# HLH-14 is a *C. elegans* Achaete-Scute protein that promotes neurogenesis through asymmetric cell division

**C. Andrew Frank, Paul D. Baum* and Gian Garriga†**

Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720-3204, USA
*Present address: Gladstone Institute of Virology and Immunology, University of California, San Francisco, San Francisco, CA 94141-9100, USA
†Author for correspondence (e-mail: garriga@uclink4.berkeley.edu)

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## Summary

**Achaete-Scute basic helix-loop-helix (bHLH) proteins promote neurogenesis during metazoan development. In this study, we characterize a *C. elegans* Achaete-Scute homolog, HLH-14. We find that a number of neuroblasts express HLH-14 in the *C. elegans* embryo, including the PVQ/HSN/PHB neuroblast, a cell that generates the PVQ interneuron, the HSN motoneuron and the PHB sensory neuron. *hlh-14* mutants lack all three of these neurons. The fact that HLH-14 promotes all three classes of neuron indicates that *C. elegans* proneural bHLH factors may act less specifically than their fly and mammalian homologs. Furthermore, neural loss in *hlh-14* mutants results from a defect in an asymmetric cell division: the PVQ/HSN/PHB neuroblast inappropriately assumes characteristics of its sister cell, the hyp7/T blast cell. We argue that bHLH proteins, which control various aspects of metazoan development, can control cell fate choices in *C. elegans* by regulating asymmetric cell divisions. Finally, a reduction in the function of *hlh-2*, which encodes the *C. elegans* E/Daughterless bHLH homolog, results in similar neuron loss as *hlh-14* mutants and enhances the effects of partially reducing *hlh-14* function. We propose that HLH-14 and HLH-2 act together to specify neuroblast lineages and promote neuronal fate.**

Key words: *Caenorhabditis elegans*, HLH-14, HLH-2, bHLH, Proneural, Neuroblast

## Introduction

Basic helix-loop-helix (bHLH) transcription factors play integral roles in a number of developmental processes, including neurogenesis. Neural bHLH proteins have been characterized in organisms as diverse as nematodes, flies and vertebrates. These factors can assume a variety of tasks, depending upon when and where they are expressed. For example, proneural bHLH factors are expressed early in neurogenesis and are both necessary and sufficient to promote neuroblast lineages. By contrast, neuronal differentiation bHLH factors are expressed later, in neuronal precursors or postmitotic neurons, and help to fine tune neuronal fates (reviewed by Bertrand et al., 2002).

The first proneural genes identified were the Achaete-Scute (A-S) Complex genes in *Drosophila*. Genes in this family include *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*lsc*) and *asense* (*ase*), and are required for external sense organ development (Garcia-Bellido, 1979; Gonzalez et al., 1989; Villares and Cabrera, 1987). Later work in *Drosophila* identified *atonal* (*ato*), the founding member of another proneural bHLH gene family, as crucial for the development of internal sense organs, the chordotonal organs (Jarman et al., 1993). Other fly Atonal family members are also involved in sense organ development and include *absent of MD neurons and olfactory sensilla* (*amos*) and *cousin of atonal* (*cato*) (Goulding et al., 2000a; Goulding et al., 2000b; Huang et al., 2000).

Proneural genes can act as developmental switches that control neural fate. In general, proneural gene expression promotes the generation of neuroblasts at the expense of adjacent cell types, like epidermal cells. Flies lacking *ac* and *sc* function, for example, are missing most of their mechanosensory and chemosensory organs (Bertrand et al., 2002; Garcia-Bellido, 1979) because of a failure to select sensory organ progenitors from the ectoderm. Ectopic expression of A-S genes in the ectoderm can induce the development of ectopic sensory organs at the expense of dermal cells (Dominguez and Campuzano, 1993; Rodriguez et al., 1990). Similarly, flies lacking *ato* gene function are missing their chordotonal sensory organs, and ectopic expression of Atonal family members promotes the formation of extra chordotonal organs (Chien et al., 1996; Goulding et al., 2000b; Huang et al., 2000; Jarman et al., 1993).

In vertebrates, members of the A-S, Atonal and Neurogenin (Ngn) bHLH families all exhibit proneural characteristics. Again, specification of neuroblast cell fate is a crucial function of these factors. For example, the A-S protein Ash1 is present in most, if not all, vertebrates (Allende and Weinberg, 1994; Ball et al., 1993; Ferreiro et al., 1993; Frowein et al., 2002; Verma-Kurvari et al., 1996). Mice lacking functional Mash1 (mouse Ash1) have neurogenesis defects in the ventral telencephalon and olfactory sensory epithelium (Casarosa et al., 1999; Guillemot et al., 1993; Horton et al., 1999). These defects are correlated with an absence of progenitor cells, consistent with Mash1 promoting neurogenesis.

*C. elegans*, like *Drosophila* and vertebrates, has a number

of neural bHLH proteins. To date, the most extensively characterized is LIN-32, the sole *C. elegans* Atonal family member (Ledent et al., 2002; Zhao and Emmons, 1995). Reminiscent of Drosophila *atonal* mutants, *lin-32* mutants lack some sensory organs (Portman and Emmons, 2000; Zhao and Emmons, 1995). In particular, *lin-32* mutant males lack rays, peripheral sensory organs of the male tail that are important for sensing hermaphrodites during mating (Zhao and Emmons, 1995). Ectopic expression of LIN-32 under a ubiquitous heat-shock promoter is sufficient to generate ectopic ray papillae structures (Zhao and Emmons, 1995).

bHLH proteins usually activate transcription of target genes as heterodimers with members of the E/Daughterless (DA) bHLH family (Cabrera and Alonso, 1991; Johnson et al., 1992; Massari and Murre, 2000). Heterodimer formation is mediated by the helices of the bHLH proteins, while the basic regions are important for binding to DNA sequences with an E-box motif, CANNTG. In *C. elegans*, both LIN-32 and the A-S protein HLH-3 can bind to the *C. elegans* E/DA homolog, HLH-2, in the presence of E-box motifs (Krause et al., 1997; Portman and Emmons, 2000; Thellmann et al., 2003). Both LIN-32 and HLH-3 are expressed in many of the same neuronal lineages as HLH-2 and probably require heterodimerization for the proper execution of some of these lineages (Krause et al., 1997; Portman and Emmons, 2000; Thellmann et al., 2003).

In this study, we characterize a new *C. elegans* A-S family member, HLH-14. We find that *hlh-14* function is required for the production of specific neurons, notably three lineally related neurons: the PVQ interneuron, the HSN motoneuron, and the PHB sensory neuron. Like other A-S factors, HLH-14 is expressed in neuronal precursors and has proneural characteristics. Yet HLH-14 does not have a strictly proneural role; it appears to act in neuronal differentiation as well. Additionally, genetic data suggest that *hlh-14* and *hlh-2* act together in neurogenesis.

Surprisingly, we find that loss of *hlh-14* function causes an asymmetric cell division defect. Specifically, in *hlh-14* mutants, the PVQ/HSN/PHB neuroblast appears to assume characteristics of its sister cell, the hyp7/T blast cell. Taken together with previous studies in nematodes, we propose that *C. elegans* proneural genes play slightly different roles from their *Drosophila* or vertebrate counterparts. In particular, *C. elegans* proneural genes such as *hlh-14* can promote neurogenesis, in part, by regulating asymmetric cell divisions. Additionally, *C. elegans* proneural genes lack the specificity of their homologs and promote neuroblast lineages that generate neural cells of disparate function.

## Materials and methods

### *C. elegans* strains

Nematodes were maintained as previously described (Brenner, 1974). Strains were kept at 20°C unless otherwise noted. This study uses standard *C. elegans* nomenclature (Horvitz et al., 1979). The wild-type strain N2 was used unless otherwise noted.

Strains with the following mutant alleles, chromosomal aberrations or transgenic arrays were used in this work. Unreferenced strains were generated in the course of this study.

#### Linkage Group (*LG*) *I*

*unc-13*(*e51*) (Brenner, 1974), *hlh-2*(*bx115*) (Portman and Emmons, 2000), *ynIs45*[*flp-15::gfp*] (Li et al., 1999), *nuIs11*[*osm-10::gfp*] (Hart et al., 1999) and *kyIs39*[*sra-6::gfp*] (Troemel et al., 1995).

#### *LG II*

*bli-2*(*st1016*) (Nonet et al., 1997), *lin-4*(*e912*) (Horvitz and Sulston, 1980), *dpy-10*(*e128*) (Brenner, 1974), *clr-1*(*e1745*) (Way and Chalfie, 1988), *hlh-14*(*gm34*), *hlh-14*(*ju243*) (W.-M. Woo and A. Chisholm, personal communication), *gmIs20*[*hlh-14::gfp*], *mIn1* (Edgley and Riddle, 2001), *maDf4* (V. Ambros, personal communication), *ccDf5* (Chen et al., 1992).

#### *LG III*

*gmIs12*[*srb-6::gfp*] (N. Hawkins, personal communication) (Troemel et al., 1995), *gmIs21*[*nlp-1::gfp*] (Li et al., 1999).

#### *LG IV*

*kyIs179*[*unc-86::gfp*] (Gitai et al., 2003), *ham-1*(*n1811*) (Desai et al., 1988; Guenther and Garriga, 1996), *ced-3*(*n717*) (Ellis and Horvitz, 1986).

#### *LG V*

*gmIs22*[*nlp-1::gfp*] (Li et al., 1999).

#### Extrachromosomal arrays

*gmEx281*[*hlh-14::gfp*], *leEx887*[C50B6.8::*gfp*] (Mounsey et al., 2002).

### Isolation of *hlh-14* mutants and cloning of *hlh-14*

*hlh-14*(*gm34*) was isolated in a genetic screen for mutants with missing or misplaced HSN motoneurons (G.G., unpublished), and *hlh-14*(*ju243*) was isolated in a screen for mutants with morphological defects (W.-M. Woo and A. Chisholm, personal communication). As the two alleles displayed similar defects and mapped to the same region of *LG II*, a complementation test was performed, confirming that they are allelic.

*hlh-14*(*gm34*) was genetically mapped between *bli-2* and *lin-4* on *LG II*. From worms of the parental genotype *hlh-14*(*gm34*)/*bli-2*(*st1016*) *lin-4*(*e912*), 4/11 Bli nonLin recombinant progeny segregated the *hlh-14*(*gm34*) mutation. Corroborating this map position, the deficiency *maDf4* fails to complement *hlh-14*(*gm34*), but the deficiency *ccDf5* complements *hlh-14*(*gm34*). Few *C. elegans* cosmid clones are in the region corresponding to these mapping data. Rescue of Hlh-14 phenotypes was achieved with injection of two of the cosmids in this region, F22C7 and C18A3, both of which contain the *hlh-14*-coding region, C18A3.8.

### Detection of *hlh-14* mutant lesions

To detect lesions in *hlh-14* mutants, we PCR amplified the genomic region of *hlh-14* from mutant genomic DNA and sequenced the amplicons. To obtain mutant genomic DNA, we picked about five mutant worms into the cap of a PCR tube containing 10 μl of lysis buffer (10 mM Tris pH 8.2, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.05% gelatin). The tubes were briefly centrifuged to bring down the lysis buffer and worms and then placed on dry ice for 10 minutes. The tubes were thawed at room temperature and then placed in the PCR machine for a lysis reaction (60 minutes at 60°C, followed by 15 minutes at 95°C). Lysate (3 μl) was used as a genomic DNA template for PCR reactions.

To amplify the *hlh-14*-coding region for sequencing, the primers C18A3.8-L1 (5′ AGACAATGCAAATTGGGAGG 3′) and C18A3.8-R1 (5′ GCTAATTGACTCTCGTCCGC 3′) were used. The resulting 5.9 kb product was used as a template to reamplify the region with the primers C18A3.8-L2 (5′ TACATCGCCTGCAGTAGTGG 3′) and C18A3.8-R2 (5′ TTGGTATGGGAGGAGAGTGC 3′), yielding a 5.2 kb product.

To sequence the *hlh-14*-coding region, the primers bg1-s1 (5′ ATACCTCCCACATTTTGG 3′), bg1-s2 (5′ CACCACCGTCTT-

CCCT 3′), bg1-s4 (5′ TCACAAGTAGTATTCTTCC 3′) and bg1-s5 (5′ CATAGAAGTACACATGATTG 3′) were used. Sequencing reactions with bg1-s2 and bg1-s5 reliably detected the *hlh-14* lesions.

### *hlh-14* 5′ and 3′ RACE

To perform 5′ and 3′ RACE, 5′ and 3′ *C. elegans* cDNA libraries were made using the SMART™ RACE cDNA Amplification Kit protocol (Clontech). Using these libraries as a template, 3′ RACE PCR was performed using the primers BG1-GSPL2 (5′ CAGAAATGAGAGAAGAACGCAAGCG 3′) and Clontech's UPM primer mix. This reaction amplified the 3′ cDNA end common to all *hlh-14* cDNAs.

5′ RACE was performed using a number of different primers. Using library template, the UPM primer mix and the primer BG1-GSPR1 (5′ TGCTGTTGTTCATCGTGTAGTCGG 3′), we amplified the 5′ end of one *hlh-14* transcript, designated *hlh-14* Short; this cDNA represents the shortest *hlh-14* transcript. We believe this is the 5′ end of the most common *hlh-14* transcript because it is the only one we can amplify without a second nested reaction. The 5′ ends of two other transcripts were detected with nested reactions. They were amplified with a primary PCR reaction using library template, the UPM primer mix and the primer BG1-GSPR3 (5′ GCTAATGGTGAGAGGAAAGGCGGG 3′). The resulting amplicon was used as a template to detect the 5′ ends of the less common *hlh-14* transcripts. The primers for this second, nested reaction were Clontech's NUP primer and BG1-GSPR4 (5′ GGATATTGGCACAAACCGTATTGGC 3′).

### Generation of *hlh-14::gfp* transgenes

To generate the full-length *hlh-14::gfp* transgene, the primers BG1GFP-L4 (5′ AAATGTCGACCAACATGCAAAAGCTAATGGG 3′) and BG1GFP-R2 (5′ CCAAGGATCCATGGTGTGGATAATTGGAATATGA 3′) were used to amplify the promoter and coding regions of *hlh-14*, using the cosmid F22C7 as a template. The resulting 8.4 kb genomic product and the GFP vector pPD95.77 (A. Fire, S. Xu, J. Ahnn and G. Seydoux, unpublished) were double digested with *Sal*I and *Bam*HI and ligated together. The resulting construct was co-injected into *hlh-14*(*gm34*)/*mIn1* animals at a concentration of 0.5 ng/μl with the pRF4 plasmid (Mello et al., 1991) at a concentration of 50 ng/μl. Rescued *hlh-14*(*gm34*) animals were found among the transgenic progeny, establishing the extrachromosomal array line *gmEx281*. Rescued animals were identified by the absence of the *mIn1* GFP balancer, the presence of the array (pRF4-bearing Rol progeny), and the absence of Hlh-14 morphological defects. Prior to array integration, *hlh-14*(*gm34*); *gmEx281* animals were backcrossed to wild-type animals, and *gmEx281* was recovered in a wild-type background.

The extrachromosomal array *gmEx281* was integrated into the genome by UV irradiation. L4 stage *gmEx281* worms were washed four times with M9 and placed on a NGM agar plate without bacteria. The worms were irradiated in a UV Stratalinker at a strength of 250 μJ×100 and allowed to recover on bacteria at 15°C overnight and lay eggs the next day. Approximately 150 F1 progeny were cloned to individual plates and allowed to produce F2 progeny; two or three F2 animals per F1 plate were cloned to new individual plates. F3 progeny were then scored for 100% transgenic animals (pRF4 Rol phenotype). In this way, the integrated array *gmIs20* was generated.

### Detection and analysis of specific neurons

The HSN neurons were detected in larvae using the *unc-86::gfp* reporter, *kyIs179* (Gitai et al., 2003). The HSN neurons were detected in adult worms using the serotonin staining procedure as described (Garriga et al., 1993). The PHB neurons were detected with two different GFP reporters. The *srb-6::gfp* reporter (Troemel et al., 1995), which was integrated onto *LG III* to form the strain *gmIs12* (N. Hawkins, personal communication), detected both the PHA and PHB phasmid neurons. The *nlp-1::gfp* reporter (Li et al., 1999) *gmEx285* was integrated onto *LG III* and *LG V* to form the arrays *gmIs21* and *gmIs22*, respectively; these arrays specifically detected the PHB neurons. The PHA neurons were detected using the *flp-15::gfp* reporter *ynIs45* (Li et al., 1999) and the *osm-10::gfp* reporter *nuIs11* (Hart et al., 1999) (PHA-specific in adults). The PVQ neurons were detected using the *sra-6::gfp* reporter *kyIs39* (Troemel et al., 1995).

All neurons were visualized using a Zeiss Axioskop compound microscope. Some images were captured using Elite Chrome 100 color film (Kodak) and developed into slides. Other images were captured with a Hamamatsu ORCA-ER digital camera and saved as OpenLab files. Images were formatted using Adobe Photoshop.

### Lineage analysis of living embryos

Lineage analysis was performed for two experiments: determining that *hlh-14::gfp* is expressed in the PVQ/HSN/PHB neuroblast lineage; and determining the cell lineage defect of *hlh-14*(*gm34*) embryos. For both experiments, embryos were mounted on 5% agar pads in 2 μl of M9 buffer and examined on a Zeiss Axioskop compound microscope by Nomarski optics. Specific cells were identified relative to nearby landmark cell deaths (Sulston et al., 1983). In the analysis of *hlh-14*(*gm34*) embryos, lineaging began at the four-cell stage and was followed for only one of the two bilaterally symmetric lineages. Lineages were observed until the comma stage of development.

### RNA interference and analyses

RNA interference experiments were performed on two genes, *hlh-14* and *hlh-2*. *hlh-14* sequences were amplified from *C. elegans* cDNA library template (see 5′ and 3′ RACE Methods) using the primers BG1-LHIS1 (5′ GAATCTGCAGCAATGGGTCTGAGCTCAGATTTTC 3′) and BG1-RHIS3 (5′ CAAAAAGCTTTTAATGGTGTGGATAATTGGAATATG 3′), yielding a 0.7 kb product. *hlh-2* sequences were amplified from *C. elegans* genomic DNA using the primers HLH-2L (5′ GTTGACTACAATCATCAATTCCCACC 3′) and HLH-2R (5′ TTAAAACCGTGGATGTCCAAACTGC 3′), yielding a 0.8 kb product.

PCR products were cloned into the TA cloning vector, pGEM T-Easy (Promega). This vector is equipped with T7 and SP6 promoters flanking the site of DNA insertion. Following the protocols of Promega's Ribomax in vitro transcription kit, sense and antisense RNAs were made using the T7 and SP6 sites. Sense and antisense RNAs (1 μl of each) were run side by side on a 1.7% agarose gel to estimate relative concentrations. Based on this estimation, equimolar amounts of sense and antisense RNA were mixed with PBS (1× final concentration), incubated at 65°C for 15 minutes, and then incubated at 37°C for 30 minutes to complete the annealing reaction. To confirm the annealing reactions had worked, 1 μl of each ssRNA species was run on a 1.7% agarose gel next to 1 μl of dsRNA. A shifted banding pattern indicated successful annealing.

To control for general effects of RNAi, we injected dsRNA molecules of genes used in a separate study. Control RNAi injections did not phenocopy *hlh-14* and *hlh-2* dsRNA injections. To make control dsRNA molecules, we obtained cDNA phage clones from Yuji Kohara: yk394g5 (*egr-1*), yk73c3 (*chd-3*) and yk72d6 (*chd-4*). cDNAs were excised from phage following protocols provided by Yuji Kohara. After excision, the cDNAs were in the pBluescript plasmid, which is equipped with T7 and T3 transcription sites flanking the sites of cDNA insertion. Using both the T7 and T3 sites, sense and antisense transcripts were made for each clone, according to the protocol described in Promega's RiboMax in vitro transcription kit. Annealing and RNA integrity analyses were performed as described above.

For RNA interference, dsRNA was always injected into young adult worms at the maximum possible concentration; it was never diluted after the annealing reactions. Injected animals were picked to plates seeded with bacteria, allowed to lay eggs for 24 hours, and then transferred to fresh plates. Progeny laid after the first 24 hours were likely to display RNAi defects.

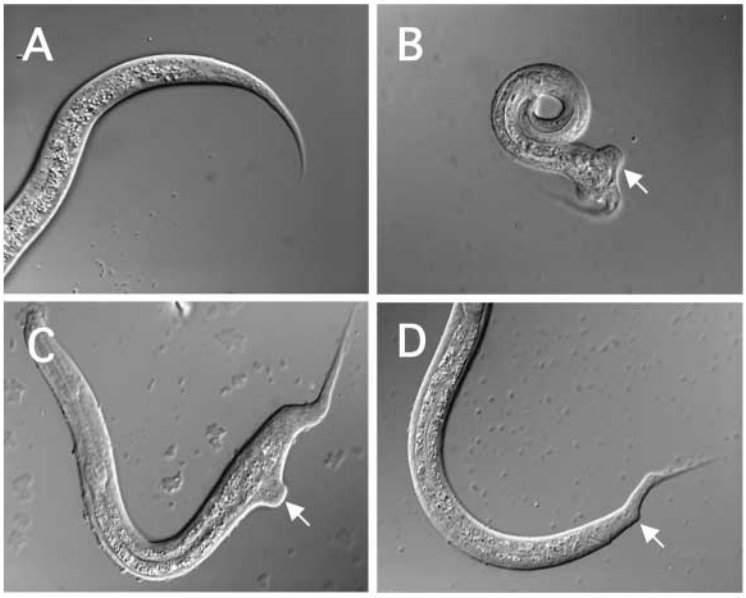


**Fig. 1.** Grossly visible phenotypes of *hlh-14* mutants. (A) The tail of a wild-type *C. elegans* larva. (B) An *hlh-14*(*gm34*) larva. *hlh-14* mutant worms can have disorganized, lumpy tails (arrow) and, less often, lumps at other positions along the AP axis. The cellular bases for these morphological defects are unclear. (C,D) *hlh-14*(*RNAi*) larvae. *hlh-14*(*RNAi*) larvae can display a range of morphological defects. (C) A severely affected *hlh-14*(*RNAi*) animal with a large posterior bulge (arrow). (D) An animal mildly affected by *hlh-14*(*RNAi*) treatment, showing only a small bump in the tail (arrow). Some *hlh-14*(*RNAi*) animals have a normal body structure, but still display neuronal phenotypes, indicating that these two phenotypes can be uncoupled.

## Results

### *hlh-14* cloning and characterization of mutations

*hlh-14* was initially defined by two mutations, *hlh-14*(*gm34*) and *hlh-14*(*ju243*). *hlh-14*(*gm34*) was isolated in a genetic screen for mutants with missing or misplaced HSN motoneurons (G.G., unpublished), and *hlh-14*(*ju243*) was isolated in a screen for mutants with morphological defects (W.-M. Woo and A. Chisholm, personal communication). On a gross level, *hlh-14* mutants have morphologically disorganized posteriors (Fig. 1B), an Unc (Uncoordinated) ventral coiling phenotype, and an Egl (Egg-laying defective) phenotype. Both *hlh-14* alleles can be maintained in homozygous strains, but homozygotes have a high degree of larval lethality and are slow to develop. Homozygotes that escape lethality and reach adulthood have low fertility (data not shown).

We cloned *hlh-14* by conventional mapping and rescue experiments (see Materials and methods). In the *C. elegans* genome database, *hlh-14* is identified as the open reading frame C18A3.8 and encodes a basic helix-loop-helix (bHLH) protein similar to members of the A-S family (Fig. 2). We detected three different forms of *hlh-14* mRNA expressed in *C. elegans* by performing 5′ and 3′ RACE (Fig. 2A; see Materials and methods). The three forms predict three different HLH-14 proteins, each containing a unique N terminus, but all containing the same bHLH domain and the same novel C terminus (Fig. 2B).

Both *hlh-14* mutations are lesions in the bHLH domain (Fig. 2C). *hlh-14*(*gm34*) is a missense mutation, changing a conserved leucine to a phenylalanine in the N-terminal helix. *hlh-14*(*ju243*) is a nonsense mutation in the C-terminal helix, changing a glutamine codon to an ochre stop codon. Several observations suggest that both *hlh-14* alleles severely reduce or eliminate gene function. First, both alleles are recessive, generate similar phenotypes and fail to complement one another (data not shown). Second, the deficiency *maDf4* fails to complement both *hlh-14*(*gm34*) and *hlh-14*(*ju243*); hemizygous *hlh-14*/*maDf4* and homozygous *hlh-14* mutants display similar defects (Table 1). Third, reduction of *hlh-14* function by RNA interference (RNAi) generates similar, although weaker, phenotypes as the *hlh-14* mutations (Fig. 1C,D; Tables 2-4). Finally, the *hlh-14* molecular lesions are predicted to reduce or eliminate *hlh-14* function. The *hlh-14*(*gm34*) leucine to phenylalanine missense mutation alters a highly conserved leucine (Fig. 2C). An identical leucine to phenylalanine mutation is found in the *C. elegans* bHLH loss-of-function mutant *lin-32*(*e1926*) (Zhao and Emmons, 1995). The *hlh-14*(*ju243*) nonsense mutation is predicted to eliminate the C terminus of the second helix, which contains highly conserved residues (Fig. 2C).

### HLH-14::GFP is expressed in the cells of the PVQ/HSN/PHB neuroblast lineage

To determine which cells express HLH-14, we generated a full-length *hlh-14::gfp* translational fusion (see Materials and methods). *hlh-14*; *hlh-14::gfp* animals are rescued for the morphological defects and the larval lethality observed in *hlh-14* mutants, indicating that this fusion is functional (data not shown). The *hlh-14::gfp* transgene expresses GFP in the nuclei of several cells in the developing embryo. Prior to morphogenesis, a horseshoe-shaped pattern of cells expresses HLH-14::GFP in the anterior embryo (data not shown). The expressing cells appear to be neuroblasts but have not been positively identified. During morphogenesis, HLH-14::GFP assumes a complicated, diverse pattern, and no HLH-14::GFP

**Table 1. *hlh-14* loss induces PHB neuron loss**

| | Number of PHB neurons/side* | | | |
|---|---|---|---|---|
| Genotype | 0 | 1 | 2 | *n* |
| Wild type | 0% | 100% | 0% | 100 |
| *hlh-14*(*gm34*) | 100% | 0% | 0% | 62 |
| *hlh-14*(*ju243*) | 100% | 0% | 0% | 65 |
| *hlh-14*(*gm34*)/*maDf4* | 99% | 1% | 0% | 74 |
| *ham-1*(*n1811*) | 3% | 74% | 23% | 274 |
| *hlh-14*(*gm34*); *ham-1*(*n1811*) | 100% | 0% | 0% | 40 |
| *ham-1*(*n1811*) *ced-3*(*n717*) | 0% | 7% | 93% | 54 |
| *hlh-14*(*gm34*); *ham-1*(*n1811*) *ced-3*(*n717*) | 100% | 0% | 0% | 28 |

**srb-6::gfp*-expressing PHB neurons in the tails of L1 larvae.

*srb-6::gfp* is expressed in PHA and PHB. When *hlh-14* mutants contained a single GFP-expressing cell/side, we scored this side as containing a single PHA, but lacking PHB (0 PHB neurons/side). Similarly, when *ham-1* mutants contained an extra GFP-expressing cell/side, we scored this side as having two PHB neurons. These assumptions were confirmed using PHA- and PHB-specific markers (see Results, Materials and methods, and Table 4).

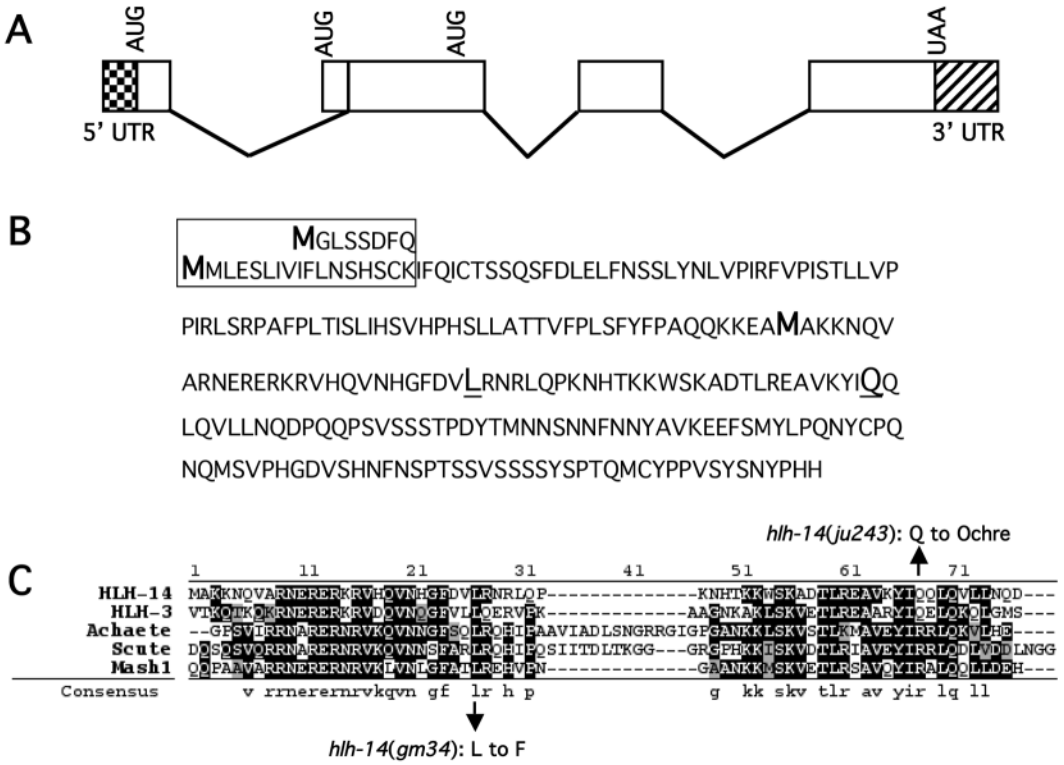


**Fig. 2.** *hlh-14* gene structure, predicted protein sequence and comparison with other A-S family members. (A) Schematic representation of *hlh-14* mRNA. By 5′ and 3′ RACE, we detected three different *hlh-14* cDNAs, predicting three different forms of HLH-14 protein (Materials and methods). Analyses of these cDNA species indicate that *hlh-14* mRNA is not trans-spliced. The three forms of mature *hlh-14* mRNA have unique start codons (AUG) but encode the same bHLH domain and the same novel C terminus. The shortest form encodes a 148 amino acid protein. The 5′ end of the corresponding short cDNA is the only 5′ end we could amplify out of a RACE cDNA library without performing a second, nested PCR reamplification (Materials and methods). Therefore, it is likely that this form is the most abundant form of *hlh-14* mRNA in *C. elegans*. In the corresponding protein, there are only eight amino acids N-terminal of the bHLH domain (MAKKNQVA). (B) Primary amino acid sequences of HLH-14. The three different start methionines are enlarged. For the two longer forms of HLH-14, alternative beginning peptides are boxed. The leucine and glutamine residues changed, respectively, by the *hlh-14*(*gm34*) missense mutation and the *hlh-14*(*ju243*) nonsense mutation are enlarged and underlined. Both lesions are in the conserved bHLH domain. (C) Alignment of the bHLH domains of HLH-14, HLH-3 (*C. elegans*), Achaete (*Drosophila*), Scute (*Drosophila*) and Mash1 (Ascl1; mouse). Residues shaded in black are identical. Lightly shaded residues are similar to corresponding black residues. Consensus residues are present in at least three of the five proteins aligned. The residues affected by the *hlh-14*(*gm34*) and *hlh-14*(*ju243*) mutations are detailed.

is visible during postembryonic development (data not shown).

In the posterior embryo, we first detect HLH-14::GFP in the bilaterally symmetric blast cells ABplapppa and ABprapppa. Respectively, we call these cells the left and right PVQ/HSN/PHB neuroblasts (Fig. 3B,D) because they generate the PVQ interneurons, the HSN motoneurons, and the PHB sensory neurons. A centrally located posterior blast cell expresses HLH-14::GFP a little later. We have tentatively identified this centrally located cell as C.aapa (Fig. 3F).

Around 230 minutes of development, each PVQ/HSN/PHB neuroblast divides to generate an anterior PVQ neuroblast and a posterior HSN/PHB neuroblast. We see HLH-14::GFP expressed in both of these daughter cells (Fig. 3F). At 280 minutes of development, the HSN/PHB neuroblast divides to produce a small daughter cell that dies and a larger daughter cell, the HSN/PHB precursor. Both cells contain HLH-14::GFP (Fig. 3H,J). Finally, around 310 minutes of development, the PVQ neuroblast divides to produce a posterior daughter cell that dies and the PVQ neuron. Again, we see HLH-14::GFP in both of these cells (Fig. 3L).

### *hlh-14* mutants are missing HSN motoneurons and PHB sensory neurons

In hermaphrodite larvae, a left/right bilaterally symmetric pair of HSN motoneurons is found at the middle of the animal, near the presumptive gonad. By Nomarski optics, we only rarely found the HSNs in *hlh-14* mutant worms; when we did find them, they were displaced posteriorly, having failed to complete their normal migratory routes (data not shown). We corroborated this observation using an HSN reporter, *unc-86::gfp* (Gitai et al., 2003). *unc-86* encodes a POU homeodomain protein, and this promoter fusion always expresses GFP in the HSNs of wild-type larvae (Fig. 4A). By contrast, we never saw HSNs expressing GFP in *hlh-14*(*gm34*); *unc-86::gfp* larvae (Fig. 4B; Table 2).

Staining the HSNs with an anti-serotonin antibody (see Materials and methods) also revealed the loss of HSNs in *hlh-14* mutants (Table 2). On the rare occasions this antibody did detect the HSNs in *hlh-14* mutants, they had failed to migrate properly (data not shown). We conclude that *hlh-14* mutants are either missing their HSN neurons, or at best, they produce profoundly defective HSN neurons. In either case, *hlh-14* appears important for HSN development.

The PHB sensory neuron is the sister cell of the HSN. As *hlh-14* mutants lack HSNs, we hypothesized that they might also lack PHBs. To test this possibility, we used the reporter *srb-6::gfp*, which expresses GFP in both the PHA and PHB phasmid neurons, sensory neurons that are located in the tail. *srb-6* encodes a seven transmembrane receptor protein

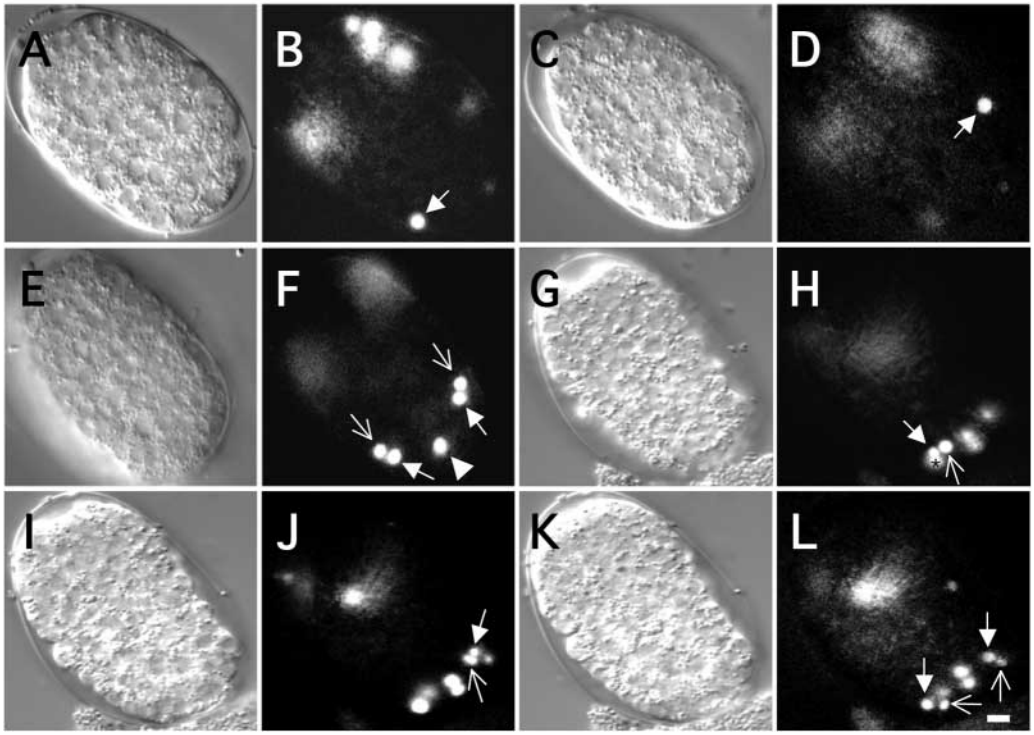


**Fig. 3.** HLH-14::GFP is expressed in the PVQ/HSN/PHB neuroblasts and their descendants. All images in this series show a ventral view. Anterior is at the upper left corner and posterior is at the lower right corner. Images in this series reveal posterior embryonic HLH-14::GFP expression. Nomarski photomicrographs (A,C,E,G,I,K) and the corresponding fluorescence photomicrographs (B,D,F,H,J,L) of wild-type embryos containing a *gmIs20*[*hlh-14::gfp*] transgene. (A,B) HLH-14::GFP is expressed in the right PVQ/HSN/PHB neuroblast, ABprapppa (arrow). (C,D) GFP expression of the same embryo shown in A and B, showing a different focal plane. HLH-14::GFP is expressed in the left PVQ/HSN/PHB neuroblast, ABplapppa (arrow). (E,F) HLH-14::GFP is expressed in the daughter cells of the PVQ/HSN/PHB neuroblasts: the anterior PVQ neuroblasts (open arrows) and the posterior HSN/PHB neuroblasts (closed arrows). We have tentatively identified the central GFP-expressing cell as the neuroblast C.aapa (arrowhead). (G,H) HLH-14::GFP is expressed in the anterior daughter of the right HSN/PHB neuroblast, a cell fated to die (closed arrow), and in its larger sister cell, the right HSN/PHB precursor (open arrow). The asterisk indicates the right PVQ neuroblast, which is out of focus. (I,J) HLH-14::GFP is expressed in the daughters of the left HSN/PHB neuroblast (arrows analogous to H). (K,L) HLH-14::GFP is expressed in the daughters of the PVQ neuroblast, the anterior PVQ neurons (closed arrows) and their posterior sisters, which are fated to die (open arrows). Scale bar: 5 μm. (M) Cell lineage depicting the PVQ/HSN/PHB neuroblast and its descendants.

expressed in several sensory neurons (Troemel et al., 1995). Wild-type *srb-6::gfp* animals always had two GFP-expressing neurons on each side of the tail, one PHA and one PHB (Fig. 4C; Table 1). By contrast, *hlh-14*; *srb-6::gfp* larvae almost always had only a single GFP-expressing neuron per side, consistent with *hlh-14* mutants missing their PHB neurons (Fig. 4D, Table 1).

To eliminate the possibility that *hlh-14* mutants lacked PHAs and not PHBs, we used more specific markers (data not shown). With the PHB-specific neuropeptide-like promoter fusion *nlp-1::gfp* (Li et al., 1999), we found that *hlh-14* mutants lacked PHB neurons. With the PHA-specific promoter fusions *flp-15::gfp* (Li et al., 1999) and *osm-10::gfp* (Hart et al., 1999) (PHA-specific in adults), we found no alteration in the number of PHA neurons in *hlh-14* mutants.

### *hlh-14* mutations are epistatic to mutations that disrupt the HSN/PHB neuroblast division

*hlh-14* mutants have the opposite neuronal phenotype as *ham-1* mutants. *ham-1* encodes a novel protein involved in executing the asymmetric divisions of neuroblasts (Guenther and Garriga, 1996). In *ham-1* mutants, the HSN/PHB neuroblast divides symmetrically, inappropriately generating two HSN/PHB precursor cells, and subsequently, extra HSN and PHB neurons. This phenotype can be seen using *srb-6::gfp*, where PHB neuron duplications generate extra GFP-expressing phasmid neurons about 25% of the time in *ham-1* mutants (Table 1). In *ham-1 ced-3* double mutants, the phenotype is more striking (Table 1). Concurrent removal of the novel protein HAM-1 and the caspase CED-3, which is

**Table 2. *hlh-14* loss induces HSN neuron loss**

| | Number of HSN neurons/side | | |
|---|---|---|---|
| Genotype | 0 | 1 | *n* |
| Wild-type adults* | 0% | 100% | 100 |
| *hlh-14*(*gm34*) adults* | 83% | 17% | 23 |
| *hlh-14*(*RNAi*) adults* | 27% | 73% | 180 |
| Wild-type larvae† | 0% | 100% | 66 |
| *hlh-14*(*gm34*) larvae† | 100%‡ | 0%‡ | 28 |
| *hlh-14*(*RNAi*) larvae† | 45%‡ | 55%‡ | 130 |

*HSNs stained with an anti-serotonin antibody in adults.
†HSNs expressing *unc-86::gfp* in L1 larvae.
‡It is possible that additional HSN neurons did express *unc-86::gfp* in *hlh-14* mutant larvae, but were completely migration defective. These HSNs would have been located in the tail among other neurons expressing *unc-86::gfp* and would not have been detected and counted. Supporting this possibility, many of the HSNs detected by anti-serotonin staining were located the tail, having failed to migrate.

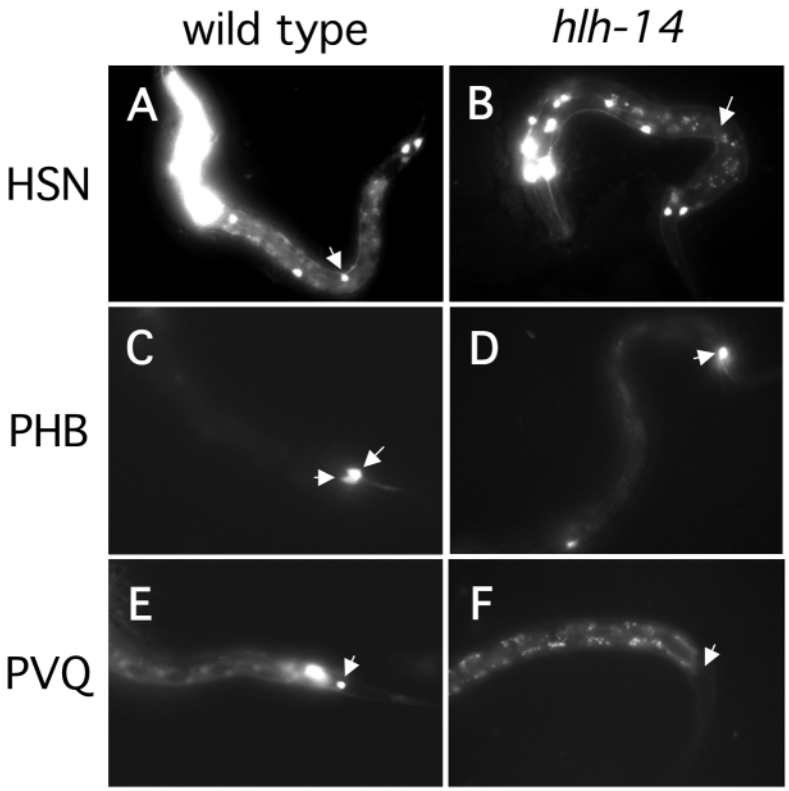


**Fig. 4.** *hlh-14* mutants are missing HSN, PHB, and PVQ neurons. Images in this series show lateral views of either wild-type (A,C,E) or *hlh-14* mutant (B,D,F) larvae expressing GFP reporters to visualize the HSN, PHB or PVQ neurons. (A,B) Anterior is towards the left and ventral is upwards. (A) GFP expression in an *hlh-14*(*gm34*)/*mIn1*; *kyIs179*[*unc-86::gfp*] larva. As this worm is only heterozygous for the *hlh-14*(*gm34*) mutation, it appears wild type and expresses GFP in the HSN neuron visible in this focal plane (arrow). The GFP expression in the pharynx of this worm indicates the presence of the *mIn1* balancer, and hence, a wild-type copy of the *hlh-14* gene. (B) GFP expression in an *hlh-14*(*gm34*); *kyIs179* larva. No HSNs are visible by GFP expression. The arrow indicates the position where the HSNs would be in a wild-type animal. (C) GFP expression in the tail of a wild-type *gmIs12*[*srb-6::gfp*] larva. GFP is visible in both phasmid neurons, PHA and PHB (two arrows), sensory neurons in the tail. (D) GFP expression in an *hlh-14*; *gmIs12* larva. Only one phasmid neuron (PHA) is visible; PHB is missing. (E) GFP expression in an *hlh-14*(*gm34*)/*mIn1*; *kyIs39*[*sra-6::gfp*] larva. As this worm is only heterozygous for the *hlh-14*(*gm34*) mutation, it appears wild type and expresses GFP in the PVQ neuron visible in this focal plane (arrow). The GFP expression slightly anterior to PVQ is due to the presence of *mIn1*. (F) GFP expression in an *hlh-14*(*gm34*); *kyIs39* larva. No PVQs are visible by GFP expression. The arrow indicates the position of the PVQs in a wild-type animal.

necessary for normal programmed cell death (Ellis and Horvitz, 1986; Yuan et al., 1993), can increase the penetrance of extra PHB neurons to over 90% (Guenther and Garriga, 1996).

We reasoned that if *hlh-14* mutations were affecting the overall ability to determine neuronal fate, *hlh-14* should be epistatic to *ham-1*. Indeed this is the case, as *hlh-14*; *ham-1* double mutants were always missing their PHB neurons (Table 1). To rule out the possibility that the effects we saw in *hlh-14* mutants were simply due to inappropriate programmed cell death, we examined *hlh-14*; *ham-1 ced-3* triple mutants. As with *hlh-14* single mutants, these triply mutant animals were always missing their PHB neurons (Table 1). We conclude that *hlh-14* mutations are epistatic to mutations that alter the HSN/PHB neuroblast division. Furthermore, *hlh-14* mutants are not missing neurons because of inappropriate programmed cell death. Therefore, *hlh-14* appears to be a vital factor in determining the HSN/PHB neuroblast fate, or the fate of a cell that generates the HSN/PHB neuroblast.

### *hlh-14* mutants are missing PVQ neurons

Considering the HLH-14::GFP expression pattern, we hypothesized that the PVQ neurons might be missing or defective in *hlh-14* mutants. Analysis of the tails of *hlh-14* mutants by Nomarski optics suggested that the PVQs were missing (data not shown). To test this hypothesis directly, we used the PVQ-specific GFP reporter, *sra-6::gfp* (Troemel et al., 1995). Wild-type *sra-6::gfp* animals always had PVQ neurons expressing GFP (Fig. 4E; Table 3). By contrast, *sra-6::gfp*; *hlh-14*(*gm34*) animals never had PVQ neurons expressing GFP (Fig. 4F; Table 3).

### *hlh-14* mutants have a cell division defect in the PVQ/HSN/PHB neuroblast lineage

The preceding data are consistent with HLH-14 acting to promote the PVQ, HSN and PHB fates. HLH-14 could act directly in these neurons. However, as HLH-14 is an A-S family member, it could play a proneural role, acting earlier to ensure the proper execution of the entire PVQ/HSN/PHB neuroblast lineage. To address this possibility, we directly observed the cell division patterns of *hlh-14*(*gm34*) mutant embryos.

We examined two PVQ/HSN/PHB neuroblast lineages in *hlh-14*(*gm34*) embryos. In wild-type embryonic lineages (Fig. 5A), the ABpl/rappp cell divides to generate the PVQ/HSN/PHB neuroblast and the hyp7/T blast cell around 170 minutes after fertilization (Sulston et al., 1983). This division occurred at the right time in each *hlh-14*(*gm34*) mutant lineage. Approximately 230 minutes after fertilization in wild-type embryos, the PVQ/HSN/PHB neuroblast divides to generate the PVQ neuroblast and the HSN/PHB neuroblast (Fig. 5A). This division also appeared to occur normally in *hlh-14*(*gm34*) embryos. Approximately 280 minutes after fertilization in wild-type embryos, the HSN/PHB neuroblast

**Table 3. *hlh-14* loss induces PVQ neuron loss**

| | Number of PVQ neurons/side* | | |
|---|---|---|---|
| Genotype | 0 | 1 | *n* |
| Wild type | 0% | 100% | 100 |
| *hlh-14*(*gm34*) | 100% | 0% | 46 |
| *hlh-14*(*RNAi*) | 91% | 9% | 46 |

**sra-6::gfp*-expressing cells in the tails of L1 larvae.

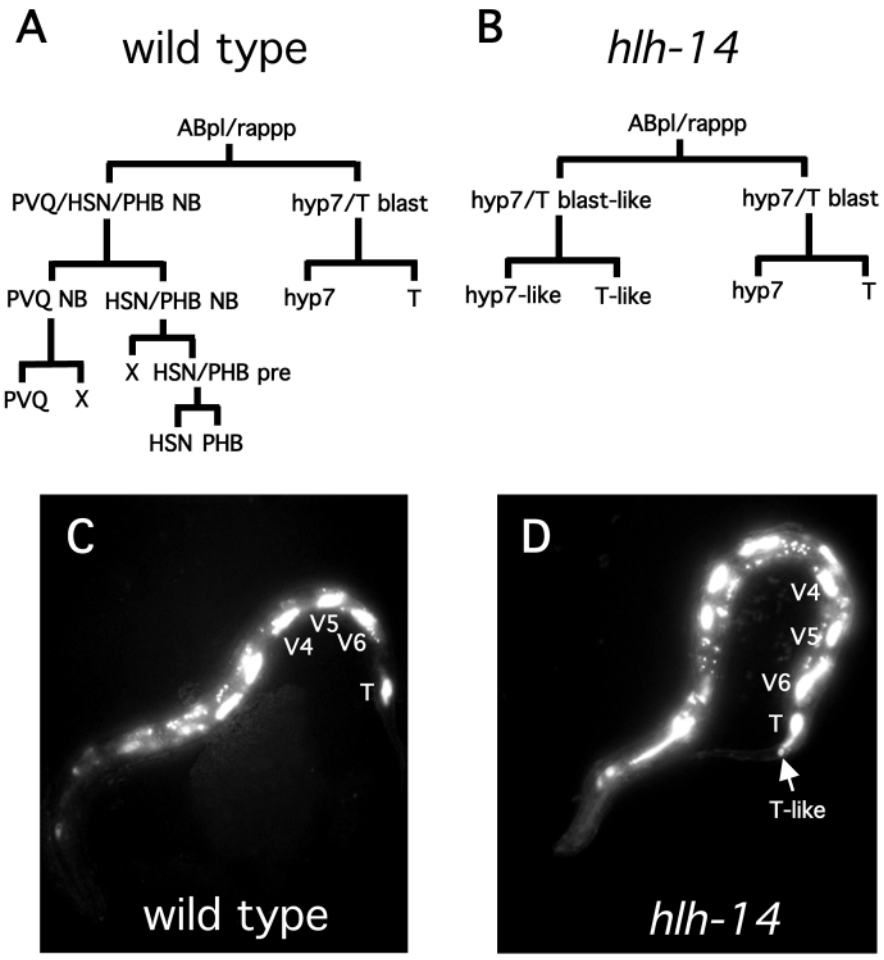


**Fig. 5.** Cell lineage defects in *hlh-14* mutants. (A) In wild-type embryos, the ABpl/rappp cell divides to generate the PVQ/HSN/PHB neuroblast and the hyp7/T blast cell. The PVQ/HSN/PHB neuroblast subsequently undergoes a series of divisions, ultimately generating the PVQ, HSN and PHB neurons. The hyp7/T blast cell divides only once during embryogenesis, generating a hyp7 cell and the T blast cell, a seam cell that divides postembryonically. (B) In *hlh-14* mutants, the ABpl/rappp cell divides at the appropriate time. However, the presumptive PVQ/HSN/PHB neuroblast does not undergo a series of divisions to generate the PVQ, HSN and PHB neurons. Instead, it divides just once, like its sister cell, the hyp7/T blast cell. In *hlh-14* mutants, it is possible that the PVQ/HSN/PHB neuroblast is transformed into a hyp7/T blast-like cell, a model shown in this lineage. (C) A wild-type L1 larva containing a C50B6.8::*gfp* reporter construct, which expresses GFP in the ten hypodermal seam cells, H0-H2, V1-V6 and T (Mounsey et al., 2002). (D) An *hlh-14* mutant L1 larva expressing the C50B6.8::*gfp* reporter. In addition to expressing GFP in the ten hypodermal seam cells, this larva expresses GFP in an extra cell in the tail, presumably a T-like cell (arrow). In 38% of the of *hlh-14* mutants, we observed this ectopic GFP expression ($n$=60 sides scored). We never observed this extra cell in wild-type animals bearing C50B6.8::*gfp*.

divides, and at ~310 minutes, the PVQ neuroblast divides (Fig. 5A). Neither of these divisions occurred in the *hlh-14*(*gm34*) embryos.

In the course of our cell lineage analyses, we never saw any inappropriate cell death corpses (data not shown). This observation is consistent with the idea that programmed cell death is not responsible for missing neurons in *hlh-14* mutants. We conclude that in *hlh-14* mutants, cell division is abnormal in both daughters of the PVQ/HSN/PHB neuroblast; specifically, the daughter cells fail to divide.

### In *hlh-14* mutants, the PVQ/HSN/PHB neuroblast may be transformed into its sister cell

We questioned why the HSN/PHB neuroblast and the PVQ neuroblast both failed to divide in *hlh-14* mutants. They may have withdrawn from the cell cycle prematurely, or they may have inappropriately assumed the fates of other cells. Considering this latter possibility, we noted that in *hlh-14* mutant embryos, the division pattern of the presumptive PVQ/HSN/PHB neuroblast was similar to the normal division pattern of its sister cell, the hyp7/T blast cell (Fig. 5A,B). We hypothesized that the PVQ/HSN/PHB neuroblast may adopt the fate of its sister cell in *hlh-14* mutants. Such a cell fate transformation would signify a defect in asymmetric cell division, which is defined as the process by which sister cells adopt distinct fates (Horvitz and Herskowitz, 1992).

If the PVQ/HSN/PHB neuroblast were transformed into an extra hyp7/T blast cell, then *hlh-14* mutants should have extra T cells, a type of dermal blast cell in the tail. To test whether *hlh-14* mutants have extra T cells, we used a C50B6.8::*gfp* reporter. C50B6.8 is a *C. elegans* gene that is expressed in the ten hypodermal seam cells, including the T cells (Mounsey et al., 2002) (Fig. 5C). C50B6.8 encodes a protein with a domain similar to the ligand binding domains of nuclear hormone receptors (Mounsey et al., 2002). With this GFP reporter, we observed that *hlh-14* mutants often have extra GFP-expressing cells in the tail, presumably extra T (or T-like) cells (Fig. 5D). This observation is consistent with the PVQ/HSN/PHB neuroblast assuming a hyp7/T blast cell fate in *hlh-14* mutants.

Notably, these supernumerary T-like cells are smaller than normal T cells (Fig. 5D). They are also located slightly medial to the seam cells, which is consistent with the positions of the presumptive PVQ/HSN/PHB neuroblast daughter cells in *hlh-14* mutants. Collectively, our observations indicate that the PVQ/HSN/PHB neuroblasts can adopt characteristics of hyp7/T blast cells in *hlh-14* mutants.

### HLH-14 determines cell fate in the descendants of the PVQ/HSN/PHB neuroblasts

HLH-14 appears to determine PVQ/HSN/PHB neuroblast fate, a proneural characteristic. Yet HLH-14::GFP is expressed not only in the PVQ/HSN/PHB neuroblast but also in its

descendants. In addition to HLH-14 acting like a proneural factor, we wondered whether HLH-14 might also act like a neural differentiation bHLH factor and determine the fates of cells late in this neuroblast lineage.

To examine this possibility, we took advantage of the partial loss-of-function phenotype of *hlh-14*(*RNAi*). *hlh-14*(*RNAi*) treatment does not cause a complete loss of PVQ, HSN or PHB neurons (Tables 2-4). Therefore, we were able to test whether the loss of one neuron type was correlated with the loss of another neuron type in *hlh-14*(*RNAi*) animals. A correlation between HSN loss and PVQ loss, for example, could indicate a defect in their common precursor, the PVQ/HSN/PHB neuroblast. A lack of correlation could indicate a role for HLH-14 later in the cell lineage, perhaps in the neurons themselves.

We first looked for a correlation between HSN and PHB loss in *hlh-14*(*RNAi*) animals. We subjected worms bearing the *nlp-1::gfp* transgene, which expresses GFP in the PHB neurons, to *hlh-14*(*RNAi*) treatment. We isolated F1 progeny as adults and double stained them with anti-serotonin and anti-GFP antibodies to examine the HSNs and PHBs. A large majority of the time, HSN loss (or presence) correlated with PHB loss (or presence; 69/78 sides scored correlated). Occasionally, however, one cell or the other was lost (9/78 sides scored). Additionally, several of the HSN neurons that were detected in *hlh-14*(*RNAi*) animals were not fully migrated, and some had axon outgrowth defects (data not shown). Together, these results indicate that in addition to determining the fates of HSN and PHB precursors, HLH-14 plays roles in determining the fates of the HSN and PHB neurons themselves.

In a similar experiment, we looked for a correlation between HSN and PVQ loss in *sra-6::gfp*; *hlh-14*(*RNAi*) worms. We found that HSN loss is not at all correlated with PVQ loss in these animals (21/54 sides scored correlated). The PVQ neurons are lost significantly more often than the HSN neurons (Tables 2, 3), accounting for most of the differences we observe. The PVQ neuroblast or PVQ neuron appear to be especially sensitive to *hlh-14* loss, by our assay. We conclude that although *hlh-14* may play a role in determining PVQ/HSN/PHB neuroblast fate, it also appears to determine the fates of the descendants of the neuroblast.

### Loss of *hlh-2* function can cause neuron loss and exacerbate *hlh-14(RNAi)* neuron loss

Neural bHLH proteins often regulate transcription by forming heterodimers with the E/Daughterless (DA) proteins, a ubiquitously expressed family of bHLH proteins. Therefore, we wondered whether the *C. elegans* E/DA family member, HLH-2, might function with HLH-14 in the PVQ/HSN/PHB neuroblast lineage. Indeed, previous work demonstrated that HLH-2 is expressed in this lineage (Krause et al., 1997).

To date, no strong loss-of-function *hlh-2* mutants exist or have been reported, so we used RNAi treatment to see what effects *hlh-2* loss could exert on PVQ, HSN and PHB neuron development. Consistent with previous studies, *hlh-2*(*RNAi*) treatment caused nearly complete embryonic lethality (Kamath et al., 2003; Krause et al., 1997). However, a small percentage of animals escaped this lethality. Like *hlh-14* mutants, these *hlh-2*(*RNAi*) escapers had gross phenotypes, including posterior morphological defects and larval lethality (data not shown). Most relevant to our study, they often lacked PVQ, HSN and PHB neurons (Table 4).

**Table 4. *hlh-2* loss induces PVQ, HSN, and PHB neuron loss**

| Genotype | Percent of neurons present (sides scored) | | |
|---|---|---|---|
| | PVQ* | HSN† | PHB‡ |
| Wild type | 100% (100) | 100% (66) | 100% (100) |
| *hlh-2*(*RNAi*) | 25% (32) | 23% (22) | 50% (14) |
| *hlh-2*(*bx115*) | N/D | 100% (30) | 100% (106) |
| *hlh-14*(*RNAi*) | 9% (46) | 55% (130)§ | 51% (304)¶ |
| *hlh-2*(*bx115*); *hlh-14*(*RNAi*) | N/D | 43% (135)§ | 35% (326)¶ |

N/D, not determined. Because *hlh-2* and *kyIs39*[*sra-6::gfp*] both reside on *LG I*, we were unable to construct the strains needed to collect these data.

**sra-6::gfp*-expressing cells in the tails of L1 larvae.

†*unc-86::gfp*-expressing HSN neurons in L1 larvae. Some HSNs may not have been counted by this assay. See Table 2 for explanation.

‡*nlp-1::gfp*-expressing cells in the tails of L1 larvae.

§$P$ =0.05 using a two-sample Z test.

¶$P$<0.0001 using a two-sample Z test.

Partial loss-of-function *hlh-2* mutations generate no phenotypes on their own, but a prior study demonstrated that they can exacerbate the male tail ray loss phenotype of hypomorphic *lin-32* mutants; HLH-2 and LIN-32 act together to execute neuronal fates in the ray lineage (Portman and Emmons, 2000). We wondered if the mutation, *hlh-2*(*bx115*), could also enhance *hlh-14*(*RNAi*), which produces a partial loss-of-function phenotype (Fig. 1C,D; Tables 2-4). Although *hlh-2*(*bx115*) mutants have a normal number of HSN and PHB neurons, *hlh-2*(*bx115*); *hlh-14*(*RNAi*) animals lack more HSN and PHB neurons than *hlh-14*(*RNAi*) animals, showing that a weak *hlh-2* mutant can enhance partial *hlh-14* loss (Table 4).

## Discussion

We have described a role in neuronal development for a *C. elegans* A-S bHLH family member, HLH-14. *hlh-14* mutants lack three lineally related neurons: the PVQ interneuron, the HSN motoneuron and the PHB sensory neuron. Based on GFP reporter data and cell lineage analyses, we hypothesize that these neurons are missing because their common precursor, the PVQ/HSN/PHB neuroblast, fails to differentiate properly, and instead adopts characteristics of its sister cell. It also appears that HLH-14 acts in conjunction with the *C. elegans* E/Daughterless homolog, HLH-2, in regulating neuronal fate in this cell lineage.

### *hlh-14* and other Achaete-Scute family genes: similarities and differences

Aside from its conserved sequence, there are a number of similarities between *hlh-14* and previously characterized A-S genes. The most obvious similarity is that *hlh-14* mutants lack neurons. A-S family members in *Drosophila* specify external sense organs. In the absence of A-S genes, neuronal cell types needed for the function of these organs are lost (Garcia-Bellido, 1979; Gonzalez et al., 1989; Villares and Cabrera, 1987). Vertebrate A-S family members generate a wide variety of neuronal precursors, including the progenitors of the cerebral cortex and progenitors in the ventral telencephalon (Casarosa et al., 1999; Guillemot et al., 1993; Horton et al., 1999). In this study, we have clearly established that *hlh-14*

function is required for normal PVQ, HSN, and PHB neuron development.

Yet a close look at the types of neurons specified by *hlh-14* reveals an important difference between *hlh-14* and other A-S genes. While *Drosophila* and mammalian A-S genes appear to specify neuroblast lineages dedicated to generating neuronal cells of a particular type or coordinated function, *hlh-14* specifies a neuroblast lineage dedicated to generating three disparate types of neuron: an interneuron (PVQ), a motoneuron (HSN) and a sensory neuron (PHB). There is no known coordinated function that these three neurons perform. Why is *hlh-14* less specific than fellow A-S family members in this regard? One possibility is that *C. elegans* must adapt the function of neural bHLH genes such as *hlh-14* in order to generate its diverse collection of 302 neurons (of 118 distinct types) out of only 959 somatic cells. Such adaptation may allow *hlh-14* to specify lineally related, yet functionally distinct, collections of neurons.

A second similarity between *hlh-14* and other A-S-like genes is the genetic interaction between *hlh-14* and *hlh-2*, the *C. elegans* E/DA homolog. In *Drosophila*, heterodimers between DA and A-S family members are essential for neurogenesis (Cabrera and Alonso, 1991). In *C. elegans*, the A-S factor HLH-3 can bind E/DA HLH-2 in vitro, and the expression patterns of HLH-3 and HLH-2 overlap in a number of neuronal lineages (Krause et al., 1997; Thellman et al., 2003). Taken together, four facts suggest that HLH-14 and HLH-2 act together in the PVQ/HSN/PHB lineage to regulate neuronal development. First, A-S proteins, as well as other types of bHLH proteins, are known to interact physically with E/DA family members to form functional heterodimers in a number of organisms (Cabrera and Alonso, 1991; Johnson et al., 1992; Krause et al., 1997; Massari and Murre, 2000). Second, both HLH-14 and HLH-2 are expressed in the PVQ/HSN/PHB lineage (this study) (Krause et al., 1997). Third, loss of function of either gene results in the loss of neurons in this lineage. Fourth, a weak *hlh-2* mutant can enhance the partial neuronal loss defects of *hlh-14*(*RNAi*).

Another similarity that HLH-14 shares with certain A-S family members is that it possesses proneural characteristics. Not only is *hlh-14* necessary for neuron development, but also it is expressed early in neurogenesis. We see *hlh-14::gfp* expressed in the PVQ/HSN/PHB neuroblast, the first cell in this lineage solely dedicated to generating neurons.

### Individual neural bHLH factors can assume multiple roles in *C. elegans*

Even though *hlh-14* has proneural characteristics, a notable difference between *hlh-14* and some A-S genes is that *hlh-14* is not strictly proneural. Numerous pieces of evidence point to this conclusion. First, *hlh-14::gfp* expression is not restricted to early neuroblasts. We observe *hlh-14::gfp* expression in the lineal descendants of the PVQ/HSN/PHB neuroblast, including the PVQ neuron, a postmitotic cell. Second, *hlh-14* mutants do not display wholesale neuron loss for all the neurons we examined. For example, the HSNs are not always lost. Furthermore, of the HSNs that are generated, many fail to migrate and project their axons properly. Finally, HSN loss is not perfectly correlated with PHB and PVQ loss in *hlh-14*(*RNAi*) animals. Collectively, these results demonstrate that *hlh-14* plays important roles in the descendants of the PVQ/HSN/PHB neuroblast, not just the neuroblast itself.

There are other A-S genes that are not strictly proneural. For example, the *Drosophila* gene *asense* has proneural characteristics; its ectopic expression induces the generation of ectopic sense organs (Dominguez and Campuzano, 1993). However, unlike other proneural factors, *asense* is not normally expressed early in neuroblast lineages. It is expressed later, in the precursors of sensory organ cells (Dominguez and Campuzano, 1993; Gonzalez et al., 1989). Among A-S genes that have been characterized, *hlh-14* appears unique in the sense that it is expressed early in a lineage to establish neuroblast fate, but nevertheless persists in subsequent generations to execute the fates of neuronal precursors and neurons.

This characteristic of *hlh-14* is shared by other *C. elegans* neural bHLH factors. For example, the *C. elegans* Atonal homolog, LIN-32, both establishes ray neuroblast fate and helps execute the fates of cells at multiple steps in the ray lineage (Portman and Emmons, 2000; Zhao and Emmons, 1995). The *C. elegans* NeuroD bHLH homolog, CND-1, is expressed in both neuronal precursors and postmitotic neurons and appears to be important to keep these precursors from withdrawing from their lineages (Hallam et al., 2000). The only other neural bHLH that has been described in any detail in *C. elegans* is the A-S protein HLH-3. It has not been reported how HLH-3 promotes neurogenesis, but embryonic expression of an *hlh-3::gfp* transgene is extensive and shows considerable overlap with HLH-2 expression in neuronal lineages (Krause et al., 1997). Therefore, it seems plausible that HLH-3 could affect both early and late events in neuronal development.

It is unclear why some *C. elegans* bHLH factors act at several steps in neuronal lineages, seemingly unlike their *Drosophila* or vertebrate counterparts. It is not because nematodes have fewer neural bHLH proteins at their disposal; indeed, there are five A-S-like genes in *C. elegans*, but only four in *Drosophila*, and three in mouse (Ledent et al., 2002). The fact that individual *C. elegans* bHLH factors appear sufficient to drive both early and late events in neural development contrasts with the progressive determination model formulated from studies in *Drosophila* and vertebrates (Dambly-Chaudiere and Vervoort, 1998; Ghysen and Dambly-Chaudiere, 1989; Jan and Jan, 1994). According to this model, neuronal lineages consist of cells whose fates become more restricted with each generation. A sequential cascade of bHLH factors helps drive this process, with proneural factors defining neuroblast lineages and signaling through Notch to promote the expression of differentiation bHLH factors that promote later developmental events. In *C. elegans*, however, a single neural bHLH protein appears capable of functioning in each of these steps, at least in some cell lineages.

### *C. elegans* bHLH mutations can disrupt asymmetric cell divisions

An additional difference between *hlh-14* and other A-S family members comes from analyzing Hlh-14 mutant phenotypes. The loss of neurons is expected for the loss of an A-S family member like *hlh-14*. What is unexpected is the inappropriate duplication of a lineally related sister cell. In *hlh-14* mutants, there appear to be duplications of the hyp7/T blast cell at the

expense of its sister cell, the PVQ/HSN/PHB neuroblast. While these duplications are not completely penetrant, they do occur with reasonable frequency, as assayed by the presence of extra T-like cells in *hlh-14* mutants (Fig. 5D). The incomplete penetrance may be due to incomplete or partial transformations of PVQ/HSN/PHB neuroblast fate.

Asymmetric cell division is defined as any division in which a mother cell gives rise to two daughter cells of distinct fates (Horvitz and Herskowitz, 1992). It is a fundamental mechanism employed by metazoans to generate cellular diversity. The vast majority of *C. elegans* non-germline cell divisions are asymmetric (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977; Sulston et al., 1983). Normally, ABpl/rappp divides asymmetrically, generating the PVQ/HSN/PHB neuroblast and the hyp7/T blast cell. Our data suggest that this asymmetric division is disrupted in *hlh-14* mutants because the PVQ/HSN/PHB neuroblast can be partially transformed into its sister cell.

The disruption of an asymmetric cell division is not unprecedented for the loss of a neural bHLH factor in *C. elegans*. This phenomenon is what one observes in the V5.pa cell lineage in *lin-32* mutants and in the NSM cell lineage in *hlh-3* mutants. In wild-type animals, the V5.pa cell becomes a neuroblast called the postdeirid neuroblast. However, in *lin-32* mutants, lineage analysis shows that V5.pa can assume the fate of its sister cell, the V5.pp hypodermal blast cell (Zhao and Emmons, 1995). In *hlh-3* mutants, the sister cell of the NSM neuron can forego a programmed cell death fate and assume an NSM-like fate instead (Thellmann et al., 2003).

Why might some nematode asymmetric divisions become symmetric upon losing a bHLH factor? The answer is unclear, but it seems instructive to consider the cellular context in which neurons are generated in different organisms. In *Drosophila*, proneural genes are first expressed in the neuroectoderm in order to select neuronal precursors. Without proneural activity, neuroblast lineages are not selected, resulting in an absence of neuronal organs and excess dermal cells. In vertebrates, proneural genes are first expressed in neuroepithelial cells. Proneural expression induces the selection of neuronal precursors that then delaminate from the neuroepithelium and divide a finite number of times to generate neuronal cell types. Some neuroepithelial cells that are not selected to be neuroblasts delaminate and generate glia instead. Loss of proneural function can lead to a loss of neurons, but extra glia in vertebrates.

By contrast, *C. elegans* does not select most of its neuroblast lineages from populations of dermal cells. Instead, *C. elegans* works within the context of its cell lineage, which generates only 959 somatic cells in the adult (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977; Sulston et al., 1983). Considering this, and considering our work, it seems possible that neural bHLH factors can act like a switch between one of two sister cell fate decisions in nematodes. This is not entirely dissimilar from what has been observed in *Drosophila* and vertebrates, where proneural bHLH factors act like a switch between neuroblast and ectodermal or epithelial/glial cell fates. The difference is that in flies and vertebrates cell fate is regulated spatially, while in *C. elegans*, cell fate may be distributed asymmetrically at mitosis. As a result, loss of a neural bHLH factor can result in the disruption of an asymmetric cell division.

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