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The POU transcription factor UNC-86 controls the timing and ventral guidance of *C. elegans* axon growth

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Abstract

The *in vivo* mechanisms that coordinate the timing of axon growth and guidance are not well understood. In the *C. elegans* hermaphrodite specific neurons, the *lin-4* microRNA controls the stage of axon initiation independent of the UNC-40 and SAX-3 ventral guidance receptors. *lin-4* loss-of-function mutants exhibit marked delays in axon outgrowth, while *lin-4* overexpression, leads to precocious growth in the L3. Here we show that loss of the POU transcription factor UNC-86 not only results in penetrant ventral axon growth defects in the HSNs, but also causes processes to extend in the L1, three stages earlier than wild-type. This temporal shift is not dependent on UNC-40 or SAX-3, and does not require the presence of *lin-4*. We propose that *unc-86(lf)* HSN axons are misguided due to the temporal decoupling of axon initiation and ventral guidance responses.

Keywords

timing; axon growth; axon guidance; UNC-86; Brn3; DCC; UNC-40

Introduction

To generate functional neural circuits, axonal growth cones must migrate appropriately to their targets. Several conserved families of extracellular cues—including the netrins, slits, semaphorins, and ephrins—as well as their corresponding receptors specify the direction of these migrations (Guan and Rao, 2003). While a great deal is known about the expression and downstream effectors of these guidance families (Butler and Tear, 2007; Polleux et al., 2007; O'Donnell et al., 2009), the ways in which these signaling pathways are coordinated spatially and temporally with axon growth are less well understood. *In vitro* experiments have revealed the growth-promoting features of guidance cues like netrin (Kennedy, 2000), revealing that axons can extend in response to extrinsic spatial patterning cues. However, observations from the *C. elegans* hermaphrodite specific motor neurons (HSNs) have shown that growth can also be initiated independently of the *unc-6*/netrin and *slt-1*/slit ventral signals (Desai et al., 1988; Wadsworth et al., 1996; Zallen et al., 1998; Hao et al., 2001; Adler et al., 2006; Olsson-Carter and Slack, 2010), suggesting that independent mechanisms ensure the correct timing of axon initiation relative to the activation of these guidance pathways.

The *C. elegans* heterochronic pathway was first defined for its role in directing stage-specific cell-division patterns in the hypodermal seam cells (Moss, 2007), although subsets

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of heterochronic genes were later shown to control developmental timing in the vulva and neurons as well (Hallam and Jin, 1998; Aurelio et al., 2003; Johnson et al., 2007). In the HSNs, three members of the heterochronic pathway, the *lin-4* microRNA (miRNA) and its targets *lin-14* and *lin-28*, direct when HSN axons extend (Olsson-Carter and Slack, 2010). Axon outgrowth is delayed in *lin-4* mutants, while it is initiated one stage too early upon *lin-4* overexpression or in *lin-14* or *lin-28* loss-of-function mutants (Olsson-Carter and Slack, 2010).

Here we identify an additional gene that controls the timing of HSN axon outgrowth, the neuronally expressed POU homeodomain transcription factor UNC-86. POU proteins are required for a range of developmental events including the maintenance of vertebrate stem cell identity and the temporal specification of *Drosophila* neuroblasts (Herr et al., 1988; Pearson and Doe, 2004; Yu et al., 2007), and one family, UNC-86/Acj6/BRN3, controls axon growth and pathfinding in *C. elegans*, *Drosophila*, and vertebrates (Certel et al., 2000; Erkman et al., 2000; Sze et al., 2002; Komiyama et al., 2003; Badea et al., 2009). In the HSNs, *unc-86* is constitutively expressed after cell cycle exit (Finney and Ruvkun, 1990) and was previously predicted to act with the zinc-finger transcription factor SEM-4 and the unidentified EGL-45 protein to promote late HSN maturation events, including axon outgrowth ((Desai et al., 1988; Basson and Horvitz, 1996)).

Here we show that *unc-86* null mutants exhibit penetrant defects in both growth and ventral guidance, with HSN axons extending approximately three stages prematurely along the anterior/posterior axis. This precocious axon initiation is independent of the two well-conserved guidance pathways—*unc-6*/netrin and *slt-1*/slit—that normally orient HSN ventral growth (Desai et al., 1988; Wadsworth et al., 1996; Zallen et al., 1998; Hao et al., 2001; Adler et al., 2006). Based on these findings, we predict that the pathfinding errors in *unc-86* mutants are not due to changes in the expression of *unc-6* or *slt-1* pathway members, but instead stem directly from a shift in the relative timing of axon initiation and activation of ventral guidance responses.

Results

unc-86 is necessary for ventral axon guidance in the HSNs

The HSNs are born in the tail and migrate anteriorly to the mid-body during embryonic development (Desai et al., 1988). In the L1 larval stage, the neurons extend short neurites that are restricted ventrally after approximately 4 hours (Adler et al., 2006), and by the end of L2, they have formed a dynamic leading edge. The HSNs complete ventral migration toward the ventral nerve cord (VNC) by early L3, and at the L3/L4 transition, select a single ventral axon that later turns anteriorly (Fig. 1)(Desai et al., 1988; Adler et al., 2006; Olsson-Carter and Slack, 2010).

To investigate a potential function for *unc-86* during HSN axon development, left HSNs (HSNLs) were visualized at the L4 larval stage in two presumptive *unc-86* null mutants using the *lin-4::GFP(zals1)* reporter strain. HSNL axons were visible in mutant animals at this stage (Fig. 2A), but they exhibited pronounced guidance defects (Fig. 2B-C). While wild-type HSNs first extend axons ventrally (Fig. 1)(Desai et al., 1988; Adler et al., 2006; Olsson-Carter and Slack, 2010), nearly all mutant HSNs initiated outgrowth in the anterior or posterior directions (Fig. 2B-C), reminiscent of the guidance defects observed in UNC-6/Netrin and SLT-1/Slit pathway mutants (Desai et al., 1988; Wadsworth et al., 1996; Zallen et al., 1998; Hao et al., 2001; Adler et al., 2006). For *unc-86(e1416)*, there was a statistically significant preference for extending in the anterior versus posterior direction ($p=0.008$, two-sample z-test; Fig. 2B), a preference that was not observed in *unc-86(n846)* ($p=0.098$). The majority of mutant HSNLs extended only a single axon, although a small percentage grew a

second short process of less than or equal to three times the HSN longitudinal diameter [9.8% *unc-86(n846)* (n=51; Standard Error of Proportion (S.E.P.)=4.5%) and 5.3% *unc-86(e1416)* (n=57; S.E.P.=3.5%)]. In both mutants, the HSN cell bodies were also dorsally displaced relative to the wild-type position in the L4 (Figs. 1, 2C), reflecting a failure in ventral cell migration.

The left HSN was initially chosen for these experiments because it has previously served as a model for postembryonic HSN differentiation events, including axon guidance and synapse formation (Wightman et al., 1997; Shen and Bargmann, 2003; Shen et al., 2004). Moreover, HSNL axons grow anteriorly as part of a much smaller axon bundle than seen for the right HSN (HSNR) (White et al., 1986; Garriga et al., 1993), potentially simplifying studies of HSN growth cone migrations. However, when we investigated HSNR axon guidance in *unc-86* mutants, we observed the same penetrant ventral growth defects as those seen for HSNLs, with 95.7% (S.E.P.=6.05) of *unc-86(n846)* and 95.2% (S.E.P.=4.15) of *unc-86(e1416)* HSNRs initiating outgrowth laterally. For this reason, we opted to collect and pool HSNR and HSNL data for experiments described in Figures 3-6.

***unc-86* controls the timing of HSN axon outgrowth**

While HSN axons initially failed to extend ventrally in both *unc-86(n846)* and *unc-86(e1416)* mutant strains, approximately one-third did display detectable ventral outgrowth at some point during their trajectories (*unc-86(n846)*: 34.2% (n=73; S.E.P. 5.4%); *unc-86(e1416)*: 29.2% (n=96; S.E.P. 4.6%)). This finding introduces the possibility that the pathfinding defects seen in *unc-86* mutants do not stem from complete failures in ventral guidance pathway(s) *per se*, but may arise from a change in the location or timing of ventral signaling events relative to HSN axon initiation. In fact, when tracking HSN development in mixed-stage *unc-86(n846)* animals, we observed HSN axons extend prematurely in the L1, prior to the stage when the *unc-6*/netrin guidance response is normally activated in the HSNs (Adler et al., 2006).

To further characterize this timing defect, we scored the effects of the two *unc-86* null mutations on HSN morphology in animals expressing GFP under the *lin-4* or *unc-86* promoters (Olsson-Carter and Slack, 2010); (Adler et al., 2006). Since *unc-86* is constitutively expressed in the HSNs and *lin-4* is up-regulated during the L1 (Finney and Ruvkun, 1990; Baumeister et al., 1996; Adler et al., 2006; Olsson-Carter and Slack, 2010), the *unc-86::myr-GFP* strain enabled us to score cell morphology at earlier developmental time-points. For the purposes of this study, axons were defined as processes extending greater than or equal to the length of three HSN cell diameters (measured along the anterior/posterior axis) to distinguish them from immature neurites normally observed at this stage, and animals were scored as positive for outgrowth if at least one of the two HSNs extended an axon.

In mixed L1-stage populations, *unc-86(lf)* animals expressing the *unc-86::myr-GFP* (Fig. 3A-C) or *lin-4::GFP* (Fig. 3D) reporters exhibited precocious axon outgrowth relative to wild-type, and as was observed for the L4-stage mutants, these axons failed to extend ventrally (Fig. 3E). Since progressively longer HSN processes were also seen in subsequent larval stages (Fig. 4D; data not shown), it is unlikely that these L1-stage axons were retracted and later re-initiated at the L3/L4 transition.

UNC-86 is not required for the expression and membrane localization of UNC-40

The UNC-40/DCC Netrin receptor is necessary for HSN ventral polarization, migration, and axon initiation (Desai et al., 1988; Wadsworth et al., 1996; Adler et al., 2006). In wild-type animals, *unc-40* is transcriptionally up-regulated in the HSNs during the L1, the same stage

that UNC-6/Netrin is detected in cells of the ventral nerve cord (VNC) (Desai et al., 1988; Chan et al., 1996; Wadsworth et al., 1996; Adler et al., 2006). Initially UNC-40/DCC is distributed throughout the HSN plasma membrane, but later in the L2 it accumulates primarily at the ventral surface, a shift in localization that is *unc-6*-dependent (Adler et al., 2006).

Like UNC-86, netrin signaling is necessary for ventral guidance but not growth of HSN axons (Desai et al., 1988; Wadsworth et al., 1996; Adler et al., 2006), and in mutants for either UNC-6/Netrin or its receptor UNC-40/DCC, axons often extend only along the anterior/posterior axis (Desai et al., 1988; Wadsworth et al., 1996; Adler et al., 2006). This suggested that UNC-86 may function by positively regulating the expression of *unc-6* and/or *unc-40*. UNC-86 cannot serve as a direct transcriptional regulator of UNC-6/Netrin since *unc-86* and *unc-6* are not co-transcribed in the same cells during embryonic or larval development (Baumeister et al., 1996; Wadsworth et al., 1996). However, UNC-86 and UNC-40 are both present in the HSNs, and thus *unc-86* could control ventral guidance by directly promoting *unc-40* expression.

To address this possibility, we generated two independent lines that each expressed an unintegrated construct containing the endogenous *unc-40* promoter fused to the *unc-40* minigene, the SL2 trans-splice site, YFP and the *unc-54* 3'UTR (construct was a gift from D. Colón-Ramos (Colón-Ramos et al., 2007)). The *unc-40* minigene was derived from both genomic and cDNA, and is able to rescue the gross *unc* and *dpy* phenotypes observed in *unc-40(e271)* mutants (D. Colón-Ramos, personal communication). *unc-54* codes for a *C. elegans* muscle myosin class II heavy chain, and its 3'UTR is commonly used in transcriptional fusion constructs (Okkema et al., 1993). Since YFP was downstream of the SL2 site and under the control of the *unc-54* 3'UTR, its expression could only be used to mark *unc-40* transcription initiation and not later post-transcriptional regulatory events.

In the HSNs, *unc-40* transcription was not observed in wild-type or *unc-86(n846)* animals during embryogenesis, but was up-regulated in both genetic backgrounds in mixed-stage L1 animals (Fig. 4A-C). It was then maintained for the remainder of larval development (Fig. 4A-B), confirming that the *unc-86(n846)* ventral guidance defects cannot be explained by the loss of *unc-40* transcription. Since the YFP construct was not stably integrated into the genome, differences in the proportion of HSNs with detectable YFP could reflect changes in the timing and/or level of transgene expression. We did not observe precocious YFP in *unc-86(n846)* animals, however, suggesting that the increase in YFP-expressing HSNs could be due to a release of *unc-86*-mediated inhibition.

Pathfinding errors can be caused by misregulation of guidance family members at both the transcriptional and post-transcriptional levels (Butler and Tear, 2007; Polleux et al., 2007; O'Donnell et al., 2009). Thus, the ventral guidance phenotypes in *unc-86(n846)* could also stem from errors in UNC-40 translation or cellular localization, leading to the inability of cells to detect the UNC-6 ventral signal. We tested this possibility in a strain expressing an UNC-40::GFP translational fusion construct under the control of the *unc-86* promoter (gift from C. Bargmann; (Adler et al., 2006)), which has previously been shown to rescue the *unc-40(e271)* axon guidance defects in the HSNs (Adler et al., 2006). In L1- and L2-stage *unc-86(n846)* animals, UNC-40::GFP was expressed and targeted to the plasma membrane (Fig. 4D) and its membrane distribution in the L1 was comparable to that observed for wild-type (Fig. 4D). While it was difficult to directly compare L2-stage patterns of UNC-40::GFP localization due to the markedly different morphologies of mutant and wild-type HSNs (Fig. 4D), we could conclude that *unc-86(lf)* outgrowth defects were unlikely to be due to general deficiencies in UNC-40 synthesis or membrane targeting.

Precocious axon outgrowth in *unc-86(lf)* is not dependent on signaling through the UNC-40/DCC and SAX-3/Robo guidance receptors

Netrin family members can guide as well as promote axon extension (Ishii et al., 1992; Serafini et al., 1994; Wadsworth et al., 1996; Adler et al., 2006), and thus the pathfinding and premature growth phenotypes observed in *unc-86* mutants could result from ectopic activation of the Netrin pathway. These defects could also stem from the possible up-regulation of *unc-40* in *unc-86(n846)* HSNs (Fig. 4A-B), which may effectively enhance sensitivity to weaker anterior/posterior netrin sources present during early larval development (Wadsworth et al., 1996; Levy-Strumpf and Culotti, 2007). However, when we scored double mutants lacking *unc-86* and either *unc-6/netrin* or *unc-40/DCC*, we found that HSNs continued to initiate outgrowth along the anterior/posterior axis at the L1 stage (Fig. 5A-B), confirming that the *unc-86(lf)* premature growth phenotype is not dependent on netrin signaling.

A second guidance pathway containing the SLT-1/Slit ligand and SAX-3/Robo receptor has also been shown to promote ventral growth of HSN axons, with approximately one-third of HSNs extending only laterally in the putative null mutant, *sax-3(ky123)* (Zallen et al., 1998; Hao et al., 2001). This pathway is also important embryonically in guiding the posterior cellular migrations of the two CAN neurons to their positions just anterior to the HSNs (Hao et al., 2001), introducing the possibility that signaling through the SAX-3/Robo receptor could also promote and guide early larval HSN axonal migrations along the anterior/posterior axis in *unc-86(n846)* animals. Yet in *unc-86(n846); sax-3(ky123)* double mutants, we continued to observe both the premature L1-stage outgrowth (Fig. 5C) and failed ventral pathfinding (Fig. 5D) defects seen in the HSNs of *unc-86(lf)* single mutants. We therefore concluded that the *unc-86(lf)* phenotypes are not caused by ectopic signaling through SAX-3/Robo.

Delayed axon growth in loss-of-function mutants for the *lin-4* microRNA is suppressed by *unc-86(lf)*

Three members of the *C. elegans* heterochronic pathway, including the *lin-4* miRNA and its targets, the LIN-14 transcription factor and LIN-28 RNA-binding protein, also control the timing of HSN axon outgrowth independent of the Netrin and Slit guidance pathways (Olsson-Carter and Slack, 2010). *lin-4* is up-regulated in the HSNs during the L1, leading to the down-regulation of LIN-14 and LIN-28 during the L2 and L3 larval stages, respectively. When *lin-14* and *lin-28* expression persists in *lin-4* loss-of-function mutants, axon extension is delayed in L4 and adult animals (Olsson-Carter and Slack, 2010). Conversely in *lin-14(lf)* or *lin-28(lf)*, outgrowth occurs approximately one stage prematurely in the L3. To assess the interaction between *unc-86* and the heterochronic pathway, we generated *unc-86(n846); lin-4(e912)* double mutants and scored for axon growth. Animals still extended axons precociously in the L1 (Fig. 6A), demonstrating that *unc-86(n846)* can suppress the *lin-4(e912)* retarded axon growth phenotype and that *unc-86* functions either in parallel to or downstream of *lin-4*.

However, *unc-86* is unlikely to be a direct *lin-4* target. Not only did we fail to find candidate *lin-4* binding sites in the *unc-86* 3'UTR using currently accepted parameters (Bartel, 2009), but expression of GFP reporter constructs containing either the *unc-54* or *unc-86* 3'UTRs also displayed no significant differences in average pixel intensities at the L2, L3, and L4 stages relative to L1 values (Fig. 6B). This suggests that post-transcriptional regulation of *unc-86* expression—at least that mediated through the 3'UTR—is minimal, consistent with the findings from antibody staining that UNC-86 protein is not down-regulated during development but is instead constitutively present after cell cycle exit (Finney and Ruvkun, 1990; Baumeister et al., 1996).

We next investigated whether *unc-86* might affect expression patterns of the *lin-4* targets *lin-14* and *lin-28*. The *lin-14::GFP* and *lin-28::GFP* reporter strains described previously (Olsson-Carter and Slack, 2010) still displayed down-regulation at the L2 and L3 stages, respectively, in *unc-86(n846)* (Fig. 6C-D). In addition, mean pixel intensity values at these time points were not significantly different for wild-type and *unc-86(n846)*, showing that there was no detectable change in *lin-14* and *lin-28* transcription in the absence of *unc-86*.

Discussion

We have shown that loss of the conserved POU domain gene *unc-86* results in penetrant defects in timing of outgrowth and ventral guidance of HSN axons, and propose that the axon misroutings stem directly from a shift in the relative timing of growth versus guidance responses during early larval development. Ultimately, these pathfinding errors in combination with cell migration defects lead to devastating effects on HSN function, since processes are no longer positioned to synapse appropriately onto the vulval muscle cells (Figs. 2C, 4C) and stimulate egg-laying (Schafer, 2006).

***unc-86* is a novel postmitotic neuronal heterochronic gene that functions independently of UNC-40/DCC and SAX-3/Robo**

In *Drosophila* mitotic neuroblast (NB) lineages, the POU family members Pdm1/2 function as part of a transcriptional cascade that specifies the temporal identities of neuronal progeny (Pearson and Doe, 2004). In the work presented here, we have shown that the POU protein UNC-86 also controls developmental timing. In contrast to *Drosophila* Pdm1/2, however, *unc-86* is not expressed in HSN precursor cells, is only up-regulated in the HSNs after cell cycle exit, and does not appear to affect the timing of early HSN differentiation events such as cell birth or anterior migration (Desai et al., 1988; Finney and Ruvkun, 1990; Baumeister et al., 1996).

unc-86 is also distinct from members of the *C. elegans* heterochronic pathway, the set of genes that controls the relative timing of stage-specific developmental events in the hypodermis and other tissues (Hallam and Jin, 1998; Johnson et al., 2005; Moss, 2007). Not only is *unc-86* expressed specifically in neuronal lineages (Finney and Ruvkun, 1990; Baumeister et al., 1996), but its expression does not appear to be developmentally regulated based on immunostaining and analysis of transcriptional reporters (Fig. 6B; (Finney and Ruvkun, 1990; Baumeister et al., 1996)). Ultimately, it may function as part of a separate timing mechanism. The *lin-4* miRNA and its targets *lin-14* and *lin-28* act as a developmental switch that controls HSN axon initiation (Olsson-Carter and Slack, 2010), and epistasis analysis reveals that *unc-86* acts either down-stream of or in parallel to *lin-4*. However, reporter assays suggest that *unc-86* is unlikely to be targeted by *lin-4* directly, and the intensity and pattern of down-regulation of *lin-14* and *lin-28* are the same for *unc-86(n846)* as for wild-type.

Constitutive overexpression of *lin-4* with the *unc-86* promoter or loss-of-function mutations in *lin-14* or *lin-28* only shift outgrowth one larval stage earlier (Olsson-Carter and Slack, 2010), suggesting that other factor(s) must prevent premature axon growth in the L1 and L2. Here we have shown that *unc-86* functions as one of these inhibitors. Yet because it continues to be expressed at the L3/L4 transition—the time when HSN axon initiation normally occurs in wild-type animals—additional factors must be required to release its inhibition of axon growth at this later stage of development. One gene that could play such a role is the zinc-finger transcription factor *sem-4*. *sem-4* is also required for larval-stage HSN maturation (Desai et al., 1988; Basson and Horvitz, 1996), and has previously been shown to act with *unc-86* and other transcriptional regulators to control development of the *C. elegans* touch cells (Mitani et al., 1993). However, precocious axon outgrowth was not observed in

sem-4(n1378) animals (0% L1-stage outgrowth (n=32; S.E.P. 3.8%)), and axon initiation in the L4 occurred in the ventral direction, as seen in wild-type (data not shown).

Transcription factors like UNC-86 have traditionally been thought to direct the timing of axon outgrowth by modulating the expression of guidance cues and/or their receptors (Polleux et al., 2007). While we cannot exclude the possibility that *unc-86* null mutants extend axons prematurely due to loss and/or ectopic expression of spatial patterning genes like the wnts (Zinovyeva et al., 2008), the observation that precocious L1-stage axon initiation does not require signaling through UNC-40/DCC or SAX-3/Robo, the two receptors known to be required for HSN ventral growth, is consistent with the independent regulation of axon growth and guidance in these neurons (Adler et al., 2006; Olsson-Carter and Slack, 2010). If this is indeed the case, the misroutings of *unc-86(lf)* HSN axons could simply be due to functional changes in these or other unidentified guidance systems. Alternatively, they could stem directly from the shift in timing of axon initiation relative to the establishment of netrin and/or slit signaling.

***unc-86* is required for late, but not early, differentiation events in the HSNs**

unc-86 is not detected in HSN precursor cells (Finney and Ruvkun, 1990) and appears to be only partially necessary for HSN specification (Desai et al., 1988). In addition to exhibiting normal anterior migration relative to the CAN neurons by the L1 stage (Desai et al., 1988), *unc-86(lf)* HSNs express a number of genes known to be up-regulated during HSN development, including the *lin-4* miRNA (Figs. 1-2, 6)(Olsson-Carter and Slack, 2010) and the *unc-40/DCC* netrin receptor (Fig. 4C). These observations coupled with the previous finding that *unc-86* is required for serotonin synthesis in the HSNs (Desai et al., 1988; Sze et al., 2002) are consistent with a role for *unc-86* in late postmitotic maturation (Desai et al., 1988). It also likely functions cell-autonomously, since it is not expressed in cells neighboring the HSNs or in neurons of the ventral cord (Finney and Ruvkun, 1990; Baumeister et al., 1996). In fact, previous studies have employed the *unc-86* promoter to test cell autonomy and/or drive gene expression in the HSNs (Wu et al., 2001; Gitai et al., 2003; Shen and Bargmann, 2003; Adler et al., 2006; Olsson-Carter and Slack, 2010).

However, since *unc-86* is known to be broadly important in *C. elegans* neuronal lineage and identity specification (Finney et al., 1988; Finney and Ruvkun, 1990; Baumeister et al., 1996; Duggan et al., 1998), the possibility cannot be excluded that *unc-86(lf)* mutant phenotypes reflect a transformation in cell fate. HSN identities could be shifted to those of the two chemosensory PHB phasmid neurons, since they are descendents of the same progenitor cell as the HSNs but do not express *unc-86* (Finney and Ruvkun, 1990; Hall and Altun, 2008). Yet while *unc-86(lf)* mutant HSNs migrate anteriorly from their place of birth in the tail, extend a single long process, and fail to produce the serotonin neurotransmitter in the adult (Desai et al., 1988; Sze et al., 2002), the PHBs remain in the posterior of the animal, extend axons during embryogenesis, and have been reported to synthesize serotonin (Sawin et al., 2000; Hall and Altun, 2008). Other potential candidates are the two PVQ interneurons, which are also present in the HSN lineage and fail to express *unc-86* (Finney and Ruvkun, 1990; Hall and Altun, 2008). However, these cells are also positioned in the tail and complete axon outgrowth by the beginning of L1 (Hall and Altun, 2008), earlier than observed in *unc-86(lf)* HSNs.

An alternative possibility is that failures in the execution of later events in HSN development—such as serotonin synthesis—stem simply from defects in earlier maturation steps, including axon guidance. However, previous evidence suggests that the HSN axon misroutings in *unc-86(lf)* animals are not necessarily caused by the absence of ventral cell migration, which normally occurs just prior to axon initiation at the L3 stage (Desai et al., 1988; Adler et al., 2006). In loss-of-function mutants for the ENA/VASP protein UNC-34 as

well as several other genes, the HSN cell bodies fail to migrate properly but still exhibit ventral outgrowth (Desai et al., 1988; Adler et al., 2006). On the other hand, axon extension prior to ventral cell migration could alter the competence of the HSNs to migrate normally in the L3 (Desai et al., 1988; Adler et al., 2006). Currently, the factors affecting the decision to migrate versus initiate axon growth are not well understood in the HSNs or other neurons (Yu and Bargmann, 2001; da Silva and Dotti, 2002).

MODEL: *unc-86* guides axons by controlling the relative timing of axon growth and activation of ventral guidance pathways

During the L1, wild-type HSNs break symmetry in response to the ventral UNC-6/Netrin source, but they have not yet exhibited later features of netrin signaling required for axon guidance, including cytoplasmic thickening and redistribution of UNC-40 at the ventral surface (Adler et al., 2006). *unc-86(lf)* mutants do not exhibit evidence of precocious *unc-40* expression or membrane localization (Fig. 4A-B, 4D), suggesting that they initiate HSN axon outgrowth before the netrin response is fully activated in the L2. The same may be true for signaling through SAX-3/Robo, since the receptor is normally not up-regulated in the HSNs until the L2 larval stage (Zallen et al., 1998).

Similar to the findings presented here, loss of the murine UNC-86 homolog Brn-3.2 leads to defects in retinal ganglion cell (RGC) axon guidance and optic nerve morphology that are reminiscent of netrin signaling mutants without markedly affecting the expression of *Netrin-1* or its receptor, DCC (Erkman et al., 2000). An actin-binding protein, AbLim, was identified through differential expression analysis as a potential Brn-3.2 target, providing a direct link between Brn-3.2 and genes involved in the cytoskeletal rearrangements required for axonal elongation (Erkman et al., 2000). Interestingly, the *C. elegans* AbLim homolog, UNC-115, not only promotes axon outgrowth but also acts downstream of UNC-40/DCC (Lundquist et al., 1998; Gitai et al., 2003), suggesting that UNC-86 may control HSN axon initiation at least in part by inhibiting *unc-115* expression until the appropriate developmental stage. Other candidates for downstream targets of *unc-86* include the *Enabled* homolog *unc-34* (Gitai et al., 2003; Krause et al., 2003; Lebrand et al., 2004), the Rac GTPase *ced-10* (Gitai et al., 2003), and the actin regulator *mig-10*/lamellipodin (Manser et al., 1997; Krause et al., 2004; Adler et al., 2006). *unc-34*, *ced-10*, and *mig-10* have all been shown to function as effectors in the netrin and slit guidance pathways (Yu et al., 2002; Gitai et al., 2003; Adler et al., 2006; Chang et al., 2006; Quinn et al., 2006).

Taken together, these data support a model in which *unc-86* cell-autonomously inhibits cytoskeletal factors required for axon growth, and loss of inhibition in *unc-86(lf)* leads to premature axon initiation. Moreover, *unc-86(lf)* HSN axons are likely misrouted along the longitudinal axis because processes extend at a stage when the Netrin and Slit signaling pathways are not fully active. In summary, we propose that the *unc-86(lf)* HSN pathfinding defects stem directly from a shift in the timing of outgrowth relative to ventral guidance responses. Since the *unc-86* family is not only important for *C. elegans* axon development but also plays key roles in axon pathfinding in *Drosophila* and higher animals, this model could represent a novel and conserved mechanism for generating complex neural networks using a limited set of guidance molecules.

Experimental Procedures

Nematode Strains

Animals were cultured at 20 °C using standard methods (Brenner, 1974). The wild-type strain was N2 Bristol, mutant alleles included *unc-40(e271)*, *unc-40(e1430)* (marked with *dpy-5(e61)*), *unc-86(e1416)*, *unc-86(n846)*, *unc-6(ev400)*, *sax-3(ky123)*, and *lin-4(e912)*.

Previously described reporters were *lin-4::GFP(zaIs1)* (Boehm and Slack, 2005), *lin-14::GFP(zaIs2)* (Olsson-Carter and Slack, 2010), *lin-28::GFP* Line 10-2 (Olsson-Carter and Slack, 2010), and *unc-86::myr-GFP(kyIs262)* and *unc-86::unc-40::GFP(kyEx1212)* (Adler et al., 2006, gifts from C. Bargmann). The independent *unc-40::SL1::YFP* Lines 1c and 6 were generated by injecting 10 ng/μl *unc-40* minigene::*SL2::YFP* (construct was a gift from D. Colón-Ramos; (Colón-Ramos et al., 2007)) and 5 ng/μl of the *myo-2::dsRed2* injection marker into young adult N2 animals as previously described (Mello and Fire, 1995). For Line 6, 85 ng/μl EcoR1-cut pBluescript SK was also added to minimize toxicity associated with the co-injected constructs. *unc-86::GFP* Line 2 containing the *unc-86* 3'UTR was produced using an injection mix containing 1 ng/μl of the *unc-86::GFP::unc-86* transcriptional fusion construct, 5 ng/μl *myo-2::dsRed2* marker (Olsson-Carter and Slack, 2010), and 44 ng/μl of EcoR1-cut pBluescript SK. For *unc-40::SL1::YFP* Line 6 and *unc-86::GFP::unc-86* Line 2, EcoR1-cut pBluescript was column-purified using the Qiagen QIAquick PCR purification kit.

Generating the *unc-86::GFP::unc-86* construct—The *unc-86* 3'UTR was PCR-amplified from N2 genomic DNA using the Novagen Hot Start DNA polymerase, a 50°C annealing temperature, and U86UTRFOR (5'-GAATTCTTTTCGTTTTCGTGAACACATTTTC) and U86UTRREV (ACTAGTGAATTATATTTATTTTACGAGGCAT) primers flanked by EcoR1 and Spe1 restriction sites, respectively. The PCR product was then inserted into pCR2.1-TOPO using the Invitrogen TOPO TA Cloning Kit. A pCR2.1-TOPO clone containing the sequence-verified *unc-86* 3'UTR was digested with EcoR1 and Spe1, and the resulting UTR fragment was ligated to the EcoR1/Spe1-cut pPD95.67 GFP expression vector backbone. To generate *unc-86::GFP::unc-86*, the pSM-86 construct (gift from C. Bargmann, Rockefeller University, New York, NY (Gitai et al., 2003); (Adler et al., 2006)) was digested with Sph1 and Xma1, and the 5.1 kilobase promoter fragment (Baumeister et al., 1996) was inserted into the Sph1/Xma-cut pPD95.67 construct containing the *unc-86* 3'UTR. All digested DNA was gel-purified using the Qiagen QIAquick Gel Extraction Kit, and ligations were performed with NEB T4 DNA ligase.

Microscopy

Animals were immobilized with 5 mM levamisole on 2% agarose pads (Figs. 2-6) or directly on a slide (Fig. 1), and scored on the Zeiss Axioplan 2 using the Endow (Chroma #41017; Figs. 1-3, 4D, 5-6) and/or Yellow (Chroma #41028; Fig. 4A-C) GFP bandpass filter sets. Images were acquired with the Zeiss Plan-Apochromat 100x/1.4 N.A. objective, AxioCam MRm camera and the automated AxioVision (v. 4.6) multidimensional acquisition module, and they were processed using AxioVision (v. 4.6) or Adobe Photoshop CS3 software.

For Fig. 6B-D, fluorescence data were acquired after HSNs were first identified by DIC. Average pixel intensity values for these cells were determined using the Zeiss AxioVision AutoMeasure module, and image acquisition and processing parameters were identical for all animals expressing the same reporter construct. The HSNs were selected primarily based on pixel intensity, but separation lines were drawn when necessary. Pixel intensity values of axonal processes were not included in our analyses. For *unc-86::GFP* Line 2, HSNs which failed to express detectable transgene due to genetic mosaicism were also excluded. The two-sample t-test was used to determine the significance of the difference between mean values.

Staging and scoring the HSNs

Staging was performed as described previously (Ambros and Horvitz, 1987; Olsson-Carter and Slack, 2010). HSNs were identified based on overall morphology, position relative to

other cells and anatomical structures, and expression of cell-specific markers (White et al., 1986), and if cells could not be conclusively identified using these criteria, they were not scored. To distinguish axons from more immature neurites, an HSN process was scored as an axon if it had extended a distance of at least three times the anterior/posterior cell diameter. Unless indicated otherwise, animals were considered positive for axon outgrowth or reporter gene expression if the phenotype was observed in at least one of the two HSNs. In Figs. 2B, 3E, 5B, and 5D, initial axon guidance was assigned by determining the direction in which axons first extended at least 1.5 anterior/posterior cell diameters. When neurons extended more than one process or a branched axon, the direction of extension of the longest process or branch was scored. In the rare case where a neuron extended multiple processes or branches of the same length, it was excluded from analysis.

For *lin-4::GFP* experiments, HSNs were more clearly visualized by preferentially knocking down GFP in non-neuronal cells (Simmer et al., 2002) using the GFP L4440 RNAi feeding vector (gift from A. Fire) and previously established methods (Timmons and Fire, 1998). The standard error of proportion was calculated for all proportional data, and the significance of the difference between two proportions was determined using the two-sample z-test.

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Fig. 1. Wild-type HSNs initiate axon growth in the ventral direction

Top: Cartoon representation of an L4-stage animal, with the CAN neuron and excretory canal dorsal to the HSN. Image not drawn exactly to scale. **Bottom:** Images of a representative L4-stage wild-type HSN expressing *lin-4::GFP(zal1)* that extends its axon ventrally and then anteriorly. Left: DIC merged with GFP. Right: GFP only. The arrow points to the excretory canal, arrowheads designate the HSN axon paths, the scale bars represent 5 μm, and anterior is to the left and ventral is down. The CAN neuron is in a distinct focal plane and is thus undetectable. The GFP images were pseudocolored green.

Fig. 2. UNC-86 is required for the ventral growth of HSN axons

A: The proportion of left HSNs with detectable axon outgrowth, visualized with the *lin-4::GFP(zals1)* reporter strain. $n > 50$ for each genotype. **B:** The percentage of HSNL axons scored in (A) extending in the direction indicated, and representing the first direction in which axons extended a distance of 1.5 anterior/posterior cell diameters. ***: $p < 0.0001$ when comparing proportions between wild-type and the indicated mutants, calculated using the two-sample z-test. For (A) and (B), error bars represent standard error of proportion. **C:** Representative images of a single *unc-86(n846)* (upper two panels) or *unc-86(e1416)* (bottom panel) L4-stage HSN with ventral guidance defects. Cell somas were often directly adjacent to the excretory canal, a more dorsal position than in wild-type (top panels; data not shown). For *unc-86(n846)*, images of the same animal were acquired using a GFP bandpass filter (upper image) or DIC (merged with GFP, lower image). Arrows point to the excretory canal, just ventral to the CAN neuron. Arrowheads designate the HSN axon paths, the scale bars represent 5 μ m, and anterior is to the left and ventral is down. GFP images for *unc-86(n846)* were pseudocolored green.

Fig. 3. *unc-86* controls the timing and guidance of HSN axon outgrowth

A-B: Representative wild-type HSN (A) or *unc-86(n846)* (B) expressing the *unc-86::myr-GFP* reporter at the L1 stage. No axon has extended in wild-type, while *unc-86(n846)* exhibits precocious outgrowth. For (A) and (B), scale bars represent 5 μ m, and anterior is to the left and ventral is down. **C-D:** The proportion of wild-type and *unc-86* mutant animals with axon outgrowth in mixed L1 populations using *unc-86::myr-GFP* (myr-GFP) (C) or *lin-4::GFP* (GFP) (D) reporter strains. $n > 50$ for all genotypes. **E:** The proportion of HSN axons scored in (C-D) with extension along the anterior/posterior (A/P) or dorsal/ventral (D/V) axes for the indicated genetic backgrounds. Assigned axes represent the first direction in which axons extended a distance of 1.5 anterior/posterior cell diameters. For C-E, error bars represent standard error of proportion, and ***: $p < 0.0001$ for the difference between wild-type and *unc-86(n846)* (C-D) or A/P and D/V axon outgrowth (E), calculated using the two-sample z-test.

Fig. 4. The UNC-40/DCC receptor is expressed and membrane-targeted in *unc-86(n846)*

A-B: The proportion of wild-type or *unc-86(n846)* animals with detectable *unc-40::SL2::YFP* expression in mixed populations at each larval stage (A: Line 1c; B: Line 6). Error bars represent standard error of proportion, and for each condition, $n \geq 50$ (A) or ≥ 25 (B). ***: $p < 0.0001$. **: $p = 0.007$. *: $p < 0.05$ (0.043 for L1, 0.035 for L2, and 0.033 for L4). p-values were calculated using the two-sample z-test. **C:** For Line 6, representative L4-stage HSNs from wild-type (upper row) and *unc-86(n846)* (lower row) animals. Images of the same animal were acquired using the Yellow GFP (YFP) filter or DIC, and YFP-expressing cells were pseudocolored green. Left: YFP; Right: YFP/DIC merge. Arrowheads indicate the CAN neuron, while arrows refer to the HSN. Note that in *unc-86(n846)*, the HSN cell body is dorsally displaced relative to the developing vulva, reflecting a failure in ventral migration. **D:** A strain expressing the UNC-40::GFP translational fusion construct was crossed into *unc-86(n846)* and scored for membrane localization. Wild-type and *unc-86(n846)* L1-stage animals display UNC-40 membrane targeting with no preferential ventral localization (arrowhead). In the L2, UNC-40 has accumulated primarily at the ventral surface (arrowhead) in wild-type animals. In L2-stage *unc-86(n846)* mutants, UNC-40 is distributed throughout the precociously extending axon (arrowheads), with preferential accumulation in the ventral/posterior cell soma and a posterior membrane protrusion (arrow). For **C** and **D**, scale bars represent 5 μm , and anterior is to the left and ventral is down.

Fig. 5. Precocious HSN axon initiation in *unc-86(lf)* is not dependent on signaling through the UNC-40/DCC or SAX-3/Robo guidance receptors

A: The proportion of L1-stage HSN axon outgrowth in *unc-86(n846)* animals that also contained a mutation in either *unc-6/netrin* or its receptor *unc-40/DCC*. $n \geq 50$ for all three genotypes. **B:** The proportion of axons in (A) with initial outgrowth along the A/P or D/V axes for the genotypes indicated. **C:** The proportion of L1-stage HSN axon outgrowth in *unc-86(n846); sax-3(ky123)* double mutant animals ($n=30$). **D:** The proportion of HSN axons in (C) with initial extension along the A/P versus D/V axes. For (A)–(D), axons were visualized using the *unc-86::myr-GFP* reporter strain, and error bars represent standard error of proportion. For (B) and (D), assigned axes reflect the first direction in which axons extended a distance of 1.5 anterior/posterior cell diameters. ***: $p < 0.0001$ for the difference in the percentage of HSNs extending axons along each axis, calculated using the two-sample z-test.

Fig. 6. *unc-86(n846)* suppresses the delay in HSN axon outgrowth observed in loss-of-function mutants for the *lin-4* microRNA

A: The percentage of *unc-86(n846); lin-4(e912)* animals that extended axons during the L1 stage. Axons were visualized with the *unc-86::myr-GFP* reporter (designated myr-GFP) and *lin-4::GFP* (designated GFP). Error bars represent standard error of proportion. $n > 50$ **B:** GFP expression in the HSNs normalized to L1-stage values for two *unc-86* transcriptional fusion strains: *unc-86::myr-GFP* containing the *unc-54* 3'UTR and *unc-86::GFP* Line 2 containing the endogenous *unc-86* 3'UTR. No statistically significant differences in relative mean pixel intensity were observed between the two strains at the L2, L3, and L4 larval stages. **C:** Expression of the *lin-14::GFP(zals2)* reporter strain in a wild-type or *unc-86(n846)* mutant background at the L1 and L2 larval stages. The mean pixel intensity values were not statistically different for wild-type and *unc-86(n846)* at both stages, but significant down-regulation between L1 and L2 was observed for each strain (**: $p = 0.002$). **D:** Expression of *lin-28::GFP* Line 10-2 in a wild-type or *unc-86(n846)* mutant background. While no difference was observed between wild-type and *unc-86(n846)* values at L1 or L3, each background exhibited significant down-regulation by the L3 (***: $p < 0.0001$ for wild-type and $p = 0.0002$ for *unc-86(n846)*). For **A**, $n > 50$ for each genetic background. For **B**, **C**, and **D**, $n \geq 10$ for each time point and error bars represent standard error of the mean. p-values were calculated using the two-sample t-test.