Regulation of Gliogenesis by lin-32/Atoh1 in Caenorhabditis elegans

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ABSTRACT The regulation of gliogenesis is a fundamental process for nervous system development, as the appropriate glial number and identity is required for a functional nervous system. To investigate the molecular mechanisms involved in gliogenesis, we used C. elegans as a model and identified the function of the proneural gene lin-32/Atoh1 in gliogenesis. We found that lin-32 functions during embryonic development to negatively regulate the number of AMsh glia. The ectopic AMsh cells at least partially arise from cells originally fated to become CEPsh glia, suggesting that lin-32 is involved in the specification of specific glial subtypes. Moreover, we show that lin-32 acts in parallel with cnd-1/ NeuroD1 and ngn-1/ Neurog1 in negatively regulating an AMsh glia fate. Furthermore, expression of murine Atoh1 fully rescues lin-32 mutant phenotypes, suggesting lin-32/Atoh1 may have a conserved role in glial specification.

In the developing nervous system, diverse sets of neuronal and glial cell types arise from common progenitors in specific spatiotemporal contexts. The mechanisms that give rise to the specific cell types are highly context-dependent, and involve the coordination of different transcription factors as well as epigenetic regulation based on both timing and position (Rowitch 2004; Temple 2001; Sugimori et al. 2007; Hirabayashi and Gotoh 2010). Given the vast complexity of the system and the diversity of developmental contexts, there is still much to be known about the molecular mechanisms of glial fate determination.

Proneural genes were first discovered and studied in Drosophila, and many show functional and sequence conservation among vertebrates and invertebrates (Jan and Jan 1994; Jiménez and Modolell 1993). Such factors including the neurogenin, atonal, NeuroD and Achaete-Scute families all have basic helix-loop-helix (bHLH) motifs and were found to play major roles regulating neurogenesis during different stages of development (Bertrand et al. 2002; Ross et al. 2003; Sugimori et al. 2007). At the same time, certain proneural genes such as Neurog1 and NeuroD1 have been found to play important roles in the neuron-glia fate decision, where they independently inhibit a glial fate while promoting a neuronal one (Morrow et al. 1999; Sun et al. 2001; Tomita et al. 2000). Here, we use the C. elegans glia as a model to study the molecular mechanisms regulating gliogenesis. C. elegans glia share lineages with neurons and show functional similarity with mammalian ones (Bacaj et al. 2008; Oikonomou and Shaham 2011; Ward et al. 1975). We show that similar to what was reported in mammalian systems, loss-of-function in C. elegans Neurog1 and NeuroD1 homologs ngn-1 and cnd-1 give rise to additional glial cells, suggesting that C. elegans gliogenesis likely utilize similar mechanisms as those in mammals.

To identify additional factors involved in gliogenesis, we carried out an unbiased genetic screen and identified the role of lin-32/Atoh1 in glial specification. lin-32 was previously reported to regulate the neuronal fate specification of multiple cell lineages including neurons in the male tail (Portman and Emmons 2000; Zhao and Emmons 1995; Rojo Romanos et al. 2017); CEPD, ADE and PDE dopaminergic neurons (Doitsidou et al. 2008) and the URX oxygen-sensing neurons (Rojo Romanos et al. 2017). Its mammalian homolog Atoh1 has also been implicated in the generation of inner ear hair cells (Bermingham et al. 1999) and cerebellar granule neurons (Ben-Arie et al. 1997), where overexpression can induce transdifferentiation of glial-like support cells into functioning hair cells in the cochlea and specify differentiation of mature cerebellar granule neurons at the expense of glial production in embryoid bodies (Izumikawa et al. 2005;
Srivastava et al. 2013; Sayyd et al. 2019). Furthermore, Atoh1 exhibits functional conservation with Drosophila atonal where it also promotes a neuronal fate (Ben-Arie et al. 2000), while sensory precursors of the ato lineage generate the bulk of glia in the antenna (Jhaveri et al. 2000; Sen et al. 2005). Thus, Atoh1/lin-32 appears to play varying roles depending on developmental context. We found that lin-32 loss of function leads to increased numbers of certain glia cells such as AMsh and AMso glia, while having reduced numbers of other glia and neurons. Further investigation show that lin-32 acts in early progenitor cells and in parallel with cnd-1 and ngn-1 in glial specification. Our results suggest that lin-32 is involved in the regulation of glial specification across different cellular lineages. More interestingly, expression of murine Atoh1 can fully rescue lin-32 mutant phenotypes, indicating that our findings may represent a conserved function for this gene during gliogenesis.

MATERIALS AND METHODS

C. elegans genetics

C. elegans strains were grown on nematode growth media (NGM) plates with E. coli OP50 as their food source. Animals were grown according to standard methods at 20°C unless otherwise stated (Brenner 1974). Wild type worms were of the Bristol N2 strain. All transgenes, strains and DNA constructs used are described in Table S1. yadIs46 (Pf16f9.3::GFP) was used to visualize AMsh cells while Pttx-3::RFP was used as a co-injection marker.

The recessive allele lin-32(yad67) was isolated from a visualized EMS mutagenesis screen of over 4000 haploid genomes and was the only allele isolated in the screen with the ectopic AMsh phenotype. During backcrossing, we noticed that yad67 was on the left arm of chromosome X, and whole genome sequencing revealed that lin-32 was the most likely candidate in the region. The mutation was confirmed through rescue experiments.

Cloning and constructs

All DNA expression constructs were generated using Gateway cloning technology (Invitrogen, Carlsbad, CA) and subsequently sequenced. lin-32, ngn-1 and cnd-1 cDNA were all amplified from a homemade genomic DNA pool. Promoters of lin-32 (Forward cggccaccgattagagactag; Reverse ggttggtctgactgaaaacgacgatgtgtgag), yadIs46 (Pf16f9.3::GFP) was used to visualize AMsh cells while Pttx-3::RFP was used as a co-injection marker.

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Microscopy

Representative images were acquired with a Zeiss LSM 700 confocal microscope using a Plan-Apochromat 40x/1.4 objective. Worms were immobilized using 1.5% 1-phenoxy-2-propanol (TCI America, Portland, OR) in M9 buffer and mounted on 5% agar slides. 3D reconstructions were done using Zeiss Zen software as maximum intensity projections. A Zeiss Axio Imager 2 microscope equipped with Chroma HQ filters was used to score AMsh number defects. Any animal with more than the wild type AMshL and AMsoH glia were scored as having the defect. Each condition represented 3 experiments of at least 50 D1 animals each that were picked at random from the culture plate unless otherwise noted, in accordance with previous literature in C. elegans. Cell numbers were quantified by counting the number of red nuclei labeled by Pf16f9.3::mCherry::H2B and confirmed by referencing the whole cell morphology labeled by Pf16f9.3::GFP.

For tracking of AMsh cell number during larval development, 10 individual L4 worms per genotype were scored under the Zeiss Axio Imager 2 microscope without 1-phenoxy-2-propanol and recovered from the agar slide. They were scored again when they reached the D1 adult stage.

Statistical analysis

Data were analyzed using one-way ANOVA followed by Tukey’s HSD test, Chi-square test, two-tailed Student’s t-test, Spearman’s Rank-Order Correlation and Pearson Correlation in Graphpad Prism (Graphpad Software, La Jolla, CA).

Data availability

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Dong Yan (dong.yan@duke.edu). C. elegans strains and plasmids generated in this study are available from the lead contact without restriction. Supplemental Figures S1 and S2 as well as Strain Table S1 are available at figshare: https://doi.org/10.25387/g3.12650363.

RESULTS

Mutants of Neurog1 and NeuroD1 homologs possess additional glia cells

To study the molecular mechanisms underlying gliogenesis, we focused mainly on the AMsh glial cells, which are a pair of glia that ensheathe the dendrites of sensory neurons in the amphid sensilla, the primary chemosensory organ (Oikonomou and Shaham 2011). AMsh glia are critical for the neurons they envelop to function, and they are easy to visualize in vivo (Oikonomou and Shaham 2011). To determine whether C. elegans shares similar mechanisms with mammals during gliogenesis, we tested the functions of the homologs of two well-studied proneural genes Neurog1 and NeuroD1 during AMsh genesis. We found that loss of function mutations of C. elegans Neurog1 and NeuroD1 homologs, ngn-1 and cnd-1, caused approximately 20% of ngn-1(ok2200) and 30% of ngn-1(ok2200) animals respectively to possess more than the invariant two AMsh cells observed in wild type animals when examined during the day 1 adult stage (D1). The ngn-1(ok2200)::cnd-1(gk718) double mutants had around a 45% mutant phenotype, which is significantly higher than in either of the single mutants (Figures 1A, 1B and 1C). Furthermore, cnd-1 and cnd-1::ngn-1 mutants had higher mean numbers of AMsh cells than wild type animals, while the difference was not statistically significant in ngn-1 animals likely due to the low penetrance of phenotypes (Figure 1D). These results demonstrate that ngn-1 and cnd-1 function in parallel during AMsh formation, and the relatively low penetrance of phenotypes also suggest additional factors are involved in AMsh genesis as well (Figure 1C). Consistent with loss of function, expression of ngn-1 and cnd-1 under their own promoters strongly rescued the ectopic AMsh phenotype in their respective mutants (Figure 1E). Thus cnd-1 and ngn-1 are important in regulating glial specification, consistent with the role of Neurog1 and NeuroD1 in mammals (Sun et al. 2001; Morrow et al. 1999; Tomita et al. 2000), and supports that C. elegans may share common mechanisms with mammals during gliogenesis.

A forward genetic screen reveals that loss-of-function of lin-32 results in ectopic AMsh glia

Finding a function of ngn-1 and cnd-1 in regulating AMsh number, we decided to carry out an unbiased forward genetic screen targeting
any mutants that possessed additional AMsh glia. We isolated a mutant, yad67, that possessed more than 2 cells labeled by the AMsh marker Pf16f9.3 (Figures 2A and 2B). The yad67 mutation was identified to affect the proneural gene lin-32, a homolog of Atoh1, consisting of a point mutation in the splice donor of its second intron (Fig. S1A). Rescue experiments confirmed lin-32 to be the gene involved, as expression of lin-32 under its own promoter consisting of its upstream 2kb sequence was able to fully rescue the ectopic AMsh phenotype in yad67 mutants (Figure 2C). Interestingly, expression of murine Atoh1 under the lin-32 promoter was also able to fully rescue the mutant phenotype, suggesting that this regulation of gliogenesis may be conserved (Figure 2C). Further testing of other alleles of lin-32 (tm2044, tm1446 and u282) showed that they all recapitulated the mutant AMsh phenotype observed in yad67 animals (Figures 2D, S1A). In particular, tm2044 is likely a null allele of lin-32 due to it containing a deletion spanning most of the gene, including part of the basic helix-loop-helix (bHLH) domain vital for regulating transcription. Thus, the similar phenotypes and penetrance between yad67 and tm2044 suggest that yad67 is likely a null allele of lin-32 (Figures 2D, S1A). The tm2044 allele will be used for most genetic analyses unless otherwise stated due to its similar phenotype with yad67 and the ease of genotyping. lin-32(tm2044) mutants possessed variable numbers of AMsh glia, with numbers ranging from 2 to 7 cells (Figure 2E). Furthermore, all the cells labeled by the Pf16f9.3 marker were also colabeled by two other AMsh markers Pf53f4.13 and Pt02b11.3 (Figs. S1B-S1D), supporting the conclusion that these additional cells are AMsh glia.

To determine whether this phenotype is limited to only AMsh cells, we also examined the amphid socket (AMso) cells, which are another type of glia that come from a different cell lineage (Figure 2A) (Sulston et al. 1983). A similar mutant phenotype was observed in the AMso cells, where approximately 48% of lin-32(yad67) animals possessed more than the usual pair of AMso cell observed in wild type animals (Figures 2F and G).

Given the important role of lin-32 in gliogenesis, we used AMsh glia to test other genes that have been shown to function together with lin-32 in regulating neuron fate determination and organogenesis, including hlh-2 which can heterodimerize with lin-32 to regulate neuronal specification (Portman and Emmons 2000), the parallel stz1 and hll-2 (Zhu et al. 2014), gain of function (n302) and loss of function (n941) alleles of the potential upstream lin-12/Notch (Greenwald et al. 1983; Sundaram and Greenwald 1993; Zhong and Sternberg 2006), and a Max homeobox homolog vab-15(u781) found to also regulate hypoderms to neuron transformations similar to lin-32. None were found to have significant ectopic AMsh cell phenotypes, suggesting they may not be involved in the formation of AMsh cells (Fig. S1E). However, hlh-2(tm1768) is not a null allele and vab-15(u781) is not a confirmed null, so it is possible that these genes may still play a role.

lin-32 suppresses a glial fate in different lineages during early embryogenesis

As lin-32 controls cell type determination in different neural lineages (Rojo Romanos et al. 2017; Zhao and Emmons 1995), we hypothesized that lin-32 would likewise function early in development to suppress an AMsh glial fate. As expected, expression of lin-32 in AMsh cells (Pf16f9.3), head neurons (Pdyf-7), AMso and other socket cells (Pglr-2), neurons including the AMsh sister URB neurons (Pflp-3), hypodermis (Pdyf-7), and pharyngeal muscles (Pmyo-2) were not able to rescue the additional AMsh phenotype (Figure 3A), likely due to these promoters turning on after the ectopic glial cells already arise. This is supported by transcriptomic data showing that AMsh, AMso and URB cells show little to no expression of lin-32 (Fig. S1F) (Packer et al. 2019). One earlier promoter is Plin-26, a known regulator of glial and hypodermal cell specification that is also required for proper AMsh cell specification (Labouesse et al. 1996). It is expressed in several cells of the AB lineage starting from around 100 min into embryonic development including the parent cell of AMsh (Packer et al. 2019). However, expression of Plin-26:lin-32 was not able to rescue the additional glia phenotype in lin-32(yad67) animals (Figure 3a), suggesting that lin-32 is required earlier or in a different set of progenitor cells.
Since many types of vertebrate glial cells have the ability to divide during development as well as after injury (Horner et al. 2000; Kornack and Rakic 2001; Fields and Burnstock 2006; Rusznák et al. 2016; Noctor et al. 2001), an alternative explanation is that the ectopic AMsh cells may emerge through additional proliferation. However, we found that the number of AMsh cells did not change when their number was traced in individual mutant animals from late larva to adults (Fig. S2A). Consistent with this finding, when the penetrance of the additional glial phenotype was quantified in late larval stage and D1 adult lin-32(yad67) animals, it was found that there was no significant difference in the penetrance between L1 and D1 worms (Figure 3B). Further supporting this, the additional AMsh glia can already be observed late in embryogenesis, shortly after the AMsh reporter P[lin-32::GFP] turns on (Figure 3C). Use of an integrated rescuing lin-32::GFP reporter (Yi et al. 2000) showed that lin-32 is expressed in different cells before the comma stage, when the AMsh cells begin to develop, and is not detectable in the AMsh cells when the P[lin-32::GFP] marker turns on (Figure 3D), nor is it present in the AMsh cells in D1 adults (Fig. S2B). These results suggest that lin-32 functions earlier to inhibit a AMsh glial fate rather than preventing glial proliferation.

The proneural genes lin-32, ngn-1, and cnd-1 restrict AMsh formation through independent means

Analysis of the penetrance of the additional AMsh phenotype in all the lin-32, ngn-1, and cnd-1 double and triple mutant combinations show more severe phenotypes, which suggests that they function independently of each other to regulate AMsh cell number (Figure 3E). Furthermore, the mean number of AMsh cells increases from the single mutants to the double or triple mutants (Figure 3F). However, no significant increase in mean AMsh cell number was detected between the lin-32;cnd-1 double mutant and the triple mutant despite the increase in penetrance, which could be due to a saturation of the cells that could be affected by these transcription factors (Figure 3F). On the other hand, loss of function of either cnd-1 or ngn-1 both lead to an increase in the number of cells expressing the P[lin-32::GFP] transcriptional reporter during the bean stage of embryogenesis, suggesting possible restriction of lin-32 expression by cnd-1 and ngn-1 (Fig. S2C). Loss of function of either lin-32 or cnd-1 did not significantly affect ngn-1 expression though (Fig. S2D). While there may be interactions between these transcription factors, they may affect different cell lineages or function during different time periods to restrict an AMsh glial fate.

Dorsal CEPsh cells mis-differentiate into AMsh cells in lin-32 mutants

AMsh cells and amphi neurons extend their dendritic tips through a process of retrograde extension, where the extracellular proteins DEX-1 and DYF-7 anchor the cell at the anterior end while it migrates posteriorly (Heiman and Shaham 2009). Thus, in dyf-7 loss of function mutants, the amphi neurons and AMsh glia exhibit a process extension defect where it fails to reach the tip of the nose.
combinations of *lin-32(tm2044)*, *nrgn-1(ok2200)* and *cnd-1(gk718)* backgrounds. Data are represented as mean ± SD. One-way ANOVA, followed by Tukey’s HSD test, *P* < 0.05 **P** < 0.01. ns, not significant. Each column represents three biological replicates of at least 50 worms each time. (B) The proportion of WT and *lin-32(yad67)* animals with additional AMsh cells during the L1 larval stage and D1 adult stage. Animals were quantified when they were D1 adults. Data are represented as mean ± SD. One-way ANOVA, followed by Tukey’s HSD test, *P* < 0.05 **P** < 0.01. ns, not significant. Each column represents three biological replicates of at least 50 worms each time. (C) Confocal images of AMsh cells labeled by *Pf16f9.3::GFP* during late embryogenesis in WT and *lin-32(yad67)* animals (left). Right column are merged with Nomarski images. White arrows indicate AMsh cell bodies. Scale bar, 10 μm. (D) Merged confocal images of a *Plin-32::GFP* expression reporter and a *Pf16f9.3::mCherry::H2B* AMsh marker (left). Right column are their respective Nomarski images. White arrows indicate Amsh cell nuclei. Scale bar, 10 μm. (E) Proportion of D1 animals with additional AMsh cells in the single, double and triple mutant combinations of *lin-32(tm2044), nrgn-1(ok2200)* and *cnd-1(gk718)* backgrounds. Each dot represents one animal, n > 80. Mean ± SD is represented in red. One-way ANOVA, followed by Tukey’s HSD test, *P* < 0.05 **P** < 0.01. ns, not significant.

(Heiman and Shaham 2009). We found that all *dyf-7* mutants exhibit defects in process extension (Figure 4A). Interestingly in *lin-32*/*dyf-7* double mutants, some animals actually possess AMsh cells that properly extend their processes to the nose and no process extension defects were observed in *lin-32* mutants (Figure 4A). When they were further divided into animals with 2 AMsh cells and those with more, it was found that the group with more than 2 AMsh cells had over 60% more animals with some AMsh that reach the nose tip (Figure 4B). While no AMsh cells adhere in the *dyf-7* single mutants, a small percentage of AMsh cells adhere to the nose tip in *lin-32*/*dyf-7* mutants with only two AMsh cells, which are likely the original AMsh cells. It could be that loss of *lin-32* may also affect the cell expression profile of these original AMsh cells as well. It is also possible that at least some of the ectopic AMsh cells may originate from a different cell type or lineage that utilizes a different mechanism for process extension (Cebul et al. 2020).

Each AMsh cell arises from an asymmetric division that yields a URB neuron and an AMsh glia (Fig. S1F). Thus, it is possible that the ectopic AMsh cells in the *lin-32* mutants arise from a failure of the parent cell to divide asymmetrically or that the potential URB cell fails to take on a neuronal fate. Next, *lin-32(yad67)* animals were D1 adults. Data are represented as mean ± SD. One-way ANOVA, followed by Tukey’s HSD test, *P* < 0.05 **P** < 0.01. ns, not significant. Each column represents three biological replicates of at least 50 worms each time.
Thus, these ectopic AMsh cells may arise at the expense of the CEPsh and AMsh cell number ($\rho=-0.62$, $r=-0.65$) (Figure 4E). CEPsh marker at early developmental stages (Figure 4D). Furthermore, CEPsh and AMsh cell numbers were quantified in $lin-32$ mutants, there was a strong negative correlation between CEPsh and AMsh cell number ($\rho=-0.62$, $r=-0.65$) (Figure 4E). Thus, these ectopic AMsh cells may arise at the expense of the distantly related CEPsh cells. These results suggest that $lin-32$ can independently promoting a neuronal one, similar to Neurog1 (Sun et al. 2001).

To determine whether the ectopic AMsh cells arise from $lin-32$-expressing cell lineages, cell death was induced in $lin-32$-expressing cells of wild type and $lin-32$ mutant animals by overexpressing the apoptotic caspase CED-3 under the $lin-32$ promoter (Ellis and Horvitz 1986; Shaham and Horvitz 1996). Overexpression of $Plin-32::ced-3$ did not significantly change the number of AMsh glia in wild type animals, suggesting that these wild type AMsh glia arise from outside of $lin-32$-expressing lineages (Fig. S1F and S2G). In contrast, there was a significant reduction in AMsh number in $lin-32$ mutant animals overexpressing $Plin-32::ced-3$, but did not fully remove all ectopic glia. This suggests that the ectopic glia at least in part arise from cells of the $lin-32$ lineage, though there may also be up to roughly 50% of ectopic cells that do not arise from $lin-32$-expressing lineages (Fig. S2G).

In $lin-32$ mutants, it was observed that roughly 27% of animals had 1-2 missing dorsal CEPsh glial cells (Figure 4C), which are derived from $lin-32$-expressing lineages (Murray et al. 2012; Packer et al. 2019), but the ventral two CEPsh cells that derive from cell lineages that very weakly express $lin-32$ are still present in $lin-32$ mutants (Fig. S1F). Thus, it is possible that ectopic AMsh cells may originate from cells originally fated to become dorsal CEPsh cells. In support of this, coexpression of the CEPsh marker $Phlh-17::GFP$ with the AMsh marker $Pf16f9.3::mCherry$ in $lin-32$ mutants revealed that certain ectopic AMsh cells also express the CEPsh marker at early developmental stages (Figure 4D). Furthermore, when CEPsh and AMsh cell numbers were quantified in $lin-32$ mutants, there was a strong negative correlation between CEPsh and AMsh cell number ($\rho=-0.62$, $r=-0.65$) (Figure 4E). Thus, these ectopic AMsh cells may arise at the expense of the distantly related CEPsh cells. These results suggest that $lin-32$ can function to specify glial fate among cells that come from distant cell lineages.

**DISCUSSION**

By analyzing $nog-1$/Neurog1 and $cnr-1$/NeuroD1 mutants, we show *C. elegans* share common mechanisms with mammals in gliogenesis. We then identified the role of a proneural gene $lin-32$ in regulating glial fate specification and show that $lin-32$ functions in parallel to $nog-1$/Neurog1 and $cnr-1$/NeuroD1. Furthermore, the role of $lin-32$ in glial fate specification appears to be independent of its function in neuronal fate determination and likely acts in progenitor cells to restrict an AMsh cell fate. There is also potential functional conservation of $lin-32$ in gliogenesis, as expression of murine Atoh1 fully rescued $lin-32$ mutant phenotypes.

LIN-32 belongs to a conserved bHLH-containing proneural gene family. The first member of this family atonal was identified in *Drosophila* and is required for formation of the chordotonal organ and photoreceptors (Jarman et al. 1993; Jarman et al. 1994; Jarman et al. 1995). Furthermore, it is required for generating the majority of glia in the antenna (Jhaveri et al. 2000; Sen et al. 2005). As one of the first known transcriptional factors expressed in inner hair cells, Atoh1 is required for fate determination of those cells, and misexpression of Atoh1 in other cells such as the glial-like support cells is sufficient to generate hair cells (Bermingham et al. 1999; Ben-Arie et al. 2000; Zheng and Gao 2000; Kawamoto et al. 2003; Izumikawa et al. 2005; Srivastava et al. 2013; Sayyd et al. 2019). In the cerebellum, Atoh1 is required for cerebellar granule neuron formation in addition to other neurons types in the parabrachial, lateral lemniscal, and deep cerebellar nuclei, while not found to be important for gliogenesis (Ben-Arie et al. 1997; Wang et al. 2005). *C. elegans lin-32* was first identified as an essential gene for the development of peripheral sense organs and has been shown to be important for the

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**Figure 4** Dorsal CEPsh cells mis-differentiate into AMsh cells in $lin-32$ mutants. (A) Confocal images of AMsh cells labeled by $Pf16f9.3::GFP$ in $dyf-7$ and $dyf-7;lin-32$ (tm2044) mutants. Outlines of the head are demarcated by the dashed lines. White arrowheads indicate the cell bodies of AMsh cells that fail to extend their processes to the nose tip while white asterisks indicate the cell bodies of AMsh cells that extend their processes to the nose tip. Scale bar, 10 μm. (B) Proportion of WT, $dyf-7$ and $dyf-7;lin-32$ animals that either possess no AMsh cells that extend processes adhering to the nose tips, some AMsh cells that extend processes adhering to the nose tips, or all AMsh cells that extend processes adhering to the nose tip. $dyf-7;lin-32$ animals were split into two groups based on whether or not they possess more than the WT 2 AMsh cells. Chi-square test, $^*P<0.05$ $^*^*P<0.01$ ns, not significant. n > 120. (C) The proportion of animals with fewer CEPsh cells labeled by $Phlh-17::GFP$ in $D1$ adults. Data are represented as mean ± SD. Student’s $t$-test, $^*P < 0.05$ $^*^*P < 0.01$ ns, not significant. Each column represents three biological replicates of at least 50 worms. (D) Confocal images of $lin-32$(tm2044) animals coexpressing the CEPsh marker $Phlh-17::GFP$ (left) and the AMsh marker $Pf16f9.3::mCherry$ (center). Asterisks denote CEPsh cells while white arrowhead points to cells expressing both AMsh and CEPsh markers. Scale bar, 10 μm. (E) Correlation of CEPsh and AMsh cell number. Spearman’s Rank-Order Correlation ($\rho=-0.62$) and Pearson Correlation ($r=-0.65$). 48 D1 adult animals were quantified.
development of different neuronal lineages (Zhao and Emmons 1995; Portman and Emmons 2000; Doitsidou et al. 2008; Rojo Romanos et al. 2017). Interestingly, it was also found that lin-32 activates the transcription factor zif-11, which is required for specifying a post-embryonic neuronal identity by repressing non-neuronal genes (Lee et al. 2019). Although the authors found that loss of function of zif-11 did not significantly affect embryonic neurogenesis, it is expressed in many cells of the AB lineage and may play a role in specification of other cell types as a downstream of lin-32. These studies highlighted the diverse roles of lin-32 and its homologs in regulating neuronal and glial fate determination and sensory organ formation. Here, we uncover a function of lin-32 in negatively regulating gliogenesis during embryonic development.

The function of Neurog1 and NeuroD1 in neuronal fate determination has been extensively investigated in different model organisms (Miyata et al. 1999; Morrow et al. 1999; Hallam et al. 2000; Sun et al. 2001; Bertrand et al. 2002; Ross et al. 2003; Hirabayashi and Gotoh 2010; Guo et al. 2014). Also, crossinhibitory activities of Neurog1 and Atoh1 have been shown to be essential for the specification of dorsal interneurons in mice (Gowan et al. 2001).

Our genetic data does not support interactions between ngn-1 and lin-32 in gliogenesis, as ngn-1;lin-32 double mutants show stronger phenotypes that are consistent with independent function. However, we do find that loss-of-function in ngn-1 or cnd-1 increase the number of cells that express lin-32 in embryos, while lin-32 does not appear to be important for ngn-1 expression (Fig. S2C and S2D), suggesting that the regulatory interactions among ngn-1, lin-32 and cnd-1 may be more complicated than our current understanding.

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