Regulation of neuronal lineage decisions by the HES-related bHLH protein REF-1

Anne Lanjuina,1, Julia Claggettb, Mayumi Shibuyaa, Craig P. Hunterb, Piali Senguptaa,*

a Department of Biology, Brandeis University, Waltham, MA 02454, USA
b Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA

Received for publication 5 August 2005, revised 8 November 2005, accepted 14 November 2005
Available online 22 December 2005

Abstract

Members of the HES subfamily of bHLH proteins play crucial roles in neural patterning via repression of neurogenesis. In C. elegans, loss-of-function mutations in ref-1, a distant nematode-specific member of this subfamily, were previously shown to cause ectopic neurogenesis from postembryonic lineages. However, while the vast majority of the nervous system in C. elegans is generated embryonically, the role of REF-1 in regulating these neural lineage decisions is unknown. Here, we show that mutations in ref-1 result in the generation of multiple ectopic neuron types derived from an embryonic neuroblast. In wild-type animals, neurons derived from this sublineage are present in a left/right symmetrical manner. However, in ref-1 mutants, while the ectopically generated neurons exhibit gene expression profiles characteristic of neurons on the left, they are present only on the right side. REF-1 functions in a Notch-independent manner to regulate this ectopic lineage decision. We also demonstrate that loss of REF-1 function results in defective differentiation of an embryonically generated serotonergic neuron type. These results indicate that REF-1 functions in both Notch-dependent and independent pathways to regulate multiple developmental decisions in different neuronal sublineages.

Keywords: bHLH; ref-1; Hes; C. elegans; Asymmetry

Introduction

Basic helix–loop–helix (bHLH) proteins act in a lineage- and cell type-specific manner to regulate neurogenesis and neuronal cell fate specification (reviewed in Bertrand et al., 2002; Dambly-Chaudiere and Vervoort, 1998; Hatakeyama and Kageyama, 2004; Lee, 1997; Ross et al., 2003). Proneural bHLH proteins such as members of the Achaete-Scute and Atonal subfamilies promote the generation of neuronal fates, whereas members of the HES subfamily of bHLH proteins inhibit neurogenesis (Fisher and Caudy, 1998; Garcia-Bellido, 1979; Goulding et al., 2000; Guillemer et al., 1993; Jarman et al., 1993; Knust et al., 1987; Orenic et al., 1993; Skeath and Carroll, 1991; Zhao and Emmons, 1995). The roles of different members of this large family of proteins in different lineages and cell types are not fully understood.

Hes bHLH genes are upregulated upon Notch signaling between initially developmentally equipotential cells (Bailey and Posakony, 1995; Jarriault et al., 1995; Lecourtois and Schweisguth, 1995). HES proteins antagonize proneural bHLH protein function by either directly repressing proneural gene expression, and/or via physical interaction with proneural proteins, thereby promoting adoption of a non-neuronal fate (Alifragis et al., 1997; Chen et al., 1997; Fisher and Caudy, 1998; Giagtzoglou et al., 2003; Ishibashi et al., 1995; Orenic et al., 1993; Paroush et al., 1994; Skeath and Carroll, 1991; Van Doren et al., 1994). Thus, loss-of-function mutations in Hes genes result in the generation of ectopic neuronal cells (Ishibashi et al., 1995; Nakamura et al., 2000; Wrischnik and Kenyon, 1997), indicating that precise regulation of Hes gene expression and function is critical for patterning the nervous system.

The C. elegans genome is predicted to encode 39 bHLH domain proteins (Ledent et al., 2002). Analyses of the
functions of a subset of these genes indicate that similar to observations in other organisms, individual bHLH proteins are required for correct neurogenensis and neuronal specification in specific cell types (Frank et al., 2003; Hallam et al., 2000; Karp and Greenwald, 2004; Krause et al., 1997; Portman and Emmons, 2000; Wrzischnik and Kenyon, 1997; Zhao and Emmons, 1995). For example, mutations in the lin-32 Atonal homolog result in loss of subsets of sensory neuron types (Portman and Emmons, 2000; Zhao and Emmons, 1995), whereas mutations in the lin-22 Hes homolog result in the generation of ectopic neuroblasts (Wrzischnik and Kenyon, 1997). However, it is unclear whether these genes function in only the lineages examined, or whether they also play roles in the development of additional neuron types. In addition, the functions of a majority of bHLH proteins in C. elegans are unknown.

Six Notch interactions have been shown to be critical in patterning the C. elegans embryo. Two of these interactions occur in the E blastomere lineage and are required to create a left/right (L/R) asymmetric twist in the intestine (Hermann et al., 2000). The remaining four interactions involve daughters of the AB blastomere which gives rise to the majority of neuron types in C. elegans (Hutter and Schnabel, 1994; Hutter and Schnabel, 1995; Mango et al., 1994; Mello et al., 1994; Moskowitz and Rothman, 1996; Moskowitz et al., 1994; Priess et al., 1987; Sulston et al., 1983). These embryonic interactions are essential to create L/R asymmetry in the AB lineage, such that L/R pairs of initially equivalent blastomeres adopt distinct lineage patterns and cell fates. Later in development, L/R asymmetry is re-imposed through extensive cell rearrangement (Schnabel et al., 1997; Sulston, 1983; Sulston et al., 1983). As a consequence, many bilaterally symmetric neurons arise from AB-derived neuroblasts that are not initially L/R symmetric.

A subfamily of HES-related bHLH proteins have been recently shown to be targets of Notch signaling in the embryo (Neves and Priess, 2005). This nematode-specific subfamily consists of six genes, each of which is predicted to encode proteins containing two HES-related bHLH domains (Alper et al., 1995, 1997, 1999; Tsalik et al., 2003; Yu et al., 1997). The ref-1 gene, a member of this family, was initially identified on the basis of ectopic neuroblast expression. ref-1 was mapped with respect to genetic markers and deficiencies, and rescue of the mutant phenotype was obtained with the cosmid R05H5 containing the predicted ref-1 gene (T01E8.2). The mu220 and ok288 alleles were obtained from the Caenorhabditis Genetics Stock Center. Strains were outcrossed at least two times prior to further analyses. The presence of ref-1 alleles in marked strains was confirmed by the size or sequence of ref-1 amplicons generated from genomic DNA.

Here, we describe the role of ref-1 in the generation and differentiation of neurons derived from the AB blastomere during embryogenesis. We show that multiple neuron types are ectopically generated in ref-1 mutants consistent with the ectopic generation of an AB-derived neuroblast. Intriguingly, ectopic cells are observed only on the right side of animals, and these cells exhibit gene expression patterns characteristic of their counterparts on the left. In addition, we observe developmental defects in a serotoninergic neuron type. Taken together, these results implicate ref-1 in regulating distinct developmental decisions in different neuronal sublineages.

Materials and methods

Nematode strains

Worms were grown using standard protocols (Brenner, 1974). Integrated marker strains used in this work were the following: kyIs104 (str-1::gfp), oys144 (ocr-1::rfp), oys51 (srf-142::rfp), yzls71 (tph-1::gfp), yzls3 (lip-8::gfp), yzls18 (gey-8::gfp), ots3 (gey-7::gfp), ots131 (gey-7::rfp), nts1 (gey-3::gfp), oys114 (ara-6::gfp), kyIs140 (str-2::gfp), yzls37 (lip-13::gfp), ots158 (ser-2::gfp) (Chang et al., 2003; Kim and Li, 2004; L’Etoile and Bargmann, 2000; Li et al., 1999; Peckol et al., 1999; Sze et al., 2000; Troemel et al., 1995, 1997, 1999; Tsaklik et al., 2003; Yu et al., 1997).

Isolation of ref-1(oy40)

A strain carrying stably integrated str-1::gfp transgenes was mutagenized using EMS. The progeny of ~8000 mutagenized F1 animals were examined using a dissection microscope equipped with epifluorescence, and oy40 was identified on the basis of ectopic str-1::gfp expression. oy40 was mapped with respect to genetic markers and deficiencies, and rescue of the mutant phenotype was obtained with the cosmid R05H5 containing the predicted ref-1 gene (T01E8.2). The mu220 and ok288 alleles were obtained from the Caenorhabditis Genetics Stock Center. Strains were outcrossed at least two times prior to further analyses. The presence of ref-1 alleles in marked strains was confirmed by the size or sequence of ref-1 amplicons generated from genomic DNA.

Molecular biology methods

The ref-1 transcript was obtained by amplification from cDNA generated from oligo-d(T)-primed wild-type mRNA. The transcript includes 365 bp of ref-1 3' UTR sequences and contains a single PCR-generated T to A transition resulting in a F93Y conservative substitution. The F93 residue lies in a poorly conserved linker region between the two bHLH domains of REF-1, and the resulting in a F93Y conservative substitution. The F93 residue lies in a poorly conserved linker region between the two bHLH domains of REF-1.

The ref-1 minigene was constructed by fusing 2.0 kb ref-1 promoter sequences amplified from genomic DNA to the ref-1 transcript in a C. elegans expression vector (gift from A. Fire). The ref- [RAGGCAA] minigene was constructed by replacing the ref-1 promoter in the wild-type ref-1 minigene construct with a ref-1 promoter in which eight LAG-1/Su(H) binding sites were mutated (Neves and Priess, 2005). The functional gfp-tagged ref-1 fusion gene was constructed by fusing ref-1 genomic sequences, including 2.0 kb upstream sequences, in frame to gfp coding sequences prior to the STOP codon.

RNAi

Bacterial strains carrying the die-1(RNAi) or the control vector clones were grown overnight, diluted, and expression was induced by treatment with IPTG for 5 h at 37°C. Cultures were plated in duplicate onto worm growth plates and allowed to dry overnight. L4 larvae of wild-type or a ref-1(ok288) strain carrying stably integrated gey-5::gfp transgenes were placed on these plates and grown overnight at 20°C. Adult animals were removed 24 h later and placed onto fresh plates containing the die-1(RNAi) expressing or the control bacterial
strains and allowed to lay eggs. Adult animals were examined after 3 days at 20°C.

**Temperature-shift experiments**

Animals were grown at either 20°C or 25°C for one to two generations. Synchronized L1 larval populations were obtained by treating gravid hermaphrodites with sodium hypochlorite and allowing the embryos to hatch overnight at the same temperature in the absence of food. Larvae were then placed on food and moved to the converse temperature or maintained at the same temperature until adulthood. Adult animals were examined for the pattern of *str-1::gfp* expression.

**Embryonic expression pattern analysis**

4-cell embryos from a strain carrying integrated copies of the functional gfp-tagged *ref-1* fusion gene were collected from cut mothers using a mouth pipet. The embryos were mounted on agarose pads and viewed using an Olympus IX70 microscope. Images were acquired using DeltaVision Spectris software. Nomarski optical sections along the full z-axis (every 0.9–1.0 μm) were gathered every minute to determine the identities and invariant positions of all cells up to the 28-cell stage, and of the cells in the ABar(a/p) lineage through the next two cell divisions. Fluorescent z-series were likewise gathered after each AB-lineage cell-division. 80 min and 110 min embryos were gathered at exposure times of 0.5–0.7 s. Fig. 6D was generated using Quick Projection, which displays the maximum intensity voxels for all sections.

**Results**

**Ectopic AWB olfactory neurons are generated in *ref-1* mutants**

*oy40* animals were isolated in a screen for mutants exhibiting altered expression of the AWB olfactory neuron-specific marker *str-1::gfp* (Fig. 1A) (Troemel et al., 1997). Mapping, complementation, and sequence analyses showed that *oy40* was allelic to the previously described gene *ref-1*. The previously described *ref-1(mu220)* allele is a point mutation resulting in an R to Q substitution in the first basic domain (Fig. 1B) (Alper and Kenyon, 2001). ref-1(oy40) is also a point mutation resulting in a G82R substitution after the first bHLH domain (Fig. 1B). We obtained the *ref-1(ok288)* allele from the *C. elegans* Gene Knockout Consortium. Genomic sequences from the second to the last predicted exons are deleted in ok288, including sequences encoding part of the first and the entire second bHLH domains (Fig. 1B). Thus, ok288 is likely a null allele.

We further characterized the *str-1::gfp* expression defect in all three *ref-1* mutants. We observed ectopic *str-1::gfp* expression in all three *ref-1* mutant strains with the penetrance of the defect being highest in ok288 and lowest in *mu220* animals (Table 1). *ref-1(oy40)* is temperature-sensitive for this defect (Table 1). Ectopic expression of *str-1::gfp* was first observed at approximately the same developmental stage as wild-type expression. Ectopic expression of this marker likely corresponds to the presence of additional AWB-like neurons, since we also observed ectopic expression of another AWB marker, *odr-1::rfp* (L’Etoile and Bargmann, 2000) (Table 1), and the sensory endings of the extra neurons exhibited forked structures characteristic of AWB neurons (data not shown). Intriguingly, we noted that although in wild-type animals, the AWB neuron subtype consists of a L/R symmetrical pair of neurons, ectopic cells were present only on the right side of the animal in all three *ref-1* alleles. This phenotype was rescued by a genomic fragment containing *ref-1* coding sequences and 2 kb of 5′ promoter sequences, as well as a *ref-1* minigene (Table 1).

Markers for an entire neuronal sublineage are ectopically expressed on the right side of *ref-1* mutants

To further characterize the defects in *ref-1* mutants, we analyzed the expression of additional cell-specific markers. The AWB olfactory and ADF chemosensory neuron pairs arise from a terminal cell division of their shared ABpaaapppa precursor on the right, and the ABalppppapa precursor on the left (Fig. 2A) (Sulston et al., 1983). Markers for the ADF neurons such as *srh-142::rfp* (Peckol et al., 1999) and *tph-1::gfp* (Sze et al., 2000) were also ectopically expressed in *ref-1* mutants (Fig. 2B and Table 2). Similar to *str-1::gfp* expression, ectopic expression of these markers was observed...
only on the right side. To determine whether the generation of ectopic AWB and ADF neurons was correlated, we examined transgenic ref-1(oy40) animals expressing both str-1::gfp and srh-142::rfp. The ref-1(oy40) allele was selected for this analysis as the ectopic gene expression phenotype is incompletely penetrant in this mutant background. 30% of ref-1(oy40) animals express str-1::gfp or srh-142::rfp ectopically, thus, if the cell fate transformations are independent, we would expect that only ~30% of animals expressing one marker ectopically would coexpress the second marker ectopically. However, we found that 97% of ref-1(oy40) mutants exhibiting ectopic srh-142::rfp expression also expressed str-1::gfp ectopically on the right side (Fig. 2B and Table 3). These results indicate that the generation of ectopic AWB and ADF neurons is correlated, and likely arises due to defects at or prior to their shared precursor stage in the lineage.

To identify the stage at which REF-1 acts to mediate lineage decisions, we analyzed additional markers for cells closely related by lineage to the AWB/ADF neurons. The ABpraaap and ABalpppp precursors are generated at approximately 120° after fertilization. Following a stereotyped pattern of cell divisions, these precursors give rise to the AUA, ASJ, ASE, ADL and OLL neurons in addition to the AWB/ADF neurons (Fig. 2A). We found that markers for all these cell types were ectopically expressed on the right side to a similar extent as str-1::gfp and srh-142::rfp in ref-1(oy40) and ref-1(ok288) animals (Tables 2–4). Ectopic cells were located at approximately the same positions as their wild-type counterparts. For example, the cell bodies of OLL neurons are located anteriorly, and cell bodies of most chemosensory neurons are located posteriorly to the nerve ring, which is the primary site for signal integration (White et al., 1986). OLL-specific marker expression was always observed in a cell located anteriorly to the nerve ring, whereas chemosensory neuron-specific markers were always expressed in more posteriorly located cells (data not shown). The ADL, ASJ, and AWB neurons are three of six neuron types that fill with lipophilic dyes such as DiI or DiO (Perkins et al., 1986).

To identify the stage at which REF-1 acts to mediate lineage decisions, we analyzed additional markers for cells closely related by lineage to the AWB/ADF neurons. The ABpraaap and ABalpppp precursors are generated at approximately 120° after fertilization. Following a stereotyped pattern of cell divisions, these precursors give rise to the AUA, ASJ, ASE, ADL and OLL neurons in addition to the AWB/ADF neurons (Fig. 2A). We found that markers for all these cell types were ectopically expressed on the right side to a similar extent as str-1::gfp and srh-142::rfp in ref-1(oy40) and ref-1(ok288) animals (Tables 2–4). Ectopic cells were located at approximately the same positions as their wild-type counterparts. For example, the cell bodies of OLL neurons are located anteriorly, and cell bodies of most chemosensory neurons are located posteriorly to the nerve ring, which is the primary site for signal integration (White et al., 1986). OLL-specific marker expression was always observed in a cell located anteriorly to the nerve ring, whereas chemosensory neuron-specific markers were always expressed in more posteriorly located cells (data not shown). The ADL, ASJ, and AWB neurons are three of six neuron types that fill with lipophilic dyes such as DiI or DiO (Perkins et al., 1986).

To identify the stage at which REF-1 acts to mediate lineage decisions, we analyzed additional markers for cells closely related by lineage to the AWB/ADF neurons. The ABpraaap and ABalpppp precursors are generated at approximately 120° after fertilization. Following a stereotyped pattern of cell divisions, these precursors give rise to the AUA, ASJ, ASE, ADL and OLL neurons in addition to the AWB/ADF neurons (Fig. 2A). We found that markers for all these cell types were ectopically expressed on the right side to a similar extent as str-1::gfp and srh-142::rfp in ref-1(oy40) and ref-1(ok288) animals (Tables 2–4). Ectopic cells were located at approximately the same positions as their wild-type counterparts. For example, the cell bodies of OLL neurons are located anteriorly, and cell bodies of most chemosensory neurons are located posteriorly to the nerve ring, which is the primary site for signal integration (White et al., 1986). OLL-specific marker expression was always observed in a cell located anteriorly to the nerve ring, whereas chemosensory neuron-specific markers were always expressed in more posteriorly located cells (data not shown). The ADL, ASJ, and AWB neurons are three of six neuron types that fill with lipophilic dyes such as DiI or DiO (Perkins et al., 1986).

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>°C</th>
<th>% of animals expressing str-1::gfp in AWB neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>ref-1(oy40); str-1::gfp</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>ref-1(oy40); str-1::gfp</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>ref-1(mut220); str-1::gfp</td>
<td>25</td>
<td>91</td>
</tr>
<tr>
<td>ref-1(ok288); str-1::gfp</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>ref-1(ok288); str-1::gfp</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>ref-1(oy40); str-1::gfp</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>ref-1(ok288); str-1::gfp</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

n = 40–100 for each. Adult animals grown at the indicated temperatures were examined, except as indicated.

a The expression of stably integrated str-1::gfp and odr-1::rfp transgenes was examined. For strains carrying extrachromosomal arrays, transgenic animals from at least two independent lines were examined. pRF4 was used as the coinjection marker.
b odr-1::rfp drives expression in both the AWC and AWB neurons (L’Etoile and Bargmann, 2000). Neurons ectopically expressing odr-1::rfp also frequently filled with DiI, a characteristic of AWB neurons. However, a subset of odr-1::rfp-expressing ectopic neurons may represent AWC neurons.
c ref-1 genomic refers to ref-1 coding sequences including 2 kb of upstream and 352 bp of downstream non-coding sequences.
d gfp-tagged ref-1 refers to a ref-1 genomic fragment which includes gfp coding sequences inserted in frame into the last exon prior to the STOP codon. See Materials and methods for details.
e ref-1 genomic refers to 2 kb of ref-1 promoter sequences driving expression of a ref-1 cDNA.
f ref-1[RAGGGCA] minigene refers to a ref-1 minigene in which eight predicted LAG-1 binding sites in the promoter have been mutated (Bengue and Priess, 2000).
g lag-1(q385); str-1::gfp expression also exhibited the penetrance of ectopic expression of srh-142::rfp in the ADF neurons on the right side, although at a lower penetrance (Table 2). It is possible that the ectopic ADF neurons do not differentiate fully, such that a subset fails to express the gcy-8::gfp marker. Alternatively, only a subset of affected lineages may exhibit defects at the earlier ABpraaap/ABalpppp precursor stage, thereby accounting for a lower frequency of ectopic ADF neurons. We found that 100% and ~91% of ref-1(oy40) and ref-1(ok288) mutants with ectopic ADF marker expression, respectively, also ectopically expressed srh-142::rfp in the ADF neurons (Table 3; based on the penetrance of ectopic expression of each marker alone in this experiment, if ectopic marker expression in each neuron type is determined independently, only 49% of ref-1(ok288) and 15% of ref-1(oy40) animals ectopically expressing gcy-8::gfp would be expected to ectopically
express *srh-142*: rfp. This result suggests that ectopic expression in these neuron types was correlated, and that the lineage defect in a subset of *ref-1* mutants can be further traced back to the ABpraaa/ABalppp precursor stage during embryogenesis (Fig. 2A).

**REF-1 functions prior to the first larval stage to regulate neuronal lineage decisions**

To confirm that REF-1 functions embryonically, we carried out temperature-shift experiments using the temperature-sensitive *ref-1*(oy40) allele. Animals were grown at the permissive (20°C) or restrictive (25°C) temperature, and growth-synchronized L1 larvae were shifted to the restrictive or permissive temperature, respectively. *str-1*: gfp expression was then examined in adult animals. As shown in Fig. 3, loss of REF-1 function prior to the L1 larval, but not at later stages, results in ectopic *str-1*: gfp expression. These results are consistent with the hypothesis that REF-1 acts embryonically to regulate lineage decisions.

**Notch signaling may not be required for REF-1-dependent suppression of the ectopic neuroblast lineage**

Upon activation of Notch signaling, the intracellular domain of Notch is cleaved and translocates to the nucleus where it directs LAG-1/CFB1/Suppressor of Hairless [Su(h)]-mediated transactivation of Hes gene expression by direct binding to promoter sequences (Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000; Yoon and Gaiano, 2005). The promoter of *ref-1* contains multiple predicted LAG-1 binding sites (Neves and Priess, 2005), and mutating these sites was shown to abrogate Notch-mediated upregulation of *ref-1* gene expression in the embryo (Neves and Priess, 2005). To explore whether REF-1 functions in a Notch-mediated pathway to regulate the ectopic lineage decision, we replaced promoter sequences in the rescuing *ref-1* minigene with sequences in which all predicted LAG-1 binding sites were mutated (Neves and Priess, 2005). This minigene fully rescued the ectopic *str-1*: gfp expression phenotype (Table 1). Moreover, 94% of *lag-1*(q385) mutants also exhibited the wild-type pattern of *str-1*: gfp expression (Table 1), suggesting that REF-1 may function independently of Notch to mediate this lineage decision.

**The ectopic ASER neuron exhibits ASEL-like characteristics**

Although cells derived from the ABpraax and ABalppp precursors are bilaterally symmetric overall, the left and right ASE neurons exhibit asymmetric features in that they express distinct sets of signaling molecules and respond to different chemical stimuli (Hobert et al., 1999; Pierce-Shimomura et al., 2001; Yu et al., 1997). ASER expresses the *gcy-5* guanylyl cyclase gene whereas ASEL expresses the *gcy-7* and the *lim-6* LIM-homeobox gene. To investigate whether the ectopic ASE neuron observed on the right side

---

**Fig. 2.** Markers for multiple ABpraax or ABalppp-derived neuron types are ectopically expressed on the right side in *ref-1* mutants. (A) Shown are the bilaterally symmetrical lineages arising from the non-symmetric ABpraax and ABalppp neuroblasts on the right and left sides, respectively. Neurons for which marker expression was examined are indicated in red. The identities of additional postmitotic cell types derived from these lineages are shown, along with cells that undergo programmed cell death (indicated by X). Circles indicate precursors discussed in the text. Precursors that may be affected in *ref-1* mutants are colored yellow. (B) Ectopic expression of *str-1*: gfp (AWB—green) and *srh-142*: rfp (ADF—red) on the right side of wild-type and *ref-1*(oy40) animals is correlated. Lateral view; anterior at left. Scale bar = 10 µm.
of ref-1 mutants exhibit ASER or ASEL-like properties, we examined gcy-5:\textit{gfp} and gcy-7:\textit{gfp} expression in ref-1 mutants. Surprisingly, expression of the ASER marker gcy-5:\textit{gfp} was unaffected in these animals. Instead, we observed ectopic expression of the ASEL markers gcy-7:\textit{gfp} and lim-6:\textit{gfp} on the right side of ref-1 mutant animals (Table 4). To confirm that the ectopic ASE cell generated on the right was expressing ASEL-specific genes, we examined ref-1 mutants coexpressing gcy-5:\textit{gfp} and gcy-7:\textit{rfp}. In wild-type animals, GFP expression was only observed in a single cell on the right side and RFP expression in a single cell on the left. However, in ref-1 mutants, while we still observed a single cell expressing gcy-5:\textit{gfp} on the right, we observed two cells expressing RFP in a L/R symmetrical manner (Fig. 4 and Table 4). These results indicate that the ectopic ASE neuron expresses ASEL-like features in ref-1 mutants.

A complex regulatory cascade involving transcription factors and miRNAs has been shown to regulate the left/right decision in the ASE neurons (Chang et al., 2003, 2004; Johnston and Hobert, 2003). In particular, the DIE-1 zinc finger transcription factor acts in the ASE neuron to promote an ASEL fate such that, in die-1 mutants, the ASE neuron inappropriately expresses ASER characteristics (Chang et al., 2004). To determine whether the ectopic ASEL-like neuron on the right side of ref-1 mutants is subject to the same L/R regulatory mechanisms as the wild-type ASE neuron, we used RNAi to knockdown ref-1 function in die-1(ok26) mutants (Table 4). In both cases, we observed not only the expected misexpression of the ASER marker gcy-5:\textit{gfp} on the left side, but we also observed an additional cell expressing gcy-5:\textit{gfp} on the right. These results indicate that the ectopic ASEL-like neuron on the right side of ref-1 mutants is subject to DIE-1-mediated regulation of L/R asymmetry.

Table 2
Markers for neurons generated from the ABpraaa/ABalppp neuroblast are ectopically expressed on the right side in ref-1 mutants

| Strain* | Marker for | % of animals expressing in
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L</td>
</tr>
<tr>
<td>sre-1-142':\textit{rfp}</td>
<td>ADF</td>
<td>100</td>
</tr>
<tr>
<td>ref-1(ok288); sre-1-142':\textit{rfp}</td>
<td>ADF</td>
<td>32</td>
</tr>
<tr>
<td>ref-1(oa40); sre-1-142':\textit{rfp}</td>
<td>ADF</td>
<td>70</td>
</tr>
<tr>
<td>tph-1':\textit{gfp}</td>
<td>ADF</td>
<td>100</td>
</tr>
<tr>
<td>ref-1(ok288); tph-1':\textit{gfp}</td>
<td>ADF</td>
<td>38</td>
</tr>
<tr>
<td>ser-2':\textit{gfp}</td>
<td>OLL</td>
<td>100</td>
</tr>
<tr>
<td>ref-1(ok288); ser-2':\textit{gfp}</td>
<td>OLL</td>
<td>38</td>
</tr>
<tr>
<td>ref-1(oa40); ser-2':\textit{gfp}</td>
<td>OLL</td>
<td>81</td>
</tr>
<tr>
<td>sre-1':\textit{gfp}</td>
<td>ADL</td>
<td>98</td>
</tr>
<tr>
<td>ref-1(ok288); sre-1':\textit{gfp}</td>
<td>ADL</td>
<td>30</td>
</tr>
<tr>
<td>flp-8':\textit{gfp}</td>
<td>AUA</td>
<td>100</td>
</tr>
<tr>
<td>ref-1(ok288); flp-8':\textit{gfp}</td>
<td>AUA</td>
<td>35</td>
</tr>
<tr>
<td>gpa-9':\textit{gfp}</td>
<td>ASI</td>
<td>100</td>
</tr>
<tr>
<td>ref-1(oa40); gpa-9':\textit{gfp}</td>
<td>ASI</td>
<td>82</td>
</tr>
<tr>
<td>gcy-8':\textit{gfp}</td>
<td>ADF</td>
<td>100</td>
</tr>
<tr>
<td>ref-1(ok288); gcy-8':\textit{gfp}</td>
<td>ADF</td>
<td>84</td>
</tr>
<tr>
<td>ref-1(oa40); gcy-8':\textit{gfp}</td>
<td>ADF</td>
<td>100</td>
</tr>
</tbody>
</table>

* n = 30–100 for each. Adult animals were examined except as indicated. ref-1(ok288) animals grown at 25°C; ref-1(oa40) animals grown at 25°C. There were no differences in the expression of transgene markers in wild-type animals grown at either temperature.

# a All transgenes were stably integrated into the genome. ser-2':\textit{gfp}, sre-1-142':\textit{gfp}, flp-8':\textit{gfp}, tph-1':\textit{gfp}, and gpa-9':\textit{gfp} are also expressed in additional cell types.

b Rarely, we observed loss of expression in one cell, or two expressing cells on a side with none on the other.

c This strain also contained stably integrated sre-1-142':\textit{gfp} transgenes. Expression of sre-1-142':\textit{gfp} and sre-1-142':\textit{rfp} in the corresponding control wild-type strain was similar to those of strains expressing each transgene individually, and is not shown for brevity.

d This strain also contained stably integrated sre-1-142':\textit{gfp} transgenes. Expression of sre-1-142':\textit{gfp} and sre-1-142':\textit{rfp} in the corresponding control wild-type strain was similar to those of strains expressing each transgene individually, and is not shown.

Daughters of an additional AB-derived blastomere also exhibit defects in ref-1 mutants

The ectopic lineage observed in ref-1 mutants could arise from the generation of an ectopic neuroblast, or cell fate transformation of a lineally related or unrelated precursor(s). In the latter case, we would expect to observe loss of expression of markers for terminally differentiated cells arising from the affected precursor. Moreover, this loss should correlate with the observed ectopic expression of cell markers. To address this issue, and to explore the role of REF-1 in the generation of additional neuron types, we examined markers for cells generated from multiple AB daughter-cell blastomeres (Fig. 5).

The ectopic neuroblast likely does not arise solely from complete cell fate transformation of the ABalpp, ABplpp(a/p), ABpia(a/p), or ABpia(a/p) blastomeres, since terminal differentiation markers for subsets of cells derived embryonically from these precursors are expressed in the wild-type manner in ref-1 mutants (Table 1, 2, and 5). It has previously been shown that postembryonic lineages derived from the ABarpp(a/p), ABpia(a/p), and ABprp blastomeres are affected in ref-1 mutants (Alper and Kenyon, 2001; Neves and Priess, 2005). For example, the ABarpp-derived V6L(R) epidermal cells were shown to infrequently adopt a V5 epidermal cell-like fate in ref-1(mu220) mutants resulting in the presence of ectopic V5-derived postdeirid structures (Alper and Kenyon, 2001). We explored the possibility that ABarpp may be undergoing cell fate transformation, followed by regulatory compensation by additional epidermal cells (Sulston and White, 1980). However, no correlation was observed between the presence of ectopic postdeirids and ectopic AWB cells in ref-1(ok288) animals (data not shown). Development of additional cell types derived from these precursors was also unaffected by ref-1 mutations (Table 5), suggesting that examined precursors are not undergoing complete cell fate transformation, and that REF-1 functions may be differentially required in different sublineages. Since additional members of the ref-1 family exhibit partly overlapping gene expression patterns
with ref-1 (Neves and Priess, 2005), we also determined whether animals mutant in these genes exhibit gross neuronal developmental defects. Loss-of-function mutations are available for hlh-28 and hlh-29. However, hlh-28(tm458) and hlh-29(tm284) mutant animals exhibited wild-type patterns of dye-filling in both head and tail neurons, and exhibited wild-type expression of tph-1::rfp (data not shown), suggesting that development and differentiation of nine AB-derived neuronal subtypes are unaffected upon loss of these ref-1 family members.

Interestingly, we observed defects in the expression of a marker for the serotonergic NSM(L/R) neurons which are generated from the AB Baraa blastomere. We observed loss of expression of the tryptophan hydroxylase fusion gene tph-1::gfp in ~74% of ref-1(ok288) mutants in either the left or the right NSM neurons, and rarely in both (Table 5). Since tph-1::gfp is also expressed in the ADF neurons, we correlated loss of expression in the NSM neurons with ectopic expression in ADFR. However, expression in the NSM(L/R) neurons was lost at a similar percentage in ref-1 mutants with either one or two ADFR neurons (80% and 70% of ref-1 mutants with one or two ADFR neurons, respectively, showed loss of tph-1::gfp expression in either NSML/R) (Table 5). Thus, loss of tph-1::gfp expression in the NSM cells and generation of the ectopic neuroblast lineage are likely independent events, indicating that the AB Baraa blastomere is not undergoing complete cell fate transformation in ref-1 mutants. We are unable to rule out the possibility of a partial cell fate transformation. In addition, since we could not examine expression of markers for every AB-derived postmitotic cell type, it remains possible that the ectopic cells are generated from fate transformation of later-born precursor(s). The observed effects of ref-1 mutations on cells generated from multiple AB daughters are summarized in Fig. 5.

Table 3
Ectopic expression of markers for multiple neurons are correlated

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Marker 1 (neuron)</th>
<th>Marker 2 (neuron)</th>
<th>% of animals with ectopic Marker 1 expression that also exhibit ectopic expression of Marker 2* (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ref-1(oy40)</td>
<td>srh-142::gfp (ADF)</td>
<td>str-l::gfp (AWB)</td>
<td>97 (36)</td>
</tr>
<tr>
<td>ref-1(oy40)</td>
<td>srh-142::gfp (ADF)</td>
<td>gcy-9::gfp (ASJ)</td>
<td>91 (22)</td>
</tr>
<tr>
<td>ref-1(oy40)</td>
<td>srh-142::gfp (ADF)</td>
<td>sre-l::gfp (ADL)</td>
<td>87 (23)</td>
</tr>
<tr>
<td>ref-1(oy40)</td>
<td>gcy-8::gfp (AFD)</td>
<td>srh-142::rfp (ADF)</td>
<td>100 (4)*</td>
</tr>
<tr>
<td>ref-1(ok288)</td>
<td>gcy-8::gfp (AFD)</td>
<td>srh-142::rfp (ADF)</td>
<td>91 (11)*</td>
</tr>
<tr>
<td>ref-1(oy40)</td>
<td>srh-142::gfp (ADF)</td>
<td>gcy-7::gfp (ASEL)</td>
<td>88 (16)</td>
</tr>
<tr>
<td>ref-1(ok288)</td>
<td>srh-142::gfp (ADF)</td>
<td>gcy-5::gfp (ASER)</td>
<td>0 (12)</td>
</tr>
</tbody>
</table>

* ref-1(oy40) animals were grown at 25°C; ref-1(ok288) animals were grown at 20°C. Adult animals were examined.

Table 4
Ectopic ASE neurons on the right side express ASEL-specific markers

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Marker for</th>
<th>% of animals expressing in number of neurons on a side (L/R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lim-6::gfp*</td>
<td>ASELE</td>
<td>None</td>
</tr>
<tr>
<td>ref-1(oy40); lim-6::gfp*</td>
<td>ASELE</td>
<td>0</td>
</tr>
<tr>
<td>gcy-7::gfp</td>
<td>ASELE</td>
<td>0</td>
</tr>
<tr>
<td>ref-1(ok288); gcy-7::gfp</td>
<td>ASELE</td>
<td>0</td>
</tr>
<tr>
<td>ref-1(oy40); gcy-7::gfp</td>
<td>ASELE</td>
<td>0</td>
</tr>
<tr>
<td>gcy-5::gfp</td>
<td>ASE</td>
<td>0</td>
</tr>
<tr>
<td>ref-1(ok288); gcy-5::gfp</td>
<td>ASE</td>
<td>0</td>
</tr>
<tr>
<td>ref-1(oy40); gcy-5::gfp</td>
<td>ASE</td>
<td>0</td>
</tr>
<tr>
<td>gcy-5::gfp; gcy-7::rfp</td>
<td>ASELE</td>
<td>0</td>
</tr>
<tr>
<td>ref-1(oy40); gcy-5::gfp; gcy-7::rfp</td>
<td>ASELE</td>
<td>0</td>
</tr>
<tr>
<td>gcy-5::gfp</td>
<td>ASE</td>
<td>0</td>
</tr>
<tr>
<td>ref-1(ok288); gcy-5::gfp</td>
<td>ASE</td>
<td>0</td>
</tr>
<tr>
<td>ref-1(oy40); gcy-5::gfp; gcy-7::rfp</td>
<td>ASELE</td>
<td>0</td>
</tr>
<tr>
<td>ref-1(ok288); gcy-5::gfp</td>
<td>ASE</td>
<td>0</td>
</tr>
<tr>
<td>ref-1(oy40); gcy-5::gfp; gcy-7::rfp</td>
<td>ASELE</td>
<td>2</td>
</tr>
</tbody>
</table>

* lim-6::gfp is expressed in additional cell types.

n = 50~100 for each. ref-1(ok288) animals were grown at 20°C; ref-1(oy40) animals were grown at 25°C. There were no differences in the expression of examined transgenes in wild-type animals grown at either temperature.

Numbers shown are the average derived from two independent experiments. See Materials and methods for details.
Correct patterning of the nervous system requires that the appropriate numbers and types of neurons be generated from precursor cells in precisely delineated locations. However, equally critical for patterning is the suppression of inappropriate adoption of neuronal fate by other cell types. Thus, a regulated balance between the functions of neurogenic and proneural genes which promote neural fates, and antineural genes which antagonize neural fates is essential for neuronal patterning. Members of the bHLH protein family have been implicated in both proneural and antineural functions, and the functions of individual members of these families are well-conserved across species (Bertrand et al., 2002; Hatakeyama and Kageyama, 2004). Moreover, in both vertebrates and invertebrates, the functions of these proteins have been shown to be regulated by intercellular signaling primarily involving the Notch receptor, as well as cell-intrinsic mechanisms (Bertrand et al., 2002; Ross et al., 2003).

In *C. elegans*, the unicellular zygote divides to give rise to the AB and P1 blastomeres. The AB blastomere generates the majority of the nervous system, whereas the P1 blastomere generates other tissue types including the germline (Sulston et al., 1983). Cell–cell inductive events involving Notch-mediated signaling direct the lineage patterns and cell fates of a subset of AB daughters. In particular, L/R asymmetry in the AB progeny blastomere cell fates is directed by Notch signaling events (Hutter and Schnabel, 1994, 1995; Mango et al., 1994; Mello et al., 1994; Moskowitz et al., 1994; Priess et al., 1987). However, additional AB daughters appear to acquire their cell fates in the absence of intercellular signaling. Thus, similar to other organisms, a combination of extrinsic and intrinsic mechanisms are required to regulate the cell fates, and hence the numbers and types of neurons, generated from individual AB progeny. Somewhat surprisingly, genes that act to mediate these early patterning events are largely unknown. In particular, despite the large number of bHLH proteins predicted by the *C. elegans* genome, their roles in patterning the embryonic nervous system have remained obscure.

Previous work implicated the LIN-22 and REF-1 HES protein family members in antineural roles similar to their counterparts in other organisms. In *lin-22* mutants, the V1–V4 epidermal cells inappropriately acquired the V5 fate resulting in the generation of ectopic postdeirid neurons (Wrischnik and Kenyon, 1997). Similarly, in *ref-1* mutants,
the V6 cells infrequently adopted V5 neuroblast fate at the expense of V6 epidermal fate (Alper and Kenyon, 2001). Recently, REF-1 has also been shown to antagonize the LIN-32 proneural protein in the generation of male sensory rays (Ross et al., 2005). The functions of REF-1 in these postembryonic lineages appear to be Notch-independent. In the embryo, REF-1 was recently shown to be upregulated in response to Notch signaling in the E lineage which gives rise to intestinal cells, and in this lineage, REF-1 acts in a Notch-dependent manner (Neves and Priess, 2005). REF-1 was also upregulated in response to Notch signaling in the AB lineage, and may act in a Notch-dependent manner to regulate the development of two AB-derived epidermal cells (Moskowitz and Rothman, 1996; Neves and Priess, 2005). We have

Fig. 5. Summary of effects of ref-1 mutations on AB-derived neuronal and non-neuronal cell types. Cell types for which marker expression was examined in ref-1 mutants are shown, along with the AB-derived precursors from which they are generated. AB-derived neuron types are present ectopically on the right side in ref-1 mutants and are shown in bold. Italics indicate additional cell types affected in ref-1 mutants. Effects on cell types marked with asterisks were shown previously (Alper and Kenyon, 2001; Neves and Priess, 2005). Lack of effect on cells indicated in parentheses were inferred indirectly by dye-filling. AB-derived blastomeres shown to express ref-1:;gfp in this study are underlined. Double underlines indicate blastomeres in which ref-1:;gfp expression is maintained through subsequent cell divisions; dashed lines indicate blastomeres that express ref-1:;gfp weakly.

Table 5
Effect of ref-1 mutations on expression of markers for additional AB-derived cell types

<table>
<thead>
<tr>
<th>Strain</th>
<th>Marker for</th>
<th>Derived from</th>
<th>% showing wild-type pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>tph-1:;gfp</td>
<td>NSM L/R</td>
<td>ABBaraa</td>
<td>100</td>
</tr>
<tr>
<td>ref-1(ok288); tph-1:;gfp</td>
<td>NSM L/R</td>
<td>ABBaraa</td>
<td>26</td>
</tr>
<tr>
<td>tph-1:;gfp</td>
<td>HSNL/HSNR</td>
<td>ABBlap/ABBrap</td>
<td>100</td>
</tr>
<tr>
<td>ref-1(ok288); tph-1:;gfp</td>
<td>HSNL/HSNR</td>
<td>ABBlap/ABBrap</td>
<td>100</td>
</tr>
<tr>
<td>sra-6:;gfp</td>
<td>ASHL/ASHR ASIL/ASIR</td>
<td>ABBlap/ABBrap ABBlap/ABBrap</td>
<td>97</td>
</tr>
<tr>
<td>ref-1(ok288); sra-6:;gfp</td>
<td>ASHL/ASHR ASIL/ASIR</td>
<td>ABBlap/ABBrap ABBlap/ABBrap</td>
<td>91</td>
</tr>
<tr>
<td>str-2:;gfp; ref-1(ok288)</td>
<td>AWCL/AWCR</td>
<td>ABBlap/ABBrap</td>
<td>84</td>
</tr>
<tr>
<td>Wild-type (Dil)</td>
<td>PHAL/PHAR PHBL/PHBR</td>
<td>ABBlpp/ABBrpp ABBlpp/ABBrpp</td>
<td>96</td>
</tr>
<tr>
<td>ref-1(ok288) (Dil)</td>
<td>PHAL/PHAR PHBL/PHBR</td>
<td>ABBlpp/ABBrpp ABBlpp/ABBrpp</td>
<td>87</td>
</tr>
<tr>
<td>Wild-type (a-ODR-7 ab)</td>
<td>AWAL/AWAR</td>
<td>ABBlpp/ABBrpp</td>
<td>86</td>
</tr>
<tr>
<td>ref-1(ok288) (a-ODR-7 ab)</td>
<td>AWAL/AWAR</td>
<td>ABBlpp/ABBrpp</td>
<td>90</td>
</tr>
<tr>
<td>flp-13:;gfp</td>
<td>DD1, DD3, DD5/DD2, DD4, (DD6)</td>
<td>ABBlpp/ABBrpp</td>
<td>100</td>
</tr>
<tr>
<td>ref-1(ok288); flp-13:;gfp</td>
<td>DD1, DD3, DD5/DD2, DD4, (DD6)</td>
<td>ABBlpp/ABBrpp</td>
<td>100</td>
</tr>
</tbody>
</table>

n = 30 – 100 for each.

a The expression of stably integrated transgenes was examined. Animals were grown at 25°C and adults were examined.
b See Fig. 4 for summary.
c Since tph-1:;gfp is also expressed in ADF (Table 2), we correlated loss of expression in NSML or R with ectopic expression in ADFR. 33/53 animals exhibited ectopic expression in ADFR. Of these, 15 lacked expression in NSML and 8 in NSMR. 20/53 animals exhibited wild-type expression in one ADFL and one ADFR. Of these, 8 lacked expression in NSML, 7 in NSMR, and 1 in both NSML/R.
d str-2:;gfp is expressed stochastically in either AWCL or AWCR (Troemel et al., 1999).
e 11% did not exhibit any expression.
f Anti-ODR-7 antibody staining was carried out as previously described (Sarafi-Reinach and Sengupta, 2000).
g Expression in DD6 was variable and frequently difficult to observe. Numbers shown reflect flp-13:;gfp expression in D1–D5.
shown that mutations in ref-1 result in the generation of an entire ectopic neuronal sublineage derived from the AB blastomere embryonically, as well as defects in differentiation of the AB-derived serotonergic NSM neuron type. Interestingly, although the LAG-1 binding sites upstream of ref-1 were shown to be required for upregulation of ref-1 expression in the AB lineage (Neves and Priess, 2005), we found that these sites were dispensable for regulation of the ectopic lineage decision and that moreover, ectopic cells were not observed in lag-1 mutants, suggesting that the functions of REF-1 in this decision are Notch-independent. Thus, similar to other HES genes, REF-1 may also exhibit Notch-dependent and independent roles in different lineage decisions (Fisher and Caudy, 1998; Ohsako et al., 1994; Van Doren et al., 1994).

Our analysis of REF-1 functions suggests that REF-1 may act at multiple steps in different lineages. REF-1 is likely to act early to suppress the generation of ectopic neurons from an early neuronal or non-neuronal precursor(s). REF-1 retains strong expression in the descendants of the ABa(a/p) blastomeres, and may play a later role in the subtype specification of the NSM neuron type which derives from ABara. Similar early and late roles for correct nervous system development have been proposed for other bHLH domain proteins (Hallam et al., 2000; Jarman et al., 1993; Jarman et al., 1995; Portman and Emmons, 2000; White and Jarman, 2000). However, it is possible that the phenotypes of ref-1 mutants are not indicative of the temporal requirements for REF-1 function, but instead reflect the partly redundant roles of each of six REF-1 family members. Members of this subfamily are expressed in overlapping spatiotemporal domains (Neves and Priess, 2005), and are likely to act combinatorially and redundantly in a subset of cell types, whereas a single member may act in other cell types.

The origin of the ectopic neuronal subtypes is unclear. Although we attempted to perform mosaic analyses to determine the site(s) of action of REF-1, overexpression of ref-1 together with markers commonly used for such experiments resulted in strong embryonic lethality (our unpublished data) precluding this analysis. The constellation of ectopic neurons generated is similar to that generated by ABalppp, suggesting transformation of an entire sublineage. The complete cell fate transformation of several neuroblasts at the ABalppp developmental stage may be ruled out since we showed that there is no correlated loss of neurons generated from these neuroblasts. However, the ectopic sublineage may arise from partial cell fate conversion of an early precursor, or fate conversion of a later precursor. Alternatively, loss of REF-1 function may lead to the duplication of a precursor. Interestingly, mutations in ref-1 result in the ectopic generation of all descendants of the ABa(p) neuroblasts. This observation suggests that the lineage program of the affected precursor may be intrinsically determined, such that this neuroblast once specified, is ‘preprogrammed’ to go through a defined set and pattern of cell divisions giving rise to a defined subset of neuronal subtypes. Studies in vertebrates and Drosophila have indicated that proneural bHLH proteins direct specific developmental programs in a context-dependent manner (e.g. Chien et al., 1996; Goridis and Brunet, 1999; Gowan et al., 2001; Jarman and Ahmed, 1998; Jarman et al., 1993, 1994; Mizuguchi et al., 2001; Perez et al., 1999). The lineage program followed by the affected neuroblast may be directed by a proneural protein(s). It is also formally possible that the ectopic cells arise from the coordinated transformation or generation of many individual cell fates from unrelated lineages. Detailed lineaging experiments may resolve these issues in the future.

Unexpectedly, we observed a L/R bias in the functions of REF-1 in the regulation of the ectopic lineage decision. It is possible that the early Notch-mediated signaling events induce a REF-1-dependent lineage intrinsic memory that is then translated into L/R differences. Redundancy in ref-1 functions may account for the relatively restricted L/R defects observed in ref-1 mutants. We noted that although ectopic cells were on the right side, the ectopic ASE neurons (the only affected neuron type for which L/R markers are available) exhibit characteristics specific for ASE. However, these neurons retain the ability to adopt ASER fate in a die-1 mutant background. Recently, it has been shown that the ASE(L/R) neurons express hybrid characteristics of both neuron types prior to adopting terminal L or R fates presumably in response to one or more spatially restricted transient signaling events (Johnston et al., 2006).
et al., 2005). These events may not be available to the ectopic lineage due to either spatial or temporal constraints, resulting in the adoption of a ‘default’ ASEL-like terminal fate. Alternatively, the ectopic neurons may arise on the left and migrate over to the right. It is also possible that the presence of the wild-type lineage inhibits adoption of ASER-like characteristics by the ectopic ASE neurons on the right. These results suggest that a global L/R positioning mechanism is likely not necessary for maintenance of ASE(L/R) asymmetry.

In summary, we have described a role for a nematode-specific member of the HES subfamily of bHLH proteins in neuronal development and differentiation. Our results together with those of others indicate that members of the REF-1 subfamily function in both Notch-dependent and independent pathways to execute neuronal lineage decisions. Analyses of the functions of additional family members singly, and in combination will likely provide additional insights into the roles of these genes in patterning and L/R asymmetry decisions in multiple tissue types.

Acknowledgments

We thank Maura Berkley and Caron Gauthier for technical assistance, the Caenorhabditis Genetics Center for strains, the C. elegans Gene Knockout Consortium for the ok288 allele, Andy Fire for the C. elegans expression vectors, Jim Priess for the ref-12.0kb[RAGGCA] promoter, Gary Ruvkun for the die-1(RNAi) clone, Shohei Mitani for the hlih-28(tm458) and hlih-29(tm284) alleles, and Oliver Hobert for comments on the manuscript. This work was supported by the NSF (IBN 0129370-P.S.) and NIH (GM 069891-C. P. H.).

References


