**ngn-1/neurogenin Activates Transcription of Multiple Terminal Selector Transcription Factors in the Caenorhabditis elegans Nervous System**

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**ABSTRACT** Proper nervous system development is required for an organism’s survival and function. Defects in neurogenesis have been linked to neurodevelopmental disorders such as schizophrenia and autism. Understanding the gene regulatory networks that orchestrate neural development, specifically cascades of proneural transcription factors, can better elucidate which genes are most important during early neurogenesis. Neurogenins are a family of deeply conserved factors shown to be both necessary and sufficient for the development of neural subtypes. However, the immediate downstream targets of neurogenin are not well characterized. The objective of this study was to further elucidate the role of ngn-1/neurogenin in nervous system development and to identify its downstream transcriptional targets, using the nematode Caenorhabditis elegans as a model for this work. We found that ngn-1 is required for axon outgrowth, nerve ring architecture, and neuronal cell fate specification. We also showed that ngn-1 may have roles in neuroblast migration and epithelial integrity during embryonic development. Using RNA sequencing and comparative transcriptome analysis, we identified eight transcription factors (hhf-34/NPAS1, unc-42/PROP1, ceh-17/PHOX2A, lim-4/LHX6, fax-1/NRZE3, lin-11/LHX1, tlp-1/ZNF503, and nhr-23/RORB) whose transcription is activated, either directly or indirectly, by ngn-1. Our results show that ngn-1 has a role in transcribing known terminal regulators that establish and maintain cell fate of differentiated neural subtypes and confirms that ngn-1 functions as a proneural transcription factor in C. elegans neurogenesis.

Defects during nervous system development are implicated in numerous neurological diseases that are polygenic in nature, such as autism spectrum disorder (ASD) and schizophrenia (Ho et al. 2008; Xiao et al. 2014; Happé et al. 2006; Stachowiak et al. 2013; Zhou et al. 2016). In addition, genome-wide association studies indicate that polymorphisms linked to ASD and schizophrenia often map to non-coding regions of the genome housing control elements for gene transcription (Freitag 2007; Ripke et al. 2011). These findings suggest that some neurodevelopmental disorders may manifest as the result of changes in gene regulation.

Some of the earliest acting regulators of neurogenesis are the neurogenin genes, which code for a family of basic-helix-loop-helix (bHLH) transcription factors (Yuan and Hassan 2014; Guillelott and Hassan 2017). These proteins form obligate heterodimers via interactions between their helix-loop-helix domains (Figure 1A). These heterodimers then bind to cis-regulatory regions of other genes via their “basic” domains, which contain multiple conserved basic amino acids that recognize the canonical E-box motif “CANNTG” (Figure 1B). This interaction either enhances or suppresses transcription of downstream target genes, although the identity of such targets is not well characterized (Massari and Murre 2000; Grove et al. 2009; Waterhouse et al. 2009).
Mammals have three neurogenin genes with neurogenin 1 and 2 active during neurogenesis, and a third (neurogenin 3) required for the development of pancreatic tissue and insulin-secreting cells (Sommer et al. 1996; Ma et al. 1998; Ma et al. 1999; Gu et al. 2002; Seo et al. 2007). Neurogenin 1 promotes neural development but actively inhibits astrocyte differentiation (Sun et al. 2001). In addition, homozygous neurogenin 2 null mutant mice have developmental defects in the forebrain, dorsal root ganglia, and distal cranial ganglia (Fode et al. 1998; Hand and Polleux 2011). These animals also exhibit defective axon targeting in the corpus callosum, aberrant projections across the midline, and deafferenciation of axon bundles. Finally, injection of mouse neurogenin mRNA into Xenopus embryos caused ectopic neurogenesis in ectodermal tissue (Ma et al. 1996). Together, these data suggest a critical role for neurogenins in the regulation of neurogenesis and suggests that neurogenins likely function near the top of a proneural fate specification cascade.

In humans, neurogenin 1 is associated with multiple neurodevelopmental disorders. Two single nucleotide polymorphisms associated with schizophrenia fall within the regulatory region of the neurogenin 1 locus (Fanous et al. 2007). In addition, a patient with congenital cranial dysinnervation disorder, a neurological condition characterized by facial paralysis including hearing loss and lack of facial expression, was found to have a homozygous deletion of the neurogenin 1 gene (Schröder et al. 2013). In another study, patients with homozygous mutations in neurogenin 3 were diagnosed with hypogonadotropic hypogonadism, a disorder characterized with hypoandrogenism, which rendered the summated stacks as 32-bit images. The head, from the nose tip to the posterior end of the pharynx was outlined using a segmented line and the region outside the line deleted using the Clear Outside function. Pixel values and counts were obtained using the Analyze - Histogram function (using the pixel value range) and expressed as arbitrary fluorescence units.

Lethality assays
L4 hermaphrodites were transferred to NMG Lite plates, one worm per plate. After 24 hr, each hermaphrodite was transferred to a new plate. 24 hr after the removal of the hermaphrodite, each plate was scored for dead embryos and live L1/L2 larvae. The plates were rescreened 48 hr later for live adults, and the number of adults was subtracted from the L1/L2 larval counts to determine larval lethality. Adults were transferred until they died or until no more viable embryos were observed. Dead embryo and larvae counts were compared to wild-type worms and statistical analysis was performed using Fisher’s exact test.

Embryo laying assay
Healthy well-fed young adults were transferred to plates containing fresh OP50 E. coli and allowed to lay embryos for one hour.

MATERIALS AND METHODS

Strains and maintenance
C. elegans strains were grown on nematode growth medium plates (NGM Lite) at 20°C as described previously (Brenner 1974). N2 (Bristol) was used as the wild-type strain. The following alleles were used in the course of this study: LGI dpy-5(e907) and mab-20(tx24), LGII vab-1(dx31), LGIII cnd-1(nu29), cnd-1(gk718) and unc-119(ed3), LGIV dau-10(ok480), ngn-1(ok2200) and efn-4(bx80), LGV him-5(e1490), LGX sax-5(ky123) and adh-1(hk20). Integrated transgenes used in the course of this study were Jus76 [unc-25p::GFP + lin-15(+)], mgls18 [txc-3p::RFP], nls394 [ngn-1::GFP + lin-15(+)], otl353 [ksl-1p::GFP], sIs1442 [phth-3p::GFP + dpy-5(+)], and uIs113 [pie-1::mCherry::Histone H2B + nhr-2p::mCherry::HIS-24 + unc-119(+)]. Extrachromosomal arrays used in this study were leEx1829 [unc-42p::GFP + unc-119(+)], quEx99 [sax-3(mingene) + odr-1p::RFP], and wEx37 [ngn-1p::GFP + unc-119(+)]. The chromosomal rearrangement mbl1 [mbl14p::dpy-10(e128)]. All worms were staged for imaging by using Fiji software (Rueden et al. 2017; Schindelin et al. 2012). For quantitative imaging of leEx1829 [unc-42p::GFP + unc-119(+)] and ngn-1(ok2200); leEx1829 [unc-42p::GFP + unc-119(+)], animals, the head regions of L1 larvae (collected within an hour of hatching) were captured as an image stack using a Zeiss LSM 700 confocal microscope at 40x magnification under identical settings, ensuring no detector saturation. Images were processed in Fiji using Z-project - Sum Slices workflow, which rendered the summed stacks as 32-bit images. The head, from the nose tip to the posterior end of the pharynx was outlined using a segmented line and the region outside the line deleted using the Edt – Clear Outside function. Pixel values and counts were obtained using the Analyze - Histogram function (using the pixel value range) then processed in Microsoft Excel (sum [pixel value x pixel count]) and expressed as arbitrary fluorescence units.

Microscopy
Well-fed worms grown under normal conditions were used to characterize expression patterns. Images were captured using either a Zeiss LSM 700 confocal microscope with Zen Black imaging software, a Zeiss AxioImager Z2 fluorescent microscope with Zen Blue imaging software, or an Olympus BX61 fluorescence microscope with CellSense-Dimension software. Adult worms were staged for imaging by transferring ~20 L4 hermaphrodites to a new plate and imaging those worms 24 hr later. Worms were mounted on 2% agarose pads, anesthetized with 3.5 µl of 10 mg/ml sodium azide solution, and then immobilized under an 18 x 18mm cover slip. Images were analyzed using Fiji software (Rueden et al. 2017; Schindelin et al. 2012). For quantitative imaging of leEx1829 [unc-42p::GFP + unc-119(+)] and ngn-1(ok2200); leEx1829 [unc-42p::GFP + unc-119(+)], animals, the head regions of L1 larvae (collected within an hour of hatching) were captured as an image stack using a Zeiss LSM 700 confocal microscope at 40x magnification under identical settings, ensuring no detector saturation. Images were processed in Fiji using Z-project - Sum Slices workflow, which rendered the summed stacks as 32-bit images. The head, from the nose tip to the posterior end of the pharynx was outlined using a segmented line and the region outside the line deleted using the Edt – Clear Outside function. Pixel values and counts were obtained using the Analyze - Histogram function (using the pixel value range) then processed in Microsoft Excel (sum [pixel value x pixel count]) and expressed as arbitrary fluorescence units.
Embryos were promptly picked to agarose pads and imaged at 40x magnification on an Olympus BX61 microscope equipped with DIC optics. Developmental timing was determined by counting the nuclei visible on the uppermost surface. Embryos with greater than ~30 nuclei visible were timed by counting nuclei along the anterior-posterior and left-right midlines, and multiplying the two values.

4-dimensional time-lapse microscopy of embryonic development

Two to four cell embryos were obtained by picking young adult hermaphrodites into a watch glass filled with M9 buffer then cutting each worm at the vulva using a number 10 blade scalpel. Embryos were transferred to a freshly made 2% agarose pad then sealed under a coverslip using a small drop of immersion oil at each corner. High resolution differential interference contrast (DIC) z-stack images (1 μm steps, 27–30 slices) were collected using a 63x magnification oil immersion objective on a Zeiss AxioImager Z2 microscope equipped with motorized z-axis stage. Data stacks were captured every 2 min for 300 cycles using Zen Blue software and a microscope equipped with motorized z-axis stage. Data stacks were magnified on an Olympus BX61 microscope equipped with DIC optics. Developmental timing was determined by counting the nuclei visible on the uppermost surface. Embryos with greater than ~30 nuclei visible were timed by counting nuclei along the anterior-posterior and left-right midlines, and multiplying the two values.

RNA extraction and sequencing

Wild-type and ngn-1(ok2200) mutants and were grown in liquid culture in M9 buffer supplemented with 1 mg/ml cholesterol, and 5 mls of 50% OP50 E. coli slurry as described previously (Hudson et al. 2006). Embryos were isolated by pelleting worms and resuspending in 1.2% NaOCl in 0.5M NaOH until only embryos remained, then washed three times and resuspended in M9 buffer. 200-300 μl of embryos were added to a mortar containing liquid N2 and ground to a fine powder then total RNA extracted using RiboZol (RIBOSOL) according to the vendor’s protocol and the aqueous phase separated by centrifugation in Maxtract High Density tubes (QIAGEN, Germantown, MD). The aqueous phase was precipitated using an equal volume of 100% isopropanol plus 3 μl of 20 ng/ml glycogen, the RNA pellet washed once in isopropanol, once in 70% ethanol then resuspended in 50μl of diethylpyrocarbonate (DEPC) treated water.

Figure 1 Overview of NGN-1/neurogenin structure, binding specificity, and sequence homology. (A) Schematic of NGN-1/neurogenin dimerization and DNA binding interactions. NGN-1 is a basic-helix-loop-helix (bHLH) transcription factor and is predicted to heterodimerize with another bHLH binding partner via its conserved helix-loop-helix domains (green and blue), while interacting with DNA via the basic domains (orange). (B) Illustration of the human neurogenin 1 preferential binding motif downloaded from the JASPAR database (http://jaspar.genereg.net/matrix/MA0623.2/). This conforms to a classic E-box “CANNTG” sequence. (C) Sequence homology of human, mouse, Xenopus, Zebrafish, and C. elegans NGN-1 neurogins. Amino acids in red are 100% conserved while amino acids in orange and yellow are structurally similar. Alignment created using Jalview. (D) Diagram of the ngn-1 locus showing the size and approximate breakpoints of the ok2200 deletion allele. The 3’ end of adjacent gene smf-3 is included for context and to demonstrate that the ok2200 allele is unlikely to interfere with smf-3 gene function. Figure 1A adapted from Roschger and Cabrele (2017). Figure 1B adapted from Fornes et al. (2020).
RESULTS

ggn-1 is required for embryonic viability and neuromuscular development

Beyond the role of ggn-1 in defining MI pharyngeal motorneuron cell fate, little is known about this deeply conserved gene in C. elegans nervous system development (Nakano et al. 2010). To further characterize ggn-1, we examined worms carrying the ok2200 mutant allele, which is a 2266bp deletion/7 bp insertion in the ggn-1 locus and predicted to be a null mutation (Figure 1D). In our study, ggn-1(ok2200) mutants showed 45% embryonic lethality (Figure 2A, Table 1). ggn-1(ok2200) animals bearing a ggn-1::GFP translational transgene (Nakano et al. 2010) are rescued for embryonic developmental defects, showing significantly less embryonic lethality (10% compared to 45% in ok2200 mutants alone, \( P < 0.01 \)), indicating that the embryonic lethal phenotype is caused by the ggn-1(ok2200) allele and not a closely linked second-site mutation. ggn-1 mutant animals that survived to adulthood showed sluggish, uncoordinated movement, aberrant head movement, and precocious embryo laying (Supplemental Figure 1). Overall, these observations suggest that ggn-1 is required for a broad array of neuromuscular functions.

To further investigate the role of ggn-1 in embryonic development, we scored whole broods of ggn-1 mutants for embryonic lethality and larvae that died before reaching adulthood (Table 1). In addition to the 45% embryonic lethality there was 10% larval lethality suggesting that ggn-1 is required for one or more processes essential to larval health (Table 1, 10.2% larval lethal, \( P = 0.005 \)).

To determine when ggn-1 mutants were dying during embryonic development, we used 4-dimensional (4D) time-lapse microscopy to observe developing ggn-1(ok2200) embryos from the 2-4 cell stage through just prior to hatching (10 hr of development, Figure 2B-D).
Table 1 Summary of embryonic and larval lethal interactions between ngn-1(ok2200), cnd-1 and canonical axon guidance and cell migration mutants. *P < 0.005; **P < 0.001; ***P < 0.0001, 2-tailed student t-test and Bonferroni correction for multiple comparisons. Note that double mutants were compared against the single mutant that showed the strongest phenotype. ngn-1; vab-1 and ngn-1; sax-3 double mutants could not be isolated away from balancer chromosomes or rescuing transgenes suggesting 100% embryonic lethality in the unbalanced strains.

| Genotype                  | Embryonic Lethality (SD) | Larval Lethality (SD) | Broods | N   |
|---------------------------|--------------------------|-----------------------|--------|-----|
| wild-type                 | 0.11 (0.2)               | 0.12 (0.2)            | 4      | 823 |
| ngn-1(ok2200)             | 45.9 (4.8) ***           | 10.2 (2.2) *          | 3      | 482 |
| ngn-1; ngn-1::GFP         | 9.8 (10.6) ***           | ND                    | 5      | 471 |
| cnd-1(1u29)               | 1.5 (1.4)                | 1.0 (1.2)             | 4      | 783 |
| ngn-1; cnd-1             | 45.5 (6.1)               | 26.1 (5.2)            | 8      | 1967|
| cnd-1(gk718)             | 7.9 (1.4)                | 12.4 (2.0)            | 6      | 1464|
| ngn-1; cnd-1(gk718)      | 51.7 (5.5)               | 18.7 (1.1) **         | 6      | 851 |
| daf-18(ok480)            | 3.3 (1.0)                | 0.3 (0.5)             | 6      | 1586|
| ngn-1 daf-18(ok480)      | 64.3 (5.4)               | 17.1 (5.6)            | 4      | 715 |
| sdn-1(1zh20)            | 7.7 (5.3)                | 5.5 (3.7)             | 5      | 868 |
| ngn-1; sdn-1(1zh20)      | 11.8 (8.5) *             | 10.4 (5.9)            | 5      | 656 |
| efn-4(1bx80)            | 14.3 (2.5)               | 8.0 (3.3)             | 6      | 927 |
| ngn-1 efn-4(1bx80)       | 60.1 (15.3)              | 13.1 (1.9)            | 3      | 389 |
| mab-20(1bx24)            | 15.2 (7.2)               | 5.0 (4.1)             | 5      | 905 |
| ngn-1 mab-20(1bx24)      | 32.3 (7.3)               | 3.1 (1.0) *           | 5      | 1510|
| vab-1(1dx31)            | 6.7 (5.0)                | 18.4 (4.1)            | 5      | 763 |
| ngn-1; vab-1(1dx31)      | ND                      | ND                    |        |     |
| sax-3(1ky23)            | 70.1 (17.2)              | 10.3 (8.2)            | 3      | 635 |
| ngn-1; sax-3(1ky23)      | ND                      | ND                    |        |     |

Surprisingly, only 8% of ngn-1 mutant embryos (2/24 observed) showed defects in gastrulation consistent with errors in neuroblast migration when compared to wild-type embryos (0/29 observed). Instead, the main arrest stage observed (25% of embryos) was due to a posterior ventral rupture during elongation (6/24 ngn-1(ok2200) embryos compared to 0/29 wild type). While only 33% of ngn-1(ok2200) embryos arrested during 4D imaging when compared to 46% when measured by whole brood analysis, this discrepancy is likely a result of the small sample size (n = 24) used in this assay. These data suggest that ngn-1 plays only a minor role in neuroblast migration but may have a non-cell autonomous role in posterior epithelial integrity.

ngn-1 is required for organization of the nerve ring and neuronal cell body location

To further understand the role of ngn-1 in development of the nervous system, ngn-1(ok2200) mutants were crossed with a kal-1p::GFP reporter gene, which labels a subset of neurons and glia in the head region (Supplemental Figure 2; Supplemental Table 2; Bülow et al. 2002). In wild type animals bearing the kal-1p::GFP reporter gene, the nerve ring (a tight bundle of neuronal processes where many of the worm’s synaptic connections are found) appears as a distinct narrowing of fluorescence around the isthmus of the pharynx (arrowhead, Figure 3A, B). In ngn-1 mutants, this nerve ring architecture is completely lost (arrowhead, Figure 3C, D). In addition, numerous cell bodies were anteriorly displaced and showed aberrant dendritic projections (Figure 3D). We characterized neuronal cell body displacement by counting the number of kal-1p::GFP-positive cell bodies in three regions of the head: the corpus, the isthmus, and the terminal bulb. In wild-type animals, an average of 18.5 kal-1p::GFP-positive neurons were visible in the head region (n = 9 worms scored) and 12.8 cell bodies (70%) were located in the isthmus region, with the remaining 5.7 (30%) clustered around the terminal bulb (Figure 3E). ngn-1 mutants showed around the same number of kal-1p::GFP-positive head neurons (17.8 average, n = 7 worms scored). However, many neurons were anteriorly displaced, with an average of 7.5 neurons (42%) now located in the corpus region (P < 0.001), 8.7 neurons in the isthmus (42%, P < 0.001) and only 1.7 neurons (9%, P < 0.001) in line with the terminal bulb. These data indicate the ngn-1 has roles in controlling both nerve ring architecture and neuronal cell body positioning.

ngn-1 controls AIY neuron axon outgrowth, polarity and cell fate

To gain a better understanding of ngn-1 function at the single cell level, we used a ttx-3p::GFP reporter gene to examine axon outgrowth in the AIY interneurons (Hobert et al. 1997). In wild type animals, the AIY left and right cell bodies typically lie below and just posterior to the pharyngeal terminal bulb and extend anterior processes that meet under the pharynx then enter the nerve ring, extending toward the dorsal side where they again make contact via a gap junction (Figure 4A and 4B) (White et al. 1986). In ngn-1 mutants, 98% of animals (49/50) show anteriorly displaced AIY cell bodies (Figure 4C and 4H). In addition, 100% of ngn-1 mutants (50/50, P < 0.001) showed defective dorsal axon extension, such that the AIYL and R axons fail to meet on the dorsal side (Figure 4C – E). The average AIY axon length in ngn-1 mutants was 12.3 μm (AIYL, n = 28) and 10.2 μm (AIYR, n = 34) compared with 28 μm (AIYL and R, n = 50 each) in wild type (Figure 4G). Some ngn-1 mutants (19% of neurons scored, 17/89) showed such severe outgrowth defects that the axons remained below the pharynx, appearing as a tangle (Figure 4D). We also observed loss of ttx-3p::GFP expression in 16% of ngn-1 mutants examined, resulting in only a single GFP-positive cell being apparent (Figure 4E and 4I). ngn-1(ok2200) animals carrying the ngn-1::GFP translational reporter gene showed complete rescue of cell body displacement and axon outgrowth defects, further confirming that the phenotypes observed were due to ngn-1 loss-of-function and not a closely-linked mutation (Figure 4F – H). Overall, these data indicate that ngn-1 is required for multiple neural developmental functions including cell body positioning, axon outgrowth, and axon guidance. In addition, ngn-1 has a partly redundant role in driving ttx-3.
expression, which is the terminal selector gene for AII fate, but likely functions in parallel with one or more additional genes in this process.

ngn-1 has no obvious role in controlling canonical neuroblast migration or axon guidance genes

Considering the axon guidance phenotypes seen in ngn-1 null mutants, it is likely that ngn-1 controls the transcription of multiple genes required for neural development. We took a candidate gene approach to identifying regulatory targets of ngn-1 based on the phenotypes observed in ngn-1(ok2200) mutants (Table 1). Many of these genes play a role in neuroblast migration or axon guidance during embryonic development, and loss-of-function mutations typically exhibit mild to strong embryonic lethality as a result of defects in these processes (George et al. 1998; Zallen et al. 1998; Chin-Sang et al. 2002; Hudson et al. 2006). We generated ngn-1; candidate gene double mutants and compared embryonic and larval lethality against each single mutant. Overall, the majority of the double mutant strains showed, at best, additive but not significant increases in embryonic lethality when compared to ngn-1 alone (Table 1), suggesting that ngn-1 does not exhibit strong genetic interactions with these known axon guidance cues.

To further examine the genetic interactions between candidate axon guidance genes and ngn-1 during axon outgrowth, ngn-1; candidate gene double mutants were assayed for axon outgrowth and guidance defects in the AIY interneurons (Wang et al. 2008; Christensen et al. 2011; Hartin et al. 2015; Schwieterman et al. 2016). Each double mutant showed similar average AII axon lengths when compared to ngn-1(ok2200) alone, suggesting that the ok2200 phenotype masks defects seen in each single mutant (Supplemental Figure 3). Based on these data, it was unclear if ngn-1 has any role in the transcriptional regulation of these axon guidance cues.

RNAseq identifies multiple ngn-1 transcriptional targets

RNA sequencing (RNAseq) is a powerful genetic tool to both identify and quantify gene expression (Conesa et al. 2016; Liao et al. 2014). As our candidate gene approach to identifying ngn-1 regulatory targets was unsuccessful, we used RNAseq to identify transcript differences between wild type and ngn-1(ok2200) mutant embryos. This unbiased approach was employed to characterize components of the ngn-1 gene regulatory network downstream of ngn-1. To generate a comparative transcriptome of wild type and ngn-1(ok2200) null mutants (n = 4 replicates each), RNA was isolated from mixed-stage embryos, sequenced, and analyzed. This generated a list of 587 differentially expressed genes with a p-value \( \leq 0.05 \) (Supplemental Tables 3 and 4). We found that the majority of these genes (497/587, 85%) were upregulated in the ngn-1 mutant background, suggesting that ngn-1 acts primarily as a transcriptional repressor during embryogenesis (Figure 5A). When we looked at the 40 most significant hits from the differential expression analysis based on p-value, five genes were down-regulated, three of which are predicted to code for transcription factors; ngn-1 (as expected from the comparative transcriptome of a deletion mutant), ceh-17, and hlb-34 (Table 2A). Six additional transcription factor genes had significantly lower transcript levels in ngn-1 mutants when compared to wild type (jux-1, lim-4, lin-11, unc-42, dp-1, and nhr-23), but were outside the 40 most significant hits (Figure 5A). Conversely, the up-regulated genes were primarily of uncharacterized function (n = 15/40). Whether ngn-1 controls the transcription of these genes directly or indirectly (via transcription of one or more intermediate factors or via non-cell autonomous mechanisms) is not known.

Our RNAseq assay generated an unbiased list of all C. elegans genes that are differentially regulated in ngn-1 mutants compared with wild type. We employed tissue enrichment analysis to clarify which tissues and cells our transcriptome hits associated with. To this end, we bioinformatically compared our transcriptome results against “gold-standard” curated lists of genes associated with single cell or tissue types (Angeles-Albores et al. 2016; Angeles-Albores et al. 2018). These reference datasets were generated by Fluorescence-Activated Cell Sorting of cells isolated from worms bearing cell or tissue-specific fluorescent labels, followed by RNAseq or microarray assay to determine gene expression profiles within those cell/tissue types. The bulk of our down-regulated targets identified by RNAseq were found to be associated with individual neuron subtypes such as the SIA neurons or proneural cell lineages derived from the AB founder cells, further supporting our data indicating that ngn-1 activates or enhances transcription primarily in neuronal tissue (Figure 5B). The SIA neurons play a key role in pioneering the architecture of the nerve ring suggesting that ngn-1 dependent transcription may have a role in defining SIA fate and/or function (Rapti et al. 2017). In contrast, the genes up-regulated in our ngn-1 transcriptome were significantly enriched in the excretory, intestine, and epithelial-associated genes (Figure 5C). This raises the possibility that ngn-1 may repress the transcription of non-neural genes in neural tissues. Alternatively, ngn-1 may suppress...
transcription in non-neuronal tissues via non-cell autonomous mechanisms.

We corroborated our tissue enrichment analysis by performing a gene ontology (GO) term enrichment analysis on our up- and down-regulated target genes (Table 3). Most of the terms enriched in the down-regulated dataset were related to transcription and DNA binding, consistent with ngn-1 having a role in activating gene transcription. Conversely, genes in the up-regulated dataset were mostly associated with cuticle and innate immune response terms. This suggests that ngn-1 may suppress transcription of target genes associated with the hypodermis including the innate immune response, possibly via non-cell autonomous mechanism(s).

To help clarify our understanding of how ngn-1 might be regulating transcription at the cellular level, we performed 4D-time-lapse video analysis of ngn-1p::GFP expression in the early embryo (Figure 6). We found strong ngn-1p::GFP expression in the ABpra/p cells (arrowheads, Figure 6C), along with weaker expression in ABar descendants (open arrowheads, Figure 6C). However, we also found strong expression in the MSpa/p cells (asterisks, Figure 6C) with weaker expression in MSaa/p (arrows, Figure 6C). The ABar and ABpr founder cells primarily give rise to neurons, consistent with ngn-1’s association with CNS-specific tissue enrichment datasets (Sulston et al. 1983). Also, ngn-1 expression in the ABar lineage correlates with its role in defining the MI pharyngeal motor neuron fate (ABaraappaaa) (Nakano et al. 2010). ngn-1’s role in the MS lineage is not known. However, pharyngeal neurons, muscles and accessory gland cells are derived from the MS lineage, suggesting that ngn-1 may have roles beyond the control of nervous system development.

Figure 4  ngn-1 is required for accurate cell body positioning, axon outgrowth and cell fate specification in AY interneurons. (A) Schematic showing AYL and R interneuron cell body positioning and axon location (image adapted from www.wormatlas.org). (B – F). Confocal micrographs of ttx-3p::GFP expression in (B) wild type, (C - E) ngn-1(ok2200) mutants, and (F) ngn-1(ok2200) mutants rescued with an ngn-1::GFP transgene (note that ngn-1::GFP is not visible in young adults). Arrowheads show AYL/R cell body location. These are anteriorly displaced in ngn-1 mutants. (D) AYL/R axons fail to extend to the dorsal side. (E) Only a single AY neuron is present. (G – I) Summary of wild type and ngn-1(ok2200) axon outgrowth and AY fate specification phenotypes. n = 50 worms analyzed for each strain. ***, P < 0.001, student t-test plus Bonferroni correction for multiple comparisons. Scale bar in panel D = 10 µm.

ngn-1 regulates expression of the paired-Like homeodomain transcription factor unc-42

To validate transcriptional targets downstream of ngn-1, we selected two transcription factor genes for further analysis, unc-42 and hkh-34. Both genes are significantly down-regulated in the ngn-1 transcriptome, suggesting that ngn-1 has a role in activating their transcription.

The paired-like homeodomain transcription factor unc-42 is orthologous to several mammalian genes including human Prop1
To validate our unc-42 transcriptome result, we crossed an unc-42p-GFP transgene (Hope et al. 2004) into the ngn-1(ok2200) mutant background and imaged L1 (first larval stage) larva via confocal microscopy. Wild type worms had an average of 13 unc-42p-GFP expressing head neurons (Figure 7A - C). When unc-42p-GFP expression was imaged in the ngn-1(ok2200) background, GFP expression was significantly lower ($P < 0.05$) and restricted to less than 10 cells (Figure 7D – G). Figures 7E and 7F show the same images as Figure 7E and 7F but with the contrast enhanced to make the cells more visible. We conclude that NGN-1 has two roles in controlling unc-42 expression. First, it controls the number of cells that express unc-42. Second, it controls unc-42 expression levels by up-regulating unc-42 transcription within those cells. Whether ngn-1 is required for the actual fate specification of unc-42-positive cells is not known.

ngn-1 regulates expression of the bHLH transcription factor hlx-34

We performed a similar GFP reporter gene analysis on hlx-34, which is predicted to code for a basic-helix-loop-helix transcription factor implicated in food-dependent behavioral adaptation via the AVJ interneurons, and is significantly down-regulated in our transcriptome (McKay et al. 2004; Grove et al. 2009; Lemieux et al. 2015). In concurrence with previous work, we confirmed that hlx-34p::GFP was expressed exclusively in the AVJ interneurons (Figure 7H – J). In ngn-1(ok2200) mutants, hlx-34p::GFP expression was strongly suppressed, with only 5% of animals showing any GFP expression (Figure 7K – M; 19/386 animals scored, $P < 0.001$). To confirm and validate the identity of the ngn-1(ok2200); hlx-34p::GFP strain, we crossed hermaphrodites with wild type males and scored F1 male progeny for the presence of GFP. All F1 males (anticipated genotype ngn-1(ok2200)/+; hlx-34p::GFP/+; ngn-1(ok2200); hlx-34p::GFP) were GFP-positive, confirming that our original strain was indeed homozygous at both the ngn-1(ok2200) and hlx-34p::GFP loci. We conclude that ngn-1 is almost exclusively required for hlx-34 transcription during development, but that there is a low-level, partial redundancy in the transcriptional activation of hlx-34 that can stochastically activate transcription in around 5% of animals.

**Discussion**

**ngn-1 has multiple roles in C. elegans development**

This study sought to characterize roles for the predicted proneural transcription factor ngn-1 in neural development and to identify downstream transcriptional targets using candidate gene and comparative transcriptomic approaches. Our data demonstrate roles for ngn-1 in embryonic epithelial integrity, fate specification, cell body positioning, and overall morphology of multiple neuron classes (Figures 3 and 4). These data expand on previous work that identified a role for ngn-1 in defining MI pharyngeal motorneuron fate (Nakano et al. 2010). The pleiotropic nature of ngn-1 mutant phenotypes suggests roles for this gene in neuronal cell migration/location and architecture of key neurological structures such as the nerve ring. Surprisingly, we found almost no genetic interactions with known neuroblast migration and axon guidance genes, although the deep penetrance of ngn-1 axon guidance phenotypes potentially masks any possible relationships (Table 1, Supplementary Figure 2). That being said, the transcriptional changes revealed in our ngn-1 transcriptome point to a number of avenues that might explain some of the phenotypes observed. First, we identified eight transcription factors whose transcription are either directly or indirectly under NGN-1 control. These data were confirmed for both unc-42 and hlx-34, whose expression is strongly suppressed in ngn-1 mutants suggesting...
that NGN-1 is required for their transcriptional activation (Figure 7).
By correlation, it is highly likely that lim-4, fax-1, lin-11, ceh-17, tlp-1, and nhr-23 transcription are also under direct or indirect NGN-1 control, suggesting that NGN-1 controls, in part, a cascade of at least eight downstream transcriptional regulators (Figure 8).

**NGN-1 has a role in nerve ring assembly**

Our kal-lp::GFP reporter gene data shows that ngn-1 has a role in directing nerve ring formation (Figure 3). This structure is initiated when SIA and SIB axons, along with the CEPshV glia, cluster together to form a process bundle that pioneers formation of the nerve ring (Kennerdell et al. 2009; Rapti et al. 2017). Followon neurons enter this track and reinforce its structure, with UNC-6/Netrin expression in CEPshV required for nerve ring axon guidance. kal-lp::GFP labels at least four cells required for nerve ring formation (SIAVL/R and SIBVL/R), making it a useful reporter for nerve ring structure (Supplemental Figure 2; Supplemental Table 2; Bülow et al. 2002). LIM-4 is expressed in a small number of cells including the SIA neurons (Sagasti et al. 1999). Our transcriptionome data show that lim-4 expression is controlled, in part, by ngn-1 (Supplemental Table 3; Figure 8). In addition, SIA neuron identity is strongly correlated with genes down-regulated in our ngn-1 transcriptome (Figure 5B). We speculate that ngn-1 activation of lim-4 transcription ultimately controls SIA fate, and/or expression of guidance cues expressed in SIA neurons, which helps establish nerve ring assembly.

**The role of ngn-1 in transcriptional regulatory cascades**

In addition to its role in establishing nerve ring architecture, we show that ngn-1 has roles in AIV interneuron axon guidance. However, NGN-1 likely has broader roles in interneuron specification and axon navigation. As mentioned above, both unc-42 and fax-1 transcription are down-regulated in ngn-1 mutants (Figure 7). Previous work demonstrated that unc-42 controls fax-1 expression in some contexts but works in parallel with it in others (Much et al. 2000; Wightman et al. 2005). fax-1 is required for expression of the NMDA receptor subunits nmr-1 and nmr-2 in the AVA and AVE interneurons, for opt-3 expression in the AVE neurons, and tlp-1 and ncs-2 expression

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**Table 2 ngn-1 transcriptome most significant hits by p-value. (A) Down-regulated genes. (B) Up-regulated genes. A. ngn-1 transcriptome down-regulated genes (most significant p-value) B. ngn-1 transcriptome up-regulated genes (most significant p-value)**

| Gene ID       | Gene Name | Base mean | log2(FC) | P-value   | P-adj   |
|---------------|-----------|-----------|----------|-----------|---------|
| WBGene00003595| ngn-1     | 283       | -3.9     | 3.2E-130  | 5.2E-126|
| WBGene00021766| hex-4     | 850       | -1.9     | 9.4E-63   | 7.8E-59 |
| WBGene00001816| haf-6     | 254       | -1.3     | 2.4E-22   | 1.0E-18 |
| WBGene00004440| ceh-17    | 43        | -1.3     | 1.3E-13   | 3.6E-10 |
| WBGene00011327| hih-34    | 17        | -0.8     | 1.8E-06   | 0.00076 |

### Down-regulated genes

| Gene ID       | Gene Name | Base mean | log2(FC) | P-value   | P-adj   |
|---------------|-----------|-----------|----------|-----------|---------|
| WBGene00268211| T02H6.12  | 49        | 1.6      | 5.08E-23  | 2.77E-19|
| WBGene0007097 | B0024.4   | 489       | 1.2      | 3.30E-14  | 1.08E-10|
| WBGene0007131 | pals-26   | 221       | 1.2      | 4.24E-13  | 9.89E-10|
| WBGene0016247 | C30B5.6   | 145       | 1.1      | 5.41E-12  | 1.10E-08|
| WBGene0008739 | F13D12.3  | 190       | 1.1      | 1.30E-11  | 2.36E-08|
| WBGene0014173 | ZK970.7   | 340       | 1.0      | 6.45E-11  | 1.05E-07|
| WBGene0011446 | T04F8.8   | 79        | 1.0      | 3.85E-10  | 5.72E-07|
| WBGene0013481 | Y69H12.3  | 456       | 1.0      | 8.34E-10  | 1.14E-06|
| WBGene0018725 | kreg-1    | 77        | 0.9      | 2.05E-09  | 2.58E-06|
| WBGene0018729 | F53A9.6   | 46        | 0.9      | 4.34E-09  | 5.06E-06|
| WBGene0010135 | F55H12.4  | 288       | 0.9      | 6.43E-09  | 7.00E-06|
| WBGene0012185 | W01F3.2   | 111       | 0.8      | 1.51E-08  | 1.55E-05|
| WBGene0017127 | E04F6.8   | 75        | 0.9      | 2.10E-08  | 2.02E-05|
| WBGene0017506 | F16B4.4   | 206       | 0.8      | 2.37E-08  | 2.07E-05|
| WBGene0007454 | CO8F11.7  | 20        | 0.9      | 2.41E-08  | 2.07E-05|
| WBGene0008301 | pals-39   | 33        | 0.8      | 2.74E-08  | 2.24E-05|
| WBGene0012961 | Y47H10A.5 | 178       | 0.8      | 3.00E-08  | 2.33E-05|
| WBGene0018730 | F53A9.7   | 48        | 0.8      | 6.17E-08  | 4.59E-05|
| WBGene00021977| YS8A7A.3  | 226       | 0.9      | 1.53E-07  | 1.09E-04|
| WBGene0012593 | nspe-7    | 69        | 0.7      | 1.63E-07  | 0.00011 |
| WBGene0011772 | T14G8.4   | 34        | 0.8      | 1.96E-07  | 0.00013 |
| WBGene0004222 | ptr-8     | 127       | 0.7      | 2.29E-07  | 0.00014 |
| WBGene00138721| pals-37   | 30        | 0.7      | 4.25E-07  | 0.00026 |
| WBGene0009130 | F25H5.8   | 30        | 0.7      | 4.43E-07  | 0.00026 |
| WBGene0007132 | pals-27   | 102       | 0.8      | 5.22E-07  | 2.94E-04|
| WBGene00022231| tyr-6     | 166       | 0.8      | 6.01E-07  | 0.00032 |
| WBGene00016788| c49g7.10  | 51        | 0.7      | 6.13E-07  | 0.00032 |
| WBGene00044900| cnc-11    | 39        | 0.7      | 6.71E-07  | 0.00034 |
| WBGene00015046| nlp-34    | 158       | 0.8      | 8.23E-07  | 0.00041 |
| WBGene00003765| nlp-27    | 66        | 0.7      | 1.01E-06  | 0.00049 |
| WBGene00016147| cyp-32A1  | 83        | 0.7      | 1.06E-06  | 0.00050 |
| WBGene00013489| col-42    | 126       | 0.7      | 1.29E-06  | 0.00059 |
| WBGene00010491| K02B7.3   | 1394      | 0.4      | 1.40E-06  | 0.00062 |
| WBGene00000560| cnc-6     | 70        | 0.7      | 1.65E-06  | 0.00071 |
| WBGene0007506 | C10C5.2   | 31        | 0.7      | 2.10E-06  | 0.00086 |
in the AVK neurons. This suggests that NGN-1 sets up a regulatory cascade of transcription factors that control aspects of AVA and AVE interneuron fate and function. Also, FAX-1 is expressed throughout the life of the animal, suggesting that it functions as a terminal selector gene to maintain aspects of terminal fate and function in these neuron subtypes (Wightman et al. 2005; Hobert 2016). As such, this places NGN-1 close to the head of a neurodevelopmental cascade, activating expression of an intermediate factor followed by a terminal selector, which can subsequently maintain its own expression (Figure 8).

**The role of transcriptional repression in embryonic development**

One of the surprising discoveries from our transcriptome study was NGN-1’s extensive role as a transcriptional repressor (Table 3; Supplemental Tables 3 and 4). Of the almost 500 genes whose transcription is significantly altered in ngn-1 mutants, over 70% of these were up-regulated, indicating that NGN-1 normally functions to repress such transcription. Single-cell transcriptomics show that NGN-1 is expressed in both the AB and MS lineages at the 16-cell stage (Tintori et al. 2016). These data are confirmed by our ngn-1p::GFP time-lapse imaging (Figure 6). Whether NGN-1 represses the transcription of non-neural genes in neural lineages such as those derived from the AB founder cells, or neural genes in primarily non-neural lineages such as those derived from the MS cells (or a combination of the two) is not known. Work is ongoing to identify early acting GFP reporter genes that can be used to investigate NGN-1 transcriptional activation vs. repression at the single cell level during early embryogenesis.

**Transcriptomes as a way to investigate transcription factor function**

Transcription factors and gene regulatory networks have been investigated in *C. elegans* using multiple techniques including genetic

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**Table 3** ngn-1 transcriptome gene ontology (GO) term most significant hits by p-value. (A) GO-terms associated with down-regulated genes. (B) GO-terms associated with up-regulated genes. *A. ngn-1 transcriptome Gene Ontology analysis: down-regulated genes (most significant p-value) B. ngn-1 transcriptome Gene Ontology analysis: up-regulated genes (most significant p-value)*

| Gene Ontology Term                                      | GO-term ID | Expected | Observed | Enrichment (FC) | P-value | P-adj |
|---------------------------------------------------------|------------|----------|----------|----------------|---------|-------|
| iron binding                                            | GO:0005506 | 0.6      | 5        | 8.4            | 3.3E-05 | 0.0042|
| tetrapyrrole binding                                    | GO:0046906 | 0.77     | 4        | 5.2            | 0.0011  | 0.069 |
| RNA polymerase II regulatory region DNA binding         | GO:0001012 | 0.77     | 4        | 5.2            | 0.0011  | 0.069 |
| transcription reg. region sequence-specific DNA binding | GO:000976  | 0.84     | 4        | 4.8            | 0.0017  | 0.069 |
| organic acid metabolic process                          | GO:0006082 | 2.2      | 7        | 3.1            | 0.002   | 0.069 |
| neurogenesis                                            | GO:0022008 | 1.4      | 5        | 3.7            | 0.0026  | 0.069 |
| regulatory region nucleic acid binding                  | GO:001067  | 1        | 4        | 3.9            | 0.0039  | 0.07  |
| double-stranded DNA binding                             | GO:003690  | 1.1      | 4        | 3.7            | 0.0048  | 0.074 |
| cellular developmental process                          | GO:0048869 | 3.9      | 9        | 2.3            | 0.0062  | 0.086 |
| immune system process                                   | GO:0002376 | 3.5      | 48       | 14             | 2.1E-40 | 2.6E-38|
| response to biotic stimulus                             | GO:0009607 | 2.8      | 33       | 12             | 3.1E-26 | 1.9E-24|
| extracellular region                                    | GO:0005576 | 8.2      | 43       | 5.3            | 3.6E-19 | 1.5E-17|
| extracellular space                                     | GO:0005615 | 4.4      | 30       | 6.9            | 3.1E-17 | 9.7E-16|
| metalloendopeptidase activity                           | GO:004222  | 1.5      | 10       | 6.6            | 4.1E-07 | 1.0E-05|
| membrane                                               | GO:0016020 | 97       | 137      | 1.4            | 1.7E-05 | 0.00035|
| peptide activity                                        | GO:0008233 | 6.7      | 18       | 2.7            | 5.5E-05 | 0.0097|
| neuropeptide signaling pathway                          | GO:0007218 | 1.8      | 8        | 4.3            | 0.00011 | 0.0017|
| intrinsic component of membrane                        | GO:0031224 | 85       | 118      | 1.4            | 0.00018 | 0.0026|
| cation binding                                          | GO:0043169 | 24       | 38       | 1.6            | 0.0022  | 0.027 |
| incorrect protein topology response                     | GO:0035966 | 2.8      | 8        | 2.8            | 0.0023  | 0.027 |
| zinc ion binding                                        | GO:0008270 | 7.8      | 16       | 2.1            | 0.0025  | 0.027 |
| lytic vacuole                                           | GO:0000323 | 1.4      | 5        | 3.5            | 0.0033  | 0.032 |
| molting cycle                                           | GO:0042303 | 1.6      | 5        | 3.1            | 0.0062  | 0.055 |
| structural constituent of cuticle                       | GO:0042302 | 2.4      | 6        | 2.5            | 0.011   | 0.091 |
| protein catabolic process                               | GO:0030163 | 4.9      | 10       | 2              | 0.011   | 0.091 |
| collagen trimer                                         | GO:0005581 | 2.4      | 6        | 2.5            | 0.012   | 0.091 |

**Figure 6** ngn-1p::GFP is expressed in AB and MS lineages during early embryogenesis. (A) ngn-1-GFP expression pattern 160 min post-first cell division. (B) Parallel DIC image. (C) Panel merge. Arrowheads, ABpra/p cells; asterisks, MSpa/p cells; arrows, MSaa/p cells; open arrowheads, ABar descendants.
Figure 7 unc-42 and hlh-34 reporter gene expression is reduced or eliminated in ngn-1(ok2200) mutants. (A – C) unc-42p::GFP expression in wild type and (D – F) ngn-1 mutant L1 larvae. E’ and F’ show the same images as E and F but with contrast enhanced to show the number of cells expressing GFP. (G) Quantitative analysis of unc-42p::GFP expression in wild type and ngn-1(ok2200) mutants (* P < 0.05, student’s t-test). Error bars show standard error of the mean and gray circles show individual data points. (H – J) hlh-34p::GFP expression in wild type and (K – M) ngn-1 mutant young adults. Scale bar in panels A and H = 20 μm.
whose transcript levels changed significantly. In particular, we identified \textit{hlh-34} as a \textit{ngn-1} target (Figure 7). Although \textit{hlh-34} is only expressed in a single pair of cells (the AVJL/R interneurons), we still had the statistical resolution to identify this \textit{ngn-1} target. Overall, this suggests that comparative transcriptomics offers a powerful general approach to identifying transcription factor targets during embryogenesis. Future work will refine our transcriptomics approach by taking this to a single cell or single embryo level to provide tighter analysis of either cell lineage or developmental timing.

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