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The basic helix-loop-helix transcription factors LIN-32 and HLH-2 function together in multiple steps of a *C. elegans* neuronal sublineage

Douglas S. Portman* and Scott W. Emmons

Department of Molecular Genetics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
*Author for correspondence (e-mail: portman@aecom.yu.edu)

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SUMMARY

bHLH transcription factors function in neuronal development in organisms as diverse as worms and vertebrates. In the *C. elegans* male tail, a neuronal sublineage clonally gives rise to the three cell types (two neurons and a structural cell) of each sensory ray. We show here that the bHLH genes *lin-32* and *hlh-2* are necessary for the specification of multiple cell fates within this sublineage, and for the proper elaboration of differentiated cell characteristics. Mutations in *lin-32*, a member of the *atonal* family, can cause failures at each of these steps, resulting in the formation of rays that lack fully-differentiated neurons, neurons that lack cognate rays, and ray cells defective in the number and morphology of their processes. Mutations in *hlh-2*, the gene encoding the *C. elegans* E/*daughterless* ortholog, enhance the ray defects caused by *lin-32* mutations. In vitro, LIN-32 can heterodimerize with HLH-2 and bind to an E-box-containing probe. Mutations in these genes interfere with this activity in a manner consistent with the degree of ray defects observed in vivo. We propose that LIN-32 and HLH-2 function as a heterodimer to activate different sets of targets, at multiple steps in the ray sublineage. During ray development, *lin-32* performs roles of proneural, neuronal precursor, and differentiation genes of other systems.

Key words: Neurogenesis, *C. elegans*, Male tail, Sublineage, *lin-32*, *hlh-2*, bHLH, Proneural gene

INTRODUCTION

The development of the multiple cell types of a metazoan organism requires that precursor cells pass through a variety of intermediate cell states as cells become progressively more restricted in their developmental potential. Much remains unknown about how intermediate cell states are established during development, and how the restricted potentials of intermediates allows well-ordered transitions between states. Understanding how signals and transcription factors orchestrate changes in gene expression, and hence changes in cell states, is a major area of interest in developmental biology.

One experimental approach to addressing these questions is to study a developmental sublineage, in which the generation of multiple cell types is coupled together through the use of a stereotyped pattern of division and differentiation. A sublineage may represent a series of cell state transitions intrinsically programmed into a precursor (Chalfie et al., 1981; Sternberg and Horvitz, 1982; Sulston and Horvitz, 1977), in which a single cell gives rise to distinct differentiated progeny with fixed relative frequencies. Nematode development provides a number of examples of such sublineages, particularly in the ventral hypodermal lineages that give rise to ventral cord neurons and the vulva, and in the lateral hypodermal development of seam cells and the male tail rays (Chalfie et al., 1981; Sulston and Horvitz, 1977; Sulston et al., 1983). A lineage very similar to that which generates the rays occurs in the development of external sensory organs in *Drosophila* (Jan and Jan, 1994; Gho et al., 1999; Reddy and Rodrigues, 1999); although this program is not cell-autonomous, as it is in *C. elegans*, its similarity indicates that it may be derived from the same ancestral sublineage. In addition, development of the sensory patches in the vertebrate inner ear has been proposed to employ a similar lineage-based strategy (Adam et al., 1998), and vertebrate CNS development may use defined sublineages (Qian et al., 1998), suggesting that the use of sublineages to control cell state transitions may be a widespread developmental theme. Studying a well-defined sublineage in a genetic system provides a unique opportunity to dissect the requirements for individual cell states, state transitions, asymmetric divisions, and the establishment of differentiated cell fates by the sublineage's postmitotic descendants.

The ray sublineage in the *C. elegans* male tail is executed by each of nine pairs of hypodermal ray precursor cells, giving rise to nine rays on each side of the mature animal (Sulston and Horvitz, 1977). Each ray is a small sensillum containing two neurons and a structural cell. Together, the rays function to sense the hermaphrodite during the mating process (Emmons and Sternberg, 1997; Emmons, 1999). Specification of the ray neuroblasts has been shown to require the gene *lin-32*, a basic-helix-loop-helix (bHLH) transcription factor of the *atonal* family (Zhao and Emmons, 1995). *lin-32* has been proposed to serve a proneural function for the ray precursor cells, making them competent to become neuroblasts. Since

ectopic *lin-32* expression in anterior epidermal cells is sufficient to cause them to generate rays (Zhao and Emmons, 1995), *lin-32* may initiate the ray sublineage program.

In *Drosophila*, genetic functions in neuronal development have been separated into discrete categories, according to the progressive determination model (Ghysen and Dambly-Chaudiere, 1989; Jan and Jan, 1994; Hassan and Vaessin, 1996; Dambly-Chaudiere and Vervoort, 1998). In this model, the early specification of neuronal competence, and perhaps also neural type, is provided by the proneural genes, such as the genes of the *achaete-scute* complex and *atonal*. Later, the neuronal precursor gene *asense* is expressed only in cells that will give rise to neurons, presumably committing cells to a neuronal fate. Finally, neuronal differentiation genes are postulated to endow neurons with specific differentiated characteristics. This model of progressive determination has provided a general foundation for the progress that has been made in understanding cell state transitions in nervous system development.

In vertebrates, a number of *atonal*-class factors, including MATH1, MATH5, and the more distantly-related NeuroD and neurogenins, have been implicated in neuronal development. These factors are thought to have a variety of functions, from precursor specification and control of division and migration of precursor cells, to the establishment and maintenance of the differentiated state (reviewed by Hassan and Bellen, 2000). These studies have led to the idea that cascades of bHLH proteins with specialized functions may orchestrate neuronal development by controlling cell state transitions. According to this model, each gene activates targets that characterize a given state, as well as the next downstream gene in the cascade. This model draws heavily on the framework of the progressive determination model in *Drosophila*, and highlights the extent of conservation of the roles of bHLH proteins in neuronal development.

Here, we have further characterized the role of the *atonal* homolog *lin-32* during *C. elegans* ray development in order to better understand its functions in the cell-fate specification program. Using weak alleles and ray-neuron-specific molecular markers, we have found that, in addition to having an early role in establishing ray neuroblast fate, *lin-32* also functions later in the ray sublineage. Hypomorphic *lin-32* mutations can cause the generation of incomplete or defective ray cell groups, and *lin-32* function is also necessary for at least some of the differentiated characteristics of the three ray cell types. This suggests that *lin-32* in *C. elegans* fulfills multiple roles that require multiple factors (proneural genes, neuronal precursor genes, and differentiation genes) in *Drosophila* and vertebrates. Additionally, the ray sublineage defects of *lin-32* mutants can be enhanced by mutations that we have isolated in the gene *hlh-2*, which encodes the *C. elegans* ortholog of the E/*daughterless* family of bHLH heterodimerization partners (Krause et al., 1997). In vitro, LIN-32 and HLH-2 heterodimerize and bind to an E-box-containing sequence, and this activity is disrupted by mutations in both of these genes. Together, our results suggest that LIN-32 and HLH-2 act as a heterodimeric complex to activate specific target genes at multiple steps during ray development.

MATERIALS AND METHODS

Nematode strains and methods

All strains used contained the mutation *him-5(e1490)*V, which increases the frequency of males in self-fertile populations. Strain construction and maintenance were carried out as described by Brenner (1974). Mutants used were as follows: (LG I) *dpy-5*(*e61*), *unc-75*(*e950*), *unc-13*(*e61*), *unc-29*(*e193*), *dpy-14*(*e188*); (LG III) *pha-1*(*e2123*), *mab-5*(*bx54*); (LG X) *lin-32*(*u282*), *lin-32*(*gm239*), *lin-32*(*bx46*), *lin-32*(*e1926*), *lin-32*(*u779*), *unc-2*(*e55*). *cat-2::gfp* was expressed from the extrachromosomal complex arrays *bxEx45* (Lints and Emmons, 1999) and *bxEx38* (R. Lints and S. W. E., unpublished), which were maintained at 25°C using the selectable marker *pha-1* (Granato et al., 1994). *syEx313* was used to express *pkd-2::gfp* (Barr and Sternberg, 1999). pJS191, which expresses the adherens junction marker *jam-1::gfp*, was generously provided by J. Simske, and was used to create the extrachromosomal array *bxEx48*. Male tail phenotypes were scored using DIC microscopy in adult males mounted on agar pads (Sulston and Hodgkin, 1988).

lin-32::*gfp* reporters

A 3.8 kb genomic fragment containing the *lin-32* coding region and 2.8 kb of promoter sequence was PCR-amplified from wild-type *C. elegans* genomic DNA and cloned into the vector pCR-XL-TOPO (Invitrogen). (The sequences of all primers used in this work are available from the authors upon request.) GFP coding sequence was excised from pPD119.16 (A. Fire lab vector kit) and inserted in-frame into the unique *Acc*III site at the end of the final *lin-32* exon to create p*lin-32::gfp*. Stable extrachromosomal arrays were generated by coinjection of *pha-1*; *him-5* hermaphrodites with p*lin-32::gfp* DNA at 50 ng/μl with pBX1 (Granato et al., 1994) at 100 ng/μl. Microinjection of *C. elegans* hermaphrodites was as described (Mello and Fire, 1995). A reporter containing only the first 71 amino acids of LIN-32 (p*lin-32*ΔbHLH::*gfp*) was generated by PCR-mediated deletion of p*lin-32::gfp*; this construct was transformed into worms as above. Several transgenic lines were examined for both constructs, and all gave identical expression patterns.

Screen for *lin-32(e1926)* modifiers

To isolate modifiers of the *lin-32(e1926)* ray-loss phenotype, *him-5*; *lin-32(e1926) unc-2(e55)* hermaphrodites were mutagenized with EMS. F_2 hermaphrodites were plated individually, and F_3 males were screened by DIC microscopy for changes in ray frequency. Candidate modifiers were recovered by cloning F_3 hermaphrodite siblings. After screening 2500 mutagenized genomes, two strong enhancers, *bx108* and *bx115*, were isolated. Both mutants were outcrossed at least four times before further analysis.

PCR-based STS mapping (Williams, 1995) was used to map both mutations to chromosome I. Three-factor cross data further localized both *bx108* and *bx115* to approximately +1.8 map units on this chromosome. (Data from segregation of recombinants is available from the *Caenorhabditis* Genetics Center or www.wormbase.org.) *bx108*/*bx115*; *lin-32*(*e1926*) males had the same complete ray-loss phenotype as *bx108*; *lin-32*(*e1926*) and *bx115*; *lin-32*(*e1926*) (data not shown), indicating that the mutations did not complement each other.

hlh-2 rescue and sequencing

To determine the sequence of the *hlh-2* gene in *bx108* and *bx115* animals, the *hlh-2* open reading frame was PCR-amplified from these mutants and the products were sequenced directly. Both mutations were confirmed by sequencing at least twice.

To rescue the *hlh-2*; *lin-32*(*e1926*) enhanced phenotype, we amplified a 12.0 kb fragment containing 3.0 kb of the *hlh-2* promoter and the entire coding sequence from wild-type or *bx115* mutant animals. These fragments were cloned into the vector pCR-XL-TOPO (Invitrogen) to produce p*hlh-2*(+)-gen and p*hlh-2*(*bx115*)-gen. These DNAs were individually coinjected at 4 ng/μl (higher concentrations yielded no transformants) with pBX1 at 100 μg/ml into *hlh-2*(*bx115*); *pha-1*(*e2123*); *lin-32*(*e1926*) hermaphrodites. For both constructs, several stably transmitting lines were generated. Males were scored for ray development as above; 'rescue' was defined as reversion of the

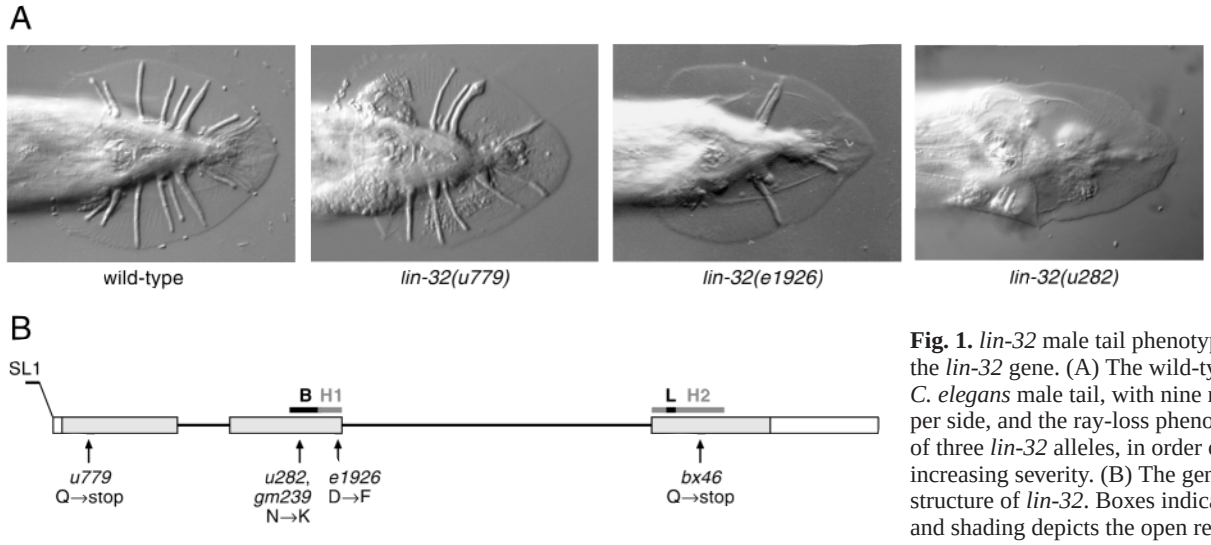

Fig. 1. *lin-32* male tail phenotypes and the *lin-32* gene. (A) The wild-type *C. elegans* male tail, with nine rays per side, and the ray-loss phenotypes of three *lin-32* alleles, in order of increasing severity. (B) The genomic structure of *lin-32*. Boxes indicate exons and shading depicts the open reading frame. B, H1, L and H2 indicate the positions of motifs in the bHLH domain. The positions of known mutations are shown with their associated molecular changes. Note that this structure contains an extra upstream exon compared to that described by Zhao and Emmons (1995); details of this exon will be described elsewhere (D. S. P., C. Zhao and S. W. E., unpublished).

rayless phenotype of *hlh-2(bx115); lin-32(e1926)* to the partial ray-loss phenotype of *lin-32(e1926)*.

RNAi 'soaking'

Double-stranded RNA was prepared as previously described (Fire, 1998); instead of injecting animals, L1 larvae from a *him-5* culture were incubated overnight at 20°C in a 5 μl drop of dsRNA solution in the cap of an inverted microfuge tube. Larvae were then transferred to plates seeded with *E. coli* OP50 and raised to adulthood as usual. Adult males were scored for their ray phenotype by DIC microscopy as described above.

HLH-2 antibody staining

Rabbit anti-HLH-2 polyclonal antisera were generously provided by M. Krause (Krause et al., 1997). To assist in the identification of hypodermal cells, we used nematodes expressing the reporter *jam-1::gfp*, which labels adherens junctions. Briefly, synchronized animals were placed in a small drop of water on a polylysine-coated slide and flattened under a coverslip. The slides were frozen on a metal block cooled to –70°C, and the coverslips were quickly snapped off with a razor blade. Slides were then immersed in –20°C methanol for 10 minutes, followed by brief incubations in methanol:3.7% buffered formaldehyde (Miller and Shakes, 1995) at 7:3, 1:1, and 3:7. The specimens were then fixed in 3.7% buffered formaldehyde for 20 minutes at room temperature. Slides were washed three times in TTB (100 mM Tris, pH 7.5, 0.1% (v/v) Tween 20; Miller and Shakes, 1995) with 0.9% (w/v) NaCl, followed by incubation with anti-HLH-2 antisera at 1:400 overnight at room temperature. Slides were washed again and treated with Cy3-conjugated goat anti-mouse antibodies at 1:1000 for 2 hours at room temperature. DAPI was used to identify cell nuclei.

Expression and purification of LIN-32 and HLH-2 from *E. coli*

To generate bacterial expression constructs, *lin-32* and *hlh-2* cDNAs were subcloned into the expression vector pET15b. A *lin-32* cDNA lacking the first 71 amino acids, but containing the entire bHLH domain, was generated by RT-PCR from total *C. elegans* RNA. (Experiments using a full-length *lin-32* cDNA gave identical results to those shown in Fig. 7.) A full-length *hlh-2* cDNA was provided by M. Krause and A. Corsi. These cDNAs were subcloned into pET15b to generate pET15b-*hlh-2* and pET15b-*lin-32*. Mutations corresponding to *hlh-2*(*bx108*), *hlh-2*(*bx115*) and *lin-32*(*e1926*) were introduced into these constructs using a PCR-based method (Horton, 1993) and verified by sequencing. Constructs were transformed into the *E. coli* expression host BL21-CodonPlus(DE3)-RIL (Stratagene); protein production was induced by the addition of 1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) to the culture, followed by growth for 3 hours at 30°C (LIN-32) or 37°C (HLH-2). Cultures were spun down and lysed by sonication; His_6-tagged protein was purified from the supernatant using the Ni-NTA Spin kit (Qiagen). Recombinant proteins were assessed for purity and concentration with SDS-PAGE and the Bio-Rad protein assay reagent.

Electrophoretic mobility-shift assay

EMSA was performed essentially as described by Krause et al. (1997). The probe was an E-box-containing sequence from the *hlh-2* promoter: 5′-ACCTAATTTTTTCACCTGCTGCTCCAGCCC-3′ (the E-box hexanucleotide is underlined). Sense and antisense probes were synthesized chemically, end-labeled with ^{32}P (Sambrook et al., 1989), annealed, and purified using the QIAQuick nucleotide removal kit (Qiagen).

RESULTS

Hypomorphic *lin-32* alleles disrupt ray development

lin-32 encodes a basic helix-loop-helix protein necessary for the development of the sensory rays in the *C. elegans* male tail (Zhao and Emmons, 1995), and mutations in *lin-32* disrupt ray development with varying expressivity (Fig. 1; Table 1). The strongest alleles, *u282* and *gm239*, cause the loss of nearly all rays as a result of a non-conservative change in the DNA-binding basic domain of LIN-32. A change in the first helix of the HLH domain, *lin-32(e1926)*, results in a less severe phenotype, though most rays are still absent. Weaker ray loss is seen with the allele *lin-32*(*u779*), which presumably causes truncation of 18 amino acids from the N-terminal end of LIN-32 as a result of a change in the site of translational initiation, leaving the bHLH domain intact (D. S. P., C. Zhao, and S. W. E., unpublished). None of these alleles is likely to be null, as *lin-32*(*u282*)/*Df* is embryonic

Table 1. Ray frequencies in selected mutant backgrounds

Genotype	Number of rays per side
Wild-type	9.0
lin-32(*u779*)	4.3
lin-32(*bx46*)	2.1
lin-32(*e1926*)	1.8
lin-32(*u282*)	0.05
lin-32(*gm239*)	0.05
hlh-2(*bx108*); *lin-32*(*e1926*)	0.0
hlh-2(*bx115*); *lin-32*(*e1926*)	0.0
hlh-2(*bx108*)	9.0
hlh-2(*bx115*)	9.0
hlh-2(*bx108*)/+; *lin-32*(*e1926*)	0.3
hlh-2(*bx115*)/+; *lin-32*(*e1926*)	0.5
hlh-2(*bx108*); *lin-32*(*u779*)	0.2
hlh-2(*bx115*); *lin-32*(*u779*)	0.4
mab-5(*bx54*)	2.1
hlh-2(*bx108*); *mab-5*(*bx54*)	1.6

All data are presented as the average number of rays per side as scored using DIC microscopy. At least 100 sides were scored for each listed strain.

lethal (Zhao and Emmons, 1995), as is interference with *lin-32* function by RNAi (data not shown).

lin-32 reporter genes are expressed until the terminal division of the ray sublineage

During the L3 larval stage, each of the nine ray neuroblasts begins the execution of the ray sublineage (Sulston and Horvitz, 1977; Fig. 2). Three successive asymmetric divisions give rise to the three cell types of a mature ray: two neurons (RnA and RnB) and the ray structural cell (Rnst). In the adult male, the cell bodies of the ray neurons lie in the tail. Each has a dendritic process extending to a single ray, and sends an axon into the preanal ganglion (Sulston et al., 1980). The ray structural cell extends a process that is ensheathed by the surrounding hypodermis to form the ray structure that is visible by light microscopy. The ray structural cell is both necessary and sufficient to generate a ray: ablation of the two ray neurons does not affect ray morphogenesis, but ablation of the ray structural cell prevents ray formation (Sulston and White, 1980; Zhang and Emmons, 1995). The fates of the ray structural cell and the ray neurons are determined cell-autonomously, as ablation of individual cells during the ray sublineage does not affect the fates of neighboring cells (Sulston and White, 1980).

As shown previously, *lin-32* is required for the Rn.a cell to adopt the neuroblast fate, and, consistent with this idea, a *lin-32::lacZ* reporter gene was shown to be expressed in all nine Rn cells (Zhao, 1995). Though we have not been able to obtain antibodies against LIN-32, we have carried out additional studies with both translational and transcriptional *lin-32::gfp* reporter fusions. These constructs suggest that the *lin-32* promoter may continue to be active in the nine Rn.a neuroblasts and their progeny, Rn.aa and Rn.ap (Fig. 2D,E). We have also observed GFP fluorescence in cells of the next generation (data not shown). It is possible that this latter expresssion could result from perdurance of GFP. However, we do not see GFP perdurance in Rn.p, which has a fluorescent signal only for a short period of time after Rn division. This raises the possibility that *lin-32* continues to be expressed in Rn.a descendants after the specification of ray neuroblast fate, and may have functions later in ray development as well.

lin-32 mutations cause multiple types of ray sublineage defects

Strong *lin-32* alleles almost completely eliminate ray development by preventing the Rn.a cell from adopting a neuroblast fate (Zhao and Emmons, 1995). Instead, this cell remains in the hypodermal state and divides only once, resulting in the clonal loss of all three cells of each ray. With weaker alleles of *lin-32*, rays form with a higher frequency (Table 1). In these cases, ray loss might similarly result from an early failure to specify the Rn.a neuroblast fate. However, in light of *lin-32* reporter expression later in the sublineage, we wondered if *lin-32* might have functions beyond Rn.a specification. If this were the case, weak *lin-32* alleles might cause some ray loss by individually disrupting the ray structural cell fate, rather than causing the complete clonal loss of a ray. To address this issue, we observed the division patterns of several Rn cells in *lin-32(e1926)* males and found only two types of lineage: the wild-type ray sublineage and the early *lin-32(u282)*-type ray neuroblast defect, in which Rn.a divides

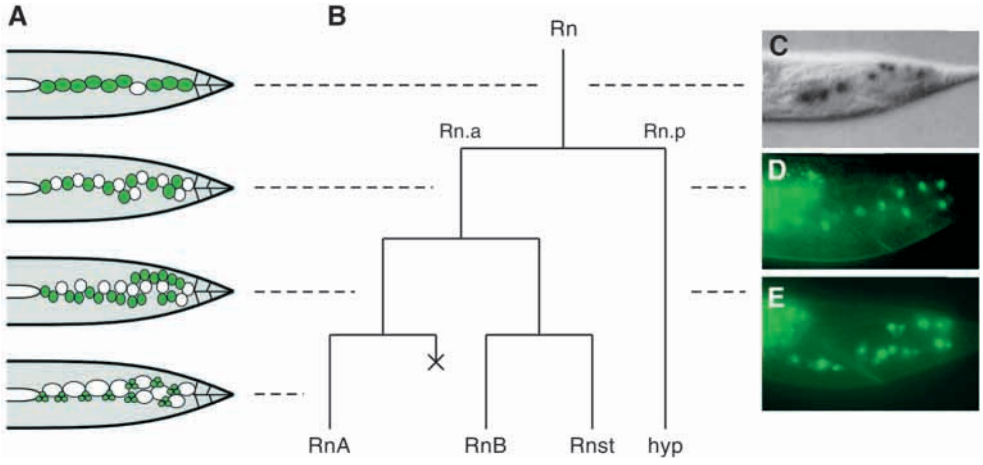

Fig. 2. *lin-32* reporter gene expression during the ray sublineage. (A) The positions of ray precursor cells and their descendants in the lateral hypodermis, from mid-L3 to mid-L4. Green shading indicates the ray precursor cells (Rn cells), the ray neuroblasts (Rn.a cells) and their descendants. (B) The ray sublineage, beginning with the ray precursor cell, Rn, in mid-L3. The anterior daughter of the ray precursor cell is the ray neuroblast, Rn.a, which divides twice to give rise to the two ray neurons (RnA and RnB), the ray structural cell (Rnst) and a cell that undergoes programmed cell death (×). (C,D,E) Expression of *lin-32* reporter genes. (C) Expression of *lin-32::lacZ* in Rn cells, taken from Zhao (1995). *lin-32::gfp* expression is only weakly detected in Rn cells (not shown), perhaps owing to the longer latency period of GFP. (D) *lin-32::gfp* expression in nine Rn.a cells. (E) *lin-32::gfp* expression in the nine pairs of Rn.aa and Rn.ap cells.

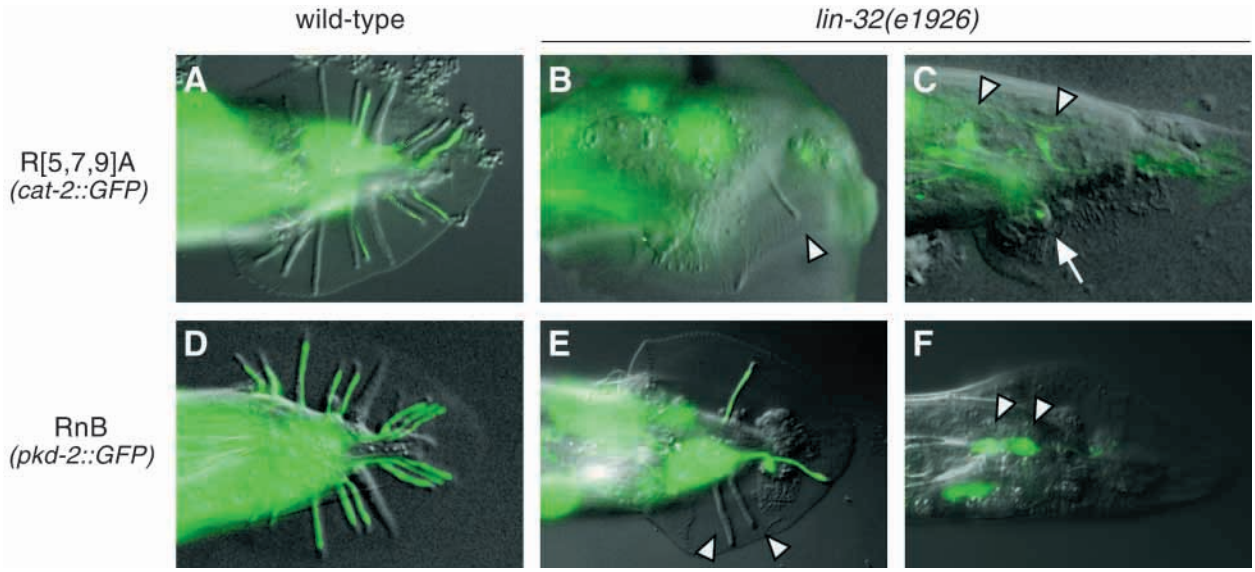

Fig. 3. *lin-32*(*e1926*) can uncouple development of individual ray cell fates from the complete ray sublineage. (A) *cat-2::gfp* expression in wild-type males marks the A-type neurons of rays 5, 7 and 9 (Lints and Emmons, 1999). (B) Ray 5 in a *lin-32(e1926)* male (arrowhead) lacking a GFP-marked A-type neuronal process. (C) Two GFP-expressing cell bodies (arrowheads) in the absence of any rays in a *lin-32(e1926)* male. Note that the more anterior cell body extends a process that terminates in a small protuberance (arrow) at the edge of the fan; this may be an incompletely differentiated ray structural cell. (D) The B-type neurons of most rays express *pkd-2::gfp* (ray 6 expresses this marker only rarely, perhaps owing to the lack of a required regulatory region in the reporter construct). (E) Two rays that lack *pkd-2::gfp*-expressing RnB neurons in *lin-32(e1926)* males (arrowheads); the most posterior ray on the lower side has a GFP-expressing neuron. (F) Two RnB cell bodies in a *lin-32(e1926)* male expressing *pkd-2::gfp* (arrowheads) yet lacking rays formed by associated ray structural cells.

only once to give two hypodermal cells (Zhao and Emmons, 1995). However, since direct observation of large numbers of cell lineages is cumbersome, we wished to have another means of assaying ray development, to allow us to detect potentially less-frequent defects.

To see whether ray neurons could be generated in the absence of their cognate ray structural cells in *lin-32(e1926)*, and vice versa, we used molecular markers for each of the two ray neuron types, RnA and RnB. The reporter gene *cat-2::gfp*, which marks the expression of tyrosine hydroxylase in dopaminergic cells, allowed us to visualize the A-type neurons of rays 5, 7 and 9 (Lints and Emmons, 1999; Fig. 3A). *pkd-2::gfp* is a reporter for a *C. elegans* homolog of the vertebrate polycystic kidney disease gene and is expressed in the B-type neurons of all rays, though with a low frequency in the B-type neuron of ray 6 (Barr and Sternberg, 1999; L. Jia, R. Lints and S. W. E., unpublished data; Fig. 3D).

Using these markers to score for the presence of A- and B-type neurons in *lin-32* mutants, we found that *lin-32* mutations can indeed uncouple differentiation of the ray structural cell from ray neurons descended from the same ray neuroblast. In *lin-32(e1926)* males, rays lacking differentiated A- or B-type neurons (expressing *cat-2* or *pkd-2* reporters, respectively) are observed with significantly higher frequencies than would be expected from transgene loss alone (Fig. 3B,E). For example, in *lin-32(e1926)* males, ray 5 forms without a GFP-marked A-type neuron with a frequency of 26% (n=54); in wild-type, this number is 12% (n=172), presumably as a result of transgene mosaicism. Similarly, rays lack a GFP-expressing B-type neuron process with a frequency of 71% (n=122) in *lin-32(e1926)* males, as opposed to 19% (n=828) in wild type. We also frequently observed the reverse phenotype, namely, mature A- and B-type neurons expressing *cat-2::gfp* or *pkd-2::gfp* in the absence of their lineally related ray (Fig. 3C,F), which never occurs in wild-type animals. Taken together, these results demonstrate that *lin-32* function is required for the development and differentiation of individual ray cell types downstream of the ray neuroblast Rn.a. Moreover, these later functions of *lin-32* are separable, since defects can occur in either ray neurons or ray structural cells, but not necessarily both.

Scoring the frequencies of ray formation and reporter gene expression in these two strains (Table 2) revealed that *lin-32* mutations affected the development of all three ray cell types. In addition, we found that the strong *lin-32* allele *gm239*, which causes almost complete loss of rays as scored by DIC microscopy, also occasionally allows the development of individual ray cell types (Table 2). By our measurement criteria, we found that the development of the ray structural cell was more sensitive to loss of *lin-32* function than was either of the ray neurons. In *lin-32(e1926)*, each adult male side contained 0.79 rays competent to express *cat-2::gfp* (i.e., ray 5, 7 or 9), yet 1.3 GFP-expressing neurons were observed per side. Similarly, only 1.4 rays which would be predicted to contain *pkd-2::gfp*-expressing RnB neurons formed in *lin-32(e1926)* males (*pkd-2::gfp* expression is usually not detectable in ray 6), but 2.1 *pkd-2::gfp*-positive B-type neurons were observed per side. Similar differences in ray versus ray neuron development frequency were also observed in *lin-32(gm239)* males (Table 2). These data indicate that different fates within the ray sublineage have different requirements for *lin-32* function, suggesting that *lin-32* is activating different target genes in different cells.

It seems likely that the expression of *cat-2* and *pkd-2*

reporter genes that we have observed in ray neurons that lack ray structural cells is in cells that are in the lineally-appropriate position for expressing these reporters, namely Rn.aaa and Rn.apa. Alternatively, it is possible that these reporters are being activated in Rn.aa or Rn.ap cells that have prematurely exited the cell cycle. Because of the relatively low frequency of these events, it has not been experimentally feasible for us to distinguish between these possibilities by direct observation of reporter genes during the ray sublineage. However, either of these cases is consistent with a requirement for *lin-32* at multiple steps in the ray sublineage.

lin-32 mutations cause differentiation defects in ray neurons and structural cells

Closer examination of *lin-32(e1926)* males revealed additional defects affecting both ray neurons and structural cells that form in this mutant. As shown above, ray neurons often develop in *lin-32* males in the absence of rays, and by inference, in the absence of properly differentiated ray structural cells. In these cases, these 'rayless' ray neurons sometimes have dendritic processes that extend to a small protrusion at the side of the body (Fig. 3C, arrow). This protrusion may result from a ray structural cell that has failed to attach to the cuticle and/or the surrounding hypodermis to initiate the development of a well-formed ray. When rays do form in *lin-32* males, they are often defective in their shape and can lack an opening to the external environment (Fig. 4A). This suggests that postmitotic Rn.app cells require *lin-32* function to differentiate as well-formed ray structural cells.

The ray neurons that formed in *lin-32(e1926)* males were sometimes found to have defects in the number, size and path of their processes. Abnormal process outgrowth was seen in both A- and B-type neurons; for example, neurons were seen with processes growing in the wrong direction (Fig. 4B) or growing past normal turning points (Fig. 4C). Sometimes, multiple small neurites were seen emerging from a single cell body (Fig. 4D). The latter phenotype is similar to those that have been associated with a variety of mutants (Peckol et al., 1999), and may be an indirect effect of other defects caused by *lin-32*. Together, this variety of phenotypes demonstrates that *lin-32* function is required for the elaboration of the differentiated characteristics of all three ray cell types, which is the final step in ray development.

Latent *hlh-2* mutations enhance *lin-32* ray loss

To better understand the role of *lin-32* in the ray developmental program, we sought to identify mutations that suppressed or enhanced the ray loss phenotype of *lin-32(e1926)*. This genetic screen resulted in the isolation of two mutants, *bx108* and *bx115*, that eliminate all rays in *lin-32(e1926)* males (Fig. 5A and Table 1). While both of these alleles have semidominant effects in a *lin-32* background (Table 1), neither results in a mutant phenotype in an otherwise wild-type background, in either males (Fig. 5B) or hermaphrodites (not shown). We mapped both of these mutations to a small interval on chromosome I that contained the gene *hlh-2*, which encodes the protein CeE/Da, the *C. elegans* ortholog of the *Drosophila*

Table 2. Frequency of expression of GFP reporter genes

Genotype	Number of 'competent' rays	Number of GFP-positive cells
A-type neurons (*cat-2::GFP*)		
Wild-type	3.0	2.4
lin-32(*e1926*)	0.79	1.3
lin-32(*gm239*)	0.047	0.53
hlh-2(*bx115*); *lin-32*(*e1926*)	0.0	0.08
B-type neurons (*pkd-2::GFP*)		
Wild-type	8.1	7.3
lin-32(*e1926*)	1.1	2.5
lin-32(*gm239*)	0.066	1.2
hlh-2(*bx115*); *lin-32*(*e1926*)	0.0	1.1

Data are shown as number of competent rays per side, compared with number of GFP-expressing cells per side. 'Competent' rays are defined as 5, 7 or 9 for *cat-2::gfp* (Lints and Emmons, 1999). For *pkd-2::gfp*, the rays competent to express the reporter are 1-5 and 7-9, plus ray 6 with a frequency of approximately 10%. Hence, in wild-type males, 8.1 rays are competent to express the reporter. In the mutants, this number represents the number of rays 1-5 and 7-9 that formed, plus 10% of the occurrences of ray 6. All data shown are mean counts per side; more than 100 sides were scored for each mutant/reporter combination.

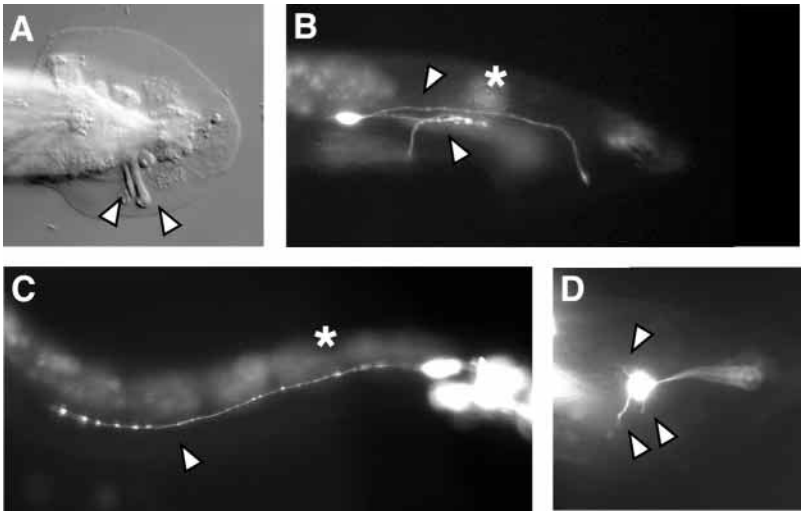

Fig. 4. Ray neurons and structural cells are sometimes defective in *lin-32(e1926)* males. (A) Two morphologically defective rays (arrowheads), likely arising from defects in the ray structural cell. Ray 5 is small and lacks a well-defined opening to the environment, and ray 6 is bulbous at its end and improperly tapered. (B) An RnA neuron (likely R5A) whose cell body is positioned too far anteriorly (normally the R5A soma is located at the position indicated by the asterisk). Also, the cell body is extending two processes posteriorly (arrowheads), and none anteriorly. Normally, RnA neurons extend one dendritic process posteriorly, and one axon anteriorly. Interestingly, the lower process grows posteriorly, then reverses course, growing anteriorly, then ventrally, and finally terminating in the preanal ganglion, where ray neurons normally synapse (Sulston et al., 1980; L. Jia, R. Lints, J. Lipton, and S. W. E., unpublished data). (C) An axon from a B-type ray neuron which has grown too far anteriorly and laterally, without a ventral turn (arrowhead); normally, all B-type axons turn ventrally by the point indicated by the asterisk. (D) An A-type neuron with a well-formed dendritic process, but lacking an axon. Instead, three small neurites can be seen extending from the cell body (arrowheads).

daughterless gene and the vertebrate E protein family (Krause et al., 1997). These proteins, which are also bHLH transcription factors, have been shown to be general heterodimerization partners for cell-type-specific bHLH proteins involved in muscle and nervous system development (Littlewood and Evan, 1998; Murre et al., 1989). Because CeE/Da had been proposed to heterodimerize with the LIN-32 protein (Krause et al., 1997), *hlh-2* was an excellent candidate gene for our enhancing mutations. We sequenced the *hlh-2* gene in these mutants, and found missense changes in both *bx108* and *bx115* animals (see below). In addition, the enhanced ray loss phenotype of *bx115; lin-32(e1926)* males was rescued by transformation with the wild-type *hlh-2* genomic DNA (Fig. 5C). Together, these results prove that the ray-loss enhancers *bx108* and *bx115* are mutant alleles of *hlh-2*.

Because both *hlh-2* mutants are semidominant for the enhancement of the *lin-32* phenotype (Table 1), it was important to determine whether the mutant phenotype resulted from loss or gain of *hlh-2* function. We were unable to use genetic deficiencies to distinguish between these possibilities, owing to the lack of a deficiency spanning *hlh-2*. *bx108* and *bx115* are not likely to be null alleles, based on their sequence alterations (see below), and the fact that *hlh-2(RNAi)* causes highly penetrant embryonic lethality (Krause et al., 1997). However, based on the following criteria, both *hlh-2* mutants are highly likely to be partial loss-of-function alleles. First, as noted above, *hlh-2*(+) supplied from a transgene was able to rescue the enhanced ray loss phenotype of *hlh-2(bx115); lin-32(e1926)* males, demonstrating that the defect does not result from increased *hlh-2* activity. Second, transgene expression of *hlh-2(bx115)* also rescued this phenotype (data not shown), suggesting that *bx115* retains some level of wild-type function. Third, the *hlh-2* alleles do not have an allele-specific interaction with *lin-32(e1926)*, as the phenotypes of the *lin-32* hypomorphic alleles *bx46* and *u779* were also enhanced by both *hlh-2* alleles (Table 1 and data not shown). *hlh-2(bx108)* also enhanced the ray loss of the hypomorphic allele *mab-5(bx54)*. *mab*-5 is a Hox gene required for the activation of *lin-32* in ray precursor cells (Chow and Emmons, 1994; Kenyon, 1986; D. S. P., C. Zhao and S. W. E., unpublished data), and the hypomorphic allele partially disrupts this function. Enhancement of *mab-5(bx54)* indicates that our *hlh-2* mutations do not depend on the presence of a *lin-32* mutation to affect ray development. Finally, postembryonic interference with *hlh-2* function by RNAi (see Materials and Methods) resulted in partial ray loss in a wild-type background (Fig. 5D) and slight enhancement of the *lin-32(e1926)* ray loss defect (data not shown). Together, these results indicate that *hlh-2* has functions required for ray development in wild-type animals, and that *bx108* and *bx115* mutants reduce, but do not eliminate, its activity. The semidominance of the *hlh-2* phenotypes in *lin-32* mutants likely represents a heightened sensitivity to the level of *hlh-2*(+) gene dosage in these sensitized backgrounds (i.e., *hlh-2* haploinsufficiency), and indicates that the products of these genes may interact functionally (see below).

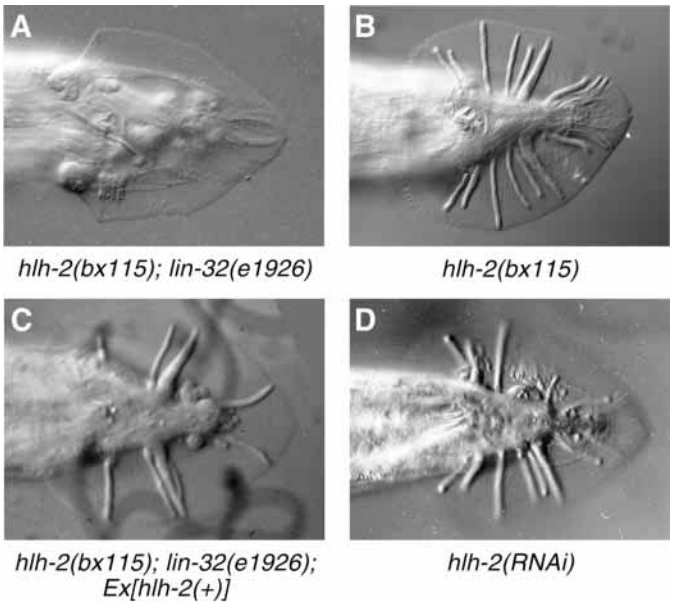

Fig. 5. *hlh-2* mutations enhance ray loss in *lin-32(e1926)* males. (A) The tail of an *hlh-2(bx115); lin-32(e1926)* adult male. No rays are formed in this background. (B) The tail of an *hlh-2(bx115); lin-32*(+) male, in which all nine rays develop normally. (C) Expression of wild-type *hlh-2* from an extrachromosomal array partially restores rays to *hlh-2(bx115); lin-32(e1926)* males, rescuing the *hlh-2* defect but not the *lin-32* defect. (D) Postembryonic RNA interference (RNAi) of *hlh-2* leads to ray loss in a wild-type background. The right (upper) side lacks rays 4 and 6.

hlh-2 also functions at several steps in the ray sublineage

The ray loss phenotype of *hlh-2; lin-32(e1926)* double mutants is stronger than that seen from strong *lin-32* alleles alone. *hlh-2* mutations could cause this phenotype by enhancing all of the ray sublineage defects caused by *lin-32* mutations, or by affecting only a subset of these. To examine these possibilities, we used *cat-2* and *pkd-2* reporter genes to score the frequency of the development of RnA and RnB neurons in *hlh-2(bx115); lin-32(e1926)* males. We found that *hlh-2(bx115)* enhanced defects in all three ray cell types (Table 2), and that both classes of defect (rays lacking GFP-expressing neurons, and neurons lacking rays) could be observed. This suggests that *hlh-2* function is likely to be required for the multiple roles of *lin-32* during the ray sublineage and that *hlh-2* mutations do not abolish ray development in *lin-32(e1926)* solely by enhancing an early defect in the establishment of the neuroblast fate in Rn.a. Direct observation of ray lineages in double mutant males confirmed that partial ray sublineages, in which one branch (e.g. Rn.aa) divides, while the other does not, do occur in this background (data not shown). Moreover, the development of RnA and Rnst, as assayed by reporter gene expression and ray development, respectively, seems to be more sensitive to reductions in the level of *hlh-2* function than development of RnB (Table 2), suggesting that different branches of the ray sublineage have different genetic requirements for the function of *hlh-2*, as they seem to for *lin-32*.

hlh-2 is expressed during the ray sublineage

Krause et al. (1997) have shown that *hlh-2* has a dynamic, restricted pattern of expression during *C. elegans* development, in contrast to the largely ubiquitous expression of E/*daughterless* factors in other organisms. To determine whether HLH-2 protein is present during the ray sublineage, where it could function as

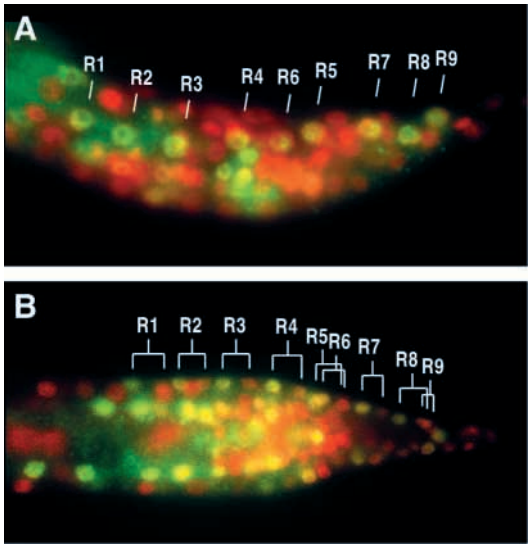

Fig. 6. HLH-2 is expressed during the ray sublineage. (A,B) Anti-HLH-2 antibody staining of late L3-stage male tails. HLH-2 immunofluorescence is shown in green; DAPI-labeled nuclei are in red. (A) An Rn-stage male tail. All nine Rn cells have nuclear HLH-2 staining. (B) An Rn.x-stage male tail. Only the anterior cell of each Rn.a/p pair, the ray neuroblast Rn.a, stains with HLH-2 antibodies.

a heterodimerization partner for LIN-32, we used antibodies raised against HLH-2 to determine its localization during male tail development. HLH-2 was not detectable in the seam cells that give rise to the ray precursor cells before the mid-L3 stage (data not shown). Expression in the seam was first apparent in each of the nine Rn cells (Fig. 6A); upon the division of these cells, HLH-2 could be detected only in the anterior daughter, the ray neuroblast (Fig. 6B). We also observed later expression of HLH-2 in ray sublineage cells that we believe to be both Rn.aa and Rn.ap, but have been unable to unambiguously identify individual cells because of the higher levels of HLH-2 expression directly underlying the hypodermis, in the male sex muscle cell lineages. The coexpression of HLH-2 protein and *lin-32::gfp* reporter genes during the ray sublineage supports the possibility that these proteins function as a heterodimeric complex during ray development.

bx108 and *bx115* mutations disrupt the HLH domain of HLH-2

To better understand how the mutants we isolated affect *hlh-2* function, we sequenced the *hlh-2* gene from *bx108* and *bx115* animals (Fig. 7A). We found that both alleles contain missense changes in the helix-loop-helix domain, which has been shown to mediate the dimerization of bHLH proteins (Littlewood and Evan, 1998). The *bx108* mutation, R316H, is a change in the first residue of the first helix of the bHLH domain. As inferred from structural studies of the bHLH domain of the orthologous protein E47 (Ellenberger et al., 1994), this position is one of a stripe of basic residues in helix 1 that is closely apposed to helix 2 of the dimerization partner, and has been proposed to be important for heterodimer formation (Ellenberger et al., 1994; Shirakata et al., 1993). The *bx115* mutation, V352M, changes a conserved valine in helix 2. In the E47 structure, this residue interdigitates with residues of two other helices in the four-helix bundle of the dimer (Ellenberger et al., 1994); again, this change seems likely to affect the formation or stability of a dimeric species involving HLH-2. Both of these positions are conserved within the E/*daughterless* subgroup of bHLH proteins, but not among bHLH proteins in general (Atchley and Fitch, 1997; Krause et al., 1997), suggesting that they are important for the specific heterodimerization properties of the E/*daughterless* family. Because neither of the mutations dramatically changes the character of the amino acid residue at its position, we suggest that the *bx108* and *bx115* mutations disrupt *hlh-2* function by reducing, but not abolishing, its dimerization affinity, or perhaps by altering its dimerization specificity.

LIN-32 and HLH-2 can heterodimerize and mutations in each protein affect this activity

To determine whether LIN-32 and HLH-2 proteins can heterodimerize and bind to DNA, and to test the predictions of the structural information above on the effects of the *hlh-2* mutants, we sought to examine their functions in vitro. Wild-type and mutant His_6-tagged LIN-32 and HLH-2 proteins were produced in *E. coli*, purified, and tested for their ability to bind a consensus E-box-containing sequence using an electrophoretic mobility-shift assay (Fig. 7B). Both LIN-32 and HLH-2 alone (lanes 2 and 4) were able to bind to the probe as homodimers, though HLH-2 binding to this probe was easily seen only on longer exposures (not shown). Our observation of HLH-2 homodimerization is consistent with that previously shown (Krause et al., 1997); however, homodimerization of a

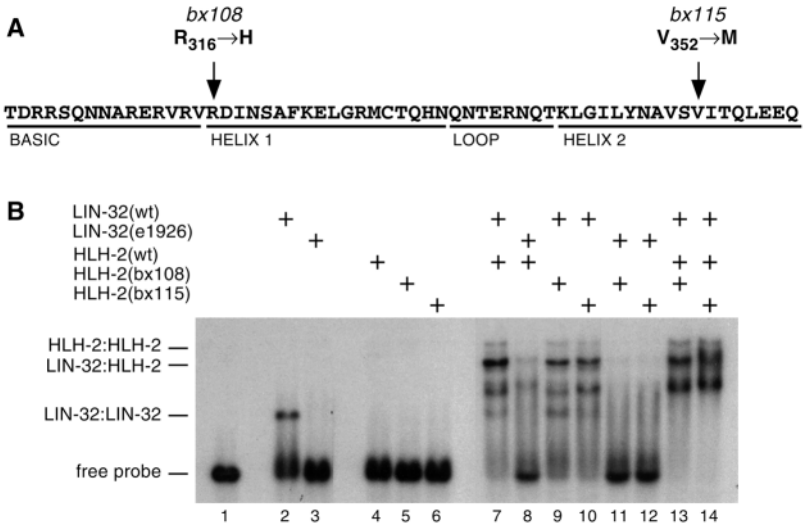

Fig. 7. *hlh-2* mutations interfere with LIN-32:HLH-2 heterodimerization. (A) The primary sequence of the bHLH domain of HLH-2, indicating the missense changes of the two *hlh-2* alleles *bx108* and *bx115*. (B) Electrophoretic mobility-shift assay. Each lane contains wild-type and/or mutant LIN-32 and/or HLH-2 proteins, as indicated. The positions of the three shifted complexes (LIN-32 and HLH-2 homodimers and LIN-32:HLH-2 heterodimers) is shown. In lane 4, HLH-2 homodimerization is detectable as a very weak band; on overexposure (not shown), the signal can be seen more clearly.

cell-type-specific bHLH protein such as LIN-32 is unusual. Homodimerization of either protein was undetectable when we tested the mutant versions (the *e1926* mutant of LIN-32, and *bx108* or *bx115* of HLH-2; lanes 3, 5 and 6).

Binding activity was strongest, however, when wild-type LIN-32 and HLH-2 were combined, and the mobility of the resulting shifted band indicates that this strongly binding species is a LIN-32:HLH-2 heterodimer (lane 7). Heterodimer binding was considerably reduced by the *e1926* mutation of LIN-32 (lane 8), though some binding was still detectable, consistent with the low level of ray formation (20%) in *lin-32(e1926)* mutants. When either of the HLH-2 mutants was tested with wild-type LIN-32 protein, only a slight decrease in binding was seen (lanes 9 and 10), consistent with the lack of any mutant phenotype associated with our *hlh-2* alleles in a wild-type genetic background. However, binding activity was undetectable when either of the HLH-2 mutants was combined with the LIN-32 mutant (lanes 11, 12); in vivo, this combination results in nearly complete loss of all ray cell types. The excellent correlation between in vitro binding activities and mutant phenotypes strongly suggests that the biological activities of *lin-32* and *hlh-2* occur within the context of a LIN-32:HLH-2 heterodimeric complex.

As noted above, the *hlh-2* mutants are semidominant for enhancement of the *lin-32* ray loss phenotype. We tested whether this might be the result of a dominant-negative effect by investigating whether mutant HLH-2 proteins could interfere with the formation of the wild-type LIN-32:HLH-2 heterodimer in vitro. No inhibition of binding was observed when either HLH-2 mutant was combined with wild-type LIN-32 and HLH-2 (lanes 13, 14), suggesting that the semidominance of our mutants is not a dominant-negative (antimorphic) effect. These results are in agreement with our in vivo genetic experiments, and are consistent with the idea that the semidominance of our mutants results from an increased sensitivity to *hlh-2* gene dosage in a *lin-32* mutant background.

DISCUSSION

We have shown that the genes *lin-32* and *hlh-2*, both of which encode bHLH transcription factors, are required for multiple steps in the developmental sublineage that generates the three cell types of each mature ray in the *C. elegans* male tail. Previously, loss-of-function and ectopic expression analyses have shown that *lin-32* function is both necessary and sufficient for hypodermal seam cells to enter the ray sublineage, leading to the idea that *lin-32* functions as the proneural gene for the rays (Zhao and Emmons, 1995). Here, we have used weak loss-of-function alleles of *lin-32* and *hlh-2* in combination with specific markers for ray cell fates to show that these genes are also required for later steps. In these mutants, we have observed partial ray sublineages and clonal groups in which some, but not all, cells have fully differentiated. These results demonstrate that the development of individual ray cell types can be uncoupled from each other by loss of *lin-32* and *hlh-2* function, indicating that these genes have separable functions required at different points in ray development. Thus *lin-32*, in addition to having a proneural-like competence function in the ray neuroblast, specifies later aspects of ray cell determination and differentiation as well.

That the roles of a single gene can span these multiple categories seems to be at odds with the ideas of the *Drosophila* progressive determination model (Ghysen and Dambly-Chaudiere, 1989; Jan and Jan, 1994; Dambly-Chaudiere and Vervoort, 1998). Interestingly, however, *atonal* has recently been shown to have multiple roles in the development of the photoreceptor cell R8. *atonal* was originally described as the proneural gene for R8 and the mechanosensory chordotonal organs (Jarman et al., 1993, 1995), acting to specify the neuronal competence of their precursors. *atonal* also has additional later roles in R8 development, being required for its ability to recruit neighbors by inductive signalling, and for proper guidance of R8's axon (White and Jarman, 2000). Although the cell signalling events in the fly eye do not have direct parallels in *C. elegans* ray development, the role of *atonal* in these processes could be seen as the activation of the expression of cell-type-specific characteristics, namely, genes required for R8's inductive functions. Along with the axonal outgrowth function, these roles of *atonal* are quite similar to those that we have described for *lin-32*. Additionally, Hallam et al. (2000) have shown that the *C. elegans* NeuroD ortholog *cnd-1*, also an *atonal*-class gene, is required for multiple steps in the development of ventral cord motor neurons. This finding is in contrast to the functions that have been suggested for vertebrate NeuroD genes, which are thought to act only in the late specification of differentiated neural types (reviewed by Hassan and Bellen, 2000). It therefore seems possible that a general property of *atonal*-class genes is that they function at multiple steps in neuronal lineages.

Our mutations in the gene *hlh-2*, which encodes the worm ortholog of *Drosophila daughterless* and the vertebrate E proteins (Krause et al., 1997), represent the first opportunity for a genetic dissection of *hlh-2* function, as mutants have not been previously described. *hlh-2* has been proposed to function in neuronal development in *C. elegans*, based on the specific expression of HLH-2 protein in most, if not all, neuronal precursors during embryogenesis, and the ability of the protein to heterodimerize in vitro with a *C. elegans achaete-scute*-like bHLH protein, HLH-3 (Krause et al., 1997). Our loss-of-function mutations in *hlh-2* demonstrate that this gene is indeed required for neurogenesis in the male tail. These alleles seem only to weakly disrupt function, so that a phenotype is revealed only in a sensitized background – in this case, *lin-32*. The *bx108* and *bx115* alleles presumably leave the roles of *hlh-2* in embryonic neurogenesis intact. Stronger *hlh-2* loss-of-function alleles may be lethal, since *hlh-2(RNAi)* has been shown to cause embryonic arrest (Krause et al., 1997), and may therefore have been missed in traditional forward-genetic screens for neuronal defects.

How do *lin-32* and *hlh-2* function in multiple steps of ray development?

Our evidence for partial defects in the ray sublineage comes largely from the finding that the expression of specific reporter genes that mark the ray A- and B-type neurons is uncoupled from the formation of a ray. Often, *cat-2::gfp* or *pkd-2::gfp* marker expression occurred in the absence of a well-formed ray; we also sometimes observed rays lacking marker-expressing neurons. This indicates that *lin-32* and *hlh-2* have at least two separable functions later in the ray sublineage, one in Rn.aa or its descendants, and another in Rn.ap or its

descendants. The absence of specific differentiated ray neurons and structural cells in *lin-32* and *hlh-2; lin-32* mutants can be explained in two ways. One possibility is that the cell in question was never born, as a result of the failure of its interim precursor to divide. Alternatively, the precursor may have divided and given rise to a cell lineally equivalent to the 'missing' cell, but this cell would have failed to fully adopt some or all of the differentiated characteristics of the mature cell type. These two possibilities are not mutually exclusive, and it seems likely that both of these defects occur in these mutants. Our observation of ray sublineages suggests that in at least some cases in *lin-32* and *hlh-2; lin-32* mutants, one of the intermediate neuroblasts (Rn.aa or Rn.ap) can fail to enter the last mitosis, while leaving the other unaffected; this would indicate a role for *lin-32* and *hlh-2* in specifying the fates of these intermediate cells, and for promoting their terminal division. However, we also believe that the alternative possibility, the failure of one of the two postmitotic siblings to fully differentiate, explains some of the loss of marker expression. In the case of the posterior branch of the ray sublineage, which gives rise to RnB and Rnst, we sometimes observed the loss of one differentiated cell type (usually the ray structural cell, but sometimes the ray neuron) without an effect on the other (Fig. 3; Table 2). If this were the result of a premature exit from the cell cycle by Rn.ap, then our results would indicate that this cell can prematurely differentiate and adopt the fate of either a ray neuron or a ray structural cell. Though this may sometimes occur, an alternative explanation is that one sister cell at the final division (Rn.apa or Rn.app) differentiated properly, while the other did not. This seems likely in the cases in which we observed small protrusions at the side of the fan that may represent undifferentiated Rn.app cells. This model suggests that the functions of both *lin-32* and *hlh-2* are required to specify both RnB and Rnst differentiation independently, and that a loss of one of these functions can occur without disruption of the other.

Based upon the genetic evidence we have presented here, we cannot determine how direct the functions of *lin-32* and *hlh-2* are on the steps for which they are required. Indeed, it is possible that the LIN-32:HLH-2 heterodimer is acting only at an early step, perhaps in ray precursor cells, to activate a variety of targets, each required for a different subsequent step of ray development. These intermediates might then be segregated as determinants into different branches of the sublineage, allowing them to function in the proper cells at the proper time. Since *lin-32* and *hlh-2; lin-32* mutations can disrupt these steps separately, it is clear that the multiple functions of these genes are independent of each other to at least some degree, and that failure to activate one target or set of targets can occur without serious effects on another. The alternative hypothesis, that LIN-32:HLH-2 complexes might be activating different targets at different times, seems more likely to us, and is supported by the observation that the expression of *lin-32* reporter genes and HLH-2 protein continues until the final division of the ray sublineage. Clarification of these possibilities will await the identification of direct LIN-32:HLH-2 targets, and determination of the point in the ray sublineage at which they are activated.

If a LIN-32:HLH-2 complex is indeed acting at different temporal steps, how is its activity controlled so that it activates the appropriate targets at distinct points during ray development? At least two possibilities, not mutually exclusive, seem reasonable: first, either or both proteins could be posttranslationally modified in a lineage-specific manner, changing their specificity for target choice or interaction with other factors; second, LIN-32:HLH-2 target genes might require the activity of cell-type-specific cofactors for activation, allowing activation of targets to be coupled to regulated cofactor expression. Precedent for both of these ideas exists. Phosphorylation of several bHLH proteins has been shown to be important for their function (Littlewood and Evan, 1998), and acetylation of MyoD is required for its activity (Sartorelli et al., 1999). Also, bHLH proteins have been shown to interact with other types of transcription factors, particularly LIM-domain-containing proteins (Bao et al., 2000).

The LIN-32:HLH-2 complex may also have roles other than the direct activation of target genes. A number of bHLH proteins have been shown to interact with or recruit histone acetyltransferase activity (reviewed by Massari and Murre, 2000), leading to the idea that bHLH proteins can modify chromatin structure at target loci. An appealing but speculative notion is that the LIN-32:HLH-2 complex may act in ray precursor cells to modify the genome such that it is made competent for the expression of neuronal genes.

hlh-2 function in *C. elegans*

The two alleles of *hlh-2* that we isolated as enhancers of *lin-32(e1926)* ray loss are both weak loss-of-function alleles, based on the criteria discussed earlier. Why do *hlh-2* mutants have no defects in otherwise wild-type animals? We suggest that these mutations in the HLH domain of *hlh-2* sensitize it to losses of function in its partners. Presumably, HLH-2 heterodimerizes with a variety of proteins during development, including the *C. elegans achaete-scute*-like factors HLH-3, -4, 6, and -14 (Krause et al., 1997; Ruvkun and Hobert, 1998) and the *C. elegans twist* homolog CeTwist (HLH-8) (Corsi et al., 2000; Harfe et al., 1998). Our *hlh-2* mutants might only affect these functions in the context of lowered function of its other partners. The *hlh-2* mutations that we have isolated do not selectively disrupt *hlh-2* function in ray formation, as *hlh-2* mutations also enhance *lin-32* defects in the development of the posterior touch cells (data not shown).

Dynamic expression of *hlh-2* in the male tail

During male tail development, we found that HLH-2 protein is not expressed in the lateral hypodermis until the Rn stage. Its activation in these cells coincides with that of our *lin-32* reporter genes, and suggests that both *lin-32* and *hlh-2* may share common mechanisms for spatial, temporal and sex-specific expression in the lateral hypodermis. Spatially, this control is likely to involve the functions of the Hox genes *mab-5* and *egl-5*, which are necessary for ray development (Chow and Emmons, 1994; Kenyon, 1986; Salser and Kenyon, 1996; Zhang and Emmons, 2000). Less is known about the temporal and sex-specific factors that direct the expression of *lin-32* and *hlh-2*. Mutations in the sex-determination gene *mab-3* disrupt ray development (Shen and Hodgkin, 1988); however, they seem not to affect expression of either *lin-32* or *hlh-2* (Yi et al., 2000).

lin-32 and lineage identity specification

The results presented here have shown that when early *lin-32*-

and *hlh-2*-dependent events in the ray sublineage are allowed to occur, later requirements for these genes are unmasked. An appealing idea might be that these early events specify lineage identity (i.e., the production of a ray), while later events carry out this program by endowing the progeny of the ray neuroblast with specific characteristics. However, it seems that *lin-32* is necessary but not sufficient to specify lineage identity. Although ectopic *lin-32* expression does generate ectopic rays (Zhao and Emmons, 1995), *lin-32* also functions in a variety of other neural lineages. For example, *lin-32* is required to generate the posterior touch cells (Chalfie and Au, 1989), the postdeirid (a lateral neural structure) and other neurons; all of these structures develop through lineages quite distinct from the ray sublineage. Interestingly, lineage identity specification seems to be at least partly under the control of the heterochronic genes, as mutations in the gene *lin-28* can transform the postdeirid into a ray (Ambros and Horvitz, 1984). We conclude that the early *lin-32* function in the Rn cells acts to set in motion the program that generates a ray (i.e., neuroblast specification), but this function is clearly exerted in the context of other factors that help determine the specific nature of the neural program.

bHLH cascades and the roles of other genes in the ray sublineage

The multiple functions of *lin-32* and *hlh-2* in the ray sublineage raise the possibility that, in contrast to neuronal development in flies and vertebrates, there may be no cascade of bHLH proteins in ray development. Indeed, of the several other *atonal* and *achaete-scute*-like genes we have examined, none seem to be expressed during the ray sublineage (D. S. P. and S. W. E., unpublished data), though this survey has not been exhaustive. Nevertheless, other transcription factors are almost certainly involved during this process, as the transcriptional specificity for determining the states of intermediate cell types and terminally differentiated cells is highly unlikely to come from *lin-32* and *hlh-2* alone. Further genetic and molecular analysis of ray development should reveal these additional factors and their roles.

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