RESEARCH ARTICLE

Piecemeal regulation of convergent neuronal lineages by bHLH transcription factors in *Caenorhabditis elegans*

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**ABSTRACT**

Cells of the same type can be generated by distinct cellular lineages that originate in different parts of the developing embryo (‘lineage convergence’). Several *Caenorhabditis elegans* neuron classes composed of left/right or radially symmetric class members display such lineage convergence. We show here that the *C. elegans* Atonal homolog *lin-32* is differentially expressed in neuronal lineages that give rise to left/right or radially symmetric class members. Loss of *lin-32* results in the selective loss of the expression of pan-neuronal markers and terminal selector-type transcription factors that confer neuron class-specific features. Another basic helix-loop-helix (bHLH) gene, the Achaete-Scute homolog *hhl-14*, is expressed in a mirror image pattern relative to *lin-32* and is required to induce neuronal identity and terminal selector expression on the contralateral side of the animal. These findings demonstrate that distinct lineage histories converge on the same neuron class can also have very distinct lineage histories. For example, the four bilaterally symmetric cephalic CEP sensory neurons, composed of a bilaterally symmetric ventral neuron pair (CEP left and CEP right) and a bilaterally symmetric dorsal neuron pair (CEPD left and CEPD right), share similar overall morphology, similar patterns of synaptic connectivity (White et al., 1986) and similar molecular composition (Taylor et al., 2021). However, the CEPD pair and CEV pair derive from different neuroblast lineages (Sulston et al., 1983). Members of other radially symmetric neuron classes are also phenotypically indistinguishable, despite distinct lineage histories; for example, the IL1, IL2 and OLQ neuron classes are composed of ventral and dorsal pairs that each have distinct lineage histories (Sulston et al., 1983). Even neuron classes that are only composed of two bilaterally symmetric neurons can be derived from two lineally distinct neuroblasts. For example, the left and right ASE neurons derive from different embryonic blastomeres (ABa and ABp) (Sulston et al., 1983). Thus, distinct lineage histories can converge on similar neuronal identities. Similar lineage convergence phenomena have also recently been observed in vertebrates (Cao et al., 2019; Chan et al., 2019; McKenna et al., 2016; Wagner et al., 2018). For example, specific types of excitatory and inhibitory neurons of the mouse central nervous system (CNS) develop through multiple, convergent trajectories (Cao et al., 2019).

How is such convergence achieved? Based on studies of neuronal identity control, one point of convergence of distinct lineage histories are terminal selector transcription factors, which are post-mitotically expressed master regulators of neuron identity (Hobert, 2016). For example, the two lineally distinct ASE neurons both eventually turn on the terminal selector CHE-1, which instructs ASE neuron identity (Hobert, 2014). Similarly, all six members of the IL2 neuron class, despite their distinct lineage histories, co-express the terminal selectors *unc-86*, *sox-2* and *elf-1*, which cooperate to control the expression of IL2 identity features (Shaham and Bargmann, 2002; Vidal et al., 2015; Zhang et al., 2014). Similarly, the left and right CAN neurons, which display nonsymmetric, distinct lineage histories, co-express the *ceh-10* homeobox gene, required to specify the identity of both these neurons (Forrester et al., 1998; Wenick and Hobert, 2004). These observations indicate that different lineage histories converge on the expression of similar terminal selectors and, therefore, that terminal selectors are integrators of distinct lineage histories. What then are the molecular factors that converge in a lineage-specific manner to drive individual terminal selectors in a neuron type-specific manner?

Recent single cell RNA (scRNA) analysis of developing embryos revealed molecular correlates to distinct lineage histories, such that the precursors of cells with similar terminal identities were shown to display distinct gene expression profiles, exactly as predicted by their distinct lineage histories (Packer et al., 2019; Sulston et al., 1983). In this paper, we show that the *C. elegans* Atonal homolog...
lin-32 (Zhao and Emmons, 1995) is differentially expressed in distinct lineages of cells that phenotypically converge on the same neuronal cell classes and selectively affects the specification of only some class members. Moreover, we discovered that the Achaete-Scute homolog hlh-14 (Frank et al., 2003) displays a mirror image expression pattern relative to lin-32 and functions on the contralateral side of the animal.

Our analysis of basic helix-loop-helix (bHLH) transcription factors was originally motivated by our quest to understand how the expression of terminal selector transcription factors is controlled. Terminal selectors have emerged as key regulators of neuronal identity throughout the entire nervous system (Hobert, 2016), yet we understand little about how their expression is induced in the embryo (Baumeister et al., 1996; Bertrand and Hobert, 2009; Christensen et al., 2020; Mittani et al., 1993; Murgan et al., 2015). Based on a previously reported effect of lin-32 on the expression of the terminal selectors unc-86/Brn3 (Baumeister et al., 1996) and mec-3 (Mittani et al., 1993), we sought to investigate the role of lin-32 further. In a number of different organisms, from flies to worms to vertebrates, Atonal orthologs have mostly been characterized for their proneural activity that imposes neuronal identity on neuroectodermal progenitor cells (Baker and Brown, 2018; Bertrand et al., 2002). Loss of such proneural activity results in conversion from a neuronal to an ectodermal skin cell fate. Such proneural functions have been defined for lin-32 in the context of a number of peripheral sense organs in C. elegans, including the postdeirid, Q and male ray lineages (Chalfie and Au, 1989; Portman and Emmons, 2000; Zhao and Emmons, 1995; Zhu et al., 2014). These proneural functions are evidenced by lineage changes in which neuroblasts that normally divide to generate multiple distinct neuron types instead convert to skin cells, leading to a failure to generate a number of neuron types (Chalfie and Au, 1989; Portman and Emmons, 2000; Zhao and Emmons, 1995; Zhu et al., 2014). However, other effects on neural lineages have also been observed in lin-32 mutants. For example, after its proneural role early during lineage specification lin-32 has also been implicated in controlling later aspects of neuronal differentiation in the Q and ray lineages (Miller and Portman, 2011; Portman and Emmons, 2000; Zhu et al., 2014). Cell identity transformations are also evident in a subset of dopaminergic neuron-producing lineages (Doitsidou et al., 2008) as well as in glia cells (Zhang et al., 2020). lin-32 has also been shown to affect the differentiation and/or function of a number of additional neuron types, including oxygen sensory neurons (Rojo Romanos et al., 2017), touch receptor neurons (Mittani et al., 1993), anterior ganglion neurons (Baumeister et al., 1996; Shaham and Bargmann, 2002) and AIB interneurons (Hori et al., 2018). However, it was left unclear whether these defects are proneural lineage defects, cellular differentiation defects, cell identity transformation defects or some combination thereof.

Here, to facilitate a comprehensive analysis of lin-32 function, we begin by describing the expression pattern of gfp-tagged LIN-32. Although the expression of lin-32::gfp is consistent with a number of previously described functions of lin-32, we identified sites of expression that led us to explore previously unknown functions of lin-32. One consistent theme of lin-32 function is its effect on the expression of terminal selector-type transcription factors. Our findings for lin-32, as well as for another bHLH family of the Atonal superfamily, hlh-14, provide insights into the molecular basis for the convergence of distinct lineage histories on similar cellular differentiation events, via the regulation of terminal selector expression.

RESULTS

Embryonic expression pattern of lin-32

The embryonic expression of lin-32 has so far only been described using a 5’ promoter fusion, which may lack cis-regulatory elements (Murray et al., 2012). We analyzed a transgenic strain carrying a fosmid in which the lin-32 locus was 3’-terminally tagged with gfp, as well as a CRISPR/Cas9-engineered reporter allele in which gfp was also inserted at the 3’ end of the gene (kindly provided by the Greenwald lab, Columbia University, NY, USA), both of which yielded similar expression patterns. Embryonic expression was analyzed using 4D microscopy (Schnabel et al., 1997). We focused on the AB blastomere- and MS blastomere-derived lineages, which produce all but two (PVR and DVC) of the 302 neurons of the C. elegans nervous system. We found that lin-32 was expressed in a number of different neuronal lineages in the AB lineage, but not the MS lineage, which produces several pharyngeal neurons (Fig. 1). The earliest expression was observed at the 128-cell stage in the daughters of ABalapa blast cells. In other lineages, the onset of lin-32 expression occurred shortly after the 128-cell stage, whereas, elsewhere, it started as late as a mother neuroblast, which generates two terminally differentiating daughters. In several cases, we were unable to record the terminal division of a neuroblast because of the movement of embryos in the egg shell and, therefore, we may have missed expression in postmitotic cells. If postmitotic expression were to exist, it would be transient because we observed no expression of lin-32 in any embryonically generated neuron in first larval or later-stage animals. This fosmid reporter-based expression is largely similar to that of the promoter fusion (Murray et al., 2012), but pushes that expression pattern to one additional round of cell cleavage.

Consistent with the expression of Atonal homologs in other organisms (Bertrand et al., 2002; Jarman and Groves, 2013), the vast majority of lineages that expressed lin-32 produced sensory neurons. Several aspects of the embryonic expression of lin-32 were in agreement with previously reported lin-32 mutant phenotypes. For example, we observed lin-32 expression (Fig. 1) in lineages that give rise to sensory neurons in the anterior ganglion, consistent with previous studies that reported lin-32 to control expression of the unc-86/Brn3 terminal selector (Baumeister et al., 1996; Shaham and Bargmann, 2002). Similarly, we observed expression in lineages that give rise to URX and CEPD neurons, and in lineages that give rise to AIB neurons, which are all neurons wherein differentiation defects have been observed in lin-32 mutants (Doitsidou et al., 2008; Hori et al., 2018; Rojo Romanos et al., 2017).

Pronuclear functions of lin-32

Previous work had shown that lin-32 has proneural functions in a number of postembryonically generated sensory neuronal cell types, including the sex-shared postdeirid lineage and the Q lineage, as well as in male-specific neuronal lineages (Chalfie and Au, 1989; Portman and Emmons, 2000; Zhao and Emmons, 1995). One common feature in these postembryonic lineages appears to be that the loss of lin-32 results in obvious lineage-patterning defects, in which neuroblasts stop dividing and transform into hypodermal cells. The embryonic expression of lin-32 that we described above prompted us to ask whether such neuroblast-to-hypodermal conversions are also observed in embryonically generated neurons of lin-32-null mutant animals (i.e. carrying the tm1449 deletion allele; Fig. 1). Nomarski optics-based lineage tracing of embryonic cell lineages was performed using 4D microscopy with SIMI BioCell (Schnabel et al., 1997) until 300 min of embryonic development. This analysis revealed no obvious cell-division defects or transformations into hypodermal fates (as would be
evidenced by changes in nuclear morphology and migratory patterns) in any of the lineages that normally express lin-32.

For a more granular assessment of cell fate, we examined the expression of different sets of pan-neuronal cell-fate markers. First, we used a nuclear-localized reporter transgene for the pan-neuronal gene \textit{rab-3} (Stefanakis et al., 2015). We counted the number of nuclei and, with the exception of the ventral nerve cord, observed a reduction in overall pan-neuronal gene expression throughout embryonically generated head and tail ganglia (Fig. 2A). To confirm this observation, we used the \textit{'UPN'} driver hybrid reporter construct, in which the \textit{cis}-regulatory elements of several pan-neuronally expressed genes are fused with one another (Yemini et al., 2021). We observed that expression of this pan-neuronal marker gene was also lost in many different normally \textit{lin-32}-expressing lineages (Fig. 2B, Fig. 3). Lastly, we also examined the existence of subnuclear granules (‘NUN bodies’), another pan-neuronal identity feature (Pham et al., 2021), observing a reduction in the number of cells containing this subnuclear structure (Fig. 2C).

The hybrid pan-neuronal marker was expressed from the recently described NeuroPAL transgene, which also contains a large number of additional neuron type-specific markers (Yemini et al., 2021). These markers allowed us to assess more specifically which neurons lose their identity and to correlate these losses with normal sites of \textit{lin-32} expression. We observed pan-neuronal and cell type-specific marker losses in AIB and URX neurons, which were previously reported to display differentiation defects in \textit{lin-32} mutants (Hori et al., 2018; Rojo Romanos et al., 2017) (Fig. 3). Within the anterior ganglion, we observed losses of pan-neuronal and cell-specific
markers of the radially symmetric IL1 and IL2 sensory neuron classes, consistent with lin-32 expression in the lineages that generate these neurons. We independently corroborated the IL1 and IL2 neuron losses with different marker transgenes (klp-6 for IL2 and flp-3 for IL1) (Fig. S2).

In a previous screen for mutants that affect dopaminergic cell fate, we had identified alleles of lin-32 and reported that lin-32 affects the expression of the dopaminergic marker dat-1::gfp in CEPD neurons (Doitsidou et al., 2008). Here, we found that lin-32 is expressed in the lineage that gives rise to CEPD neurons. Using NeuroPAL, we showed that lin-32 affects expression of not only the dat-1 marker, but also other cell-identity markers, in addition to pan-neuronal gene expression in CEPD neurons. These results indicate that lin-32 also acts as proneural factor in this lineage (Fig. 3).

**lin-32 is required for terminal selector expression**

To examine the nature of these differentiation defects further, we asked whether lin-32 controls the expression of the terminal selector-type transcription factors known to specify the identity of the neurons affected by lin-32 (Hobert, 2016). We found that the expression of unc-86, the identity regulator of IL2 and URX neurons (Shaham and Bargmann, 2002; Zhang et al., 2014), is indeed lost in lin-32 mutants (Fig. 4). Expression of ceh-43/Dlx and ceh-32/Six3, candidate terminal selectors for IL1 neurons (Reilly et al., 2020), and expression of lin-11/Lhx1, a candidate terminal selector for AVJ neurons, was affected in their respective lineages (Fig. 4). Similarly, expression of ceh-43, the terminal selector for CEPD neurons (Doitsidou et al., 2013), was lost in CEPD neurons of lin-32 mutants (Fig. 4), consistent with the loss of terminal CEPD identity markers in lin-32 mutants (Fig. 3) (Doitsidou et al., 2008), and expression of the AIB identity regulator unc-42 (Bhattacharya et al., 2019), was affected in AIB neurons (Fig. 3).

Given that terminal selectors, such as unc-86, unc-42 and ceh-43, do not affect pan-neuronal gene expression (Berghoff et al., 2021; Bhattacharya et al., 2019; Doitsidou et al., 2013; Hobert, 2016), we conclude that lin-32 independently regulates two aspects of the neuronal differentiation programs of cells such as IL2, IL1 or AIB neurons: (1) pan-neuronal identity; and (2) the acquisition of neuron type-specific features via regulation of terminal-selector transcription factors.

**lin-32 affects terminal identity markers in subsets of neuronal class members**

The effect of lin-32 on a number of different neuron classes reveals an interesting phenomenon. All six IL2 class members are very similar neurons based on process projection patterns, synaptic connectivity (White et al., 1986) and molecular markers (Taylor et al., 2020 preprint), and their common identity is specified by the terminal selectors unc-86, sox-2 and cfi-1 (Shaham and Bargmann, 2002; Vidal et al., 2015; Zhang et al., 2014). However, we found that loss of lin-32 affected the differentiation of the dorsal and lateral IL2 pairs more extensively than the ventral IL2 pairs (Figs 3 and 4). The subclass-selective effect of lin-32 correlates with the intriguing phenomenon that all six IL2 neurons derive from distinct lineages yet converge on the same neuron type. The dorsal and lateral IL2 pairs are all generated within the ABala lineage branch, whereas the ventral pairs are generated by the lineally distal ABalpp and ABarap branch (Fig. 1). In the ABala branch, where loss of lin-32 shows an effect, lin-32 is normally expressed early in the lineage, whereas, in the branches that produce the ventral pairs in a lin-32-independent manner, lin-32 is expressed much later (Fig. 1).

Another case of subclass-specific expression defects was observed in the dopaminergic CEP neuron class, composed of a dorsal pair (CEPDL/R) and a ventral pair (CEPDR/R). These two pairs are anatomically very similar (White et al., 1986) and, in the terminally differentiated state, molecularly indistinguishable (Taylor et al., 2021). However, each pair derives from distinct embryonic neuroblasts (Fig. 1). As described above, we found that lin-32 has a proneural function in CEPD neurons (Fig. 3). However, even though lin-32 is expressed in CEPV neurons (albeit much later
Fig. 3. Proneuronal activities of *lin-32* in specific lineages. (A) The terminal, neuron type-specific and pan-neuronal identities of many neurons that express *lin-32* during development are affected in *lin-32(tm1446)*, as revealed here using the cell-fate marker strain NeuroPAL (*otIs669*). Boxes with different color codes highlight the ganglia for neurons (schematized at the top of the figure) exhibiting fate defects. Neurons (indicated by 1, 2, 3, etc.) that differ in wild-type versus mutant animals are indicated by arrows (see Materials and Methods for notes on cell identification); the locations where neuron colors are missing from mutants are circled by a dashed line. Neighboring neurons with similar identity in mutant and wild-type animals are labeled with corresponding numbers (some neuron colors appear to differ between wild type and mutant because some neurons lie out of the plane of focus; see Materials and Methods and Fig. S1). The neighboring neurons are used as landmarks to ID the cell of interest (see Materials and Methods). (B) The NeuroPAL reporter-fluorophore combinations that were responsible for coloring each neuron type. Cell-fate alterations are determined by changes in this color code. With this code, lost colors can be directly mapped back to losses of reporter expression, thereby determining mutant effects on cell identity. (C) Data for the terminal and pan-neuronal fate defects shown in A, indicating the percentage of animals that display the wild-type NeuroPAL color code for the indicated neuron class. No instances were observed in which the terminal markers were affected but the pan-neuronal identity remained intact. The same effects of *lin-32* on *dat-1* expression in CEPD (i.e. loss of expression) and CEPV (i.e. no effect detected) have been previously reported (Doitsidou et al., 2008). Circles indicate bilateral left-right homologs of the respective neuron class (L>0, expression only in left neuron; 0<R, expression only in right neuron; L=R, expression in both neurons, i.e. wild type). Scale bars: 10 µm.
than in the CEPD neuron-producing lineage; Fig. 1), we detected no defect in the generation or differentiation of CEPV neurons in lin-32-null mutants. Pan-neuronal marker expression and terminal identity markers were unaffected (Fig. 3), as was expression of the terminal selector ceh-43 (Fig. 4).

Strikingly, lin-32 also selectively affects lineally convergent neuron class members in left/right symmetric neuron class members. Within the ABala lineage branch, the left and right AVH neurons are generated from two nonsymmetric precursor cells: ABalapaaa (giving rise to AVHL) and ABalappap (giving rise to AVHR) (Fig. 5A). Another neuron pair generated by these two different blast cells are the left CAN (CANL) and the right CAN (CANR) neurons (Fig. 5A). Similiar to the six IL2 neurons, each of these two left/right symmetric neurons pairs is again an example of lineage convergence, where nonbilaterally symmetric lineage histories funnel into the generation of indistinguishable left/right neuron pairs. We observed that lin-32 was expressed in a left/right asymmetric manner in the lineages that give rise to AVHL/R and CANL/R neurons. In the lineage branch that gives rise to the left AVH and the left CAN, lin-32 was expressed throughout the lineage, whereas it was expressed only very late in the postmitotic AVHR and not at all in CANR or the lineage that gives rise to it (Fig. 5). As stated above, we observed that lin-32 did not affect the cellular cleavage pattern that gives rise to these cell types. However, we discovered that loss of lin-32 affected, in a left/right

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**Fig. 4. Effect of lin-32 on the expression of candidate terminal selectors.** (A,B) Loss of candidate terminal selector expression in lin-32(tm1446)-null mutants. Reporters were: lin-11[ot958(lin-11::gfp::FLAG)], ceh-32[ot1040(ceh-32::gfp)], unc-86[ot879(unc-86::nNeonGreen)], otIs339 (ceh-43fosmid::gfp) and unc-42 [ot958(unc-42::gfp)]. (A) Representative images of wild-type and mutant phenotypes; red dashed outlines indicate location of the defective cell; blue dashed outlines indicate outline of pharynx; red arrows indicate nerve ring. (B) Percentage of animals that displayed indicated marker gene defect in the left (L) or right (R) neuron of a bilateral neuron pair. NeuroPAL (otIs696) was used in the background of all of the strains mentioned herein in order to identify neurons expressing the terminal selector. Circles indicate bilateral homologs of the respective neuron class (L>0: expression only in left neuron; 0<R: expression only in right neuron; L=R: expression in both neurons, i.e. wild type). Scale bars: 10 µm.
asymmetric manner, the expression of terminal selector-type transcription factors that define the molecular identity of neuron pairs within the ABAla lineage. Specifically, *lin-32* affected the expression of *unc-42* and *hlh-34*, the joint identity regulators of the AVH neuron class (Berghoff et al., 2021), in only the left AVH neuron (AVHL), where *lin-32* expression begins as early as the great-grandmother of AVHL, but not in the right AVH neuron (AVHR), where we observed no *lin-32* expression (Fig. 5A,B).

**Fig. 5.** Left/right asymmetric neuronal defects in *lin-32* mutants. (A) ABalapa descendants are the first blast cells to show expression of *lin-32*. The ABalapa lineage gives birth to two neurons, AVHL and CANL, among many others (red circle, neuron; yellow circle, glia). Conversely, their right contralateral homologs derive from the ABalapp lineage, which expresses *lin-32* later and more selectively. (B) Representative images of *lin-32* gene expression in the 7th (mitotically active cells) and 10th (postmitotic cells) cleavage stages. The lower panels show the ball models for the same embryonic stages shown in the images in the upper panels. Color-coded boxes represent different regions of the embryo. The names of the terminal and blast cells are indicated in the color-coded boxes. (C) The effect of *lin-32(tm1446)* was determined using terminal selector reporters for AVH and CAN (left images); *lin-32* is asymmetrically expressed in the ancestors of these neurons. The red-dashed outlines indicate the position of cells with defects; the white-dashed lines indicate outline of pharynx. The percentage of animals that displayed respective reporter gene expression is shown on the right. Circles indicate bilateral homologs of the respective neuron class (L>0: expression only in left neuron; 0<R: expression only in right neuron; L=R: expression in both neurons, i.e. wild type). Scale bar: 10 µm.
The same left/right symmetric effect was observed in the CAN neuron pair. CANL/R identity is specified by the Prd-type homeobox gene (Forrester et al., 1998; Wenick and Hobert, 2004) and may operate together with the Distal-less ortholog ceh-43 (Reilly et al., 2020). We found that lin-32 affects expression of the CANL/R identity regulator ceh-10 as well as its candidate co-factor ceh-43/Dlx in CANL, but not in CANR (Fig. 5C).

The Achaete-Scute homolog hlh-14 provides a mirror image of lin-32 function

We considered the possibility that another bHLH transcription factor may provide a mirror-image function of lin-32 in controlling the identity of neurons that are contralateral to those affected by lin-32. Unlike Drosophila or vertebrates, *C. elegans* only encodes a single Atonal ortholog (Baker and Brown, 2018; Zhao and Emmons, 1995). The next closely related bHLH genes are *ngn-1* and *cnd-1*, the single Neurogenin and NeuroD orthologs of *C. elegans* that, together with Atonal, form the Ato superfamily of bHLH family (Baumeister et al., 1996). Future work will determine whether the neuronal identity transformations observed in lin-32 mutants in the anterior deirid lineage are explained entirely by the loss of unc-86 expression.

We observed another cell identity transformation in lin-32 mutant lineages that generate the four radially symmetric OLQ and URY neurons (Fig. 9A). These neurons derive from four neuroblasts: ABalapapp, ABalapppp, and the two bilaterally symmetric ABp[i]/rpaapppp neuroblasts. All of these neuroblasts were lin-32 positive and maintained lin-32 expression throughout ensuing divisions. As in the anterior deirid lineage, we found no proneural functions of lin-32 in these lineages (i.e. we observed no obvious defects in cellular cleavage pattern in the lineage until the 300-min stage; neither did we observe loss of pan-neuronal reporter expression. Instead, we observed another apparent neuronal identity transformation: OLQ neurons lost characteristic marker gene expression and instead gained expression of URY marker genes (Fig. 9B-E). As in the anterior lineage, we sought to extend this observation by analyzing the expression of potential cell-identity regulators in this lineage. A potential URY identity regulator is the homeobox gene *ceh-32/Six3*, which is expressed in URY but not OLQ (Reilly et al., 2020). We observed that, in lin-32 mutants, OLQ neurons gained *ceh-32/Six-3* expression (Fig. 9F). This is a situation that is conceptually similar to the anterior deirid lineage, in which lineally related cells that all
express lin-32 transform their identity to that of one alternative cell type. Taken together, we conclude that lin-32, either directly or indirectly, regulates the expression of terminal selector-type transcription factors that play a role in promoting specific neuronal identities and suppressing alternative identities.

**DISCUSSION**

We found that lin-32 acts as a proneural gene in several distinct, embryonically generated lineages, mirroring its previously described function in postembryonic development (Zhao and Emmons, 1995). One distinguishing feature of the proneural role of lin-32 in postembryonic versus embryonic development is that, in postembryonic development, a loss of the proneural activity of lin-32 results in a termination of neuroblast divisions and conversion of the neuroblast to that of an ectodermal skin cell (Zhao and Emmons, 1995). In contrast, during embryogenesis, we found no evidence for the loss of lin-32 affecting cellular cleavage patterns, or obvious conversions to hypodermal fate. What we did observe was a
loss of expression of pan-neuronal features, a loss of cell identity-controlling transcription factors (terminal selectors) and, consequently, losses of expression of cell-specific identity features.

Our observation that \textit{lin-32} affects the expression of terminal selectors, as well as pan-neuronal features provides support for the ‘coupling hypothesis’ of proneural gene function, proposed during the late 1990s (Brunet and Ghysen, 1999). Early studies on proneural gene function in both flies and vertebrates had shown that, whereas proneural genes affect generic neuronal features, individual bHLH genes also confer specificity to the neuronal cell type that they generate (reviewed by Baker and Brown, 2018; Bertrand et al., 2002). Brunet and Ghysen proposed that distinct proneural bHLH proteins share the ability to induce generic neuronal properties, but each have the ability to also control the expression of neuron type-specific effector genes, thereby coupling these two distinct aspects of neuronal differentiation (Brunet and Ghysen, 1999). As an example, they discussed the ability of not only the proneural mouse \textit{Mash1} genes to activate the expression of generic neuronal features, but also the homeobox gene \textit{Phox2} to confer specific noradrenergic traits on developing neurons (Brunet and Ghysen, 1999). Phox2 is a terminal selector of noradrenergic fate (Brunet and Pattyn, 2002; Coppola et al., 2010) and, hence, provides the first example of what we abundantly corroborate here in multiple neuronal contexts: that the proneural \textit{lin-32} gene controls the expression of multiple distinct homeobox terminal selectors in a lineage-specific manner to specify distinct neuronal identities.

One intriguing aspect of the proneural function of \textit{lin-32} is the highly selective and lineage-specific effect that it has on specific members of individual neuron classes. This observation provides a molecular correlate to the phenomenon of lineage convergence: cells with different lineage histories acquire similar terminal identities. Lineage convergence is widespread in the \textit{C. elegans} nervous system (Sulston et al., 1983) and, with the advent of technologies that allow the lineages of more complex organisms to be determined, has now also been observed in vertebrates (Cao et al., 2019; Chan et al., 2019; McKenna et al., 2016; Wagner et al., 2018). In the \textit{C. elegans} nervous system, divergent lineages of individual neuron class members converge on similar gene expression profiles via the activation of terminal selectors of neuronal identity in individual class members. For example, the six IL2 sensory neurons arise from different, nonhomologous lineages but are all specified by the combinatorial activity of three terminal selectors: \textit{unc-86}, \textit{cfl-1} and \textit{sox-2} (Shaham and Bargmann, 2002; Vidal et al., 2015; Zhang et al., 2014). Likewise, the lineally diverse CEP neuron class members are all specified by the combinatorial activity of the terminal selectors \textit{ast-1}, \textit{ceh-43} and \textit{ceh-20}. 

![Fig. 7. Left/right asymmetric neuronal defects in \textit{hlh-14} mutants. (A) Lineage diagram of the expression of \textit{lin-32} and \textit{hlh-14} fosmid reporters in the ABala lineage. \textit{hlh-14} mirrors the expression of \textit{lin-32} in ABalapa descendants in the ABalapp lineage. The blue dashed lines indicate sublineages that divide in a symmetric manner to produce symmetric cell fates, as shown in B. Red circle, neuron; yellow circle, glia. (B) A more focused version of the diagram in A, showing that \textit{hlh-14} is expressed in the ABalapp descendants that give birth to AVHR and CANR, a mirror image of \textit{lin-32} expression (see Fig. 5 in the main text). (C) Representative image of \textit{hlh-14} gene expression at the 7th cleavage where mitotically active blast cells ABalappa/p (the great grandmother of AVHR and CANR) start expressing \textit{hlh-14} (top panel), as also illustrated by the ball model for the same embryonic stages (bottom panel). (D) The effects of \textit{hlh-14}(tm295) were quantified for terminal selector reporters of CAN (left) and AVH (right), as also quantified for \textit{lin-32} mutants (also see Fig. 5C in the main text). The wild-type images are from Fig. 5C in the main text and are shown here for comparison only. The white-dashed lines indicate outline of pharynx. Circles indicate bilateral homologs of the respective neuron class (L>0: expression only in left neuron; 0<R: expression only in right neuron; L=R: expression in both neurons, i.e. wild type).]
We have shown here that different class members are specified by distinct upstream inputs. Specifically, \textit{lin-32} controls the generation of distinct subsets of members from a given neuron class. In the most extreme cases, we observed that the left neuron of indistinguishable bilaterally symmetric neuron classes is controlled by \textit{lin-32}. Intriguingly, the right neuron of these neuron classes is controlled by a distinct bHLH gene, \textit{hlh-14}. Although we have not taken our study here to the \textit{cis}-regulatory level, it is conceivable that the \textit{cis}-regulatory control regions of terminal selectors serve as ‘integration devices’ that sample distinct lineage inputs.

Apart from proneural functions in a number of different cellular contexts, we also identified roles of \textit{lin-32} in distinguishing the execution of distinct neuronal differentiation programs, such that the loss of \textit{lin-32} leads to a conversion of one neuronal fate to that of another neuron. We observed such homeotic identity transformations in multiple distinct lineal contexts, and we propose that these are also the result of lost terminal selector expression. Previous work has shown that, in multiple different cellular contexts, terminal selectors can act in a mutually antagonistic and competitive manner, such that removal of one selector may now enable a different selector to exert its function (Arlotta and Hobert, 2015). For example, loss of the \textit{mec-3} homeobox gene in the ALM neuron allows UNC-86/BRN3 to pair up with a different transcription factor, PAG-3, to now promote BDU neuron fate in the ALM neuron (Gordon and Hobert, 2015; Way and Chalfie, 1988). In analogy to these cases, it is conceivable that the lost expression of some terminal selectors in \textit{lin-32} allows other terminal selectors to promote alternative fates. For example, \textit{unc-86}/\textit{Brn3} may antagonize the expression of dopamine neuron-specifying terminal selectors in the deirid lineage and, therefore, the loss of \textit{unc-86}/\textit{Brn3} in \textit{lin-32} mutants may lead to the induction of dopaminergic neuron fate.

Fig. 8. \textit{lin-32} mutants display neuronal identity fate transformation in the anterior deirid lineage. (A) Expression of \textit{lin-32} in lineages giving birth to anterior deirid neurons. The expression of \textit{lin-32} overlaps with the previously reported \textit{unc-86}/\textit{Brn3} expression (Finney and Ruvkun, 1990). The gray line indicates that we did not detect this cell during lineaging of \textit{lin-32::gfp} expression. (B) The neuronal identities of anterior deirid neurons were observed using NeuroPAL ofls669. In \textit{lin-32(tm1446)} mutants, there are ectopic neurons expressing ADE-specific terminal markers, whereas the terminal markers for FLP, RMG and sometimes for the ADA neurons are lost. (C) The combination of different reporter colors (indicated in the square) in the NeuroPAL strain used to mark cells in this lineage. (D) Percentage of different phenotypic categories observed in \textit{lin-32(tm1446)} compared with wild type using NeuroPAL reporters. Circles indicate bilateral homologs of the respective neuron class (L=0, expression only in the left neuron; 0<R, expression only in the right neuron; L=R, expression in both neurons, i.e. wild type; 2R or 2L, expression in an additional neuron on the left or right). (E) \textit{unc-86}/\textit{Brn3} expression (reporter allele \textit{ot879}) is lost in FLP, ADE and RMG neurons in \textit{lin-32} mutants (image panels). The percentage of animals that displayed expression of the tagged \textit{unc-86} reporter allele is shown on the right. Circles indicate bilateral homologs of the respective neuron class (L=0: expression only in left neuron; 0<R: expression only in right neuron; L=R: expression in both neurons, i.e. wild type). Scale bars: 10 µm.
Similarly, lin-32 may promote the expression of a terminal selector in the OLQ neuron that not only induces OLQ identity, but may also repress ceh-32-induced URY fate; hence, loss of lin-32 may result in an OLQ-to-URY identity transformation.

Moving beyond lin-32, we have also examined here the expression pattern of other Ato superfamily genes (nng-1/Neurogenin and cnd-1/NeuroD), as well as proneural AS-C homologs, thereby providing a broad, panoramic view of how these genes may affect neurogenesis in C. elegans. We observed patterns that are reminiscent of those reported in flies and vertebrates (Baker and Brown, 2018; Bertrand et al., 2002; Hassan and Bellen, 2000): (1) individual Ato and AS-C homologs are selectively expressed in distinct neuronal lineages; and (2) with some notable exceptions (Masoudi et al., 2018), they are expressed transiently during early stages of neurogenesis and, therefore, reflect a transient regulatory state that is located between neuronal fate specification and terminal differentiation.

In conclusion, we have provided insights into how terminal differentiation programs in the nervous system, controlled by terminal selector transcription factors, are coupled to earlier developmental events and, specifically, to the lineage history of a cell. Our findings demonstrate that terminal selectors are key integrators of lineage history and provide novel perspectives on how bHLH genes function to pattern the nervous system.

MATERIALS AND METHODS
Strains
Strains were maintained by standard methods (Brenner, 1974). Previously described strains used in this study were as follows:

Mutant alleles: lin-32(tm1446) (Doitsidou et al., 2008) and hhl-14(tm295) (Poole et al., 2011).

Reporter alleles: lin-11[ot958(lin-11::gfp::FLAG)] (Reilly et al., 2020), ceh-32[ot1040(ceh-32::gfp)] (kindly provided by Cyril Cros, Fig. 9. OLQ-to-URY identity transformation in lin-32-null mutants. (A) Lineage diagrams of the expression of lin-32 in lineages giving birth to URY and OLQ neurons. (B) Terminal identity of OLQ and URY neurons marked by NeuroPAL (otts669). In lin-32(tm1446) mutants, there are ectopic signals for the terminal markers of URY (indicated by arrows), whereas the OLQ specific signals are lost. Specifically, we observed potential duplicates of URYDR and URYVR in these mutants. The white-dashed circles outline the position of defective cells. (C) The combination of different reporter colors mixed in the NeuroPAL strain used to mark URY and OLQ neurons. (D) Correlation between the gain in URY terminal markers and loss of OLQ terminal markers. URY and OLQ are lineal cousins and their gain-loss correlation is statistically significant (Pearson correlation, r=−0.534). The negative correlation value reflects the anticorrelated relationship between the observation of both types of neurons (i.e. observing URY is correlated with not observing its lineal cousin OLQ). (E) Percentage of NeuroPAL color codes observed in lin-32(tm1446) compared with wild type, using the NeuroPAL reporter (otts669). The OLQ color code in ventral and dorsal OLQ neurons is partially lost in lin-32 mutants. URY color codes are observed in additional cells in lin-32 mutants. Circles indicate bilateral homologs of the respective neuron class (L>0, expression only in left neuron; 0<R, expression only in right neuron; L=R, expression in both neurons, i.e. wild type; 2R or 2L, expression in an additional neuron on left or right). (F) Ectopic OLQ expression of the ceh-32 reporter allele (ot1040), normally expressed in URY indicated by red arrows (and identified via NeuroPAL). The percentage of animals that displayed ceh-32 reporter allele expression is shown on the right. Circles indicate bilateral homologs of the respective neuron class (L>0: expression only in left neuron; 0<R: expression only in right neuron; L=R: expression in both neurons, i.e. wild type). Scale bars: 10 µm.
Columbia University, NY, USA), unc-86[ot879(unc-86::nNeonGreen)] (Serrano-Saiz et al., 2018) and unc-42[ot986::gfp] (Berghoff et al., 2021).

Reporter transgenes: IsIs394 (nmy-1::gfp) (Nakano et al., 2010), otIs339 (ceh-4::GFP::gfp) (Doitsidou et al., 2013), otIs70/3[ls(5p-3::mCherry); mlIs13 (IIs(klp-6::gfp)]] (kindly provided by Maryam Majeed, Columbia University, NY, USA), NeuroPAL (otIs669) (Yemini et al., 2021), otIs356 [Is(rub-3::NLS::tagRFP)] (Serrano-Saiz et al., 2013), lqIs54 [ceh-10p:: GFP::lin-13(765)] X (kindly provided by Erik Lundquist, University of Kansas, Lawrence, USA) and U1L692: unc-119(ed3); Is[Ex[flh-34::gfp, unc-119]]+ (Cunningham et al., 2012).

Fosmid-based reporters for hlh-3, hlh-14, lin-32 and end-1 were generated by insertion of gfp at the 3′ end of their respective loci using fosmid recombineering (Sarov et al., 2012). Fosmid names are indicated in the figures. All fosmids were injected into N2 as complex arrays at a concentration of 20 ng/µl with 5 ng/µl of tts-3::mCherry as a co-injection marker and up to 85 ng/µl of OP50 DNA, and then chromosomally integrated. The array names were: otIs594 (lin-3::GFP::gfp), otIs648 (hlh-3::GFP::gfp), otIs713 (hlh-4::GFP::gfp) and otIs813 (cnd-1::GFP::gfp).

Microscopy

For fluorescence microscopy, worms were paralyzed by 25 mM sodium azide (NaN₃) and mounted on a 5% agarose pad on glass slides. Images were acquired using an axiostage (Zeiss, AxiOl Image Z.2) or a LSM 800 laser point scanning confocal microscope (Zeiss). Representative images are maximum projections of z-stacks. Image reconstruction was performed using Fiji software (ImageJ) (Schindelin et al., 2012).

4D microscopy and SIMI BioCell (Schnabel et al., 1997) was used, as previously described, to analyze embryonic-lineage defects of mutant animals as well as bHLH fosmid reporter expression patterns during embryogenesis. Briefly, gravid adults were dissected on glass slides and a single two-cell-stage embryo was mounted and recorded over 8 h of embryonic development. Nomarski stacks were taken every 30 s and embryos were illuminated with LED fluorescence light (470 nm) at set time points during development. The recording was performed with a Zeiss Imager Z1 compound microscope, using the 4D microscopy software Steuerprg (Caenotec).

Neuron identification using NeuroPAL

We used NeuroPAL, a transgene that expresses 39 neuron type-specific markers and four pan-neuronal drivers (Yemini et al., 2021), to assess the proper execution of cellular differentiation programs. We previously provided proof-of-concept studies for this approach (Yemini et al., 2021) and, herein, we further detail this approach for lin-12 mutant animals. First, differences in neuronal differentiation were assessed by scoring of transgene color codes; these were reviewed on a per ganglia basis and, thus, the method reduced to only reviewing ganglionic groups of ~20-30 neurons. Given the approximate positional stereotypy of neurons within the worm nervous system, the problem of cell ID can often be even further reduced by focusing on subquadradants of these ganglia. Within each ganglion (or ganglionic subquadrant), many non-altered neurons serve as identifiable landmarks. These identifiable landmarks provide a map that orients researchers so that they can pinpoint mutant-based alterations in any of the remaining potentially altered neurons. Upon first review, we attempted to find by eye all those neurons that expressed LIN-32 within their lineage (looking for cell-autonomous changes). Whenever we were unable to find any potential matching cell in the vicinity that could account for the neuron we were looking for, we noted the neuron as altered. This reduced the question for each of these neurons to the following: was the cellular color code missing or had the cell simply changed its coloring? Missing color codes were easy to spot because the NeuroPAL colormap remained the same but was simply missing a cell. Conversely, cells with altered coloring stood out because they had no corresponding equivalent in the known color map. Lastly, cell nuclear morphology (shape and size) provided additional information for verifying identity-related hypotheses. For accurate capturing of the color code, it was also important to analyze image stacks, rather than images taken at a single focal plane. This was because neuronal color codes that were out of the plane of focus could be distorted by individual colors being more affected than others (see Fig. S1 for an example).

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Competing interests

The authors declare no competing or financial interests.

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