and vertebrates.

Materials and methods

Construction and use of UAS-zfh1 transgene

A full length *zfh1* cDNA including 130bp 5′ UTR and 320bp 3′ UTR was amplified by PCR and cloned into the pUAST vector. The construct was sequenced to ensure that the reading frame and coding sequence were correct, and multiple *UAS-zfh1* transgenic lines were created by using standard methods.

Drosophila stocks and genetics

zfh1 nomenclature is as per Flybase (http://flybase.net/.bin/fbidq.html?FBgn0004606). All crosses were done at room temperature (~22°C) unless stated otherwise. Wild-type and *hs-zfh1* embryos at stage 11/12 were subjected to a 15 min heat shock at 37°C and then fixed at stage 16 for antibody staining using standard methods (see below). The following *Drosophila* stocks were used in this study:

UAS-zfh1
UAS-mycEGFP (Miguel-Aliaga and Thor, 2004)
UAS-EGFP
UAS-activated tkv (Das et al., 1998)
apterous-tau:lacZ (Lundgren et al., 1995)
islet-tau:myc (Thor et al., 1999)
hs-zfh1 (Lai et al., 1993)
$zfh1^{2}$ (Lai et al., 1993)
$zfh1^{5}$ (Lai et al., 1993)
$zfh1^{75.26}$ (Broihier et al., 1998)
$hb9^{kk30}$ (Broihier and Skeath, 2002)
$nkx6^{\Delta 25}$ (Broihier et al., 2004)
isl^{1} (Frank and Rushlow, 1996)
$lim3^{2}$ (Stathakis et al., 1995)
22B dMP2-gal4 (A.G. and S.T., unpublished)
eagle-gal4 (Akiyama-Oda et al., 2000)
gcm-gal4 (Booth et al., 2000)
sca-gal4 (Pearson and Doe, 2003)
elav-gal4 (Pearson and Doe, 2003)

Antibody staining and DiI labeling

We used the following antibodies at the indicated dilutions: rabbit anti-Zfh1 (1:5000) (Lai et al., 1991), guinea pig anti-Zfh1 (1:300; Jim Skeath), rat anti-Hb9 (Odden et al., 2002), guinea pig anti-Hb9 (Broihier and Skeath, 2002), rabbit anti-phosphoMad (pS1, 1:300; Peter Ten Dijke), rabbit anti-Muscle Myosin (1:500; Dan Kiehart), mouse anti-Beta-Galactosidase (1:500; Promega), mouse anti-Tau (1:300; Sigma), mouse anti-FasII monoclonal 1D4 (1:200), mouse anti-FasIII monoclonal 7G10 (1:5), rat anti-Islet (1:500) (Broihier and Skeath, 2002), guinea pig anti-Lim3 (1:500) (Broihier and Skeath, 2002), mouse anti-Eve monoclonal 2B8 (1:20), guinea pig anti-Eve (1:1000; East Asian Distribution Center for Segmentation antibodies), mouse anti-Repo (1:20), mouse anti-22C10 (1:5), rabbit anti-GFP (1:500; Torrey Pine Biolabs), mouse anti-GFP (1:500; Roche). Secondary antibodies were purchased conjugated to Alexafluor488, Rhodamine Red, or Cy5 (Jackson Immunoresearch Laboratories; Molecular Probes) and used at 1:200. Embryos were fixed and stained using standard methods (Odden et al., 2002) and imaged on a BioRad Radiance Confocal Microscope. DiI neuroblast labeling and processing were performed as previously described (Schmid et al., 1999).

Acknowledgments

We thank Amy Sheehan for technical assistance; Zhi-Chun Lai, Jim Skeath, Dan Kiehart, and Peter Ten Dijke for fly stocks or antibodies; and current members of the Doe lab for helpful comments. This work is supported by an NIH Genetics Training Grant (M.L.) and NIH HD27056 (C.Q.D.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.12.009.

References

Aberle, H., Haghighi, A.P., Fetter, R.D., McCabe, B.D., Magalhaes, T.R., Goodman, C.S., 2002. Wishful thinking encodes a BMP type II receptor that regulates synaptic growth in *Drosophila*. Neuron 33, 545–558.

Akiyama-Oda, Y., Hotta, Y., Tsukita, S., Oda, H., 2000. Mechanism of glia-neuron cell-fate switch in the *Drosophila* thoracic neuroblast 6-4 lineage. Development 127, 3513–3522.

Arber, S., Han, B., Mendelsohn, M., Smith, M., Jessell, T.M., Sockanathan, S., 1999. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron 23, 659–674.

Bassez, G., Camand, O.J., Cacheux, V., Kobetz, A., Dastot-Le Moal, F., Marchant, D., Catala, M., Abitbol, M., Goossens, M., 2004. Pleiotropic and diverse expression of ZFHX1B gene transcripts during mouse and human development supports the various clinical manifestations of the "Mowat–Wilson" syndrome. Neurobiol. Dis. 15, 240–250.

Booth, G.E., Kinrade, E.F., Hidalgo, A., 2000. Glia maintain follower neuron survival during *Drosophila* CNS development. Development 127, 237–244.

Bossing, T., Brand, A.H., 2002. Dephrin, a transmembrane ephrin with a unique structure, prevents interneuronal axons from exiting the *Drosophila* embryonic CNS. Development 129, 4205–4218.

Bossing, T., Udolph, G., Doe, C.Q., Technau, G.M., 1996. The embryonic central nervous system lineages of *Drosophila melanogaster*: I. Neuroblast lineages derived from the ventral half of the neuroectoderm. Dev. Biol. 179, 41–64.

Broihier, H.T., Skeath, J.B., 2002. *Drosophila* homeodomain protein dHb9 directs neuronal fate via crossrepressive and cell-nonautonomous mechanisms. Neuron 35, 39–50.

Broihier, H.T., Moore, L.A., Van Doren, M., Newman, S., Lehmann, R., 1998. zfh-1 is required for germ cell migration and gonadal mesoderm development in *Drosophila*. Development 125, 655–666.

Broihier, H.T., Kuzin, A., Zhu, Y., Odenwald, W., Skeath, J.B., 2004. *Drosophila* homeodomain protein Nkx6 coordinates motoneuron subtype identity and axonogenesis. Development 131, 5233–5242.

Cheesman, S.E., Layden, M.J., Von Ohlen, T., Doe, C.Q., Eisen, J.S., 2004. Zebrafish and fly Nkx6 proteins have similar CNS expression patterns and regulate motoneuron formation. Development 131, 5221–5232.

Clark, S.G., Chiu, C., 2003. *C. elegans* ZAG-1, a Zn-finger-homeodomain protein, regulates axonal development and neuronal differentiation. Development 130, 3781–3794.

Das, P., Maduzia, L.L., Wang, H., Finelli, A.L., Cho, S.H., Smith, M.M., Padgett, R.W., 1998. The Drosophila gene Medea demonstrates the requirement for different classes of Smads in dpp signaling. Development 125 (8), 1519–1528.

Dickson, B.J., 2002. Molecular mechanisms of axon guidance. Science 298, 1959–1964.

Frank, L.H., Rushlow, C., 1996. A group of genes required for maintenance of the amnioserosa tissue in *Drosophila*. Development 122, 1343–1352.

Fujioka, M., Lear, B.C., Landgraf, M., Yusibova, G.L., Zhou, J., Riley, K.M., Patel, N.H., Jaynes, J.B., 2003. Even-skipped, acting as a repressor, regulates axonal projections in *Drosophila*. Development 130, 5385–5400.

Gorczyca, M.G., Phillis, R.W., Budnik, V., 1994. The role of tinman, a mesodermal cell fate gene, in axon pathfinding during the development of the transverse nerve in *Drosophila*. Development 120, 2143–2152.

Higashijima, S., Shishido, E., Matsuzaki, M., Saigo, K., 1996. eagle, a member of the steroid receptor gene superfamily, is expressed in a subset of neuroblasts and regulates the fate of their putative progeny in the *Drosophila* CNS. Development 122, 527–536.

Isshiki, T., Pearson, B., Holbrook, S., Doe, C.Q., 2001. *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell 106, 511–521.

Keleman, K., Dickson, B.J., 2001. Short- and long-range repulsion by the *Drosophila* Unc5 netrin receptor. Neuron 32, 605–617.

Lai, Z.C., Fortini, M.E., Rubin, G.M., 1991. The embryonic expression patterns of zfh-1 and zfh-2, two *Drosophila* genes encoding novel zinc-finger homeodomain proteins. Mech. Dev. 34, 123–134.

Lai, Z.C., Rushton, E., Bate, M., Rubin, G.M., 1993. Loss of function of the *Drosophila* zfh-1 gene results in abnormal development of mesodermally derived tissues. Proc. Natl. Acad. Sci. U. S. A. 90, 4122–4126.

Landgraf, M., Bossing, T., Technau, G.M., Bate, M., 1997. The origin, location, and projections of the embryonic abdominal motorneurons of *Drosophila*. J. Neurosci. 17, 9642–9655.

Landgraf, M., Roy, S., Prokop, A., VijayRaghavan, K., Bate, M., 1999. Even-skipped determines the dorsal growth of motor axons in *Drosophila*. Neuron 22, 43–52.

Lundell, M.J., Lee, H.K., Perez, E., Chadwell, L., 2003. The regulation of apoptosis by Numb/Notch signaling in the serotonin lineage of *Drosophila*. Development 130, 4109–4121.

Lundgren, S.E., Callahan, C.A., Thor, S., Thomas, J.B., 1995. Control of neuronal pathway selection by the *Drosophila* LIM homeodomain gene apterous. Development 121, 1769–1773.

Marques, G., Bao, H., Haerry, T.E., Shimell, M.J., Duchek, P., Zhang, B., O'Connor, M.B., 2002. The *Drosophila* BMP type II receptor wishful thinking regulates neuromuscular synapse morphology and function. Neuron 33, 529–543.

Miguel-Aliaga, I., Thor, S., 2004. Segment-specific prevention of pioneer neuron apoptosis by cell-autonomous, postmitotic Hox gene activity. Development 131, 6093–6105.

Muraoka, O., Ichikawa, H., Shi, H., Okumura, S., Taira, E., Higuchi, H., Hirano, T., Hibi, M., Miki, N., 2000. Kheper, a novel ZFH/deltaEF1 family member, regulates the development of the neuroectoderm of zebrafish (*Danio rerio*). Dev. Biol. 228, 29–40.

Novotny, T., Eiselt, R., Urban, J., 2002. Hunchback is required for the specification of the early sublineage of neuroblast 7-3 in the *Drosophila* central nervous system. Development 129, 1027–1036.

Odden, J.P., Holbrook, S., Doe, C.Q., 2002. *Drosophila* HB9 is expressed in a subset of motoneurons and interneurons, where it regulates gene expression and axon pathfinding. J. Neurosci. 22, 9143–9149.

Pearson, B.J., Doe, C.Q., 2003. Regulation of neuroblast competence in *Drosophila*. Nature 425, 624–628.

Postigo, A.A., 2003. Opposing functions of ZEB proteins in the regulation of the TGFbeta/BMP signaling pathway. EMBO J. 22, 2443–2452.

Postigo, A.A., Dean, D.C., 1997. ZEB, a vertebrate homolog of *Drosophila* Zfh-1, is a negative regulator of muscle differentiation. EMBO J. 16, 3935–3943.

Postigo, A.A., Dean, D.C., 1999. ZEB represses transcription through interaction with the corepressor CtBP. Proc. Natl. Acad. Sci. U. S. A. 96, 6683–6688.

Postigo, A.A., Ward, E., Skeath, J.B., Dean, D.C., 1999. zfh-1, the *Drosophila* homologue of ZEB, is a transcriptional repressor that regulates somatic myogenesis. Mol. Cell. Biol. 19, 7255–7263.

Prokop, A., Landgraf, M., Rushton, E., Broadie, K., Bate, M., 1996. Presynaptic development at the *Drosophila* neuromuscular junction: assembly and localization of presynaptic active zones. Neuron 17, 617–626.

Schmid, A., Chiba, A., Doe, C.Q., 1999. Clonal analysis of *Drosophila* embryonic neuroblasts: neural cell types, axon projections and muscle targets. Development 126, 4653–4689.

Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J., Technau, G.M., 1997. The embryonic central nervous system lineages of *Drosophila melanogaster*: II. Neuroblast lineages derived from the dorsal part of the neuroectoderm. Dev. Biol. 189, 186–204.

Schneider, V.A., Granato, M., 2003. Motor axon migration: a long way to go. Dev. Biol. 263, 1–11.

Sekido, R., Takagi, T., Okanami, M., Moribe, H., Yamamura, M., Higashi, Y., Kondoh, H., 1996. Organization of the gene encoding transcriptional repressor deltaEF1 and cross-species conservation of its domains. Gene 173, 227–232.

Sepp, K.J., Schulte, J., Auld, V.J., 2001. Peripheral glia direct axon guidance across the CNS/PNS transition zone. Dev. Biol. 238, 47–63.

Shirasaki, R., Pfaff, S.L., 2002. Transcriptional codes and the control of neuronal identity. Annu. Rev. Neurosci. 25, 251–281.

Skeath, J.B., Doe, C.Q., 1998. Sanpodo and Notch act in opposition to Numb to distinguish sibling neuron fates in the *Drosophila* CNS. Development 125, 1857–1865.

Spana, E.P., Kopczynski, C., Goodman, C.S., Doe, C.Q., 1995. Asymmetric localization of numb autonomously determines sibling neuron identity in the *Drosophila* CNS. Development 121, 3489–3494.

Stathakis, D.G., Pentz, E.S., Freeman, M.E., Kullman, J., Hankins, G.R., Pearlson, N.J., Wright, T.R., 1995. The genetic and molecular organization of the Dopa decarboxylase gene cluster of *Drosophila melanogaster*. Genetics 141, 629–655.

Su, M.T., Fujioka, M., Goto, T., Bodmer, R., 1999. The *Drosophila* homeobox genes zfh-1 and even-skipped are required for cardiac-specific differentiation of a numb-dependent lineage decision. Development 126, 3241–3251.

Takagi, T., Moribe, H., Kondoh, H., Higashi, Y., 1998. DeltaEF1, a zinc finger and homeodomain transcription factor, is required for skeleton patterning in multiple lineages. Development 125, 21–31.

Tanabe, Y., William, C., Jessell, T.M., 1998. Specification of motor neuron identity by the MNR2 homeodomain protein. Cell 95, 67–80.

Tessier-Lavigne, M., Goodman, C.S., 1996. The molecular biology of axon guidance. Science 274, 1123–1133.

Thaler, J., Harrison, K., Sharma, K., Lettieri, K., Kehrl, J., Pfaff, S.L., 1999. Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. Neuron 23, 675–687.

Thor, S., Thomas, J.B., 1997. The *Drosophila* islet gene governs axon pathfinding and neurotransmitter identity. Neuron 18, 397–409.

Thor, S., Andersson, S.G., Tomlinson, A., Thomas, J.B., 1999. A LIM-homeodomain combinatorial code for motor-neuron pathway selection. Nature 397, 76–80.

Vactor, D.V., Sink, H., Fambrough, D., Tsoo, R., Goodman, C.S., 1993. Genes that control neuromuscular specificity in *Drosophila*. Cell 73, 1137–1153.

Varela-Echavarria, A., Tucker, A., Puschel, A.W., Guthrie, S., 1997. Motor axon subpopulations respond differentially to the chemorepellents netrin-1 and semaphorin D. Neuron 18, 193–207.

Wacker, I., Schwarz, V., Hedgecock, E.M., Hutter, H., 2003. zag-1, a Zn-finger homeodomain transcription factor controlling neuronal differentiation and axon outgrowth in *C. elegans*. Development 130, 3795–3805.