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C. elegans ZAG-1, a Zn-finger-homeodomain protein, regulates axonal development and neuronal differentiation

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SUMMARY

Neurons acquire distinct cell identities and implement differential gene programs to generate their appropriate neuronal attributes. On the basis of position, axonal structure and synaptic connectivity, the 302 neurons of the nematode *Ceanorhabditis elegans* are divided into 118 classes. The development and differentiation of many neurons require the gene *zag-1*, which encodes a δEF1/ZFH-1 Zn-finger-homeodomain protein. *zag-1* mutations cause misexpression of neuron-specific genes, block formation of stereotypic axon branches, perturb neuronal migrations, and induce various axon-guidance, fasciculation and branching errors. A *zag-1*-GFP translational reporter is expressed transiently in most or all neurons during embryogenesis and in select neurons during the first larval stage. Analysis of the *zag-1* promoter reveals that *zag-1* is expressed in neurons and specific muscles, and that ZAG-1 directly represses its own expression. *zag-1* activity also downregulates expression of genes involved in either the synthesis or reuptake of serotonin, dopamine and GABA. We propose that ZAG-1 acts as a transcriptional repressor to regulate multiple, discrete, neuron-specific aspects of terminal differentiation, including cell migration, axonal development and gene expression.

Key words: *C. elegans*, Zn-finger-homeodomain, Axon branching, Neuronal differentiation

INTRODUCTION

Cell signaling and cell-intrinsic processes orchestrate the specification and differentiation of distinct neuron types (Edlund and Jessell, 1999). For example, a gradient of sonic hedgehog establishes the expression pattern of different transcriptional regulators along the dorso-ventral axis in the developing, vertebrate ventral spinal cord. The unique complement of factors in each domain determines, in part, the subsequent fate adopted by each neuron. Presumably, it also initiates transcriptional cascades that generate the appropriate characteristics for an individual neuron type, such as axonal structure, synaptic connections and differentiated gene-expression profile. Although factors, such as LIM-homeodomain and ETS-domain proteins, which contribute to the establishment of axonal-projection patterns and connectivity, have been identified in several organisms (reviewed by Hobert and Westphal, 2000; Shirasaki and Pfaff, 2002), the repertoire of factors needed to generate attributes for most neuron types is largely unknown.

The nervous system of *C. elegans* consists of relatively few neurons but it contains a great diversity of neuron types. The 302 neurons of the adult hermaphrodite are grouped classically into 118 classes, based on position, axonal morphology and connectivity (White et al., 1986). Subsequent studies indicate that individual neurons in some classes exhibit different gene-expression profiles, revealing the potential for a still greater number of distinct neuron types (Troemel et al., 1999; Yu et al., 1997). Several genes controlling the formation of neuron-specific characteristics and functional properties have been discovered. Some genes specify the fate of a neuron from an alternative or default state: mutation of the LIM-homeodomain gene *lim-4* causes AWB to adopt an AWC-like fate (Sagasti et al., 1999); and mutation of the forkhead domain gene *unc-130* induces ASG to adopt an AWA-like fate (Sarafi-Reinach and Sengupta, 2000). Other genes define the characteristics of different neuron types: the homeodomain gene *unc-42* regulates axonal development and neuron-type-specific expression of several glutamate and chemosensory receptors (Baran et al., 1999; Brockie et al., 2001); the homeodomain gene *unc-30* controls differentiation of GABAergic D-type motor neurons and *unc-30* mutants have defects in axon pathfinding, synaptic connectivity and expression of the glutamic acid decarboxylase UNC-25 and GABA vesicular transporter UNC-47 (Eastman et al., 1999; Jin et al., 1994).

δEF1/ZFH-1 Zn-finger-homeodomain proteins are distinguished by two arrays of highly similar C2H2-type Zn-finger domains and a centrally located homeodomain, and can act as transcriptional repressors through recruitment of the corepressor C-terminal binding protein (CtBP). Although *Drosophila* has one δEF1/ZFH-1 homolog (Fortini et al., 1991), vertebrates have two, one of which is most similar to

the eponymous chick δEF1 (Funahashi et al., 1993) (also known as Nil-2-a, TCF8, ZEB, BZP, AREB6, MEB1 and ZFHEP) (Sekido et al., 1996), the second, SIP1 (also known as ZEB-2), can associate with Smad proteins (Postigo and Dean, 2000; Verschueren et al., 1999). *Drosophila zfh-1* is expressed in embryonic mesoderm, mesodermally derived tissues and motor neurons (Lai et al., 1991), and is needed for development of gonadal mesoderm, heart and other tissues derived from mesoderm (Broihier et al., 1998; Lai et al., 1993; Su et al., 1999). Mouse δEF1 is expressed in notochord, somites, limb, neural-crest derivatives and some CNS regions, and is required for thymus and skeletal development (Higashi et al., 1997; Takagi et al., 1998). Although δEF1 is a negative regulator of muscle differentiation in vitro (Postigo and Dean, 1997), mouse δEF1 knockouts lack muscle and CNS defects. Mouse SIP1 is needed for development and migration of specific neural-crest cells (Van De Putte et al., 2003).

Here we report the genetic and molecular characterization of the lone *C. elegans* δEF1/ZFH-1 homolog, ZAG-1. *zag-1* is essential for many aspects of neuronal differentiation and is expressed widely and dynamically during formation of the nervous system and in some muscles. ZAG-1 represses its own expression by interacting with conserved sequences in its promoter and, possibly, introns. We propose that *zag-1* acts as a transcriptional repressor during the late stages of neuronal differentiation to establish several neuron-specific characteristics, including correct cell position and axon structure, and proper expression of cell-surface proteins, transmembrane receptors, ion channels, and biosynthetic enzymes and reuptake transporters for neurotransmitters.

MATERIALS AND METHODS

Genetics

Worms were raised at 20°C and cultured as described by Brenner (Brenner, 1974). The following strains were used. *LGIV: lin-1(e1275), vab-2(e96), dpy-13(e184), unc-86::gfp(kyIs179)* (Gitai et al., 2003); *LGV: him-5(e1490), sra-6::gfp(oyIs14)* (Sarafi-Reinach et al., 2001), *nmr-1(akIs3)* (Brockie et al., 2001); *LGX: lin-15(n765);* undetermined linkage: *lin-11::gfp(mgIs21)* (Hobert et al., 1998), *unc-25::gfp(juIs75)* (Jin et al., 1999).

We recovered *pag-3* and *zag-1* alleles in a screen for mutants with defects in PVQ, which will be described in detail elsewhere. In brief, *sra-6::gfp(oyIs14)* parental generation (P0) animals were treated with either ethyl methanesulfonate (EMS) or N-ethyl-N-nitrosourea (ENU) (De Stasio et al., 1997), F2 progeny that exhibited behavioral defects, such as an uncoordinated movement (Unc) or egg-laying defective (Egl) phenotype, were transferred individually to new plates and their progeny examined using epifluorescence microscopy to identify mutants with PVQ cell-fate specification, axonal development and other defects. We picked Uncs and Egls to enrich for animals with potential abnormalities in the structure or function of the nervous system.

Five *pag-3*(*zd48, zd49, zd63, zd111* and *zd120*) and two *zag-1(zd85* and *zd86)* alleles were recovered following EMS treatment and one *pag-3(zd124)* allele was found following ENU treatment. All six *pag-3* mutations mapped to *LGX* and failed to complement *pag-3(ls20)* for the Unc phenotype. Two-factor crosses of *zd85* and *zd86* indicated linkage to *lin-1 IV*. Complementation testing indicated that *zd85* and *zd86* are allelic and defined a new gene, *zag-1*. We performed three-factor crosses to map *zag-1* further. From *zag-1(zd85)/lin-1 dpy-13* heterozygotes, 24 out of 27 Lin non-Dpy recombinants segregated Zag-1 animals and from *zag-1(zd86)/lin-1 dpy-13* heterozygotes, 20 out of 21 Lin non-Dpy recombinants segregated Zag-1 animals. From *vab-2/lin-1 zag-1(zd85)* heterozygotes, 2 out of 40 Zag non-Lin recombinants segregated Vab-2 animals and 30 out of 35 Lin non-Zag recombinants segregated Vab-2 animals. Thus, *zag-1* maps between *vab-2* and *dpy-13*.

zag-1 rescue

Germline transformation (Mello and Fire, 1995) of *zag-1* mutants was performed by co-injecting test DNA (10-50 μg ml^{-1}) and *sur-5::SUR-5-GFP* reporter DNA (50 μg ml^{-1}), pTG96, which labels most nuclei with GFP (Yochem et al., 1998). Transformed F1 and F2 animals were identified by GFP expression from pTG96. The cosmids F28F9, F47C12, W02C12, W03D2, F49F1, R07C12 and R08D10 were tested, either individually or in pools, for rescue of the Unc phenotype of *zag-1(zd85)* animals. Three out of five extrachromosomal array lines containing F28F9 and none of the lines harboring the other cosmids were rescued. Subclones of F28F9 were constructed using pBluescript II KS(–). The 10 kb *Kpn*I-*Sal*I genomic fragment (pSK55; position 3,851,820-3,861,821 of chromosome *IV*) rescued (3/3 lines), whereas an 8.8 kb *Pst*I-*Pst*I fragment (pSK54; position 3,852,925-3,861,764) failed to rescue (0/2 lines).

GFP transgenics

Transcriptional and translational reporter lines were generated using GFP-expression vectors provided by A. Fire. DNA sequences upstream of each gene were amplified from N2 genomic DNA using PCR, and cloned in-frame into an appropriate GFP vector. For *dat-1::gfp*, DNA sequences –3671 to +1 were amplified (+1 is first base of ATG) and cloned into *Sph*I and *Msc*I sites of pPD95.77. For *talin::gfp*, DNA sequences –2070 to +6 were amplified and cloned into *Sph*I and *Bam*HI sites of pPD95.77. For *tph-1::gfp*, DNA sequences –1748 to +9 were amplified and cloned into *Hin*dIII and *Msc*I sites of pPD95.81. For *unc-129::gfp*, DNA sequences –3087 to +6 were amplified and cloned into *Xba*I and *Msc*I sites of pPD95.75. Sequences were included in downstream primers to generate a *Bam*HI, *Msc*I or *Stu*I (*unc-129::gfp*) site. *glr-1::gfp*, *mec-4::gfp* and *odr-2::cfp* were derived from previously described reporters (Chou et al., 2001; Lai et al., 1996; Maricq et al., 1995) by replacement of 'wild type' GFP sequences with GFP S65C or CFP (GFP Y66W, N146I, M153T and V163A) coding sequences with synthetic introns.

Using PCR and standard cloning methods, we introduced an *Xba*I site at the end of exon 7 (TCTACCTAG changed to TCTAGATAG) and fused GFP gene and *unc-54* 3′ untranslated region (UTR) sequences from pPD95.77 to *zag-1* genomic sequences. *zag-1::ZAG-1-GFP* is predicted to produce a full-length ZAG-1-GFP fusion protein that includes a substitution of arginine for the C-terminal threonine. We made *zag-1::ZAG-1(213)-GFP*, which is expected to generate a ZAG-1(213)-GFP fusion protein containing the N-terminal 213 amino acids of ZAG-1, by cloning the GFP gene and *unc-54* 3′UTR sequences from pPD95.77 into the *Bam*HI site in exon 2.

We engineered a *Sma*I site at codon 7 (GCCATGC changed to GCCCGGG) and generated *zag-1::gfp* transcriptional reporters using pPD95.81 and the following *zag-1* upstream genomic fragments: pSK73, –4030 (*Kpn*I) to +21; pSK62, –2925 (*Pst*I) to +21; pSK109, –1623 (*Sna*BI) to +21; pSK110, –736 (*Msc*I) to +21; and pSK111, –321 (*Eco*RV) to +21. We mutated specific CACCT motifs present in *zag-1::gfp* promoter (pSK109) using the Promega GeneEditor in vitro Site-Directed Mutagenesis System to create pSK183, –1114A (CAGGTG changed to CAGATG) (E box); pSK184, –918A (AGGTG changed to AGATG); pSK185, –895T (CACCT changed to CATCT); and pSK186, –918A, –895T. Mutations were confirmed by sequencing.

Extrachromosomal arrays were generated by germline transformation of *lin-15(n765)* animals with GFP-reporter DNA (50 μg ml^{-1}) and *lin-15(+)* gene (pSK1) (50 μg ml^{-1}), and stable chromosomal integration was induced by treatment with trimethyl

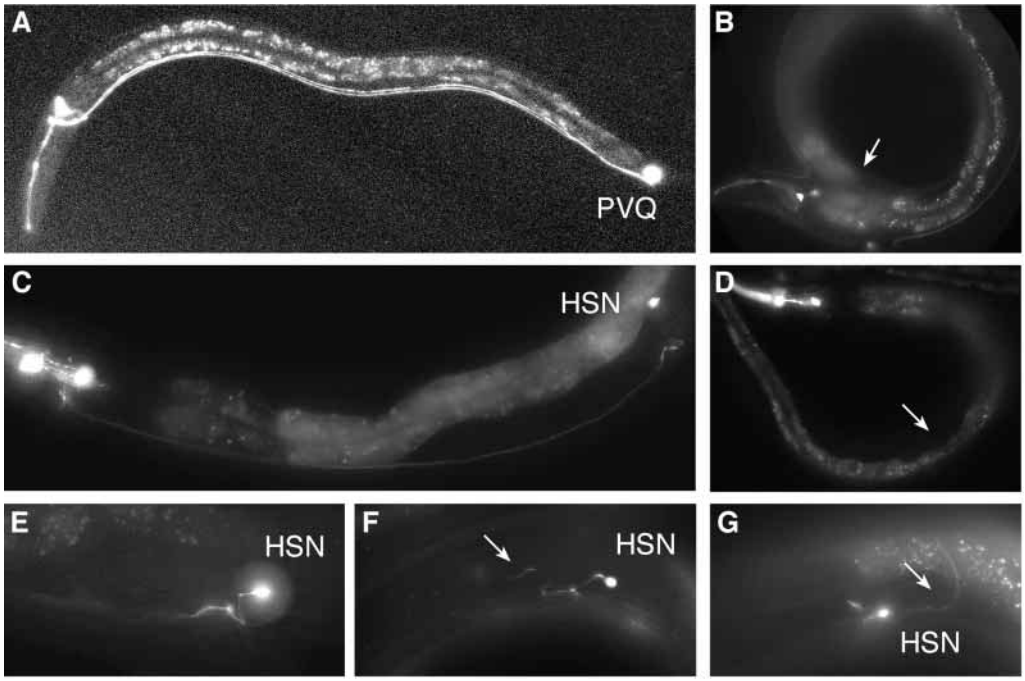

Fig. 1. (A) Wild-type, *sra-6::gfp* adult hermaphrodite. (B) *zag-1(zd85); sra-6::gfp*; arrow indicates position of PVQ. (C) Wild-type *tph-1::gfp.* (D) *zag-1(zd85) tph-1::gfp*; arrow indicates position of HSN. (E) Wild type *tph-1::gfp*. (F,G) *zag-1(zd86); odr-2::cfp*; arrows indicate abnormal axons. Although not visible in these images, HSN axons in *zag-1* animals typically enter the ventral nerve cord. Anterior is leftwards; dorsal is towards the top.

psoralen (TMP) and UV. *lin-15(n765)* mutants exhibit a temperature-dependent, multivulva phenotype that can be rescued by pSK1 (Clark et al., 1994). The two *zag-1* translational reporters (*zdIs39, zdIs40*) were created using the dominant *rol-6(su1008)* roller marker (pRF4); a similar expression pattern was observed using *lin-15(+)* marker. Each integrant was backcrossed three times with N2. The strain name, reporter name, allele designation, linkage group (if known) and plasmid name for transgenics generated in this study are: MT4003, *glr-1::gfp(zdIs3) IV*, pSK35; MT4005, *mec-4::gfp(zdIs5) I*, pSK39; MT4010, *odr-2::cfp(zdIs10) V*, pSK37; MT4013, *tph-1::gfp(zdIs13) IV*, pSK41; MT4015, *talin::gfp(zdIs15) V*, pSK57; MT4021, *zag-1::gfp(zdIs21) IV*, pSK62; MT4031, *dat-1::gfp(zdIs31) X*, pSK101; MT4039, *zag-1::ZAG-1-GFP(zdIs39)*, pSK71; MT4040, *zag-1::ZAG-1(213)-GFP(zdIs40)*, pSK67; MT4041, *zag-1(–918A, –895T, 4.0 kb)::rfp(dsred2)(zdIs41)*, pSK209; and MT4042, *unc-129::gfp(zdIs42) IV*, pSK127.

Images were acquired using a Hamamatsu Orca CCD camera and a Leica DMRE microscope, and edited using Improvision Openlab and Adobe Photoshop.

Sequence analysis

We determined the DNA sequence of the longest predicted EST clone, yk312a9, of F28F9.1. DNA sequences were determined using the ABI PRISM BigDye Terminators Cycle Sequencing kit and either an ABI PRISM 310 Genetic Analyzer or MJ Research BaseStation 51 DNA Fragment Analyzer. The genomic organization of F28F9.1 based on the sequence of yk312a9 is different from the hypothetical gene structure in WormBase release WS99 (17 April, 2003) (http:// wormbase.org); we believe the yk312a9-based structure is correct because it is derived from cDNA sequence and the open reading frames are highly conserved in the related nematode *C. briggsae*.

To find alterations associated with *zag-1* mutations, the coding and splice-junction regions were amplified from N2, *zd85* and *zd86* genomic DNA using PCR and flanking primers. The DNA sequences of the amplified products were determined directly using either internal sequencing primers or the PCR primers and a cycle-sequencing protocol. *zd85* and *zd86* each contained a single point mutation within the F28F9.1 gene and the N2 F28F9.1 sequence was identical to that reported by the *C. elegans* Sequencing Consortium.

RESULTS

zag-1 identification

We discovered *zag-1* in a screen for mutations that disrupt PVQ development. PVQ is a pair of interneurons located in the lumbar ganglia in the tail, and each extends a single axon that runs within the ventral nerve cord (VNC) to the nerve ring in the head (White et al., 1986). An *sra-6::gfp* (serpentine receptor class A) reporter is expressed in PVQ and in amphid neurons ASH and ASI (Troemel et al., 1995), which allows direct observation of these neurons in living animals using epifluorescence microscopy (Fig. 1A). We treated *sra-6::gfp(oyIs14)* animals with the mutagens EMS and ENU and identified mutants with PVQ defects (see Materials and Methods). Expression of *sra-6::gfp* was seen uniformly in ASH and ASI but was observed rarely in both PVQs in eight mutants (Fig. 1B, Table 1, data not shown). Six mutations (*zd48, zd49, zd63, zd111, zd120* and *zd124*) are *pag-3* alleles. We reported previously that PVQ is present in *pag-3* mutants but fails to express *sra-6::gfp* (Cameron et al., 2002).

The remaining two alleles (*zd85* and *zd86*) defined a new gene, *zag-1 IV*. Both are recessive and exhibit a similar, uncoordinated, behavioral phenotype. *zag-1* worms were active but, typically, had a kinked appearance and serious difficulty moving backward. The penetrance and expressivity of the PVQ *sra-6::gfp* expression defect were incomplete (Table 1). PVQ axons, when detected, followed their normal trajectory and 67 out of 68 reached the nerve ring. We examined *zag-1* animals using DIC microscopy and observed neuronal nuclei in positions characteristic of PVQ, which indicated that the lack of GFP-labeled neurons resulted from either reduction or

Table 1. GFP transgene expression in *zag-1* mutants

Genotype	Percentage of animals			
	2 PVQ	1 PVQ	0 PVQ	*n*
sra-6::gfp(oyIs14)	100	0	0	>100
zag-1(zd85); sra-6::gfp(oyIs14)	3	39(4)	58(16)	100
zag-1(zd86); sra-6::gfp(oyIs14)	5	21(3)	74(19)	100

Genotype	Percentage of animals			
	2 HSN	1 HSN	0 HSN	*n*
tph-1::gfp(zdIs13)	100	0	0	>100
zag-1(zd85) tph-1::gfp(zdIs13)	0	8	92	100
zag-1(zd86) tph-1::gfp(zdIs13)	0	0	100	100

Neurons with wild-type cell body and axon expression were scored as positive; no detectable expression was scored as negative.

PVQs with faint cell body expression and no detectable axon were scored as having no expression; the percentage of animals with one such cell are indicated in parentheses.

In animals with a single visible PVQ or HSN, the frequency of L or R homolog being present was similar. *n*, number of animals.

elimination of *sra-6::gfp* expression rather than the absence of PVQ.

lin-11::gfp(*mgIs21)* has faint PVQ expression in young larvae (Hobert et al., 1998), permitting visualization of cell bodies but not axons. We detected both PVQ cell bodies in *zag-1; lin-11::gfp* larvae (21/21 for *mgIs21*, 19/19 for *zd85; mgIs21* and 23/23 for *zd86; mgIs21)*, confirming the presence of PVQ and revealing the proper regulation of *lin-11::gfp* expression. Together, these observations indicate that *zag-1* is needed to specify some features of PVQ-cell fate, such as the expression of *sra-6*, but not *lin-11*.

HSN differentiation requires *zag-1*

The HSN serotoninergic motor neurons are born in the tail and migrate to flank the gonad in the midbody during embryogenesis (Desai et al., 1988; Sulston et al., 1983). HSN matures during the fourth larval (L4) stage: the nucleus and nucleolus enlarge and a distinctive structure, called the 'hood', forms around the nucleus. The HSN axons go round the vulva, enter the VNC and continue to the nerve ring.

Serotoninergic neurons express the tryptophan hydroxylase TPH-1 (Sze et al., 2000). *tph-1::gfp(zdIs13)* expressed GFP in serotoninergic neurons ADF, HSN, NSM and male-specific CP and R(1,3,9) (Fig. 1C). GFP expression in HSN was observed first during late L4 and continued throughout adulthood, consistent with the immunological detection of serotonin that starts in young adults (Desai et al., 1988). We found that HSN GFP expression was rare or absent in *zag-1 tph-1::gfp* adult animals, whereas expression was unchanged in ADF, NSM, CP and R(1,3,9) (Fig. 1D, Table 1). *unc-86::gfp(kyIs179)* displays GFP expression in HSN during larval development (Gitai et al., 2003). We examined *zag-1(zd85)*; *unc-86::gfp* animals and observed both HSNs in their proper midbody location (20/20 for *kyIs179* and 18/18 for *zd85; kyIs179*). Thus, the HSNs are born, migrate to the midbody and express *unc-86* but not *tph-1* in *zag-1* mutants.

odr-2 encodes a GPI-linked, cell-surface protein that is expressed by several sensory neurons, interneurons and motor neurons but not HSN (Chou et al., 2001). CFP-expressing HSNs were never observed in wild-type *odr-2::cfp* adults (*n*=50), whereas CFP-expressing HSNs were detected in all *zag-1; odr-2::cfp* adults (*zd85*; 34/40, 2 HSNs, 6/40, 1 HSN and *zd86*; 38/40, 2 HSNs, 2/40, 1 HSN). The morphology of HSN cell bodies and axons was typically abnormal in *zag-1* mutants visualized using either *odr-2::gfp* or *unc-86::gfp*. The HSNs retained a generic neuronal appearance and, often, failed to form their characteristic hood. Although most HSN axons extended ventrally and entered the ventral nerve, their trajectory was aberrant and they often branched extensively; ectopic axons also projected from cell body (Fig. 1E-G). In summary, *zag-1* mutants have defects in multiple aspects of HSN differentiation, including axon pathfinding, hood formation and *tph-1* expression. HSN development appears wild type until midlarval stages: the HSNs are born, migrate to the midbody and correctly express *unc-86* and not *odr-2*.

zag-1 mutants lack specific axon branches

Although the axons of most neurons in *C. elegans* are relatively simple and unbranched, the axons of a few neurons have well-defined, reproducible, branched structures (White et al., 1986). The dopaminergic neurons ADE and PDE have axons with stereotypic branched structures and can be visualized using a GFP reporter to the dopamine transporter gene *dat-1* (Nass et al., 2001). ADE is a pair of ciliated neurons located behind the second bulb of the pharynx. Each ADE projects a process ventrally that extends to the ventral ganglion and a process anteriorly that splits to a form a ciliated ending and a branch that enters the ring neuropil laterally (Fig. 2A,I). We found that the anterior process always formed a branch to the ring neuropil in wild-type *dat-1::gfp(zdIs31)* animals (47/47 ADEs) but this was generated infrequently in *zag-1; dat-1::gfp* animals (8/100 ADEs *zd85; zdIs31*, 17/100 ADEs *zd86, zdIs31*) (Fig. 2B,I). The ciliated branch of the anterior process and the ventral process were unaffected, indicating that *zag-1* mutants have a specific defect in branch formation rather than a general defect in ADE-axon outgrowth.

PDE is a pair of ciliated neurons located in the posterior body. Each PDE projects a process dorsally with a ciliated ending, and a process ventrally that enters the VNC, splits and extends anteriorly and posteriorly (Fig. 2C,I). Because the PDEL and PDER axons run together in the right ventral nerve bundle, we scored the extent of growth of the longest process. The posterior branch of the ventral PDE process typically extended ~3/4 of the distance between PDE cell body and anus in wild-type *dat-1::gfp* animals (38/47 full length), but occasionally it was shorter (9/47 partial length) or failed to extend (2/47). By contrast, most *zag-1; dat-1::gfp* animals lacked the posterior branch (*zd85*; 3/100 full-length, 35/100 partial length, 62/100 no extension, and *zd86*; 1/100 full length, 26/100 partial length, 73/100 no extension) (Fig. 2D,I). The posterior branch was rarely redirected anteriorly. The anterior branch was slightly shorter than wild type in ~1/2 of the *zag-1; dat-1::gfp* animals and the ciliated dorsal process was unchanged. We conclude that *zag-1* is required for the extension of the anteriorly directed axon branches of ADE and the posteriorly directed axon branches of PDE.

The HSN axons often form one or more short branches near the vulva (White et al., 1986). The HSN axons detected in *zag-1(zd85) tph-1::gfp* animals failed to branch at the vulva (0/17 HSNs) and were sometimes short or slightly misdirected

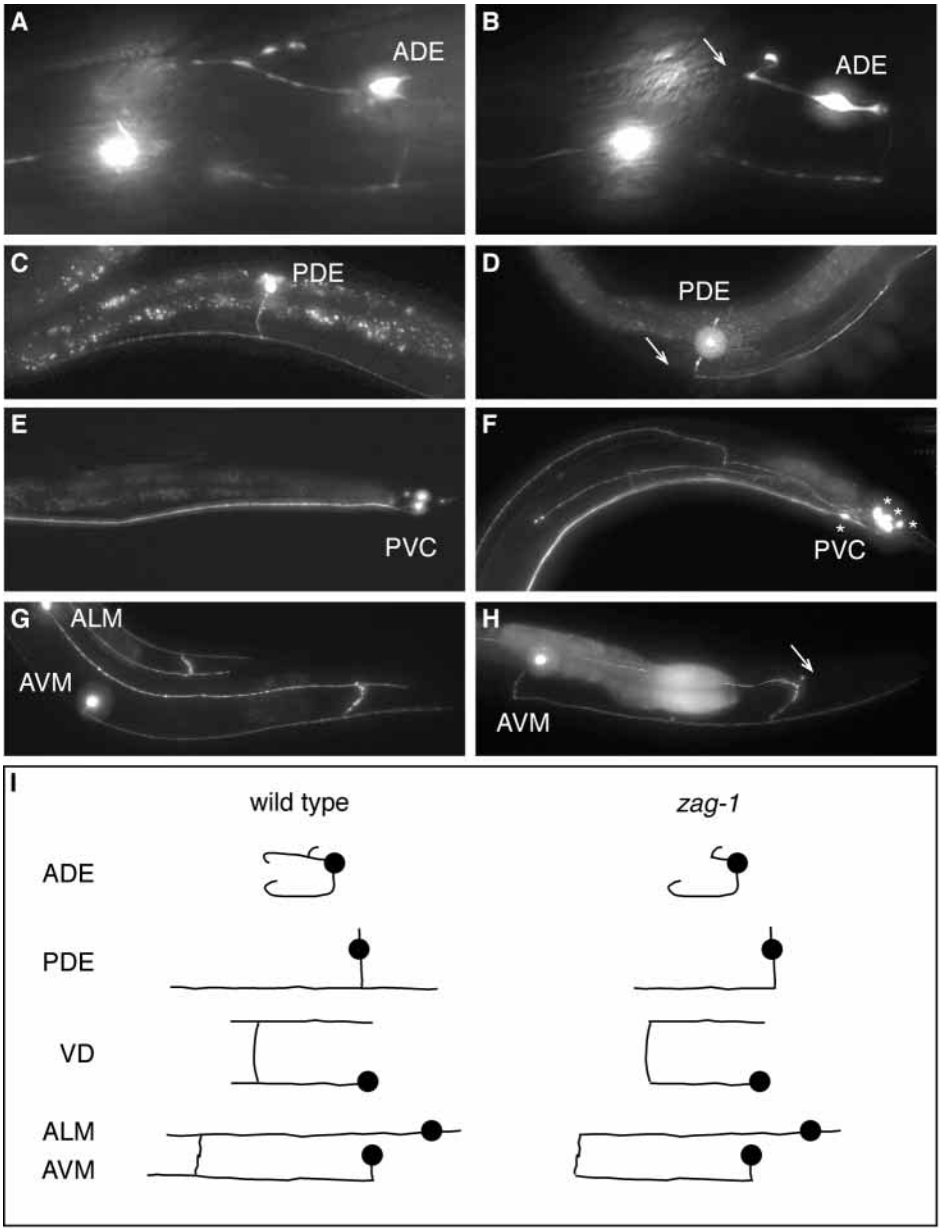

Fig. 2. *zag-1* mutations block formation of stereotypic axon branches and cause pathfinding defects. Wild-type (A,C,E,G) and *zag-1* (B,D,F,H) GFP-transgenic animals. Neurons: ADE (A,B); PDE (C,D); PVC (E,F) (additional GFP-labeled axons present in VNC); ALM and AVM (G,H). (H) AVM often terminates at the nerve ring in *zag-1* mutants, although in this image AVM is wild type. Arrows indicate locations of missing axon branches and asterisks indicate ectopic *glr-1::gfp*-expressing neurons. (I) Schematic of wild-type and *zag-1* axonal structures, not to scale. Anterior is leftwards (A,B,E,F,I), anterior is rightwards (C,D,G,H), dorsal is towards the top.

(5/17), whereas most HSNs in wild-type *tph-1::gfp* animals formed a branch near the vulva (25/31) and were rarely short or misdirected (2/200). Most HSN axons in *zag-1* mutants had severe guidance defects viewed using either *odr-2::cfp* or *unc-86::gfp* (see above). The few GFP-expressing HSNs detected in *zag-1(zd85) tph-1::gfp* animals presumably represented a population with sufficient *zag-1* activity to promote *tph-1::gfp* expression and the formation of a nearly correct axon in most cases. However, the complete absence of stereotypic branches at the vulva argues that *zag-1* is needed to shape multiple, discrete HSN-axon features, such as the extension of an axon with an appropriate nerve ring trajectory and formation of branches at the vulva.

The six DD and 13 VD motor neurons are located in the VNC. Each extends a process anteriorly that forms a collateral to the dorsal nerve cord and divides and projects both anteriorly and posteriorly (DD also extends a short process posteriorly) (Fig. 2I). DD1 and VD2 commissures extend on the left side and, typically, fasciculate, whereas DD2-6 and VD1,3-13 commissures extend on the right side. Many DD and VD axons terminated their growth prematurely, were misdirected and branched abnormally in *zag-1* mutants; the anteriorly directed branches adjacent to dorsoventral commissures were also often missing (Fig. 2I, data not shown). In addition, many DD and VD commissures extended on the wrong side. DD1 and VD2 commissures were fasciculated and extended on the left side in wild-type *unc-25::gfp(juIs75)* animals (n=63) and, infrequently, a single, additional commissure extended inappropriately on the left side (12/63). By contrast, 74% of *zag-1(zd85); unc-25::gfp* animals had one or more inappropriate commissures on the left side (19/50 one, 11/50 two, 6/50 three and 1/50 four). Conversely, DD1 and VD2 often extended on the right side (5/50 both, 35/50 one). Thus, *zag-1* is needed for the formation of anteriorly directed DD and VD axon branches as well as for several aspects of DD and VD axon guidance.

Other *zag-1* axon outgrowth defects

Several interneurons and motor neurons, including the command interneurons AVA, AVB, AVD, AVE and PVC that extend processes along the VNC, express the GLR-1 glutamate receptor (Hart et al., 1995; Maricq et al., 1995), whereas a subset of *glr-1::gfp*-expressing neurons, AVA, AVD, AVE, AVG, PVC and RIM, express the NMR-1 glutamate receptor (Brockie et al., 2001). We found that all *zag-1 glr-1::gfp(zdIs3)* animals (40/40 *zd86*) had three or more aberrant axons emanating from either the nerve ring or tail that zigzagged and branched profusely along the length of the animal, but 1 out of 30 wild-type *glr-1::gfp* animals had a single, short, inappropriate lateral process that exited the nerve ring (Fig.

2E,F). Ectopic, neuronal expression was detected in the tail and the nerve ring was disorganized and defasciculated. The VNC was also defasciculated in *zag-1; nmr-1::gfp* animals, but the growth of ectopic axons in lateral positions was uncommon. Thus, most of the aberrant processes seen in *zag-1 glr-1::gfp* animals likely arose from *glr-1::gfp*-expressing neurons that do not express *nmr-1::gfp*. *zag-1* mutations also caused variable expression of *nmr-1::gfp* in PVC and ectopic expression in an unpaired tail neuron. These results indicate that *zag-1* is required for specification and axon guidance of interneurons and motor neurons that express *glr-1* and *nmr-1*, and to prevent misexpression of *glr-1* and *nmr-1*.

AVG, an interneuron located in the retrovesicular ganglion, pioneers the right ventral nerve bundle and is thought to provide cues that promote the proper assembly and organization of VNC (R. M. Durbin, PhD thesis, University of Cambridge, UK, 1987). Ablation of the parent of AVG led to inappropriate growth of DD and VD commissures on the left side as well as extension of many longitudinal axons on the wrong side of the VNC. We found that AVG sometimes terminated its growth prematurely in *zag-1; odr-2::cfp* animals (14/40 *zd86*), but it extended completely in wild-type *odr-2::cfp* animals. As such, the disorganization of VNC and extension of DD and VD commissures on the wrong side might result in part from defects in AVG outgrowth.

ALM migration and axonal development require *zag-1*

The six touch receptor neurons, ALM, PLM, AVM and PVM, are needed for the gentle touch response (Chalfie et al., 1985) and express the MEC-4 ion channel subunit (Lai et al., 1996). The two ALMs are born and migrate to characteristic anterior body positions during embryogenesis (Sulston et al., 1983). In wild-type *mec-4::gfp(zdIs5)* animals, 49 out of 50 ALM cell bodies were found ~3/4 of the way between the second bulb of pharynx and the vulva in young adults and 1 out of 50 was found halfway or less. By contrast, 12 out of 49 ALMs in *zdIs5; zd85* and 11 out of 45 ALMs in *zdIs5; zd86* animals were positioned halfway or less, and many had a variable dorsoventral position, indicating that *zag-1* is needed for ALM to complete its posterior migration. Occasionally, *mec-4::gfp* expression in ALM was not seen in *zag-1* mutants (50/50 *zdIs5*, 49/50 *zdIs5; zd85* and 45/50 *zdIs5; zd86*).

Each ALM projects a short axon posteriorly and an axon anteriorly that runs at least to the first bulb of pharynx and forms a branch that enters neuropil and contacts the AVM branch. We found that all anterior ALM axons (50/50) had a wild-type trajectory in *mec-4::gfp(zdIs5)*, whereas only 9 out of 50 were wild type in *zdIs5; zd86* animals (Fig. 2G-I). Most anterior ALM axons (41/50) grew as far as the nerve ring and turned to join the neuropil. The nerve ring branch appeared tangled and often failed to contact the AVM branch, which was also disorganized. Anterior ALM processes in *zdIs5; zd85* animals were similarly defective. The posterior ALM process was typically absent in wild-type *mec-4::gfp* and *mec-4::gfp; zag-1* animals, which likely represents natural variation. These observations indicate that *zag-1* is required to form the ALM-axon extension past the nerve ring. Thus, *zag-1* is needed for multiple phases of ALM development, including cell migration, axonal development and *mec-4* expression. *mec-4::gfp* expression and axon morphology of PLM, the posterior homolog of ALM, were essentially wild type in *zag-1* mutants.

AVM and PVM are descendants of neuroblasts QR and QL, respectively. QR and its descendants migrate to specific positions in the anterior body, whereas QL and its descendants migrate to stereotypic positions in the posterior body. AVM and PVM were found in their correct locations in *zag-1* animals but *mec-4::gfp* expression was altered. PVM *mec-4::gfp* expression was often eliminated (25/25 *zdIs5*, 15/25 *zdIs5; zd85* and 10/25 *zdIs5; zd86)* but AVM *mec-4::gfp* expression was largely unaffected (25/25 *zdIs5*, 24/25 *zdIs5; zd85* and 24/25 *zdIs5; zd86)*. Inappropriate expression of *odr-2::cfp* was also observed occasionally in PVM (data not shown). AVM and PVM axons enter the VNC and run anteriorly, AVM continues past the first bulb of pharynx and PVM stops in the anterior body. AVM forms a branch that splits, enters the neuropil and contacts ALM branches. Only 8 out of 24 AVM processes extended past the first bulb of pharynx in *mec-4::gfp; zag-1(zd86)* compared to 25 out of 25 in wild-type *mec-4::gfp* (Fig. 2G-I). Sixteen out of 24 AVM axons turned to enter the nerve ring after passing the second bulb of pharynx and lacked an anteriorly directed lateral extension, similar to the phenotype displayed by ALM axons. AVM branches were disorganized and often failed to make contact with ALM processes. PVM axons appeared wild type. Therefore, *zag-1* mutations had differential effects on AVM and PVM. AVM usually expressed *mec-4* and had distinct axon-branch-formation defects, whereas PVM often failed to express *mec-4* and had a wild-type axon structure when detected.

zag-1 downregulates expression of neurotransmitter biosynthetic and reuptake genes

unc-25 encodes the GABA biosynthetic enzyme glutamic acid decarboxylase and is expressed in all 26 GABAergic neurons (Jin et al., 1999). The *unc-25::gfp(juIs75)* reporter, which has 1.8 kb of sequence 5′ of the ATG and coding sequences for the first 13 amino acids of UNC-25, expresses GFP in a subset of GABAergic neurons: DD, VD and RME. GFP expression in these neurons was significantly higher in *zag-1; unc-25::gfp* than in wild-type *unc-25::gfp* animals. Tryptophan hydroxylase catalyzes the first step in serotonin biosynthesis and *tph-1* is expressed in serotoninergic neurons (Sze et al., 2000). Although HSN lacked *tph-1::gfp* expression in *zag-1* mutants, expression in ADF, NSM, CP and R(1,3,9) was comparable to wild type. However, unlike wild type, faint GFP expression was detected in VC4 and VC5 in *zag-1* mutants (35/100 VC4 and 57/100 VC5 *zd85 zdIs13*). VC4 and VC5 are detected weakly and sporadically with serotonin antisera and express the vesicular monoamine transporter *cat-1* (Duerr et al., 1999). Therefore, in *zag-1* mutants, GFP expression in VC4 and VC5 apparently reflects upregulation of undetectable wild-type *tph-1::gfp* expression, rather than inappropriate expression. Last, *dat-1* encodes a dopamine transporter homolog that presumably facilitates dopamine reuptake (Nass et al., 2001). The *dat-1::gfp(zdIs31)* strain had moderate GFP expression in the eight dopaminergic neurons, ADE, PDE and CEP. Expression was greatly enhanced in these neurons by *zag-1* mutations. Together, these results indicate that *zag-1* activity downregulates the expression of neurotransmitter biosynthetic and reuptake genes.

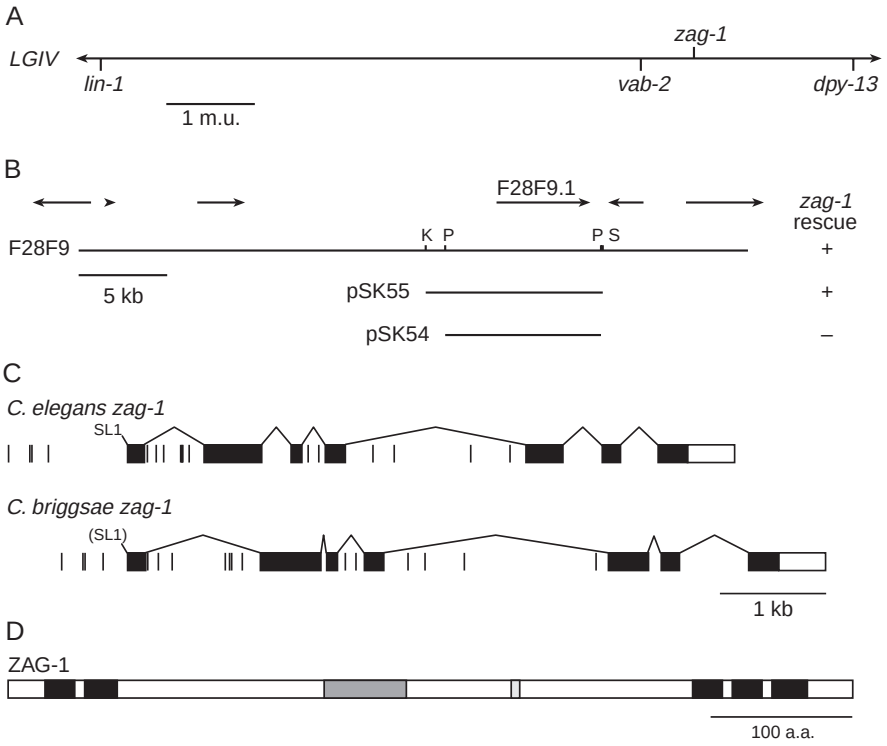

Fig. 3. Genetic and molecular analysis of *zag-1*. (A) Genetic map of *zag-1 IV* region. (B) Schematic of cosmid F28F9 and subclones. Arrows depict hypothetical gene transcripts and direction of transcription (www.wormbase.org) (+, *zag-1* rescue; –, no rescue). Restriction site abbreviations are K (*Kpn*I), P (*Pst*I) and S (*Sal*I). (C) *C. elegans* and *C. briggsae zag-1* genomic structures derived from cDNA and genomic sequences. *C. briggsae zag-1* structure is hypothetical and based on a comparison with *C. elegans* sequences. Black boxes indicate exons, white boxes depict 3′ UTR and vertical marks represent CACCT(G) motifs. VISTA server was used for some genomic sequence alignment (Mayor et al., 2000). (D) *C. elegans* ZAG-1 protein structure. Black boxes depict Zn-finger domains, the dark-gray box represents homeodomain and the light-gray box indicates PLDLT motif.

zag-1 molecular analysis

We cloned *zag-1* by genetic mapping and transformation rescue of its mutant phenotype (Fig. 3A,B). *zag-1* maps between *vab-2* and *dpy-13* on chromosome *IV* (see Materials and Methods). We tested cosmids within this 469 kb interval by germline transformation and identified a single cosmid, F28F9, that completely rescued the uncoordinated phenotype of *zag-1(zd85)* animals. A 10 kb *Kpn*I-*Sal*I subclone of F28F9, which encompasses the hypothetical gene F28F9.1, also rescued. We analyzed the nucleotide sequences of the predicted coding regions and splice junctions of F28F9.1 from wild-type and *zag-1* mutants and found that both *zd85* and *zd86* are G:C to A:T transitions that generate termination codons in exon 5. Together, these data indicate that *zag-1* is F28F9.1.

We determined the DNA sequence of a full-length cDNA (provided by Y. Kohara) to establish the *zag-1*-gene structure (Fig. 3C). *zag-1* has seven exons, a five-nucleotide 5′ UTR and, at most, a 444-nucleotide 3′ UTR. Spliced leader 1 sequences in cDNA indicated that the *zag-1* transcript is *trans*-spliced. On the basis of the DNA sequence, *zag-1* encodes a 596-amino-acid protein with five C2H2-type Zn-finger domains, two at the amino end and three at the carboxyl end, and a single homeodomain located in the middle (hence *zag-1*, Zn-finger-homeodomain, axon guidance) (Fig. 3D, Fig. 4A). The N and C-terminal Zn-finger-domain arrays are highly similar to each other, and to the Zn-finger domains in *Drosophila* ZFH-1 and vertebrate δEF1 and SIP1 proteins (Fig. 4B). Although the total number of Zn-finger domains varies in these proteins, the overall structure, namely, Zn-finger-domain clusters positioned at the N and C terminals and a centrally located homeodomain, is conserved and is a defining feature of the δEF1/ZFH-1 protein family. The Zn-finger-domain clusters of δEF1/ZFH-1 proteins bind to the consensus sequence CACCT, the E-box sequence CACCTG and tandem arrays of these motifs (Ikeda and Kawakami, 1995; Remacle et al., 1999; Sekido et al., 1997), indicating that ZAG-1 can also probably bind to CACCT sequences. The ZAG-1 homeodomain is most similar to those present in LIM homeodomain proteins, such as *C. elegans* LIM-4 and MEC-3, and the second homeodomain present in the *C. elegans* ZFH-2-related protein encoded by ZK123.3 (Fig. 4C). ZAG-1 lacks sequences characteristic of the Smad-interacting domain found in vertebrate SIP1 orthologs (Verschueren et al., 1999).

δEF1/ZFH-1 proteins share a conserved motif, PXDLS, needed for association with C-terminal binding proteins, CtBPs. The transcriptional corepressor CtBP is an NAD-dependent dehydrogenase that interacts with several DNA-binding proteins to mediate transcriptional repression (reviewed by Chinnadurai, 2002; Turner and Crossley, 2001). ZAG-1 has a CtBP-interaction motif (PLDLT) indicating that ZAG-1 can probably recruit CtBP and repress transcription.

The *zd85* and *zd86* mutations generate termination codons in exon 5 of ZAG-1. *zd85* alters the glutamine codon for residue 380 (CAG → TAG) and *zd86* changes the tryptophan codon for residue 369 (TGG → TAG). Both mutants are predicted to lack the C-terminal Zn-finger domains but retain the N-terminal Zn-finger domains, the homeodomain and the CtBP-interaction motif, and are likely to retain partial ZAG-1 function (see Discussion).

The *C. elegans* Sequencing Consortium has released preliminary genomic sequence of the nematode *C. briggsae* (www.wormbase.org), allowing identification of a *zag-1* ortholog. The *C. elegans* and *C. briggsae* ZAG-1 proteins are similar (88% identity) (Fig. 4A). The predicted amino acid sequences of the Zn-finger domains, homeodomain and CtBP-interaction motif are identical, whereas other regions contain

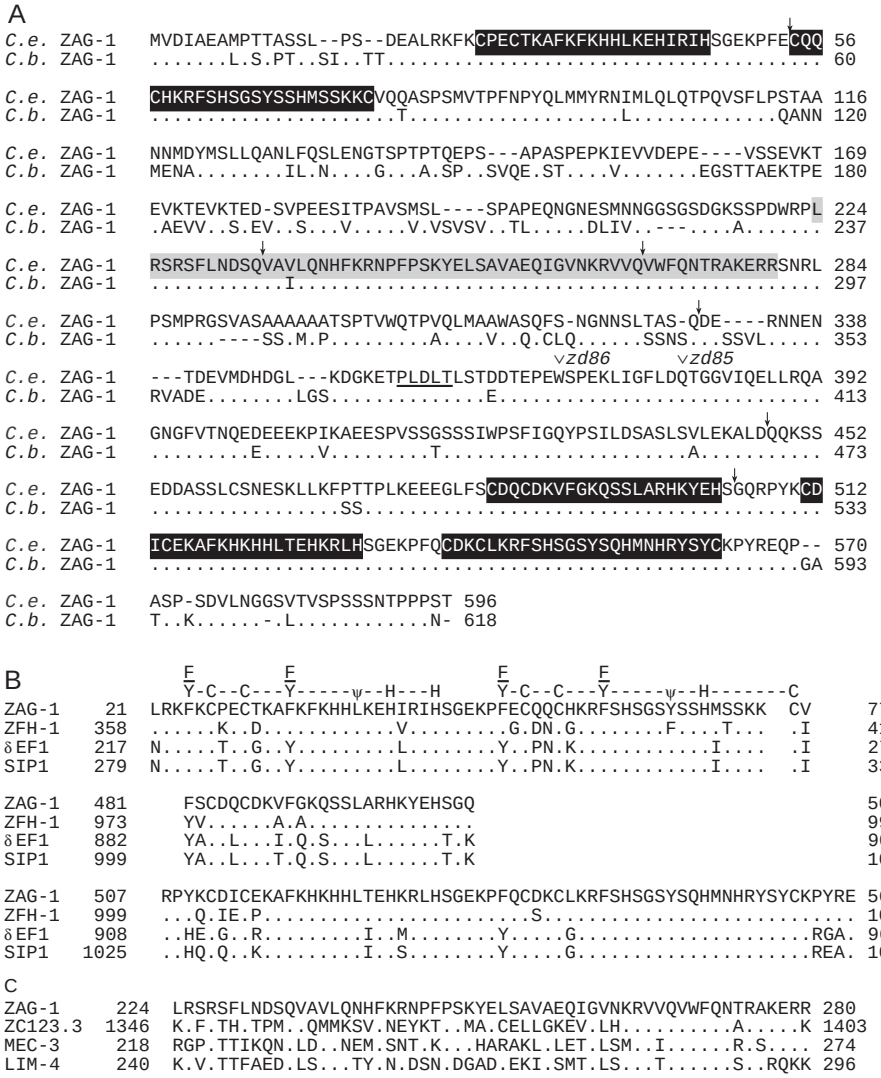

Fig. 4. (A) Alignment of predicted amino acid sequences of ZAG-1 proteins in *C. elegans* (*C. e.*) and *C. briggsae (C. b.)*. *C. elegans* sequence is based on full-length *zag-1* cDNA (GenBank Accession Number AY224511). *C. briggsae* sequence is derived from the gene structure in Fig. 3C. Hyphens indicate gaps inserted to maintain alignment and periods represent identical residues in *C. briggsae* sequence. The homeodomain and five Zn-finger domains are boxed in gray and black, respectively. PLDLT sequence required for association with CtBP is underlined. Inverted carets mark the Q and W residues altered in *zd85* and *zd86* and arrows mark the relative positions of conserved splice sites. (B) Alignment of Zn-finger domain sequences from *C. elegans* ZAG-1, *Drosophila* ZFH-1, mouse δEF1 and mouse SIP1. Modified, consensus C2H2-type Zn-finger sequence is shown on top (ψ, aliphatic amino acid). (C) Alignment of homeodomain sequences from *C. elegans* ZAG-1, ZC123.3 (ZFH-2-like protein), MEC-3 and LIM-4.

conservative substitutions and short deletions and insertions. The genomic organization of the two *zag-1* genes is similar and the positions of introns are identical (Fig. 3C, Fig. 4A). In addition to coding region, sequence conservation is present upstream of the ATG and in several introns.

Neurons and muscle express *zag-1*

We generated *zag-1* GFP translational and transcriptional transgenes to examine the expression pattern of *zag-1* (Fig. 5A). We fused 9.3 kb of *zag-1* genomic sequence (which included 4 kb of upstream sequence) to GFP coding and *unc-54* 3′UTR sequences to obtain a construct that produced a full-length ZAG-1 protein tagged with GFP at its C-terminus (Fig. 5A). This *zag-1::ZAG-1-GFP* construct rescued *zag-1* mutants (data not shown), indicating that the fusion protein is functional and expressed in a spatial and temporal pattern sufficient for rescue.

Examination of the *zag-1::ZAG-1-GFP(zdIs39)* strain revealed relatively faint GFP expression in neuronal nuclei from mid to late embryogenesis and during the L1 stage (Fig. 6A,B). We saw widespread expression in head and tail regions containing differentiating neurons beginning around the comma stage that diminished as embryogenesis continued. Expression remained in a few neurons at hatching and was typically nonexistent by midlarval stages. We also detected transient expression in the postembryonic Pn.a neuroblasts and their descendants during the L1. We conclude that *zag-1* is expressed transiently in most or all neurons during embryogenesis and in the Pn.a-derived ventral cord neurons during the L1, and that *zag-1* expression coincides generally with the time period that neurons extend axons and undergo terminal differentiation.

We constructed a translational fusion to exon 2 that expressed a GFP chimeric protein containing the first 213 amino acids of ZAG-1. *zag-1::ZAG-1(213)-GFP (zdIs40)* animals displayed a pattern of neuronal expression similar to that of *zag-1::ZAG-1-GFP(zdIs39)* animals, indicating that sequences between the end of exon 2 and 7 were unnecessary for generating the observed expression pattern. The GFP signal was detected in neuronal nuclei, showing that the first 213 amino acids of ZAG-1 are sufficient for nuclear translocation.

We examined the expression patterns of transcriptional GFP reporters containing 0.3-4.0 kb of upstream *zag-1* sequence using integrated or multiple, independent extrachromosomal array-containing lines to investigate further the regulation of *zag-1* expression (Fig. 5A). In contrast to the expression patterns displayed by the translational reporters, using the 1.6 kb, 2.9 kb and 4.0 kb upstream fragments we observed bright, persistent, GFP expression in a subset of neurons in the head and tail that began during midembryogenesis and continued throughout larval development and adulthood (Fig. 6C). These three fragments produced largely similar neuronal expression patterns, although the 4 kb fragment directed expression in additional neurons in the head, including the command

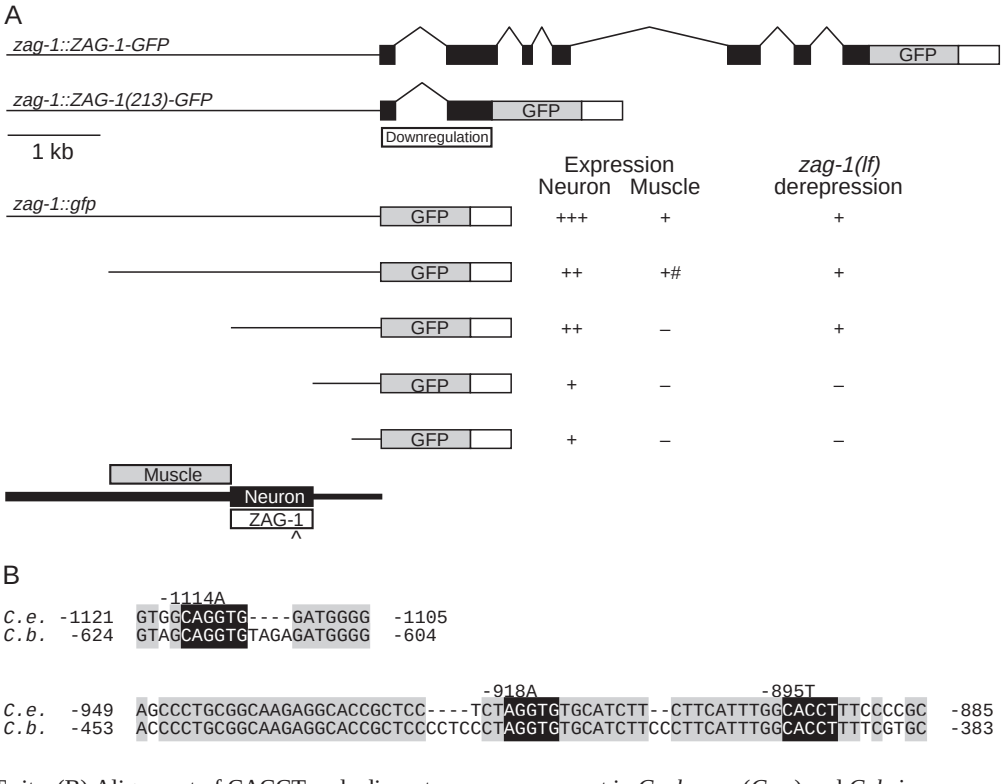

Fig. 5. (A) Schematic of *zag-1* GFP translational and transcriptional transgenes. Black boxes depict *zag-1* exons, gray boxes represent the GFP gene (coding region and synthetic introns) and white boxes indicate the 3′ UTR of *unc-54*. For neuronal GFP expression: +++, high expression in many head and tail neurons, including head command interneurons, RME, PVT and PVQ; ++, high expression in similar set of neurons, excluding head command interneurons; +, low expression in five or fewer neurons in head. For muscle GFP expression: +, high expression in anal depressor and intestinal muscles; #, transient, high expression in body wall muscles; –, no muscle expression. For *zag-1(lf)* derepression: +, high expression in command interneurons and VNC motor neurons; –, no expression in command interneurons and VNC motor neurons. Regions required for downregulation of *zag-1* expression, muscle expression, neuronal expression and ZAG-1-mediated repression are indicated. The caret indicates the location of tandem CACCT site. (B) Alignment of CACCT and adjacent sequences present in *C. elegans* (*C. e.*) and *C. briggsae* (*C. b.*) *zag-1* genomic region. Nucleotides altered in promoter mutants are indicated above the sequence.

interneurons. By contrast, the 0.3 kb and 0.7 kb fragments yielded only weak expression in a few neurons in the nerve ring. These data imply that sequences between –1.6 kb and –0.7 kb (where the first nucleotide of ATG is +1) are required for most of the expression seen in the characteristic subset of head and tail neurons.

We observed anal depressor and intestinal muscle expression starting around hatching using either the 2.9 kb or 4.0 kb 5′ fragment. No expression was seen using 1.6 kb or shorter fragments, indicating that sequences between –2.9 kb and –1.6 kb are needed for expression in these enteric muscles. The 2.9 kb fragment produced transient expression in body-wall muscles, starting before the comma stage and ending during midlarval development. The 0.3 kb, 0.7 kb, 1.6 kb and 4.0 kb fragments did not generate expression in body-wall muscle, suggesting that sequences between –2.9 kb and –1.6 kb promote body-wall-muscle expression and that sequences between –4.0 kb and –2.9 kb repress this.

The 4.0 kb *zag-1::gfp* transcriptional and *zag-1::ZAG-1(213)-GFP* translational reporters share the same 4 kb upstream sequences but differ because *zag-1::gfp* includes the coding sequences for the first seven amino acids of ZAG-1 (as do all the transcriptional reporter constructs) and *zag-1::ZAG-1(213)-GFP* includes sequences for exon 1, intron 1 and most of exon 2. Thus, exon 1 through exon 2 sequences are needed to confer the transient, widespread, weak GFP expression that is characteristic of the two translational reporters. Although there are differences in the structure and localization of the fusion proteins, we believe that transcriptional mechanisms mediated by sequence elements in intron 1 are most likely to underlie the reduced, transient expression, as detailed below. Together, these results indicate that *zag-1* is expressed broadly in the nervous system and in selected muscles. The different expression patterns of the translational and transcriptional constructs reveal the presence of multiple regulatory elements in the promoter and in the gene that either upregulate or downregulate *zag-1* expression.

zag-1 mutants lack muscle defects

Expression of *zag-1* in neurons and muscle is reminiscent of the neural and mesodermal expression of *Drosophila zfh-1* and vertebrate δEF1. *zfh-1* mutations perturb development of gonadal mesoderm, heart and other mesodermally derived tissues, and δEF1 is a negative regulator of muscle differentiation in vitro. We examined the morphology and development of body-wall muscle using the *talin::gfp(zdIs15)* reporter, which produces high GFP levels in all body-wall muscles starting during embryogenesis. GFP expression and muscle morphology were similar in wild-type *talin::gfp* and *zag-1; talin::gfp* animals. TGFβ UNC-129 is expressed in dorsal but not ventral body-wall muscles (Colavita et al., 1998); the body-wall expression pattern of *unc-129::gfp(zdIs42)* was unaffected by *zag-1* mutations. The morphology of anal depressor and intestinal muscles was unchanged in *zag-1* mutants when observed using a *zag-1::gfp* reporter. Thus, although *zag-1* is expressed in anal depressor, intestinal and body-wall muscles, we failed to detect defects in either muscle development or differentiation in *zag-1* mutants using these reporters.

ZAG-1 represses *zag-1* expression

We examined *zag-1* GFP reporter expression in *zag-1* mutants

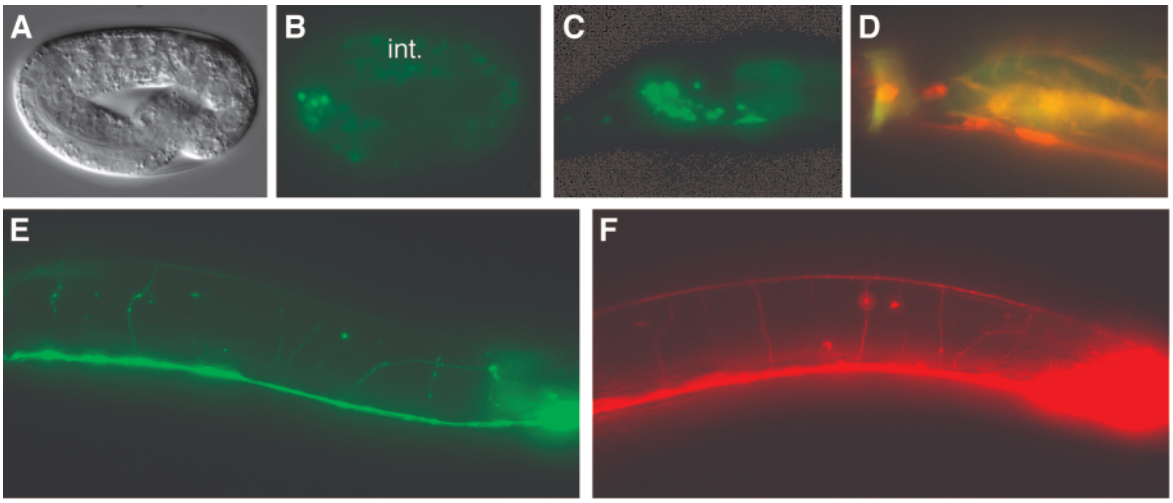

Fig. 6. *zag-1* expression pattern. DIC (A) and fluorescence (B) image of *zag-1::ZAG-1::GFP* embryo with GFP expression in nerve ring nuclei (int., intestinal autofluorescence). (C) *zag-1::gfp(zdIs21)* (2.9 kb upstream fragment) adult hermaphrodite with expression in subset of nerve ring neurons, no expression in ventral nerve cord neurons. (D) Tail region of *zag-1::gfp(zdIs21); zag-1(-918A,-895T)::rfp(zdIs41)* adult. *zag-1* promoter (4.0 kb) containing mutated CACCT sites directed RFP expression in more neurons than the wild-type promoter (GFP). Both promoters directed expression in anal depressor and intestinal muscles. (E) *zag-1(zd86) zag-1::gfp(zdIs21). zag-1* mutations induced expression in ventral cord motoneurons. (F) *zag-1(-918A,-895T)::rfp(zdIs41). zag-1* promoter with mutated CACCT sites directed RFP expression in ventral cord motoneurons. Anterior is leftwards (C), anterior is rightwards (D-F), dorsal is towards the top.

to investigate whether ZAG-1 regulated its own expression. We found that *zag-1* mutations affected neuronal expression patterns of the 1.6 kb, 2.9 kb and 4.0 kb upstream fragments but not the 0.3 kb and 0.7 kb fragments (Fig. 5A). In particular, we found that *zag-1* mutations induced bright GFP expression in the Pn.a-derived VNC motor neurons that began during midL1 and continued throughout adulthood (Fig. 6E). We also observed at least seven neurons in the tail that expressed GFP compared to only PVQ and PVT in wild type, as well as many additional GFP-expressing neurons in the head, including the command interneurons. Therefore, *zag-1* activity represses *zag-1::gfp* expression in many neurons via sequences located between –1.6 kb and –0.7 kb. The finding that the 4.0 kb upstream fragment also directed expression in the command interneurons in wild-type animals indicates the presence of multiple, positive and negative regulatory elements for command interneuron expression.

zag-1 mutations did not alter *zag-1::gfp* expression in anal depressor, intestinal and body-wall muscles. The expression pattern of the *zag-1::ZAG-1(213)-GFP* reporter, which did not rescue *zag-1*, was also unaffected by *zag-1* mutations. The inability of *zag-1* mutations to alter *zag-1::ZAG-1(213)-GFP* expression indicates that either the repression mediated by sequences in exon 1 through exon 2 is independent of *zag-1* activity or our two *zag-1* mutants retain partial activity.

δEF1/ZFH-1 proteins bind to the consensus sequence CACCT, the E box sequence CACCTG and tandem arrays of these motifs (Ikeda and Kawakami, 1995; Remacle et al., 1999; Sekido et al., 1997). Examination of *zag-1* genomic sequences from *C. elegans* and *C. briggsae* revealed several blocks of identity in addition to conserved sequences in coding regions. Several, conserved CACCT motifs are present in the 0.9 kb region that is essential for ZAG-1-mediated repression of *zag-1::gfp* expression and in the first, third and fourth introns (Fig. 3C, Fig. 5B). Because previous studies have shown that altering the CACCT sequence to CATCT either greatly reduced or eliminated binding of either N or C-terminal δEF1/ZFH-1 Zn-finger-domain clusters (Remacle et al., 1999), we introduced these mutations into the 1.6 kb promoter fragment to test whether these sites influenced *zag-1* expression. We altered the conserved E box (CACCTG) at –1114 and the two CACCT sites at –918 and –895, either singly or together, and examined GFP-reporter expression. Alteration of the E-box site had no obvious effect on expression, whereas mutation of either CACCT motif had a moderate effect. Both –918A and –895T promoter mutants produced reproducible expression in the command interneurons and, occasionally, generated expression in one or two ventral cord motor neurons in animals that contained extrachromosomal arrays. In the CACCT double mutant (–918A, –895T), the pattern of expression was strikingly similar to that observed in *zag-1 zag-1::gfp* mutants, including expression in command interneurons and most or all ventral cord motor neurons. The 4.0 kb fragment containing the –918A and –895T CACCT mutations also produced an expression pattern comparable to that observed in a *zag-1*- mutant background (Fig. 6D-F). Based on these results and published studies of the binding of δEF1/ZFH-1 proteins, we conclude that the tandem CACCT site is a ZAG-1 binding site and that ZAG-1 directly represses expression via this site.

DISCUSSION

zag-1 activity establishes several neuronal characteristics, such as cell position, axonal structure and gene-expression profile. Although *zag-1* mutations confer various defects on sensory, motor and interneurons, common or related phenotypes are evident. These include the absence of stereotypic axon branches and upregulation of neurotransmitter biosynthetic and reuptake genes. *zag-1* functions less to define neuron identity

per se and more to generate features characteristic of a particular type of neuron. The specificity and selectivity of *zag-1* phenotypes for each neuron type suggests that *zag-1* acts in combination with other cell-type-specific factors to control differentiation.

SRA-6 is a candidate chemosensory receptor, based on its predicted seven transmembrane domain topology and expression in amphid sensory neurons ASH and ASI (Troemel et al., 1995). *sra-6::gfp* provides an ideal indicator of PVQ development and differentiation, although *sra-6* function in interneuron PVQ is unclear. *zag-1* is required for specific elements of PVQ differentiation: LIM homeodomain gene *lin-11* expression and axonal development appear wild type, whereas *sra-6* expression is either reduced or eliminated. Because there is no evidence that ZAG-1 acts as a transcriptional activator, regulation of *sra-6* expression is, presumably, indirect. PVQ *sra-6* expression also requires PAG-3, a Zn-finger-domain protein that functions in neural differentiation and cell lineage (Cameron et al., 2002; Jia et al., 1997). Although *zag-1* and *pag-3* are coexpressed in other neurons, they do not share other known phenotypes.

zag-1 mutations disrupt several late differentiation features of HSN, including axon pathfinding, hood formation and *tph-1* expression. Early development of HSN appears unaffected because HSNs migrate to their correct, midbody position, express *unc-86* and do not misexpress *odr-2*. *zag-1* HSN phenotypes are remarkably similar to those of *egl-45, sem-4* and *unc-86* (Desai et al., 1988). The Zn-finger-domain gene *sem-4* controls neuronal and mesodermal development (Basson and Horvitz, 1996) and the POU-homeodomain gene *unc-86* regulates neuronal-cell lineage and differentiation (Finney et al., 1988). *egl-45, sem-4* and *unc-86* act in a genetic pathway containing both parallel and overlapping branches that controls HSN development (Desai et al., 1988). *zag-1* likely functions downstream of or in parallel to *unc-86* because *zag-1* mutations do not affect *unc-86::gfp* expression.

Ectopic HSN *odr-2* expression in *zag-1* adults reveals that *zag-1* activity blocks expression of nonHSN genes as well as promotes HSN differentiation and proper gene expression. Regulation of *odr-2*, which encodes a GPI-linked cell-surface protein, is neuron-type specific; that is, *zag-1* mutations induce misexpression of *odr-2* in HSN and, occasionally, PVM but do not otherwise appear to alter the *odr-2* expression pattern. Similarly, *zag-1* is needed for proper expression of the glutamate receptors GLR-1 and NMR-1 and the ion channel MEC-4, and to prevent misexpression of GLR-1 and NMR-1.

zag-1 activity downregulates the expression of neurotransmitter biosynthetic and reuptake genes. Although *zag-1* does not determine which neurons express *dat-1, tph-1* and *unc-25*, it modulates expression levels in the appropriate neurons, acting, perhaps, as a 'gain' switch. *zag-1* expression appears to be restricted to embryos and L1s, but upregulation of expression is still apparent in adults, which indicates that either ZAG-1 is present but undetectable in adults or ZAG-1-established expression levels are maintained.

zag-1 is essential for the formation of stereotypic axon branches of several neuron types. *zag-1* mutations block the generation of anteriorly directed branches of ADE, ALM and AVM and the posteriorly directed branch of PDE. Except for lacking these branches, the axon trajectories of these neurons appear wild type and full-length, indicating an explicit defect in the development of axon branches rather than a deficiency of axon outgrowth. Although HSN, DD and VD axon-branching defects are coupled with broader pathfinding errors, we believe that these defects reflect a specific role of *zag-1* in branching and pathfinding. The dorso-ventrally oriented ADL branches were unaffected by *zag-1* mutations (data not shown), indicating that *zag-1* is not required for all axon-branching patterns.

The dramatic guidance, branching and fasciculation defects of many *glr-1::gfp*-expressing interneuron and motor neuron axons illustrate further the role of *zag-1* in creating axonal structures. Exuberant extension and branching might reflect either a guidance defect or an inability to terminate outgrowth at appropriate targets. The defasciculation of the nerve ring and ventral nerve cord and premature termination of the AVG axon reveal that *zag-1* is also required for nerve bundle formation and axon outgrowth.

The mature axonal morphology of *C. elegans* neurons has been described in great detail, although the assembly of these axonal structures is less well understood. For example, several nonexclusive ways to sculpt the mature ALM axon structure are possible: ALM growth cone might first extend to the first bulb of pharynx and a collateral branch might later enter neuropil; ALM growth cone might first project to nerve ring, turn and enter neuropil and a collateral branch might later project anteriorly to first bulb of pharynx; and ALM growth cone might extend to nerve ring, split and form both branches concurrently. The latter two scenarios are consistent with the observation that *zag-1* mutants lack anterior but not neuropil branches. We analyzed axonal structures in young adult hermaphrodites and limited analysis of larvae revealed similar axonal defects. Our current results indicate that *zag-1* mutations block the formation of axon branches but do not rule out the possibility of inappropriate axon-branch retraction or pruning. Real-time analysis is needed to resolve the axon assembly pathway of ALM and other neurons, but this is technically difficult at present. However, regardless of assembly pathway, *zag-1* activity is needed to shape the mature axonal configuration.

HSN has two projections during the L2 and L3, one directed ventrally that will ultimately reach nerve ring and be maintained in adult, and one directed dorsally that is, presumably, retracted later because it is not found in adults (Garriga et al., 1993). The HSNs often have two processes in *zag-1* mutants, supporting the notion that *zag-1* activity sculpts the mature axonal morphology by promoting the formation of new branches, preventing extension of inappropriate processes and eliminating immature structures.

Interactions with vulval cells control HSN-axon branching (Garriga et al., 1993) and interactions with BDU guide AVM branch into the mature neuropil (Walthall and Chalfie, 1988). Similar cellular interactions are believed to guide the formation of other axon branches. *zag-1* might act in either a signaling or responding neuron to coordinate branch formation and pathfinding interactions. The specificity of axon-branch defects in *zag-1* mutants indicates the existence of a branch-formation program that functions in combination with a primary axon-guidance program to define a particular axonal-projection pattern. Such branching programs might function either during or subsequent to formation of primary axons and entail the regulation of genes that generate, recognize and transduce

spatial and temporal branching signals. The branching defects of ADE, PDE, ALM and AVM might represent a failure to activate a branching program, whereas defects of HSN, VD and *glr-1::gfp*-expressing neurons might reflect coexpression or misexpression of primary axon-guidance and branching programs.

ZAG-1 is a transcriptional repressor

ZAG-1 is a δEF1/ZFH-1 Zn-finger-homeodomain protein. Mutation of either *zag-1* or two, conserved CACCT sites in the *zag-1* promoter upregulates *zag-1::gfp* expression in many neurons, including ventral cord motor neurons and command interneurons. As Zn-finger-domain arrays from δEF1/ZFH-1 proteins bind CACCT sequences, we conclude that ZAG-1 represses *zag-1* expression by binding directly to this tandem CACCT site. Although many direct target genes of δEF1/ZFH-1 and SIP1 have been identified (reviewed by van Grunsven et al., 2001), our results provide the first example that δEF1/ZFH-1 proteins regulate their own expression. In addition, our findings that both CACCT sequences are needed for complete repression are consistent with studies of Remacle et al. (Remacle et al., 1999), which show that SIP1 binds as a monomer and contacts one CACCT site with one Zn-finger-domain cluster and the other CACCT(G) site with the second cluster.

δEF1-knockout mice have defects in skeletal and thymus development, whereas a $\delta EF1^{\Delta C727}$ mutant, which expresses a δEF1 protein lacking the C-terminal Zn-finger-domain cluster like the two ZAG-1 mutant proteins, has defects in only thymus development (Higashi et al., 1997; Takagi et al., 1998). These results suggest that $\delta EF1^{\Delta C727}$ retains sufficient activity to promote skeletal development and that δEF1/ZFH-1 binding sites can be divided into two classes: those that require the C-terminal Zn-finger-domain cluster and those that do not. We infer that our two ZAG-1 mutants also retain activity and regulate the subset of *zag-1* target genes that possess the second class of ZAG-1 binding site. Conserved CACCT(G) motifs in the first, third and fourth introns represent candidate ZAG-1-binding sites; however, *zag-1* mutations fail to alter expression of *zag-1-ZAG-1(213)-GFP*, which contains intron 1 sequences. These observations indicate that these sequences are not ZAG-1-binding sites and that other factors mediate repression or that these sequences belong to the second class of ZAG-1-binding site. Our conclusion that the truncated ZAG-1 proteins retain activity is consistent with the observation that a *zag-1* deletion mutant is not viable because of a feeding defect (Wacker et al., 2003).

Our GFP-transgene studies indicate that *zag-1* is expressed transiently in most or all neurons during embryogenesis and in the Pn.a-derived ventral cord neurons during the L1. In general, the time of *zag-1* expression coincides with the period that neurons extend axons and undergo terminal differentiation. The analysis of the *zag-1*-promoter region indicates that *zag-1* is also expressed in anal depressor, intestinal and body-wall muscles, although no obvious muscle defects were observed. Similarly, although *Drosophila zfh-1* is expressed in motorneurons, defects in motorneuron development have not been identified in *zfh-1* mutants. It is speculated that the lack of neuronal and muscle defects in δEF1-knockout mice reflects a redundancy with SIP1, which is also expressed in these tissues (Postigo and Dean, 2000). Examination of *zag-1*-null mutants and additional muscle-differentiation markers is needed to investigate further the role of *zag-1* in muscle development

ZAG-1 and other neuronal differentiation regulators

Numerous transcriptional regulators have been identified that control the generation, specification and differentiation of the 302 neurons of *C. elegans*. Mutation of these factors cause a variety of defects that affect different neuron types as well as different aspects and phases of neuronal development. Although ZAG-1 shares some activities and characteristics with known factors, the overall role of ZAG-1 in neuronal development is unique. For example, *zag-1* mutants have HSN-differentiation defects that are similar to *unc-86* mutants and PVQ-differentiation defects that are similar to *pag-3* mutants. However, unlike *zag-1* mutations, *unc-86* and *pag-3* mutations also cause some daughter cells to reiterate the lineage of their mother, demonstrating that UNC-86 and PAG-3 control the generation of particular neuron types. Touch-receptor neurons exhibit none of their unique differentiated features in LIM-homeodomain gene *mec-3* mutants (Way and Chalfie, 1988) but display only a subset of defects, such as errors in *mec-4* expression, cell migration and axon-branch formation, in *zag-1* mutants. Thus, ZAG-1 is not involved in generating neurons or in defining every trait of a particular neuron type. In contrast to *lim-4* and *unc-130* mutations, which cause specific neurons to adopt alternative or default cell fates (Sagasti et al., 1999; Sarafi-Reinach and Sengupta, 2000), *zag-1* mutations do not induce cell-fate transformations but, rather, prevent the acquisition of a subset of neuron-type characteristics. The function of *zag-1* is most similar to *lim-6*, *unc-30* and *unc-42*, which establish select aspects of late differentiation such as axonal development, synaptic connectivity and neuron-type-specific gene expression (Baran et al., 1999; Brockie et al., 2001; Hobert et al., 1999; Jin et al., 1994). However, LIM-6, UNC-30 and UNC-42 have more restricted patterns of expression compared to ZAG-1, which appears to be expressed in most or all neurons. Thus, ZAG-1 acts as a global regulator of neuronal differentiation and is the first transcription factor to be identified that controls axon-branch formation.

Although most of the identified regulators are likely to function as transcriptional activators, ZAG-1 acts as a transcriptional repressor. Other factors involved in late-neural differentiation that repress transcription directly include the homeodomain UNC-4 and Groucho-like corepressor UNC-37, which specify synaptic choice (Winnier et al., 1999). ZAG-1 might function as a temporal switch during neuronal development; that is, ZAG-1 might initiate late differentiation by turning off a ‘late-differentiation repressor’ and/or genes involved in early differentiation. The ectopic expression of *glr-1*, *nmr-1* and *odr-2* in *zag-1* mutants indicates that ZAG-1 also blocks the adoption of inappropriate neuronal characteristics. As with specification of neuron fate, combined actions of both transcriptional activators and repressors are needed to establish the characteristics of terminal differentiation. Last, the observation that a presumptive ZAG-1-binding site is conserved between *C. elegans* and *C. briggsae* indicates that a bioinformatics strategy can be used to identify potential direct gene targets of ZAG-1, which will provide further insight into *zag-1* function.

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