

## ***zag-1*, a Zn-finger homeodomain transcription factor controlling neuronal differentiation and axon outgrowth in *C. elegans***

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### **SUMMARY**

The nervous system consists of diverse subtypes of neurons, whose identities must be specified during development. One important aspect of the differentiation program of neurons is the expression of the appropriate set of genes controlling axon pathway selection. We have identified a novel Zn-finger/homeodomain containing transcription factor, *zag-1*, required for particular aspects of axonal pathfinding. In *zag-1* mutants, motorneuron commissures either branch prematurely or fail to branch at the correct point. Ventral cord interneurons show defects in the guidance towards the ventral cord and also in the ventral cord. Several neurons misexpress differentiation markers, including glutamate receptor subunits and chemosensory receptors. *zag-1* is

expressed transiently in embryonic and postembryonic neurons during differentiation as well as in some mesodermal tissues. Null mutants of *zag-1* are unable to swallow food and die as L1 larvae with a starved appearance, indicating that *zag-1* has an additional role in pharynx development. The vertebrate homolog,  $\delta$ EF1, is highly conserved and known to act as transcriptional repressor in various tissues. Our data indicate that *zag-1* also acts as transcriptional repressor controlling important aspects of terminal differentiation of neurons.

Key words: *zag-1*, *C. elegans*, Neuronal differentiation, Axon guidance

### **INTRODUCTION**

The directed outgrowth of neuronal processes is a crucial step in neuronal development because navigation errors will, eventually, lead to wiring defects in the nervous system. Different types of neurons typically send their axons along different pathways into distinct target areas. This is achieved, in part, by the differential expression of particular axon-guidance receptors, which define the spectrum of guidance cues that can be sensed (reviewed by Chisholm and Tessier-Lavigne, 1999; Grunwald and Klein, 2002; Wadsworth, 2002; Yu and Bargmann, 2001). Ectopic expression of guidance receptors, such as UNC-5, the receptor mediating repulsive responses to netrin, in cells that normally do not express the receptor leads to a redirection of their outgrowth away from the netrin source (Hamelin et al., 1993).

A number of transcription factors are implicated in various steps of neuronal differentiation, starting with the selection of neuronal precursors to the specification of neuronal subtypes (Bertrand et al., 2002; Brunet and Pattyn, 2002; Dubois and Vincent, 2001; Goulding, 1998; Lee and Pfaff, 2001; Marquardt and Pfaff, 2001; Shirasaki and Pfaff, 2002). An extensively studied family that affects aspects of neuronal differentiation are the LIM-homeodomain transcription factors, which define motorneuron-subtype identities in a combinatorial fashion (reviewed by Jacob et al., 2001; Shirasaki and Pfaff, 2002). However, expression of LIM-

homeodomain transcription factors is restricted to only a few subsets of neurons, so that LIM proteins alone cannot explain how hundreds of different neuronal subtypes are specified. A few other transcription factors are known to be involved in neuronal subtype specification (Brunet and Pattyn, 2002; Dubois and Vincent, 2001; Lee and Pfaff, 2001; Marquardt and Pfaff, 2001), but a direct link between these and genes involved in axonal pathfinding has been made rarely (Erkman et al., 2000). This leaves the problem of how a particular class of neurons express the appropriate set of axon-guidance receptors and signal-transduction components largely unresolved.

Neurons and their processes can be visualized in vivo in *C. elegans* with GFP markers (Chalfie et al., 1994). This allows new types of genetic screens, in which mutants with axon guidance defects can be isolated directly by selecting for animals with visible axon outgrowth defects in GFP-labelled neurons. We isolated mutants with defects in the outgrowth of interneuron axons in *C. elegans* (H.H., unpublished). Here we describe one of these mutants defining the gene *zag-1*. Mutants in *zag-1* show characteristic defects in the navigation of interneuron axons, ranging from fasciculation defects in the ventral cord to completely misrouted axons that extend inappropriately along the side of the animal. Furthermore, *zag-1*-mutant animals ectopically express the *glr-1::GFP* marker in additional neurons, indicating that the specification of neurotransmitter subtypes, in this case expression of the glutamate receptor *glr-1*, is compromised. Various classes of

motorneurons also show characteristic axon outgrowth defects, such as ventral cord fasciculation defects, premature and incomplete branching of commissures, and misexpression or lack of expression of cell-type-specific markers. A deletion allele of *zag-1*, which was isolated from our deletion library, has additional defects in pharynx development leading to an inability to feed and, consequently, larval lethality. *zag-1* encodes a putative transcription factor with N- and C-terminal Zn-finger clusters and a homeodomain in between, hence the name *zag-1* (Zn finger involved in axon guidance). *zag-1* is expressed transiently in a large number of postmitotic neurons, indicating that this gene plays an important role in controlling aspects of neuronal differentiation.

## MATERIALS AND METHODS

### Nematode strains

The following strains were used for mapping: *dpy-5(e61)*, *dpy-10(e128)*, *dpy-17(e164)*, *dpy-13(e184)*, *dpy-11(e224)*, *dpy-13(e184)* *unc-5(e53)*, *dpy-13(e184)* *unc-17(e113)*, DR684: mDf9/nT1 IV; +/nT1 V, DR814: *dpy-13(e184)* *ama-1(m118)* mDf8 IV/nT1 [*let-?(m435)*](IV;V), and CB4856 [N2, CB subclone of HA-8 (Tc1 pattern IX)].

The following GFP-reporter strains were used for analysis of axonal defects and misexpression: evIn82A[*unc-129::GFP*], oxIs12[*unc-47::GFP*NTX, *lin-15(+)*], wdIs6[*del-1::GFP*, *dpy-20(+)*], evIs111[F25B3.3::GFP], gvEx173[*opt-3::GFP*, *rol-6(su1006)*], kyIs51[*odr-2::GFP*, *lin-15(+)*], ccIs4251[*myo-3::DFP*, *myo-3::mitoGFP*, *dpy-20(+)*] I; *him-8(e1489)*, rhIs4[*glr-1::GFP*; *dpy-20(+)*], rhIs7[*unc-4::GFP*; *rol-6(su1006)*], rhIs11[*unc-3::GFP*; *rol-6(su1006)*], rhIs12[*sra-6::GFP*; *dpy-20(+)*], rhIs18[*epi-1::GFP* nuclear, *dpy-20(+)*], hdIs1[*unc-53::GFP*, *rol-6(su1006)*], hdIs10[*unc-129::CFP*, *glr-1::YFP*, *unc-47::DsRed*, *hsp16::rol-6*] V, hdIs14[*odr-2::CFP*, *unc-129::YFP*, *glr-1::DsRed*, *hsp-16::rol-6*], hdIs8[*him-4::GFP*, *rol-6(su1006)*].

All strains were cultured at 20°C using standard methods.

### GFP markers

To generate GFP-reporter constructs, promoter sequences were amplified by PCR and cloned into GFP vectors: *epi-1::GFP*: 2.8 kb upstream of ATG cloned in pPD96.62 (nuclear localized GFP, gift from A. Fire). All other markers (*unc-3*, *unc-4*, *unc-47*, *unc-53*, *unc-129*, *glr-1*, *him-4*, *odr-2*, *opt-3*, F25B3.3, *myo-3* are *sra-6*) were derived from previously published constructs. For some, the GFP coding region was replaced with a different GFP variant. Variants used are (A. Fire vector kit): GFP, S65C mutation; CFP, Y66W, N146I, M153T, V163A; YFP, S65G, V68A, S72A, T203Y; DsRed, DsRed from Clontech vector pDsRed.

### Mutant isolation

The *zag-1(rh315)* allele was isolated after EMS mutagenesis of rhIs4[*glr-1::GFP*] animals in a nonclonal screen for animals with axon outgrowth defects in *glr-1*-expressing interneurons. The deletion mutant *hd16* was isolated from an EMS-mutagenised library using a poison primer approach targeting the first exon (Edgley et al., 2002).

### Mapping and cosmid rescue

*zag-1(rh315)* was mapped to a region between *unc-17(e113)* and *dpy-13(e184)* on LG IV by classical genetic methods. For further mapping using single nucleotide polymorphisms *zag-1(rh315)* *dpy-13(e184)* recombinants were crossed into the CB4856 wild type strain. F2 animals having lost one of the markers were scored for the presence of a SNP marker on cosmid W03D2. Transgenic animals injected with a cosmid pool (K09B3, M02B7, F28F9, T08C8, F37C4, K02F11,

F26F6, T17A2) at 10 ng/μl each and pRF4 (*rol-6(su1006)*) at 25 ng/μl) or the individual cosmids were analysed for rescue of movement and axonal defects. PCR fragments containing the entire coding region of F28F9.1 or F28F9.4 plus 4.5 kb or 3.5 kb upstream were also tested for their rescuing abilities.

### Sequencing

PCR fragments containing either exons 1-4 or exons 5-7 were generated for sequencing to identify the mutation in *zag-1(rh315)*. cDNA clones yk168d11, yk281e5, yk312a9, yk556a12, yk621g7, and yk621g7 were excised according to the manufacturer's instructions (Stratagene) and used for restriction analysis and sequencing. The deletion in *zag-1(hd16)* was defined by sequencing a 1.7 kb PCR fragment starting ~0.75 kb upstream of the first ATG. The deletion in *zag-1(hd16)* is 516 bp in size and starting 50 bp downstream of the ATG.

### Analysis of *zag-1* expression

To generate reporter constructs we used the Gateway™ cloning system according to the manufacturer's instructions (Life Technologies). A PCR fragment containing 4.7 kb upstream region of *zag-1* was cloned into an entry vector and recombined with a destination vector containing YFP to create *Pzag-1::YFP*. Similarly, the same upstream region plus the entire coding region was fused to the N-terminus of YFP. Transgenic animals were generated by injecting 20 ng μl<sup>-1</sup> reporter plasmid plus 50 ng μl<sup>-1</sup> pRF4. Arrays were integrated using UV-irradiation, followed by two backcrosses with N2.

### Analysis of neuronal defects

Animals were grown at 20°C and analysed as either newly hatched L1 larvae or adult animals from a growing population. For taking images, animals were incubated with 100 mM NaN<sub>3</sub> in M9 buffer for 1 hour and mounted on agar pads. Stacks of confocal images with 0.3–0.4 μm vertical pitch were recorded with a Leica TCS SP2 microscope. Maximum intensity projections of all images from a given animal were generated using the Imaris 3.1 software package.

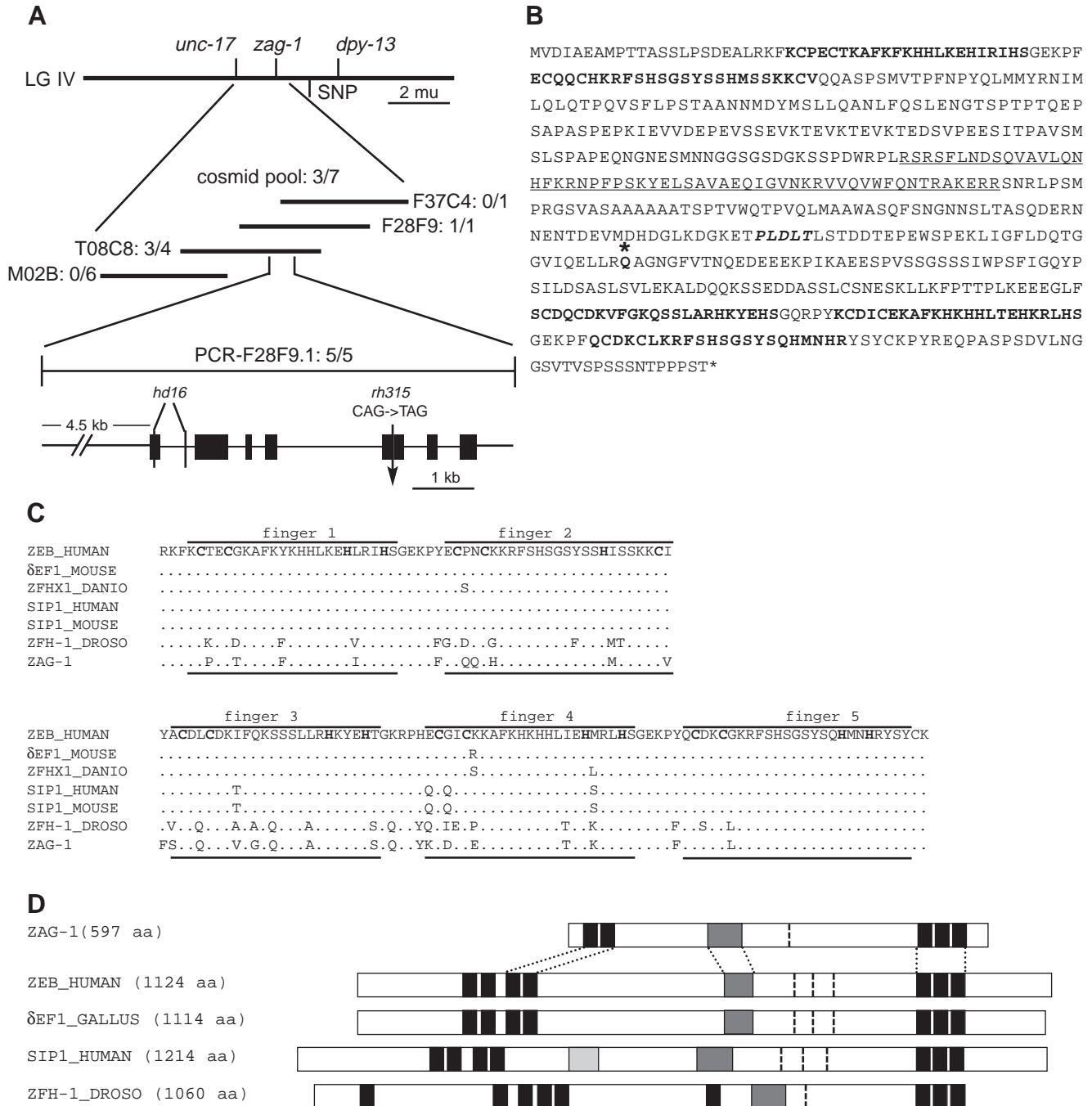
### Feeding experiments

1 ml of a 0.02% suspension of fluorescent latex beads (100 nm diameter, Molecular Probes) in OP50-containing medium was added to a mixed population of almost starving animals (progeny of a *zag-1(hd16)*/hdIs14 IV parent) on a small Petri dish. After 1 hour incubation with occasional agitation, animals were transferred to agar pads on microscope slides and observed with DIC and epifluorescence microscopy. Images were recorded with a Princeton Instruments MicroMAX cooled CCD camera using the Metamorph 4.1 Imaging software package. To investigate uptake of nonparticulate markers, 10 μM FM1-43 (Molecular Probes) in M9 buffer was applied as above and analyzed after either 1 or 4 hours.

## RESULTS

### Molecular cloning of the *zag-1* gene

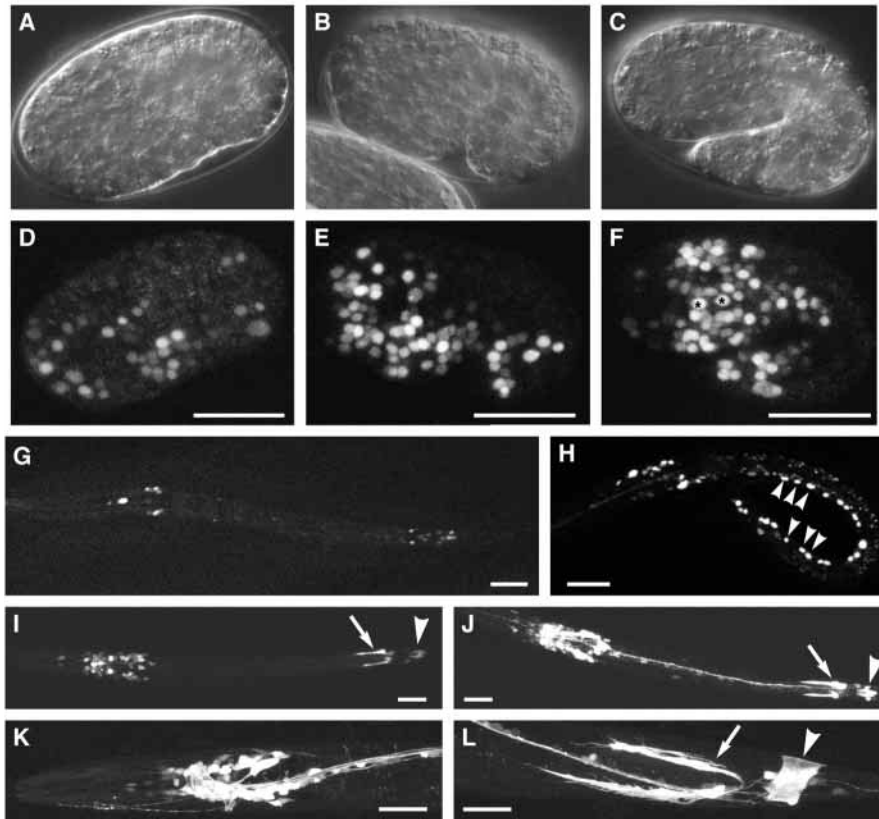
The *zag-1(rh315)* mutant was identified originally in a genetic screen for mutants with axon outgrowth defects in *glr-1::GFP* expressing interneurons. To identify the gene, we narrowed the location of the *zag-1(rh315)* mutation by standard, two- and three-factor mapping to the interval between *unc-17* and *dpy-13* on chromosome IV. The use of SNP markers helped to refine the position further, to an interval of 450 kb. Cosmid clones covering this region were used to generate transgenic lines that were assayed for their ability to complement the movement and *glr-1::GFP* misexpression defects in *zag-*



**Fig. 1.** Cloning of *zag-1*. (A) Genomic region containing the *zag-1* gene. The fraction of transgenic lines rescuing *zag-1* defects is indicated with numbers beside the cosmid names (3/4 – defects were rescued in 3 out of 4 transgenic lines). ‘Cosmid pool’ refers to a mixture of all the cosmids shown. (B) ZAG-1 protein sequence. Zn fingers are indicated in bold, the homeodomain is underlined, the CtBP1/2 corepressor binding site (PLDLT) is in bold italics and the star indicates the premature stop codon in *rh315*. (C) Sequence alignments of Zn fingers. The sequence of the Zn fingers in the chick δEF1 protein is identical to human ZEB. (D) Domain organisation of ZAG-1 and its homologs. Zn fingers are indicated in black, homeodomains in dark grey, SMAD-interacting domain in light grey and CtBP1/2 corepressor binding sites are dashed vertical lines.

*l(rh315)*. Two overlapping cosmids were found to contain the *zag-1* gene. PCR fragments containing the genes located in the region of overlap of these two cosmids were used for further rescue experiments. Of these, only the fragment corresponding to the F28F9.1 gene rescued the *zag-1*-mutant phenotype (Fig. 1A). Sequencing the coding region in the *zag-*

*l* mutant revealed a single transition, C to T in glutamine 391, that resulted in a premature stop codon, thus confirming the identity of the *zag-1* gene (Fig. 1B). Several cDNA clones were found to be identical, indicating that the gene gives rise to one major form of mRNA. Sequencing of cDNA clones revealed one difference in comparison to the sequence of



**Fig. 2.** Expression of *zag-1*. *zag-1::YFP*: DIC (A-C) and corresponding epifluorescence images (D-F) of embryos at different developmental stages. *zag-1* expression is mainly seen in neurons in the head and tail ganglia as well as in ventral cord motorneurons. Stars (F) mark two pharyngeal nuclei, probably m4 and m5 muscle cell nuclei. (G-H) Early (G) and late (H) L1 larvae; expression is seen transiently in postembryonic motorneurons (H). Arrowheads (H) indicate motorneuron nuclei in the ventral cord. *Pzag-1::YFP* in wildtype (I) and *zag-1(rh315)* animals (J-L); *zag-1* expression in neurons is not downregulated in *zag-1(rh315)*. Arrows (I,J,L) indicate intestinal muscle, arrowheads (I,J,L) indicate the anal depressor muscle. Ventral aspects in (I,J), side views in all others. Anterior is to the left, dorsal is up. Scale bars: 20  $\mu$ m.

similarity. A paralog, SIP1/ZEB2, has been identified in vertebrates, indicating that there was a gene duplication in the vertebrate lineage.

### Expression of *zag-1*

We generated several YFP-reporter constructs to study the expression pattern of *zag-1*. First we cloned the upstream regulatory region, which is sufficient to rescue the defects in *zag-1*-mutant animals, into a YFP-vector (*Pzag-1::YFP*). Second, we cloned the upstream and entire coding region into a vector fusing YFP to the C-terminus of ZAG-1 (*zag-1::YFP*). With both constructs we generated transgenic lines that were stably integrated into the genome and used for expression analysis.

We found the *Pzag-1::YFP* construct was expressed predominantly in neurons in head and tail ganglia, starting approximately midway through embryogenesis (Fig. 2A). In some of these neurons expression was maintained throughout development (Fig. 2I). Additional expression was found consistently in the intestinal and anal depressor muscles during all life stages (Fig. 2L) as well as occasionally in body-wall muscles during embryogenesis.

The *zag-1::YFP* fusion construct was used to study the expression in more detail. *zag-1::YFP* signal was detected only in the nuclei of cells, supporting the idea that *zag-1* acts as transcriptional regulator. Expression was detected in a few nuclei in the head as the embryo reached morphogenesis stage at ~300 minutes of development (Fig. 2A,D). Expression was identified in more and more nuclei as development proceeded (Fig. 2B,E), so that by the 1 1/2-fold stage a large number of neuronal cells in the head and a few cells in the pharynx expressed *zag-1::YFP* (Fig. 2C,F). At this stage expression was also prominent in motorneurons in the ventral cord and in neurons in tail ganglia. Expression was maintained during the 3-fold stage, but reduced to undetectable levels in most cells before hatching, when only a few cells in the head still expressed *zag-1::YFP* (Fig. 2G). In the L1/L2 stage expression was detected transiently in postembryonic motorneurons (Fig.

F28F9.1 predicted by the gene finder program of the Genome Sequencing Center.

The protein encoded by *zag-1* contains a N-terminal cluster of two and a C-terminal cluster of three Zn fingers, hence the name *zag* (Zn finger involved in axon guidance). The protein also contains one homeodomain in the center of the protein (Fig. 1B). The combination of Zn fingers and a homeodomain in one protein is unusual. The entire *C. elegans* genome contains only two such proteins. The other, ZC123.3, has three homeodomains and several Zn-finger clusters.

Orthologs of *zag-1* are found in *Drosophila* and vertebrates. The *Drosophila* protein is called ZFH-1 (Fortini et al., 1991). The vertebrate proteins are  $\delta$ EF1 in chick (Funahashi et al., 1993), MEB1/ $\delta$ EF1 in mouse (Genetta and Kadesch, 1996; Sekido et al., 1994), AREB6/ZEB-1/*nil-2-a* in humans (Genetta et al., 1994; Watanabe et al., 1993; Williams et al., 1991) and Zfhx1 in zebrafish (Muraoka et al., 2000). Each of these proteins has a cluster of Zn fingers at both ends and a single homeodomain in the center of the protein (Fig. 1D). The C-terminal Zn-finger cluster consists of three fingers in all proteins, whereas the number of fingers in the N-terminal cluster varies from two (ZAG-1) to five (ZFH-1, long isoform).  $\delta$ EF1 has been shown to act as transcriptional repressor and interact with a corepressor called CtBP1. The CtBP1-binding site downstream of the homeodomain (consensus sequence PLDLS/T) is also conserved in ZAG-1, and a CtBP1 homolog is found in the *C. elegans* genome (F49E10.5 Wormpep).

ZAG-1 and its homologs have a high degree of sequence identity (80% identity) in the Zn fingers (Fig. 1C). The homeodomain is also fairly well conserved (61% similarity), but sequences between these structural motifs have little

**Table 1. Axon guidance defects in *zag-1* mutants**

GFP marker	Neurons labeled	Processes analysed	Defect	% Animals defective	<i>zag-1</i> allele
evIs82A ( <i>unc-129::GFP</i> )	DA + DB motorneurons	Axons	Ventral cord defasciculation	42 ( <i>n</i> =114)	rh315
			Commissures	Outgrowth on the wrong side	93 ( <i>n</i> =95)
				Not reaching dorsal cord	84 ( <i>n</i> =100)
					Dorsal cord defasciculation
oxIs12 ( <i>unc-47::GFP</i> )	DD + VD motorneurons	Axon	Ventral cord defasciculation	43 ( <i>n</i> =100)	rh315
			Gap in ventral cord	0 ( <i>n</i> =100)	rh315
		Commissures*	Outgrowth on the wrong side	73 ( <i>n</i> =100)	rh315
			No outgrowth out of ventral cord	12 ( <i>n</i> =100)	rh315
			Not reaching dorsal cord	6 ( <i>n</i> =100)	rh315
			Missing branch	17 ( <i>n</i> =100)	rh315
			Premature/ectopic branch	30 ( <i>n</i> =100)	rh315
oxIs12 ( <i>unc-47::GFP</i> )	Motorneurons*	Axons	Ventral cord defasciculation	88 ( <i>n</i> =77)	hd16
			Gap in ventral cord	83 ( <i>n</i> =78)	hd16
		Commissures	Outgrowth on the wrong side	93 ( <i>n</i> =107)	hd16
			No outgrowth out of ventral cord	72 ( <i>n</i> =76)	hd16
wdIs6 ( <i>del-1::GFP</i> )	SABVR/L interneurons	Axons	Axon(s) loop back/change tract	50 ( <i>n</i> =104)	rh315
gvEx173 ( <i>opt-3::GFP</i> )	AVEL/R interneurons	Axons	Axon(s) extending laterally	16 ( <i>n</i> =114)	rh315
rhIs4 ( <i>glr-1::GFP</i> )	Ventral cord interneurons	Axons	Ventral cord defasciculation	55 ( <i>n</i> =147)	rh315
			Axon extending laterally	97 ( <i>n</i> =67)	rh315
hdIs10 ( <i>glr-1::GFP</i> )	Ventral cord interneurons*	Axons	Ventral cord defasciculation	70 ( <i>n</i> =64)	hd16
			Axon extending laterally	90 ( <i>n</i> =64)	hd16

\*L1 larvae analysed.

2H). Expression was maintained in a few head neurons throughout the entire life cycle.

### ZAG-1 regulates its own expression

The *Pzag-1::YFP* construct was crossed into the *zag-1(rh315)* mutant to analyze the potential effects of *zag-1* on its own expression. We found that expression of the *Pzag-1::YFP* construct in several classes of neurons, most notably motorneurons in the ventral cord, was not downregulated in *zag-1(rh315)*-mutant animals (Fig. 2J,K). These neurons expressed *zag-1* throughout the entire life cycle, indicating that downregulation of *zag-1* depends on the presence of intact ZAG-1 protein. The vertebrate homolog of ZAG-1,  $\delta$ EF1, has been shown to bind to E-box-sequence motifs (CACCTG) with each of its Zn fingers. Pairs of such motifs are found in the upstream regulatory region and the first intron of the *zag-1* gene (data not shown), indicating that this repression could be mediated directly, by ZAG-1 binding to its own promoter.

### *zag-1* mutants show defects in axonal outgrowth, branching and fasciculation

The ventral cord contains essential components of the motor circuit and consists of two axon bundles flanking the ventral midline. Interneuron axons enter the ventral cord from the anterior after exiting the nerve ring on the ventral side. Almost all axons on the left side cross over to run in the right axon tract, which leads to a highly asymmetrical ventral cord with many more axons running in the right than the left fascicle. *zag-1(rh315)*-mutant animals showed penetrant axon outgrowth defects in *glr-1::GFP*-expressing interneurons (Table 1). First, not all axons followed their normal trajectory from cell bodies in the head ganglia towards the nerve ring and further on into the ventral cord. Instead, axons extended abnormally in lateral positions, often wandering between lateral axon tracts (Fig. 3B). Occasionally, additional processes

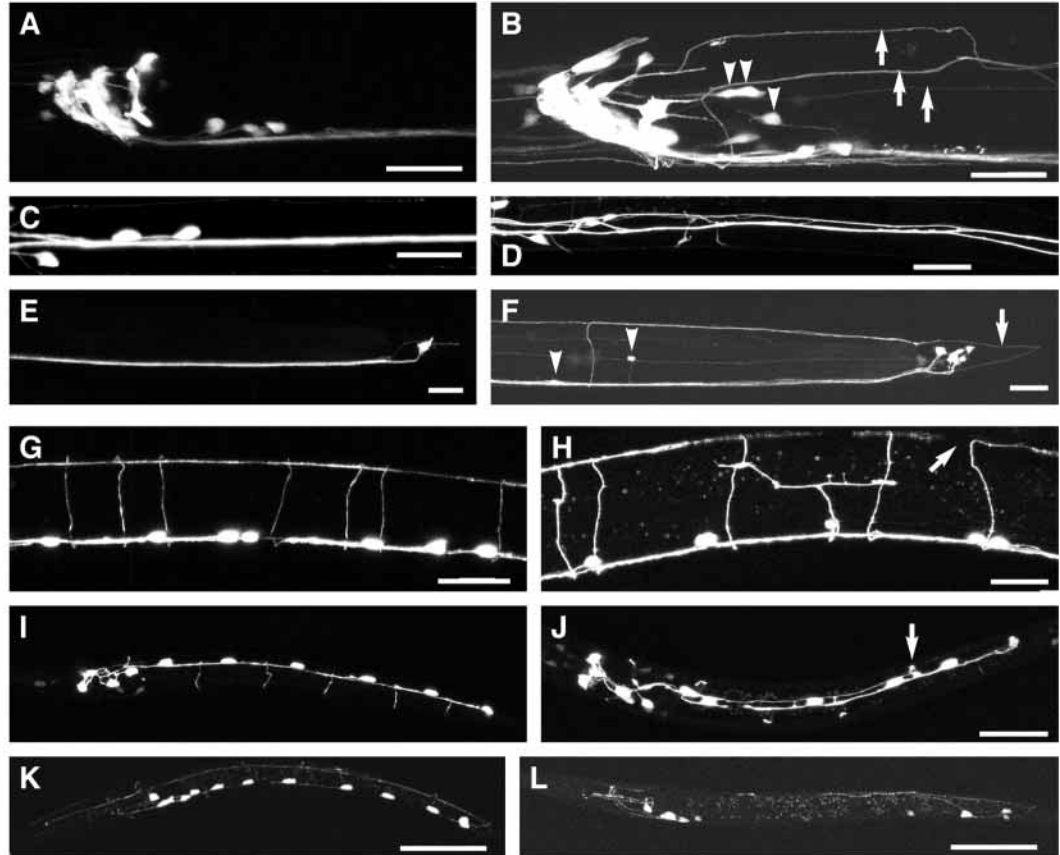
were sent out from the cell body, which sometimes formed ectopic branches. Axons reaching the ventral cord frequently had fasciculation problems in the ventral cord and crossed back and forth between the right and left axon tracts (Fig. 3D).

Defects in axon outgrowth were not restricted to *glr-1::GFP*-expressing interneurons. Trajectories of various classes of motorneuron axons were also defective (Table 1). Defects ranged from fasciculation defects in the ventral (Fig. 3J) and dorsal nerve cords to commissure outgrowth defects (Fig. 3H). Commissure defects assayed with an *unc-47::GFP* marker in D-type motorneurons fell into distinct classes. In >70% of animals one or more commissures grew on the left side, rather than the right side, towards the dorsal cord (Fig. 3J). In some animals commissures branched prematurely before they reached the dorsal cord, and in a few, commissures failed to branch on reaching the dorsal cord (Fig. 3H). Typically, in these cases it was the anterior branch that was missing. Occasionally, commissures either never left the ventral cord or failed to reach the dorsal cord, extending in lateral positions instead.

AVE-interneuron axons labeled with *opt-3::GFP* normally cross the midline to the contralateral side in the nerve ring, turn posterior and enter the ventral cord. In 16% of *zag-1*-mutant animals they never entered the ventral cord and, instead, ran in a lateral position. These axonal defects in various classes of neurons that are part of the motor circuit led to variable movement defects in *zag-1(rh315)* mutants. Animals were able to move forward and backward, but were significantly uncoordinated in their movement. In some animals, parts of the body appeared stiff and other animals tended to coil, whereas a few moved without apparent problem. This variability in movement defects reflects the variability in the axonal-outgrowth defects seen in *zag-1(rh315)*.

### *zag-1* regulates neuronal differentiation

When analysing axonal defects in *zag-1(rh315)* mutants we



**Fig. 3.** Neuronal defects in *zag-1(rh315)*. Wild-type (A,C,E,G,I,K) and *zag-1(rh315)*-mutant animals (B,D,F,H,J,L) expressing *glr-1::GFP* (A-F), *unc-47::GFP* (G-J) and *unc-4::GFP* (K,L). *zag-1(rh315)* mutants have several axon outgrowth defects and misexpression of neuronal markers. Arrowheads (B,F) indicate cells ectopically expressing *glr-1::GFP*. Arrows (B) indicate lateral axons, (F) the PDB axon, which has a characteristic and unique trajectory, (H) a missing anterior commissure branch, and (J) a commissure growing on the wrong side. Ventral aspects in (C,D,I,J), side views in all others. Anterior is to the left, dorsal is up. Scale bars: 20  $\mu$ m.

noticed that additional cells expressed the *glr-1::GFP* marker. In head, as well as tail, ganglia a number of extra cells expressed *glr-1::GFP* (Fig. 3B,F). Among these were, occasionally, motorneurons and one or two cells in the PDE cluster, most likely PVD and occasionally PVM (Table 2). Some cells, such as RID and PDB, could also be identified by the position of their cell body and axon trajectory (Fig. 3F), indicating that these neurons retained major aspects of their original identity. The same was true for motorneurons that misexpress *glr-1::GFP*. Typically, these sent out commissures towards the dorsal cord, just as normal motorneurons do (Fig. 3F).

In addition to ectopic expression of neuronal differentiation markers, we also observed the loss of expression of several markers in part of their normal expression domain. Normally, the *sra-6::GFP* reporter is expressed in the ASH and PVQ neurons. Frequently, in *zag-1(rh315)*-mutant animals only one or none of the two PVQ neurons expressed the marker (Table 2). By contrast, *sra-6* expression in the two ASH neurons was always normal. The PVQ cell nucleus is larger than the surrounding neuronal nuclei and lies immediately adjacent to the PHA and PHB neuron cell bodies that can be stained with lipophilic dyes such as DiI. In *zag-1(rh315)*-mutant animals stained with DiI, neuronal cell bodies with the characteristics of PVQ were adjacent to the PHA and PHB neurons, indicating that the failure to observe *sra-6::GFP* expression is not caused by an absence of the cells (data not shown).

Various motorneuron markers were also misexpressed in *zag-1(rh315)* mutant animals (Table 2). We found *unc-*

*129::GFP* expressed ectopically in head neurons, a defect also seen in *unc-130* mutants. Some of the DA-type motorneurons failed to express the *unc-3::GFP* and *unc-4::GFP* markers (Fig. 3L). Conversely, *unc-129::GFP* was expressed consistently at high levels in DA8 and DA9, cells that do not express this marker in adult, wild-type animals. Other motorneuron markers, such as *unc-47::GFP*, were expressed normally in GABAergic motorneurons although, occasionally, some cells had lower levels of expression. Similarly, expression of *del-1::GFP* in VA and VB motorneurons was only changed to a minor degree. A pan-neuronal marker (F25B3.3::GFP) is expressed in the normal number of motorneurons in L1 larvae, again indicating that the failure in expression of some markers is not caused by the absence of the cells (Table 2). Finally, several other neuronal markers expressed in subsets of sensory and interneurons, including *odr-2::GFP* and *opt-3::GFP*, were also expressed normally, indicating that not all classes of neurons are affected in *zag-1(rh315)*.

The putative null allele, *zag-1(hd16)*, has axon guidance defects comparable to *zag-1(rh315)* mutants when assayed with a *glr-1::GFP* reporter (Fig. 4C, Table 1). Ectopic expression of *glr-1::GFP* in motorneurons was seen in the majority of animals (64% in 0-2 cells, 36% in  $\geq 3$  cells,  $n=67$ ). With a motorneuron marker (*unc-129::GFP*) that was expressed in the normal number of cells in *zag-1(rh315)*, we saw partial loss of expression in *zag-1(hd16)*-mutant animals (43% in 7-10 cells, 57% in  $\leq 6$  cells,  $n=67$ ). With the D-type motorneuron marker *unc-47::GFP*, we saw no changes in expression but very pronounced axon outgrowth defects (Fig.

**Table 2. Misexpression of neuronal markers in *zag-1(rh315)* mutants**

GFP marker	Cell-type labeled	Cells analysed	Number of cells expressing GFP	Phenotype in wild type (n)	Phenotype in <i>zag-1(rh315)</i> (n)
rhIs4 ( <i>glr-1::GFP</i> )	Interneurons	PDB	1	0% (100)	84% (147)
		PDE cluster	1-2	0% (100)	44% (147)
		motorneurons	1-2	0% (100)	13% (147)
evIs111 (F25B3.3:: <i>GFP</i> )	Motorneurons*	DA2-7, DB3-7, DD2-5	15	94% (100)	93% (100)
			13-14	6% (100)	7% (100)
rhIs11 ( <i>unc-3::GFP</i> )	Excitatory motorneurons*	DA1-DA9, DB1-DB7	14-15	85% (100)	29% (106)
			11-13	15% (100)	66% (106)
rhIs7 ( <i>unc-4::GFP</i> )	Excitatory motorneurons*	DA3-DA9	6-7	74% (132)	3% (124)
			3-5	27% (132)	53% (124)
			0-2	0% (132)	46% (124)
evIs82A ( <i>unc-129::GFP</i> )	Excitatory motorneurons*	DA1-DA7, DB1-DB7	13-14	70% (100)	100% (104)
			11-12	30% (100)	0% (104)
		DA8, DA9	2	0% (100)	0% (104)
oxIs12 ( <i>unc-47::GFP</i> )	Inhibitory motorneurons	DD1-DD6, VD1-VD13	19	87% (103)	53% (100)
			17-18	13% (103)	44% (100)
			15-16	0% (103)	3% (100)
wdIs6 ( <i>del-1::GFP</i> )	Excitatory motorneurons	VB3-VB7, VA2-VA6	10	100% (140)	0% (135)
			9	0% (140)	100% (135)
		VB8-11, VA7-VA10	8-9	17% (140)	32% (135)
			4-7	83% (140)	68% (135)
rhIs12 ( <i>sra-6::GFP</i> )	Interneurons	PVQ	2	99% (121)	1% (149)
			1	1% (121)	20% (149)
			0	0% (121)	79% (149)

\*L1 larvae analysed.

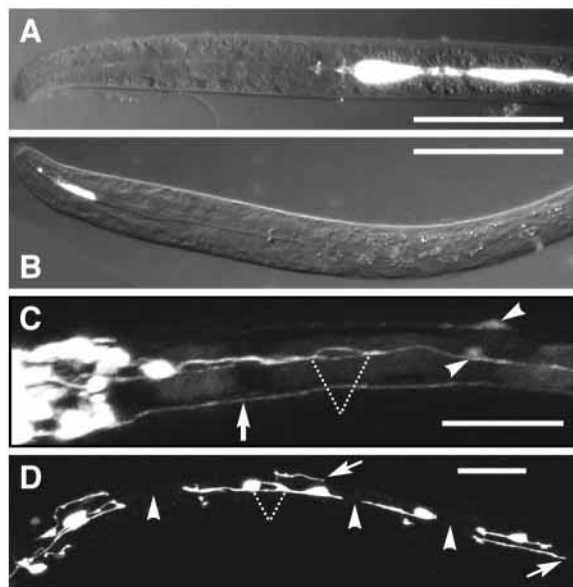
4D, Table 1), including premature termination of outgrowth that led to gaps in the ventral cord. In addition, a few motorneuron cell bodies are mispositioned in the ventral cord, most prominently DD2 lying close to DD1 in 95% of animals ( $n=77$ ). This indicates that some residual function for neuronal development is still present in *zag-1(rh315)*.

### Mesodermal development in *zag-1* mutants

The nature of the movement defects seen in *zag-1* mutants is characteristic of neuronal defects. There is no indication of a

muscle-related movement defect. Because we saw occasional *Pzag-1::GFP* expression in embryonic body-wall muscle as well as in some minor muscles (intestinal and anal depressor muscles, see above), we used several GFP-reporter constructs to look at the expression of muscle-specific markers and muscle morphology. We found no changes in either the morphology of body-wall muscle or expression of *myo-3::GFP* (myosin heavy chain), *epi-1::GFP* (laminin) and *him-4::GFP* (hemicentin). The only noticeable difference was a change in the expression of *unc-53::GFP* (a cytoskeletal adapter protein) in intestinal and anal sphincter muscles. In *zag-1(rh315)* mutants, expression of *unc-53::GFP* in the intestinal muscle was higher and in the sphincter muscle lower than in wild type, indicating that *zag-1* might modulate levels of expression of UNC-53 in these cells.

Because the *zag-1(rh315)* mutation leaves a large part of the protein intact, it is not clear whether *zag-1(rh315)* represents a



**Fig. 4.** Mesodermal and neuronal defects in *zag-1(hd16)*.

(A,B) Wild-type and *zag-1(hd16)* larvae after 1 hour incubation with fluorescent latex beads. Fluorescent material was transported into the gut in wild type (A), but remained in the anterior part of the pharynx in *zag-1* (B). *zag-1(hd16)* also has pronounced axon guidance defects. (C) *zag-1(hd16)* L1 larva expressing *glr-1::GFP*; the arrow indicates an axon extending laterally, arrowheads indicate ectopically expressing cells, and dotted lines indicate ventral cord defasciculation. (D) *zag-1(hd16)* L1 larva expressing *unc-47::GFP*; the arrows indicate commissures not reaching the dorsal cord but extending processes in a lateral position, arrowheads indicate gaps in the ventral cord, dotted lines indicate ventral cord defasciculation. Ventral aspect in C,D; side views in A,B. Anterior is to the left. Scale bars: 20  $\mu$ m.

complete loss-of-function allele. We therefore isolated a deletion allele from our deletion library using primers targeting the first exon. This 517 bp deletion removes part of the first exon (starting at codon 18) and most of the first intron, introducing a frame shift and a stop codon shortly thereafter (Fig. 1A). We expect this mutant to produce no functional product from the *zag-1* locus. We were unable to obtain homozygous mutant animals, even after two rounds of outcrossing, indicating that the *zag-1(hd16)* deletion might be lethal. Balanced *unc-17 dpy-13/zag-1(hd16)* IV and *hdIs14[unc-129::YFP]/zag-1(hd16)* IV strains indeed segregated early-larval-lethal animals. Overall, these animals have normal body morphology with all organs developed. They are severely uncoordinated and tend to coil. Even on plates with food they adopt a starved appearance and die after a few days, indicating that the animals are unable to either take up or digest food. Feeding *zag-1(hd16)* mutants with fluorescent beads (0.1  $\mu\text{m}$ ), we observed that the beads inevitably stuck in the anterior part of the pharynx (procorpus), suggesting that the animals are unable to swallow food (Fig. 4B). Similar observations were made when fluid-phase markers were used for feeding. No obvious morphological defects in the pharynx were observed by light microscopy. Pharynx-muscle-specific markers like *myo-2::GFP* are expressed normally (data not shown). Pharyngeal muscles are able to contract in *zag-1(hd16)*, however the contractions were weaker and did not lead to the characteristic opening and closing of the lumen of the isthmus. The lethality of *zag-1(hd16)* can be rescued by the *zag-1::YFP* transgene, which also rescued the neuronal defects in *zag-1(rh315)*, indicating that the lethality is, indeed, caused by deletion in the *zag-1* gene (data not shown). Transheterozygous *zag-1(rh315)/zag-1(hd16)* animals can survive (20% survivors), but grow slowly and are severely uncoordinated, indicating that they still have some feeding problems and, probably, have even stronger neuronal defects than *zag-1(rh315)* alone.

## DISCUSSION

### *zag-1* encodes a conserved Zn-finger/homeodomain transcription factor

In genetic screens for axon outgrowth mutants in *C. elegans* we identified a conserved transcription factor with an unusual structure – Zn-finger clusters at both ends of the protein flanking a homeodomain in the center of the protein. The vertebrate ortholog,  $\delta$ EF1, was identified originally as transcriptional repressor that binds to the  $\delta$ 1-crystallin enhancer core in chickens (Funahashi et al., 1991; Funahashi et al., 1993). Other vertebrate orthologs have been reported. These include: in humans, a partial sequence of nil-2-a, (Williams et al., 1991), also known as AREB6 (Watanabe et al., 1993) and ZEB (Genetta et al., 1994); in mouse, MEB1 (Genetta and Kadesch, 1996), which is identical to  $\delta$ EF1 (Sekido et al., 1994); and in zebrafish, Kheper (Muraoka et al., 2000). A single ortholog of ZAG-1/ $\delta$ EF1/ZEB is known in *Drosophila* as ZFH-1 (Fortini et al., 1991; Lai et al., 1991). It was shown that the *Drosophila* protein ZFH-1 and ZEB/ $\delta$ EF1 have the same repressor specificity and that ZFH-1 can functionally substitute for ZEB/ $\delta$ EF1 in blocking myogenic

differentiation in mammalian cell culture systems (Postigo et al., 1999). This suggests that ZFH-1 and therefore most likely also ZAG-1 is a functional homolog of ZEB/ $\delta$ EF1. A paralog of  $\delta$ EF1/ZEB, called SIP1, has been identified in mouse as Smad-interacting factor (Verschuere et al., 1999), suggesting that a gene duplication occurred in the vertebrate lineage.

$\delta$ EF1 binds to CACCT sequences and is thought to compete with basic helix-loop-helix (bHLH) transcription factors for binding to E2-boxes (sequence, CACCTG), thereby preventing gene transcription (Sekido et al., 1994). Two, independent, repression domains of  $\delta$ EF1/ZEB outside the Zn fingers have been shown to be sufficient to repress genes regulating either hematopoietic or muscle-specific differentiation pathways (Postigo and Dean, 1999a). One of these domains contains a PLDLS motif, which was shown to recruit the CtBP1/2 corepressor (Furusawa et al., 1999; Postigo and Dean, 1999b). This indicates that  $\delta$ EF1/ZEB can act in more than one way as repressor (Ikeda and Kawakami, 1995; Postigo and Dean, 1999a; Remacle et al., 1999; Sekido et al., 1997). The key sequence elements essential for  $\delta$ EF1/ZEB repressor function, the Zn-finger cluster and the CtBP-corepressor binding site, are highly conserved in ZAG-1, with a high degree of sequence identity. This makes it likely that ZAG-1 binds to the same consensus sequence as  $\delta$ EF1 and also acts as transcriptional repressor.

### *zag-1* expression and function in the mesoderm

The *zag-1* gene is expressed in mesodermal tissues including the pharynx, and the intestinal and anal depressor muscles (Fig. 2F,I,J). Expression in the pharynx is transient during embryogenesis, whereas expression in the intestinal and anal depressor muscles was apparent throughout development. *zag-1* orthologs in *Drosophila* and vertebrates are also expressed in the developing mesoderm, most notably in muscle cells (Funahashi et al., 1993; Lai et al., 1991; Takagi et al., 1998), again emphasizing the strong conservation in the expression of these proteins. *zfh-1* mutants in *Drosophila* have various defects in mesodermal tissues, including body-wall muscle, heart and gonadal mesoderm (Broihier et al., 1998; Lai et al., 1993). In *zfh-1* mutants some muscle cells are missing whereas others are either misplaced or disorganized.  $\delta$ EF1/ZEB in vertebrates has been shown to interfere with muscle differentiation in transfected cells in culture by counteracting the effect of bHLH proteins such as MyoD (Postigo and Dean, 1997; Sekido et al., 1994). The mouse  *$\delta$ EF1* (*Zfhxla* – Mouse Genome Informatics) gene is expressed in muscle cells during embryonic development, but no obvious defect in muscle-cell differentiation was detected in  $\delta$ EF1-mutant mice (Takagi et al., 1998). In *C. elegans*, *zag-1* is the only homolog of  *$\delta$ EF1/zfh-1*. With the *zag-1*-promoter-GFP construct we occasionally saw transient expression in body-wall-muscle cells during embryogenesis. However, using several muscle markers we were unable to detect any obvious defects in the differentiation of body-wall-muscle cells in *zag-1(rh315)* mutants, indicating that ZAG-1/ $\delta$ EF1 might not be essential for muscle differentiation in this animal. We did, however, find subtle changes in the level of expression of muscle-specific markers in anal depressor and sphincter muscles. *zag-1* might have a role in modulating expression of muscle genes, rather than acting as an all-or-none switch for the expression of particular target genes in muscle cells.



The *zag-1(rh315)* mutation leads to a truncated protein with the N-terminal Zn fingers and the homeodomain intact, indicating that this might be a partial loss-of-function allele. This is confirmed by the observations that neuronal defects in *zag-1(rh315)* were recessive and that transheterozygous *zag-1(rh315)/zag-1(hd16)* animals had a phenotype stronger than *zag-1(rh315)* but weaker than *zag-1(hd16)*, the putative null allele.

*zag-1(hd16)* mutants died with a starved appearance. We found that the animals were, apparently, unable to swallow (food) particles efficiently. Fluorescent beads fed to the animals stuck in the anterior part of the pharynx, the procorpus, indicating that the pharynx did not function properly. This defect could either be caused by defects in pharyngeal-muscle development or by a failure in development or function of the pharyngeal M4 neuron that has been shown to be essential for contractions of the isthmus and, consequently, for passage of food into the terminal bulb (Avery and Horvitz, 1987). One of the pharyngeal cells expressing *zag-1* is in a position consistent with it being the M4 neuron. Other pharyngeal cells expressing *zag-1* are most likely the m4 and m5 muscle cells, which leaves both possibilities (either neuronal or muscle defect) open at the moment.

In the mouse, two mutants in the  $\delta E F 1$  gene have been generated. The  $\delta E F 1^{\Delta C 7 2 7}$  mutation truncates the protein and eliminates the C-terminal Zn-finger cluster. Mutant mice have defects in thymus development and a greatly reduced number of T cells (Higashi et al., 1997).  $\delta E F 1^{\text{null(lacZ)}}$  mutants, where the entire protein is eliminated, have additional defects in neural-crest-derived skeletal elements, limb-bone and sternum development (Takagi et al., 1998), also suggesting that the truncated protein has some residual activity.

For  $\delta E F 1$  it has been shown that binding of both Zn-finger clusters is necessary for efficient transcriptional repression at some promoters (Remacle et al., 1999; Sekido et al., 1994). By contrast, the N-terminal and C-terminal fingers alone can bind to their respective target sequences, so that a truncated form of  $\delta E F 1 / Z A G - 1$  with just one cluster of Zn fingers intact might still successfully repress transcription at other target sites. Binding studies with  $\delta E F 1 / A R E B 6$  and SIP1 suggest models in which binding to target sites with either one or two Zn fingers leads to different effects on the transcription of target genes (Ikeda and Kawakami, 1995; Remacle et al., 1999). This might provide an explanation for the additional phenotypes observed with null mutants in mice and *C. elegans*.

### **zag-1 controls aspects of neuronal differentiation**

We found *zag-1* expressed predominantly in the developing nervous system. Expression started soon after neurons became postmitotic, peaked during the period when neurons differentiated and faded away in most neurons when embryogenesis was complete. Expression of *zag-1* is highly dynamic and occurs in many different classes of neurons, arguing against a role of *zag-1* in the specification of particular neuron types. This is in contrast to the function of other transcription factors like LIM-homeodomain proteins, which are expressed in a more restrictive way and are thought to act in combination to specify subtype identities of motoneurons in vertebrates (Goulding, 1998; Jacob et al., 2001). *zag-1* orthologs in *Drosophila* and vertebrates are also expressed prominently in the developing nervous system (Lai et al., 1991;

Takagi et al., 1998). However, no neuronal defects have been described in the corresponding mouse or *Drosophila* mutants (Broihier et al., 1998; Higashi et al., 1997; Lai et al., 1993; Su et al., 1999; Takagi et al., 1998). Our data show that *zag-1* plays an important role in neuronal differentiation in *C. elegans*. Because of the strong conservation in sequence and expression of the *zag-1* homologs, we strongly suspect that  $\delta E F 1 / Z E B / A R E B 6$  might have a similar, but so far undetected, role in neuronal development in vertebrates.

We found that *zag-1* expression itself was affected by the absence of functional ZAG-1 protein. The *zag-1*-promoter-GFP construct, which closely reflects the dynamic expression of *zag-1* in wild type, is not downregulated in *zag-1(rh315)* mutants during postembryonic development, indicating that *zag-1* negatively regulates its own expression and seems to shut down its own expression when it is no longer required. We found closely spaced pairs of putative ZAG-1/ $\delta E F 1$  binding sites in the promoter region of the *zag-1* gene, indicating that this effect could be due to ZAG-1 binding to its own promoter.

*zag-1*-mutant animals had several defects indicative of incomplete neuronal differentiation. First, cell-type-specific markers were not expressed properly in *zag-1* mutant animals. Affected are genes determining neurotransmitter properties, including *glr-1*, a glutamate receptor gene, and chemosensory receptor genes such as *sra-6*. These genes encode terminal differentiation products that are characteristic of some subtypes of neurons. In *zag-1* mutants, either too few cells express these markers (*sra-6*) or additional cells express the marker (*glr-1*). Typically, cells that fail to express a particular marker still express other neuronal markers and also have the characteristic appearance of neurons, indicating that neuronal cell lineages are normal and that *zag-1* does not affect the generation of neurons per se. Furthermore these changes in gene expression do not seem to reflect a switch in neuronal identity because extra neurons expressing the *glr-1* marker – like the PDB and RID neurons – send out their axons along their normal (often unique) paths. Genes controlling axonal outgrowth are still, apparently, expressed correctly in these cells, suggesting that neuronal identity has not been lost completely. Conversely, in other neurons, such as the D-type motoneurons, the expression of particular cell-type-specific markers is normal, but axon outgrowth is affected, again indicating that only some aspects of neuronal identity are disturbed.

The most prominent defects in *zag-1* mutant animals are characteristic axon outgrowth defects. Interneurons that express *glr-1* have defects in the navigation through the nerve ring towards the ventral cord and fasciculation defects in the ventral cord. Motoneuron axons also exhibit fasciculation defects in the ventral cord, as well as commissure outgrowth defects; commissures grow out on the wrong side, sometimes fail to reach the dorsal cord, and, occasionally, either branch prematurely before reaching the cord or fail to branch after reaching the dorsal cord. These defects could be caused by either a failure in the proper expression of extracellular guidance cues or be intrinsic problems of particular neurons in responding to guidance cues. The expression of *zag-1* in neurons shortly before the time of axon outgrowth suggests that *zag-1* directly or indirectly affects the transcription of genes that are important for responding to axon guidance signals. These genes could be either receptors for particular

guidance cues or components of the signal transduction machinery that integrates guidance signals.

### **zag-1 transcriptional repressor acts in a regulatory network for neuronal differentiation**

An increasing number of transcription factors are known to affect different steps of neuronal differentiation in *C. elegans*. Several genes have been identified that affect the division pattern of neuroblasts and, hence, the generation of neurons. This frequently leads to loss of particular classes of neurons, due either to premature withdrawal of neuroblasts from the cell cycle, as in *cnd-1* mutants (Hallam et al., 2000), failure to generate neuronal lineages, as in *lin-32* (Chalfie and Au, 1989), and to changes in the lineage program itself, as in *unc-86* (Chalfie et al., 1981) and *pag-3* mutants (Cameron et al., 2002). *zag-1* seems to act downstream of these early events in neuronal specification, because neurons are generated in *zag-1* mutants and seem to differentiate with neuronal properties.

Next in the hierarchy of neuronal differentiation are transcription factors that affect the differentiation of particular classes of neurons, like touch cells in *mec-3* mutants (Mitani et al., 1993) and D-type motoneurons in *unc-30* mutants (Jin et al., 1994; McIntire et al., 1993). MEC-3 has been shown to directly regulate the expression of the mechanosensory specific genes *mec-4* and *mec-7* (Duggan et al., 1998), providing a direct link between a transcription factor and terminal differentiation products of certain subtypes of neurons. There are also a few examples where neurons switch identities. The AWB sensory neuron adopts an AWC fate in *lim-4* mutants (Sagasti et al., 1999), and several different neurons adopt a CEM fate in *cfi-1* mutants (Shaham and Bargmann, 2002). All known aspects of the differentiation of particular classes of neurons are affected in mutants in the above-mentioned genes, which, again, is different from the situation in *zag-1* mutants, where neuronal identities are not completely lost or changed.

The partial loss of neuronal identity in *zag-1* mutants is more reminiscent of the situation found in *unc-42*, which affects expression of glutamate-receptor subunits in a subset of interneurons of the motor circuit (Baran et al., 1999). *zag-1* phenotypes are also reminiscent of defects in the nuclear hormone receptor *fax-1*, which is required for correct pathfinding of axons extending in the left axon tract of the ventral cord, and also correct expression of a peptide neurotransmitter precursor (Much et al., 2000). Distinct aspects of neuronal identity are also affected in *unc-4* mutants, where VA motoneurons receive input from interneurons that would normally connect to VB motoneurons but retain VA-specific output and axon trajectories (Miller and Niemeyer, 1995; Miller et al., 1992; White et al., 1992). UNC-4, a homeodomain transcription factor, is thought to be involved specifically in defining synaptic input for one class of motoneuron axons, again illustrating that different aspects of neuronal differentiation can be under independent transcriptional control.

*zag-1* probably acts as a coregulator, most likely in combination with various other transcription factors in different neurons because many features of neuronal identities are still normal in *zag-1* mutants. Dissecting the components of this transcriptional network further will lead to a better understanding of how distinct features of neurons appear during their differentiation. In vertebrates *zag-1* homologs have

been shown to block the function of bHLH proteins in myogenic development. Therefore it will be especially interesting to study the interactions between *zag-1* and neuronally expressed bHLH proteins, which constitute a fairly large family of transcription factors in *C. elegans*, with more than 20 members.

It is possible that *zag-1* acts at different levels in the differentiation pathway of neurons because it is found in many neurons only at late stages of differentiation. We found several terminal differentiation markers were expressed ectopically in *zag-1* mutants, and it is possible that *zag-1* acts directly to repress expression of some of these genes in inappropriate places. The most prominent defects in *zag-1*-mutant animals are axon guidance defects. Many of these defects affect particular guidance decisions, implying that *zag-1* might regulate genes that are essential for the response to particular guidance cues. The identification of target genes whose expression is controlled by *zag-1* might lead to the identification of novel, key regulators of axon guidance.

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