A Developmental Timing Switch Promotes Axon Outgrowth Independent of Known Guidance Receptors

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Introduction

During development, neurons must extend axons in the correct direction and at the proper time. Yet while several conserved families of ligands and receptors have been identified that control axon guidance [1–2], little is known about the temporal regulation of axon growth. The observation that guidance cues can promote axon elongation as well as turning in vivo has led to models in which the timing of receptor gene expression and/or function specifies when axons extend [1–4]. However, it is clear that additional mechanisms must ensure that axon growth and guidance are appropriately coupled. For instance, in the C. elegans hermaphrodite specific motor neurons (HSNs), the UNC-40/DCC Netrin receptor is up-regulated at the L1 larval stage and ventrally localized by the L2, yet the HSNs do not extend single axons toward the ventral UNC-6/Netrin source until early L4, nearly two stages later [5]. In addition, HSN axons growth at the correct stage in animals lacking UNC-6/Netrin or UNC-40/DCC, suggesting that the timing of axon elongation is regulated independently of Netrin signaling [5]. The C. elegans HSNs provide a convenient system for further investigating the temporal control of axon elongation. Many of the principle molecules required for C. elegans axon growth and guidance are conserved in higher animals [5–10]. In addition, the morphological features of HSN development have been very well characterized [5]. At the mid-to-late L3 larval stage, the neurons extend several short neurites ventrally, and at the L3/L4 transition, they retract all but one of these processes, the neurite selected to become the axon. In the L4, the axon completes its extension toward the ventral nerve cord (VNC) and turns anteriorly, where it forms synapses with vulval muscles and the VC4 and VC5 motor neurons before growing to the nerve ring [5,11].

We predicted that genes which regulate the timing of cell divisions might also control HSN postmitotic differentiation. The C. elegans transcription factor lin-14 has previously been shown to play a similar role during synaptic development of the DD motor neurons [12–14]. lin-14 is a member of the heterochronic pathway, a set of temporal patterning genes that was first characterized in mitotic hypodermal cells [15–18]. Several other heterochronic genes are also expressed in the C. elegans nervous system, including those encoding the lin-4 microRNA, the cytoplasmic RNA-binding protein LIN-28, nuclear hormone receptor DAF-12 and the HBL-1 transcription factor [19–25]. However, the neuronal functions and interactions of these genes with lin-14, if any, have not yet been determined.

MicroRNAs (miRNAs) represent a set of non-coding RNAs that inhibit genes post-transcriptionally by binding to partially complementary mRNA sequences [19,20,26,27]. While they are dynamically expressed during the development of invertebrate and vertebrate nervous systems, only a small number have been characterized in vivo [28–31]. Here, we show that the lin-4 miRNA together with lin-14 and lin-28 comprise a cell-autonomous switch that promotes axon growth. These genes act independently of ventral guidance receptors to ensure that axon development is properly completed before the HSNs are required in the adult egg-laying system [32].
Author Summary

During development, nerve cells extend long structures called axons which are required for communication across distant brain regions and/or with other tissues. Many of the signals controlling the direction of axon extension are well understood, but much less is known about the factors that control when this growth occurs. Ultimately, failure to coordinate the direction and timing of axon elongation could lead to dramatic defects in the way our brains function. By studying the simple soil nematode C. elegans, we have identified mutations in three timing genes that result in either premature or delayed axon growth. These genes function independently from known directional signals as well as axon position. Since two of the genes are shared with mammals, findings presented here will help expand our understanding of human axon development, and could potentially lead to novel strategies for promoting growth in injured adult nerve cells.

Results

The lin-4 miRNA promotes HSN axon elongation

Candidate heterochronic mutants were crossed into a transcrip-
tional reporter strain expressing myristoylated green fluorescent protein (GFP) under the control of the unc-86 promoter [5]. unc-86 encodes a POU homeodomain transcription factor that is expressed constitutively after HSNs exit the cell cycle and controls execution of late maturation events [33,34]. In this work, the phenotypic criterion for distinguishing HSNs from less mature neurites was initiation of the anterior turn (Figure 1A).

Loss-of-function (lf) mutants for the lin-4 miRNA displayed a penetrant retarded phenotype, in which HSN axons were not detected in L3 or L4 animals or the majority of young adults scored (Figure 1A and 1B). Sporadic neurite outgrowth was observed in lin-4(lf) HSNs after mid-L4, but was distinct from the extensive neurite outgrowth patterns that precede axon extension in wild-type animals (Figure 1A). In the few young adults with detectable axon outgrowth, processes were often morphologically abnormal (Figure 1C). Since a percentage of older adults also extended axons (data not shown), it is likely that this lin-4(lf) phenotype represents a developmental timing defect and not simply a uniform inability to initiate growth.

We predicted that if lin-4 acts as a temporal regulator of axon development, then lin-4 over-expression would lead to precocious axon elongation, opposite to the phenotype observed in lin-4(lf) mutant animals. MiRNAs can be ectopically expressed in vivo using cell- or tissue-specific enhancers or promoters [35], and ∼80 nucleotides (nt) of both upstream and downstream sequences have been shown to be sufficient for processing in vitro [36]. To over-express lin-4 in the HSNs, the lin-4 hairpin and ∼160 nt of total flanking sequence were sub-cloned behind the unc-86 promoter, and the resulting construct was injected into wild-type N2 animals. Control lines were generated using the promoter, and the resulting construct was injected into unc-86, [36]. To over-express sequences have been shown to be sufficient for processing in vivo lin-4(lf)

Previously, lin-14 loss-of-function mutations were found to be more robust in suppressing lin-4(lf) hypodermal phenotypes than those for lin-28 [15,46], suggesting that in the hypodermis, lin-4 interacts more strongly with lin-14. In addition, loss-of-function alleles for lin-14, but not lin-28, were sufficient to restore vulval development and dauer formation in lin-4(lf) mutants [46], making it likely that lin-14 genetically interacts with lin-4 in greater subset of cells. To test whether lin-14 and lin-28 function with lin-4 to control HSN axon outgrowth, lin-4(lf);lin-14(gf) and lin-4(lf);lin-28(gf) double mutants expressing the unc-86:myr-GFP reporter were scored for axon extension. Strikingly, the presence of either lin-14(gf) or lin-28(gf) almost completely suppressed lin-4(lf), resembling the observations for lin-14(gf) and lin-28(gf) single mutants (Figure 2A and 2B).

The daf-12 nuclear hormone receptor has been shown to interact with lin-14 and lin-28 in the hypodermis, and a gain-of-function allele of the gene leads to retarded seam cell development [25,47,48]. Recently, daf-12 was also found to regulate the extension of C. elegans muscle arms, membrane protrusions that are required for forming productive contacts between muscles and motor neurons [49]. We tested whether the DAF-12 receptor could also play a role in the HSN axon growth response, but failed to detect changes in the timing of elongation in gain-of-function (rh61) or null (rh61 rh411) alleles. As seen in wild-type, no axons extended during the L3 in either mutant (n = 51 for rh61 and n = 60 for rh61rh411) while nearly all displayed outgrowth in the L4 [98.1% (n = 52) for rh61 and 94.3% (n = 53) for rh61rh411]. In addition, when we crossed the daf-12 null mutant into lin-4(lf), we did not observe axon extension at the L4 (n = 54) or adult (n = 25)

The heterochronius genes lin-14 and lin-28, but not daf-12, inhibit axon extension in the HSNs

In the C. elegans hypodermis, lin-4 controls the execution of L1- and L2-stage cell division patterns by inhibiting lin-14 and lin-28, respectively [19,20,24]. Seven lin-4 complementary elements (LCs) have been identified in the lin-14 3'UTR and one has been found in lin-28, consistent with both genes being direct targets of lin-4 [37]. Unlike lin-14, lin-28 is conserved in Drosophila and vertebrates, and along with the lin-4 homolog miR-125, is widely expressed in the vertebrate nervous system [30,31,38–40]. LIN-28 has also been shown to be one of four ‘stemness’ factors that are capable of reprogramming differentiated mouse fibroblasts to a pluripotent stem cell state, in part by inhibiting processing of the let-7 pri-miRNA [38,41,42].

In animals with loss-of-function mutations in lin-14 or lin-28, axons were detected prematurely during the L3 stage (Figure 2A, 2B, 2Cii, and 2Civ). Neurite outgrowth was also precocious (Figure 2Ci and 2Ciii), indicating that these phenotypes are shared with mammals, findings presented here will help expand our understanding of human axon development, and could potentially lead to novel strategies for promoting growth in injured adult nerve cells.
stages, indicating that in contrast to lin-14 and lin-28, def-12 does not act downstream of lin-4 to control HSN axon growth.

The lin-4 miRNA and both lin-14 and lin-28 are reciprocally expressed in the HSNs

The expression pattern of lin-4 in the HSNs was determined using an integrated lin-4::GFP reporter strain [50]. GFP was first detected in the late L1 and values progressively increased in later stages until they spiked in the L4 (Figure 3A and 3D). These data highlight the dynamic tissue-specific changes in lin-4 expression and contrast with the pattern determined by northern analysis using RNA from whole animals, in which lin-4 was up-regulated in the late L1 and then remained at relatively constant levels through the remainder of larval development [51].

To characterize lin-14 and lin-28 expression, GFP was quantified in the HSNs in strains containing integrated lin-14::GFP or an extrachromosomal array of lin-28::GFP. Both reporter constructs were previously shown to rescue lin-14 and lin-28 mutant phenotypes, respectively, and the lin-14::GFP transgene was found to display expression patterns that were consistent with results from anti-LIN-14 antibody staining [24,43]. In the HSNs, lin-14 and lin-28 displayed reciprocal expression patterns to lin-4, in which they were expressed at their highest levels in the L1, and became undetectable in later larval stages [19,20,24]. However, removal of three nucleotides from the

Figure 1. lin-4(lf) displays delayed HSN axon extension. (A) In late L3 wild-type (wt) animals (top), HSNs extended multiple neurites (arrow) in the ventral direction (left). By early L4 (middle) and in the adult (right), wild-type animals extended a single HSN axon ventrally and then anteriorly. VNC: ventral nerve cord. In L3, L4, and adult lin-4(lf) animals (bottom), no neurites or axons were typically seen. (B) Percentage of L3, L4, or adult wild-type or lin-4(lf) animals in which HSN axons had completed their anterior turn. n=100 for each time point. (C) Image of one of the few lin-4(lf) adult animals with axon outgrowth (top). When the region contained by the box is magnified (bottom), variation in axon diameter is clearly visible. (D) Cell-autonomous over-expression of the lin-4 O/E construct led to precocious neurite outgrowth in early L3 (i) and axon elongation by late L3 (ii), while over-expression of the control construct resulted in neurite outgrowth, but not axon elongation, by late L3 (iii). (E) Percentage of L3- or L4-stage animals with axon extension in lin-4 over-expression (O/E) or control (CON) lines. All lin-4 O/E (Lines 3, 4, and 5) and control (Lines 2, 7, and 17) strains contained the integrated unc-86:myr-GFP reporter to visualize axon outgrowth. n=50 for each time point. In A, C and D, the arrowheads point to the PLM axon, scale bars represent 5 μm, and anterior is depicted to the left and ventral is down. In A (wt L3), Di and Diir, the arrow points to one of several neurites, and in A (wt L4 and Adult), C, and Dii, it refers to the HSN axon’s anterior turn. For B and E, error bars represent standard error of proportion.
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LCE resulted in only a modest increase in expression of a transgene containing a hypodermal promoter fused to lacZ and the lin-28 3'UTR [47]. Several additional cis regulatory elements as well as trans-acting factors, including the protein LIN-66, have now been shown to be important for regulation of lin-28 expression in the hypodermis [47,48].

To investigate whether lin-4 targets lin-28 directly in the HSNs, the changes in GFP expression relative to L1 values were determined for a lin-28::GFP transgenic line in which the LCE was deleted (Figure 4A, 4B, 4D, and 4E). Consistent with a direct regulatory role for lin-4, relative GFP levels were elevated in the absence of the LCE compared to the control (Figure 4A, 4D, and 4E). However, in contrast to the pattern observed in lin-4(lf) mutants, LIN-28::GFP expression partially declined in the L3 stage (Figure 4B). This trend was also observed in a second lin-28::GFPΔLCE line (data not shown), suggesting that lin-4 also regulates lin-28 indirectly through an LCE-independent mechanism.

In the hypodermis, lin-14 is required for maintenance of lin-28 expression in a lin-4(lf) mutant background [24]. To test whether it is also a positive regulator of lin-28 in the HSNs, GFP expression was quantified in lin-14(lf) mutants that were maintained at the restrictive 23°C temperature (Figure 4C, 4F, and 4G). Introduction of the lin-14 mutant allele resulted in a significant decrease in GFP expression in the HSNs at the L2 and L3 stages, leading to complete down-regulation of the reporter one stage earlier than in wild-type animals grown under the same conditions (Figure 4C, 4F, and 4G). This result indicates that one mechanism by which lin-4 indirectly controls lin-28 expression in the HSNs is through the down-regulation of lin-14.

lin-4 functions cell-autonomously to control HSN axon elongation

Since lin-4 was found to be expressed and to genetically interact with lin-14 and lin-28 in the HSNs, it likely acts cell autonomously.
to regulate the timing of axon development. To test this possibility directly, transgenic lines over-expressing \textit{lin-4} under the control of the \textit{unc-86} promoter (Figure 1D and 1E) were crossed into \textit{lin-4(lf)} and scored for rescue of the \textit{lin-4} retarded phenotype. The \textit{unc-86} promoter has previously been used to test autonomous function or drive transgene expression in the HSNs [5,52,53], in large part because it is not expressed in neighboring tissues, including the ventral nerve cord or cells of the egg-laying system.

Precocious HSN axon extension was observed for all three lines, likely due to the predicted over-expression of \textit{lin-4} from the multi-copy transgene (Figure 5A–5C). In addition, removal of the mature \textit{lin-4} sequence from the rescuing construct resulted in a failure to restore axon extension in \textit{lin-4(lf)} mutants (Figure 5A, 5D, and 5E), demonstrating that the effect was specific. This rescue of retarded axon growth was unlikely to be due to extrinsic signaling from other \textit{unc-86}-expressing cells like the PLMs.

Figure 3. \textit{lin-4} and its targets \textit{lin-14} and \textit{lin-28} are expressed reciprocally during larval development. (A) In an integrated \textit{lin-4::GFP} reporter strain, GFP is up-regulated in the HSNs during larval development. The y-axis depicts raw average pixel intensity values, and \( n = 10 \) for each time point. Note that the change in pixel intensity after L3 and Adult stages exceeded the dynamic range of the camera, and at the set exposure time, only pixel intensity values for L1, L2, and L3 were always within the linear range. Some images acquired for late L4 and young adult stages were oversaturated, and thus the changes in pixel intensity after L3 are likely to be under-representations of the true fold increases in GFP expression. EL1–4: Early Larval Stage 1–4, LL1–4: Late Larval Stage 1–4, YAd: Young Adult. (B) GFP expression from an integrated \textit{lin-14::GFP} reporter was down-regulated in the HSNs by the L2 stage, while in \textit{lin-4(lf)} mutants, GFP expression was maintained. \(*p\text{-value} < 0.01\) (0.005 for L2 and 0.003 for L3), \( **p\text{-value} = 0.015 \). (C) A \textit{lin-28::GFP} reporter strain (Line 10-2) was down-regulated in the HSNs during L2 and L3 stages. When it was crossed into \textit{lin-4(lf)}, GFP expression persisted throughout larval development. \( *p\text{-value} = 0.012 \). \( **p\text{-value} = 0.001\) (2.5 \( \times \) 10\(^{-5} \) for L3 and 6.3 \( \times \) 10\(^{-7} \) for L4). For B and C, \( n \geq 10 \) for each time point. Note that for each strain, pixel intensity values for L2, L3, and L4 were normalized to average L1 values and do not represent absolute fluorescence intensities. In addition, the \textit{lin-14::GFP} strain expressed GFP more weakly than the \textit{lin-28} reporter during the L1, and thus a much smaller decrease in relative pixel intensity was required for complete down-regulation of the \textit{lin-14::GFP} transgene. \( p\text{-values} \) for differences in relative pixel intensity were obtained using the two-sample t-test. In (A–C), all error bars represent standard error of the mean (S.E.M.). (D) Representative images of HSNs from L1 (top) and L4 (bottom) animals bearing \textit{lin-4}, \textit{lin-14}, or \textit{lin-28} GFP reporters in wild-type or \textit{lin-4(lf)} backgrounds as noted. Arrowhead: HSN. Scale bars represent 5 \( \mu m \), and anterior is to the left and ventral is down. doi:10.1371/journal.pgen.1001054.g003
Precocious HSN axon outgrowth was still observed upon lin-4 over-expression when HSNs were dorsally displaced relative to the PLMs, such as in unc-40(e721) mutants (data not shown; [5]). Rescue was also likely not due to the export of lin-4 to adjacent cells, since the lin-4(lf) vulval-less defect persisted in animals expressing unc-86::lin-4 (Figure 5F and 5G; [26]).

To confirm independently that functional lin-4 is generated in the lin-4 over-expression (O/E) lines, representative O/E (Line 4) and control (Line 7) lines were crossed into a lin-28::GFP sensor (Line 10-2) whose down-regulation is known to be lin-4-dependent (Figure 3C and 3D). A comparison of average pixel intensity values in L2-stage HSNs showed that GFP levels were lower in the lin-4 O/E line compared to the control, as would be expected if functional lin-4 were expressed in the HSNs (Figure 5H–5J).

Precocious axon outgrowth is not dependent on unc-40/DCC or sax-3/Robo

Axon guidance factors not only control the direction of growth cone migrations, but they also promote axon outgrowth [3]. In the HSNs, the Netrin receptor UNC-40/DCC is known to be required cell-autonomously for cellular polarization as well as for directing ventral axon extension [5]. UNC-40 is up-regulated during the late L1 stage and by L2 is localized primarily to the ventral surface in response to the release of UNC-6/Netrin from the VNC [5,55]. Interestingly, HSN axons extend two larval stages

Figure 4. Regulation of lin-28 by lin-4 and lin-14 changes over developmental time. (A) Relative GFP levels were higher for lin-28::GFP::LCE (Line 5-1) lacking the LCE than for lin-28::GFP (Line 10-2) with an intact 3’UTR at the L2, L3, and L4 stages. **p-values=0.01 (0.005 for L2, 0.01 for L3, and 0.002 for L4). (B) Relative GFP intensity was lower at the L3 stage in lin-28::GFP::LCE (Line 5-1) lacking the LCE than in lin-28::GFP (Line 10-2) crossed into lin-40(lf), *: p-value = 0.030. For A–B, changes in LIN-28::GFP levels across larval development in animals lacking the LCE or lin-4 exceeded the dynamic range of the camera. At the set exposure time, some images acquired for late L4 were oversaturated. n=10 for each strain and time point. (C) Relative lin-28::GFP (Line 10-2) expression was lower in L2- and L3-stage HSNs in lin-14(lf) compared to wild-type at 23°C. n≥9 for each lin-28::GFP condition and n≥18 for each lin-14(lf); lin-28::GFP condition. *: p-value = 0.019, **: p-value = 0.001. For A–C, data were normalized to L1 values for each strain, p-values for differences in relative pixel intensity were obtained using the two-sample t-test, and error bars represent S.E.M. (D,E) Representative L1- (D) and L4-stage (E) HSNs from lin-28::GFP::LCE (Line 5-1). (F,G) Representative L1- (F) and L4-stage (G) HSNs from lin-28::GFP (Line 10-2); lin-14(lf). For (D–G), scale bars represent 5 μm, and anterior is left and ventral is down. Arrowhead: HSN. Arrow: CAN neuron. doi:10.1371/journal.pgen.1001054.g004
either the lin-4 hairpin or a control (I') construct, acquired with GFP(BP) (H and I') or TRITC (H' and I') filters. The scale bars in B–I' represent 5 μm, and anterior is to the left and ventral is down. (J) The mean pixel intensity in 10 L2-stage HSNs from the lin-4 O/E and control strains. *: p-value = 0.021 using a two-sample t-test for the difference between two means. Error bars represent S.E.M.

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after this Netrin response [5], suggesting that Netrin signaling alone is not sufficient to induce growth. This conclusion is further corroborated by the finding that HSN axons are misguided but elongate at the appropriate time in mutants for unc-6/netrin or unc-40/DCC [5].

To determine whether the lin-4 switch functions as part of this Netrin-independent timing mechanism, lin-14[lf]; unc-86::myr-GFP animals were crossed with animals containing either a severe loss-of-function (e271) or null (e1430) allele of unc-40 [6,43,56]. In both double mutants, precocious axon outgrowth was observed at the L3 stage (Figure 6A). Premature axon extension was also observed in lin-14[lf] animals lacking functional SAX-3/Robo, a receptor which acts in parallel to UNC-40 to promote ventral growth in the HSNs (Figure 6A) [57]. While guidance defects for unc-40(e271), unc-40(e1430), and sax-3(ky123) were incompletely penetrant as previously described [5,57], the precocious phenotypes observed in all three double mutants were not confined to those animals with intact ventral outgrowth (Figure 6B).

Since our genetic and expression data demonstrated that lin-4 acts upstream of lin-14, we predicted that lin-4 – like lin-14 – also controls the timing of axon growth independent of ventral guidance. To confirm this genetically, unc-40(e271) mutants were crossed into a strain expressing the integrated unc-86::lin-4 over-expression vector and the unc-86::myr-GFP reporter. No statistical difference in the percentage of animals extending axons precociously was observed between wild-type and unc-40(e271) (Figure 6C), despite the fact that there was a dramatic reduction in the number of HSNs that extended axons ventrally in the unc-40(e271) background (Figure 6D). Precocious growth was also seen in HSNs that exhibited defects in ventral cell migration, another hallmark of unc-40[lf] mutants (data not shown; [5]). Based on these findings, we concluded that the lin-4 developmental switch acts independently of ventral guidance signals to control the timing of HSN axon extension.

**Discussion**

The lin-4 miRNA cell-autonomously controls the timing of HSN axon elongation after cell cycle exit

In the nervous system, the timing of the birth of neurons is critical for their specification [58–62]. In specific neuronal lineages, this is likely due to the coupling of terminal cell divisions with distinct differentiation programs [63,64]. However, evidence is emerging that at least in the vertebrate neocortex, determination of laminar identity is not simply controlled by the cell cycle [65,66].

In the HSNs, the temporal regulation of axon outgrowth by lin-4 also appears to be regulated postmitotically. In wild-type animals, lin-4 is initially detected in the HSNs during the first larval stage (Figure 3A), well after the neurons exit the cell cycle during embryogenesis [33]. Moreover, in lin-4[lf] mutants, HSNs are still born, express the neuronal determination gene unc-86, and complete their anterior migration prior to L1 [34,54,67], confirming that lin-4 is only required for maturation events that occur after it is normally up-regulated. Finally, the postmitotic expression of lin-4 under the unc-86 promoter [34,54] rescues the lin-4[lf] retarded axon growth phenotype, strongly suggesting that
lin-4 functions cell-autonomously after cell cycle withdrawal. While the lin-4 site of action has previously been predicted for a number of cell types, it has previously not been verified in vivo [19,20,24,47,68].

LIN-14 and LIN-28 inhibit HSN maturation

The lin-4 miRNA is required for the down-regulation of lin-14 and lin-28 in the HSNs. Over-expression of each lin-4 target leads to a delay in HSN axon outgrowth, and when LIN-14 and LIN-28 levels are maintained in lin-4(lf) mutants, HSN axon extension is not detected during larval development. A loss-of-function mutation in either gene suppresses the lin-4(lf) delay and results in axons extending ~one stage too early. Strikingly, similar one-stage shifts in the timing of a later differentiation event, the expression of the serotonin-synthesis gene tph-1, are also observed in lin-14(lf) and lin-28(lf) mutants in the presence or absence of the lin-4 loss-of-function allele (Figure S1). Since the relative timing of axon outgrowth and tph-1 expression is unaltered in these animals, it is likely that after axon initiation the overall rate and sequence of differentiation remains unchanged.

It has been proposed that in hypodermal lineages, the transcription factor LIN-14 specifies L1-stage cell divisions, while LIN-28 acts post-transcriptionally to specify L2 and/or L3 developmental events [13,37]. Limited examples from the C. elegans nervous system suggest that lin-14 and possibly lin-28 may function similarly in postmitotic cells. In the HSNs, LIN-14 and LIN-28 expression patterns are consistent with roles in preventing the progression to L2 or L3/4 fates, respectively (Figure 3B–3D). lin-14 is also required during the L1 stage to inhibit precocious synaptic remodeling in the DD-type motor neurons as well as for expression of the ventral cord maintenance factor zig-4 in the PVT interneuron [14,69]. Interestingly, lin-28 is not necessary for zig-4 expression [69], potentially because it is required to promote L2- and/or L3-specific temporal identities. Further studies will be required to confirm this possibility, as well as to determine whether lin-14 and lin-28 interact with lin-4 or other known heterochronic genes—such as hbl-1 or the let-7 miRNA—in these or other neurons [22,23,70]. However, given that the heterochronic gene daf-12 functions with lin-14 and lin-28 in the hypodermis [25,48] but does not control axon growth in the HSNs, it is clear that there is no universal mechanism governing temporal patterning across tissues.

The interactions between the lin-4 microRNA and its targets depend on cellular and developmental context

Although lin-4 functions together with two previously identified targets—lin-14 and lin-28—in the HSNs, its interactions with these genes are distinct from patterns described for other cell types. In the C. elegans hypodermis, lin-4(lf) is a more efficient suppressor of lin-4(lf) than lin-28(lf), and in the vulva, only lin-4(lf) is able to suppress the lin-4(lf) vulvalless defect [15,46]. In the HSNs, by contrast, both lin-14(lf) and lin-28(lf) can strongly suppress the delays in axon outgrowth observed in lin-4(lf) mutants.

The interactions between lin-4 and its targets are highly dynamic in the HSNs, and the importance of the LCE in lin-28 down-regulation—which potentially reflects the role of direct lin-4 binding—also varies over developmental time. After the L2 stage, for instance, relative levels of LIN-28 are lower when the LCE is removed than in lin-4(lf), revealing that lin-4 likely inhibits lin-28 through both direct and indirect mechanisms. The latter is mediated at least in part by lin-14, since lin-14 is targeted by lin-4 and promotes lin-28 expression in the HSNs. Ultimately, the differences in the interactions between lin-4, lin-14, and lin-28 at distant postembryonic stages of HSN development demonstrate the importance of tracking miRNA regulation in its in vivo context, and reveal the limitations of identifying and functionally characterizing miRNA targets in isolated cell culture systems.

The lin-4 switch controls timing of HSN axon outgrowth independent of known ventral guidance receptors

We have demonstrated that up-regulation of the lin-4 miRNA and down-regulation of its targets lin-14 and lin-28 are required for axon initiation in the HSNs. Previous work has shown that axons grow in response to extracellular matrix proteins, guidance cues, and/or trophic factors [1–4,71,72]. It is therefore likely that the
lin-4 switch functions by altering the expression, activity and/or localization of the receptors that detect these signals or by inhibiting downstream cytoskeletal remodeling events until the localization of the receptors that detect these signals or by initiation in elongation in wild-type animals [5]. Conversely, precocious axon require ventral growth, the initial direction of HSN axon continued to observe temporal shifts in HSN axon elongation in wild-type animals [5]. In this work, we have described a cell-autonomous timer that is included

Materials and Methods

Nematode strains

All animals were maintained at 20°C using standard protocols as previously described [75], unless indicated otherwise. For experiments which included temperature-sensitive lin-4(f) animals, all strains were transferred to 23°C as gravid adults and phenotypes were scored in progeny.

The wild-type strain was N2 Bristol, and genetic mutant alleles included lin-4(e912), lin-4(n179ts), lin-4(n355gf), lin-28(e719), sav-3(hy123), daf-12(h61), daf-12(h61b411), unc-40(e271), and unc-40(e1430). unc-40(e1430) was marked with dpy-5(e61). unc-40(e271) is a strong loss-of-function allele, while unc-40(e1430) is a null [6,56]. Integrated strains were as follows: unc-86::myr::GFP(pVT218) (gift from C. Bargmann, Rockefeller University, New York, NY), tph-1::GFP(mgIs42) (gift from J. Sze, Albert Einstein College of Medicine, Yeshiva University, Bronx, NY), lin-4::GFP(zaIs1), lin-14::GFP(zaIs2) and lin-4 O/E(zaIs3) [53,45,50,76]. lin-14::GFP(zaIs2) was generated from the lin-14::GFP translational reporter strain maEx166 [43], while lin-4 O/E(zaIs3) was generated from lin-4 O/E #4 (Figure 1 and Figure 5). Integrations were performed using trimethylsoralen and 365nm irradiation, and both strains were backcrossed at least three times prior to analysis. To generate stable transgenic lines, experimental plasmids were co-injected into adult N2 animals with myo-2::GFP (5–10 ng/µl) or rol-6 (75 ng/µl) injection markers as previously described [77].

The myo-2::GFP marker was used in injections of lin-28::GFP and lin-28::GFPALC, and the rol-6 marker was used for all other injections. Plasmid concentrations for injections were as follows: 10 ng/µl lin-28::GFP(pVT218) (gift from E. Moss, University of Medicine and Dentistry of New Jersey, Stratford, NJ [24]), 5 ng/µl lin-28::GFPALC, 1.5 ng/µl unc-86::lin-4, and 1.5 ng/µl unc-86::lin-4MAVATURE, and 1.5 ng/µl unc-86::dsRED2. The latter construct was used to tag the HSNs in lines expressing unc-86::lin-4 or unc-86::lin-4MAVATURE.

Plasmid construction

The Invitrogen GeneTaq Site-Directed Mutagenesis System was used during the preparation of lin-28::GFPALC and unc-86::lin-4MAVATURE, and the Invitrogen TOPO TA Cloning Kit for Sequencing was used to clone all PCR products. The LCE was removed from the lin-28 3’UTR in pVT218 through PCR using the mutagenic L28TRM2F (5’ – CGAATTCTACCTCCT- CAAAATTCTTTTTTTTTTTG) and reverse L28TR-R (5’ – TTTGAGGAGTACGGTTAGTATGTT) primers. To generate the unc-86::lin-4 construct, the lin-4 hairpin and 80-nt flanking sequences were amplified by PCR from the pVTs6 rescuing fragment (gift from V. Ambros, University of Massachusetts Medical School, Worcester, MA [19]) using XMA-LIN+ (5’– TCCCCCGGGGAAATATAAATGTCTT) and NCO-LIN+ (5’– CATGCCCCATGTACCATATTGTGAA) primers, and the resulting product was inserted into pCR4.0-TOPO. A sequence-verified lin-4::pCR4.0-TOPO clone was digested with XmaI and NolI, and the lin-4 fragment was inserted into an XmaI/NolI-cut construct containing a 5-kilobase unc-86 promoter in pSM, a modified version of the pPD49.26 Fire vector (pSM-unc86; gift from C. Bargmann, Rockefeller University, New York, NY [5]). The 21-nt mature lin-4 sequence from lin-4::pCR4.0-TOPO was removed by PCR using the mutagenic LIN4CON+ (5’– ATGCTTCCGGCGCTGTTGACTATTGTAGCT) and reverse LIN4CREV (5’– ACCAGCCGGAAGCATAACTCA- TAAACCAA) primers. Once the deletion was verified, the truncated lin-4 sequence was digested with XmaI and NolI, and sub-cloned into XmaI/NolI-cut pSM-unc86. dsRED2 was PCR-amplified from pdbRED2 (Clontech Catalog No. 632404) using NHE-RED+ (5’– CTAGCTACGATTTTCCGCAAGGAACTCGAG) and KPN-RED- (5’– GACTGGTACCTCAGAGAACGGGTTGTT) primers. The resulting product was inserted into pCR4.0-TOPO, and DNA from a sequence-verified clone was digested with NheI and KpnI and ligated to NheI/KpnI-cut pSM-unc86.

Staging and identifying the HSNs

Staging was performed on the basis of gonad morphology, size, and as previously described [16]. Larvae in which distal tip cells had initiated their dorsal turn were scored as early L4. Animals with fully turned gonad arms with three or fewer embryos were categorized as young adult. Data from older adults were not included in this study. The HSNs were identified based on morphology, focal plane, and dorsal/ventral position as well as by analyzing patterns of axon outgrowth [11]. In cases where HSNs could not be conclusively identified based on these criteria, they were not scored.

Microscopy and data analysis

Animals were anaesthetized in 5 mM levamisol directly on a slide (Figure 1A, wt L4 and adult; Figure 2C, lin-28(f) early L5) or on 2–5% agarose pads. Imaging was performed on the Zeiss Axioplan 2 microscope equipped with a mercury arc lamp, using the Endow GFP bandpass (Chroma #41017) and/or HQ TRITC (Chroma #41002c) filter sets and the Zeiss Plan-Apochrom
100 ×/1.4 N.A. objective unless otherwise indicated. For quantitative studies, HSNs were first identified by DIC, and fluorescence images were then acquired with the Zeiss AxioCam MRm camera and the automated Zeiss AxioVision (v. 4.6) multidimensional acquisition module. Acquisition parameters were optimized to ensure signals fell as close as possible to the center of the camera’s linear range, and were identical in cases where fluorescence intensity values were to be compared across samples. Image display was linear for all quantitative studies and was otherwise optimized in AxioVision (v. 4.6) or Adobe Photoshop 7.0. Average pixel intensity values were determined for the cell cytoplasm in lin-28::GFP strains and the nucleus in lin-4::GFP and lin-14::GFP strains. These regions were selected based on pixel intensity in the AxioVision AutoMeasure module, and separation lines were drawn when necessary. Since background subtraction did not alter trends observed in a representative experiment with the lin-4::GFP(ads1) reporter, it was not utilized during analysis of any images. Standard error of the mean was calculated for all average pixel intensity values, and the significance of the difference between two means was assessed with the two-sample t-test.

For the purposes of this study, an HSN process was scored as an axon if 1) it was the longest neurite and 2) it had extended ventrally and initiated an anterior (or rarely, posterior) turn or, for lin-14(gf) animals and strains depicted in Figure 6, it was at least three times the length of the HSN anterior/posterior cell diameter. If HSN axons could not be clearly resolved for any reason, they were not scored. Animals were designated as positive for HSN axon elongation or tph-1::GFP expression if the phenotype was observed in at least one of the two HSNs. The standard error of proportion was calculated for all proportional data, and the significance of the difference between proportions was determined using the two-sample t-test.

In the experiments described in Figure 6B, axons extending from the cells scored in Figure 6A were deemed misguided if they failed to grow initially at least one anterior/posterior cell diameter in the ventral direction before turning. In Figure 6D, axons scored in Figure 6C were categorized according to the direction in which they first extended at least one anterior/posterior cell diameter.

**Supporting Information**

**Figure S1** lin-4 and its targets, lin-14 and lin-28, temporally regulate tph-1 expression. (A,B) Percentage of wild-type or mutant animals which expressed the integrated tph-1::GFP transgene in at least one HSN during L4 and adult stages. (A) At the restrictive temperature (23 °C), lin-14(gf) mutant animals initiated precocious GFP expression at the L4 stage, and lin-4(gf) was sufficient to suppress the lin-4(gf) retarded phenotype in double lin-4(gf);lin-14(gf) mutants. (B) lin-28(gf) animals displayed precocious tph-1::GFP expression, and lin-28(gf) suppressed the lin-4(gf) retarded phenotype. For (A), n = 50, and for (B), n = 100 for all conditions, and error bars represent standard error of proportion. (C) Representative images of L4- and adult-stage HSNs using DIC or fluorescence microscopy. At the L4 stage, GFP was not detectable in wild-type HSNs, and was precociously expressed in lin-14(gf) or lin-28(gf) HSNs. In the adult, GFP was present in the HSNs of wild-type animals but not in lin-4(gf) mutants. Arrowhead: HSN cell body. Scale bars represent 5 μm, and anterior is to the left and ventral is down.

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**Author Contributions**

Conceived and designed the experiments: KOC FJS. Performed the experiments: KOC. Analyzed the data: KOC. Contributed reagents/materials/analysis tools: KOC. Wrote the paper: KOC FJS.

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