Atypical TGF-β signaling controls neuronal guidance in *Caenorhabditis elegans*

Highlights

- The *Caenorhabditis elegans* TGF-β type I receptor SMA-6 controls neuronal guidance
- SMA-6 acts independently of the TGF-β type II receptor to control neuronal guidance
- SMA-6 signaling limits expression of the NLR-1 neurexin-like cell adhesion receptor
- Epidermal mis-expression of NLR-1 causes neuronal guidance defects

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Atypical TGF-β signaling controls neuronal guidance in Caenorhabditis elegans

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SUMMARY

Coordinated expression of cell adhesion and signaling molecules is crucial for brain development. Here, we report that the Caenorhabditis elegans transforming growth factor β (TGF-β) type I receptor SMA-6 (small-6) acts independently of its cognate TGF-β type II receptor DAF-4 (dauer formation-defective-4) to control neuronal guidance. SMA-6 directs neuronal development from the hypodermis through interactions with three, orphan, TGF-β ligands. Intracellular signaling downstream of SMA-6 limits expression of NLR-1, an essential Neurexin-like cell adhesion receptor, to enable neuronal guidance. Together, our data identify an atypical TGF-β-mediated regulatory mechanism to ensure correct neuronal development.

INTRODUCTION

Brain development requires the generation and assembly of neurons into interconnected circuits. During these highly choreographed processes, neurons are guided by interactions with adjacent cells and the extracellular matrix, which present a variety of cell adhesion and signaling molecules. Among these are the transforming growth factor β (TGF-β) family of cytokines that are critical for metazoan development (Yi et al., 2010). TGF-β ligands control cell signaling by binding to and coordinating cell surface heteromeric type I and type II TGF-β receptor serine/threonine kinases (Shi and Massague, 2003). Upon receptor complex formation, the type II receptor phosphorylates the type I receptor kinase, which transmits the signal by phosphorylating cytoplasmic SMAD (small and mothers against decapentaplegic) proteins (Shi and Massague, 2003). Once phosphorylated, SMAD proteins form complexes that translocate to the nucleus where they interact with nuclear cofactors to regulate target gene transcription (Shi and Massague, 2003). In vertebrates, TGF-β signaling controls multiple aspects of brain development, including neuronal differentiation and migration, axon guidance, and synaptogenesis (Meyers and Kessler, 2017). In addition, the TGF-β family plays extensive roles in neuronal injury and repair (Meyers and Kessler, 2017). However, the role of TGF-β signaling in the C. elegans nervous system is not fully understood.

C. elegans encodes a single TGF-β type II receptor (DAF-4) that acts with two TGF-β type I receptors: SMA-6 to control body size/male tail development, and DAF-1 to regulate dauer formation (Figure 1A) (Georgi et al., 1990; Krishna et al., 1999). The TGF-β ligands, DBL-1 and DAF-7, control the SMA-6 and DAF-1 signaling pathways, respectively (Figure 1A) (Golden and Riddle, 1984; Suzuki et al., 1999). However, the function for other putative TGF-β ligands, TIG-2 and TIG-3, are undescribed and UNC-129 controls dorso-ventral axon guidance independently of TGF-β receptors (Figure 1A) (Colavita et al., 1998). Transcriptional output from each C. elegans TGF-β pathway is regulated by specific SMAD proteins: SMA-2/3/4 (SMA-6 signaling) and DAF-3/8/14 (DAF-1 signaling) (Savage-Dunn and Padgett, 2017).

In this study, we explored the function of the TGF-β signaling molecules in controlling development of the hermaphrodite-specific neurons (HSNs). Our experiments show that the TGF-β type I receptor SMA-6 acts independently of DAF-4 to control HSN guidance non-cell-autonomously from the hypodermis (epidermis). HSN guidance also requires functional TIG-2, TIG-3, and UNC-129 TGF-β ligands that are released from both the nervous system and muscle. We found that correct SMA-6-directed signaling restricts expression of the NLR-1 Neurexin-like cell adhesion receptor, to enable HSN guidance. Together, our study reveals the ability of a TGF-β type I receptor to control neuronal development independently of a TGF-β type II receptor.
RESULTS

The TGF-β type I receptor SMA-6 controls neuronal guidance

How TGF-β signaling coordinates gene expression programs to control nervous system development is not fully understood. Here, we used the C. elegans HSNs to explore the in vivo requirement for TGF-β signaling in neuronal migration and axon guidance. During embryogenesis, the HSNs undergo long-range posterior-anterior migration over hypodermal cells. Later, in juvenile larvae the HSNs extend anterior-directed axons over a precisely defined route within the ventral nerve cord before terminating at the nerve ring (Figure 1B) (Desai et al., 1988; Garriga et al., 1993). These developmental events are precisely regulated by...
multiple conserved guidance pathways and environmental factors (Adler et al., 2006; Desai et al., 1988; Kinunen et al., 2005; Pedersen et al., 2013; Pocock and Hobert, 2008). We examined HSN development using a transgenic strain in which the HSN cell bodies and axons are marked with green fluorescent protein (GFP) (Figure 1B). We tested the effect of independently disrupting the two recognized TGF-β pathways in C. elegans using mutants for the TGF-β type I receptors SMA-6 (body size/male tail development pathway) and DAF-1 (dauer pathway) (Figures 1A–1C). We found that SMA-6, but not DAF-1, is required for HSN migration and axon guidance (Figures 1B and 1C; Table S1). Analysis of three independently-derived sma-6 loss-of-function alleles confirmed the importance of SMA-6 for HSN development (Figure 1C). SMA-6 is primarily expressed in the hypodermis, from which it regulates body size, the pharynx and the intestine (Wang et al., 2002; Yoshida et al., 2001). To examine where SMA-6 acts to control HSN development, we restored sma-6 expression under its own promoter and tissue specific drivers in sma-6(wk7) mutant animals (Figure 1C). Expression of sma-6 cDNA under its own promoter or a hypodermal promoter (elt-3) rescued the sma-6(wk7) HSN developmental defects (Figure 1C and Table S1) (Gleason et al., 2014). Expression of sma-6 in the intestine (ges-1 promoter) or HSNs (tph-1 promoter) did not rescue HSN developmental defects (Figure 1C and Table S1) (Sze et al., 2000; Zhang and Zhang, 2012). These data show that SMA-6 functions in the hypodermis to non-cell-autonomously control HSN development.

The sole C. elegans TGF-β type II receptor DAF-4 is dispensable for neuronal guidance

The SMA-6 TGF-β type I receptor functions in a complex with the sole DAF-4 TGF-β type II receptor to control body size and male tail morphogenesis (Savage-Dunn and Padgett, 2017). Surprisingly, two widely used daf-4 deletion alleles - e1364 and ok828- exhibited wild-type HSN development (Figures 2A and 2B). These daf-4 alleles harbor large deletions that lead to premature stop codons, and in the case of e1364, elimination of kinase function (Figures 2A and S1) (Gunther et al., 2000). To confirm that DAF-4 is dispensable for HSN development, we generated two additional deletion alleles using CRISPR-Cas9 that introduce frameshifts in exon one of daf-4 (rp122 and rp123) (Figures 2A and S1) (Dokshin et al., 2018). HSNs developed normally in rp122 and rp123 animals, confirming that DAF-4 does not control HSN development (Figure 2B).

Although DAF-4 is dispensable for HSN development, it is conceivable that lack of SMA-6 causes aberrant DAF-4 receptor trafficking or dysregulation of DAF-4-directed signaling. To investigate these possibilities, we first examined the effect of disrupting TGF-β receptor recycling on HSN development. Following endocytosis, TGF-β receptors are trafficked to early endosomes, from where they are either recycled to the plasma membrane for further signaling or conveyed to the lysosome for degradation. A previous study showed that SMA-6 and DAF-4 are recycled through separate mechanisms (Gleason et al., 2014). RME-1 (Eps15 homology-domain containing/receptor mediated endocytosis-1) is a conserved protein required for trafficking of SMA-6 and DAF-4 (Gleason et al., 2014). Loss of rme-1 reduces SMA-6 levels because of inappropriate lysosomal degradation and causes accumulation of DAF-4 in intracellular vesicles (Gleason et al., 2014). In contrast, ARF-6 (ADP-ribosylation factor-6) specifically controls DAF-4 recycling, where DAF-4 incorrectly accumulates in endosomes of arf-6 mutants (Gleason et al., 2014). We found that rme-1(0b1045) mutants exhibit ~30% penetrant HSN defects, like that caused by loss of SMA-6 (Figure 2C). In addition, loss of rme-1 did not enhance sma-6(wk7) HSN defects, suggesting they act in the same genetic pathway (Figure 2C). In contrast, arf-6(tm1447) mutants exhibited wild-type HSN development (Figure 2C). Next, we examined whether the HSN developmental defects of sma-6(wk7) animals are caused by inappropriate DAF-4 signaling. To this end, we introduced mutants for transcriptional regulators acting downstream of DAF-4 in the dauer pathway into the sma-6(wk7) background. We found that neither loss of the antagonistic co-SMAD DAF-3 nor the R-SMAD DAF-14
agonist altered the sma-6(wk7) mutant HSN developmental defects (Figures 2D and 2E). Collectively, our genetic data reveal that the SMA-6 TGF-β type I receptor controls HSN development independently of signal directed by the TGF-β type II receptor DAF-4.

Three TGF-β ligands interact with SMA-6 to control neuronal guidance

Five putative TGF-β ligands (DAF-7, DBL-1, TIG-2, TIG-3, and UNC-129) are encoded by the C. elegans genome, with functions identified for only two of them (DAF-7 and DBL-1) in controlling TGF-β signaling (Golden and Riddle, 1984; Suzuki et al., 1999). We assessed which TGF-β ligand(s) control HSN development. The DAF-7 ligand acts through the DAF-1/DAF-4 receptor complex and was not required for HSN development (Figures 1 and 3A). DBL-1 acts in the body size/male tail development pathway through the SMA-6/DAF-4 receptor complex; however, dbl-1 was also dispensable for HSN development (Figures 1 and 3A). We next examined the other less studied TGF-β ligands that have not previously been associated, either functionally or physically, with a TGF-
receptor. Surprisingly, we found that knockout of any one of TIG-2, TIG-3 or UNC-129 caused HSN developmental defects (Figure 3A). Using compound mutant analysis, we examined whether these ligands act in the same genetic pathway to control HSN development. We found that all double mutant combinations and the tig-2; tig-3; unc-129 triple mutant exhibit the same penetrance and expressivity of HSN developmental defects as the respective single mutants - suggesting they act in concert to control HSN development (Figure 3A and Table S1). UNC-129 was previously shown to control DA/DB axon guidance (Colavita et al., 1998; MacNeil et al., 2009). We examined a potential overlapping role for the TIG-2 and TIG-3 ligands and SMA-6 in DA/DB development but found that UNC-129 acts independently in this context (Figure S2 and Table S1). This supports the presence of context-dependent functions for TGF-β ligands.

tig-2, tig-3, and unc-129 are prominently expressed in body wall muscle (BWM) and the nervous system (Cao et al., 2017). We examined whether the source of these secreted TGF-β ligands is important for HSN development. We performed tissue-specific transgenic rescue experiments by expressing cDNAs of each ligand in BWM or the nervous system in the respective mutant backgrounds (Figure S3) (Fujiwara et al., 1999; Okkema et al., 2002).

Figure 3. SMA-6 interacts with three, orphan, TGF-β ligands to control HSN development

(A) Quantification of HSN developmental defects in dbl-1(wk70), daf-7(e1372), tig-2(ok3416), tig-3(tm2092), and unc-129(ev554) single mutants, and all compound mutant combinations of tig-2, tig-3, and unc-129. dbl-1 and daf-7 mutant animals exhibit wild-type HSN development. Loss of either tig-2, tig-3, or unc-129 causes HSN developmental defects and the tig-2; tig-3; unc-129 triple mutant and each double mutant combination is not significantly different from each single mutant. n > 100; *p < 0.05, **p < 0.01, n.s. not significant (One-way ANOVA with Tukey’s correction). Error bars represent mean ± SEM.

(B) Quantification of HSN developmental defects in sma-6(wk7) animals in combination with either tig-2(ok3416), tig-3(tm2092), or unc-129(ev554) mutations. Each double mutant combination is not significantly different from the respective single mutant. n > 100; ****p < 0.0001, n.s. not significant (One-way ANOVA with Tukey’s correction). Error bars represent mean ± SEM.

(C–E) Input (total cell lysates) and immunoprecipitates (IP) from transfected HEK293T cells. Proteins detected with antibodies in western blots (WB) as indicated. (C) TIG-2-FLAG co-precipitates with SMA-6-MYC; (D) TIG-3-HA co-precipitates with SMA-6-MYC; (E) UNC-129-V5 co-precipitates with SMA-6-MYC. kD, kilodalton. Immunoprecipitated SMA-6-MYC is marked with a red arrow. Whole blots in Figure S4.
Figure 4. SMA-3 regulation of the NLR-1 Neurexin-like receptor controls HSN development
(A) Heatmap of whole-animal transcriptional profiling from sma-3(wk30) L2 larvae compared to wild-type. Gene names of candidate HSN regulators on the left. Each column represents individual total RNA samples. Downregulated (blue), upregulated (red), and unchanged (white).
(B) Quantification of HSN developmental defects in wild-type animals following RNAi knockdown of genes downregulated in the sma-3(wk30) mutant. n > 100; n.s. not significant (One-way ANOVA with Tukey’s correction). Error bars represent mean ± SEM.
(C) Quantification of HSN developmental defects in sma-3(wk30) animals following RNAi knockdown of genes upregulated in the sma-3(wk30) mutant. n > 100; **p < 0.01, n.s. not significant (One-way ANOVA with Tukey’s correction). Error bars represent mean ± SEM.
TGF-β control HSN guidance non-cell-autonomously from the hypodermis. We hypothesize that TIG-2 homodimers expressed (G) The TGF-β ligands TIG-2 (red), TIG-3 (green), and UNC-129 (yellow) regulate the TGF-β type I receptor SMA-6 to control HSN guidance non-cell-autonomously from the hypodermis. We hypothesize that TIG-2 homodimers expressed from neurons and TIG-3/UNC-129 homo- or heterodimers expressed from muscle interact with SMA-6. In the hypodermis TGF-β signaling through the SMA-2/3/4 transcriptional regulators limits expression of the Neurexin-like cell adhesion molecule NLR-1 to enable faithful HSN development.

Our genetic data suggest that the TIG-2, TIG-3, and UNC-129 ligands control HSN development by interacting with the SMA-6 TGF-β type I receptor. To test this assertion, we investigated whether SMA-6 interacts with TIG-2, TIG-3, and UNC-129 using co-immunoprecipitation (co-IP) experiments in human HEK293T cells (Figures 3C–3E). We found that MYC-tagged SMA-6 co-precipitated with TIG-2-FLAG, TIG-3-HA, and UNC-129-V5 (Figures 3C–3E and S4). Together, our genetic and biochemical analysis suggest that the TGF-β ligands TIG-2, TIG-3, and UNC-129 interact with the SMA-6 TGF-β type I receptor and act in the same genetic pathway to control HSN development.

SMA-6 signaling controls neuronal guidance by limiting neurexin expression

How does SMA-6-directed signaling control HSN development? To answer this question, we focused our analysis on SMA-3, an R-SMAD, which controls transcriptional output downstream of SMA-6 signaling and is important for HSN guidance (Figure 1D). We performed RNA sequencing (RNA-seq) to identify differentially expressed genes (DEGs) in sma-3(wk30) null mutant animals compared to wild-type at the L2 stage of development, a stage during which the HSN axons extend (Garniga et al., 1993). We identified 406 (FDR <0.05, absolute log2Fc > 0.585) and 2082 (FDR < 0.05) DEGs in sma-3(wk30) animals compared to wild-type (Figures 4A, S5, and S6; Tables S2 and S3). Gene ontology (GO) analysis of DEGs revealed enrichment of several biological processes, including those previously associated with the regulation of body size and male tail development (Figure S5) (Huang et al., 2007; Savage-Dunn and Padgett, 2017). However, regulation of neuronal development was not an enriched GO term, suggesting that SMA-3 controls a specific gene to control HSN development.

We manually surveyed the DEG lists for molecules with putative or known roles in cell signaling and adhesion - likely architects of HSN development. We identified eleven such DEGs, two downregulated and nine upregulated in sma-3(wk30) animals (Figures 4A, S5, and S6; Table S4). To assess functional importance of these DEGs in HSN development, we used RNA-mediated interference (RNAi) (Figures 4A–4C). For the genes downregulated in sma-3(wk30) animals, sgk-1 and mboa-6, we knocked down their expression in wild-type animals but no HSN developmental defects were detected (Figure 4B). To assess the potential impact of inappropriate expression of the nine genes upregulated in sma-3(wk30) animals, we knocked down their expression in the sma-3(wk30) mutant and asked whether HSN developmental defects were suppressed (Figure 4C). We found that RNAi knockdown of nlr-1 suppressed the HSN defects of sma-3(wk30) animals (Figure 4C).

NLR-1 is a member of the Caspr subfamily of neurexin-like proteins (Haklai-Topper et al., 2011). This family of transmembrane cell adhesion molecules mediate neuron-neuron interactions and functionally organize...
synapses (Lise and El-Husseini, 2006; Sudhof, 2008). NLR-1 contains extracellular Laminin G, epidermal growth factor, and fibrinogen-related domains, suggesting roles in cell adhesion and cell-cell signaling (Haklai-Topper et al., 2011). A C. elegans nlr-1 deletion mutant (tm2050) causes embryonic lethality (C. elegans Gene Knockout Consortium), demonstrating the essential requirement for this protein (C. elegans Deletion Mutant Consortium, 2012). Our RNAi knockdown experiments show that reducing nlr-1 expression suppresses the HSN defects caused by loss of sma-3, suggesting that SMA-6 signaling limits nlr-1 expression (Figure 4C). We tested this hypothesis by measuring nlr-1 mRNA abundance in sma-3(wk30) and sma-6(wk7) mutants compared to wild-type animals. Using qPCR analysis, we found that nlr-1 expression is increased by approximately 2-fold in the absence of either sma-3 (as revealed by RNA-seq) or sma-6 (Figure 3D). These data show that SMA-6 signaling limits nlr-1 expression.

Because of the hypodermal-specific function of SMA-6 in non-cell-autonomously regulating HSN development (Figure 1C), we predicted that overexpression of nlr-1 in the hypodermis, and not in another tissue, would cause HSN defects in wild-type animals. We found that nlr-1 overexpression in the hypodermis (elt-3 promoter), but not the intestine (ges-1 promoter), caused HSN defects like those observed in the sma-3(wk30) mutant both quantitatively and qualitatively (Figures 4E and 4F; Table S1). Importantly, hypodermal overexpression of nlr-1 did not further exacerbate sma-3(wk30) HSN defects (Figure 4E). These data reveal that SMA-6 signaling in the hypodermis prevents expression of the neurexin-like protein NLR-1 to optimize the extracellular landscape and enable correct HSN development (Figure 4G).

**DISCUSSION**

We have shown here that the SMA-6 TGF-β type I receptor controls a developmental decision independently of DAF-4 - the sole TGF-β type II receptor in C. elegans. To our knowledge, this is the first report of such a function for a TGF-β type I receptor. However, a previous report did reveal that the other C. elegans TGF-β type I receptor, DAF-1, acts partially in parallel with DAF-4 to control dauer formation (Gunther et al., 2000). Our data show that SMA-6 acts in the hypodermis to control HSN guidance. DAF-4 is also expressed in this tissue (Gunther et al., 2000), suggesting that SMA-6 regulates HSN guidance from distinct membrane domains or cellular subpopulations within the hypodermis. Intriguingly, we also show that three, orphan, TGF-β and TIG-3/UNC-129 homodimers or heterodimers form complexes with an unidentified receptor(s). A previous study found a similar non-redundant requirement for three Activin ligands (TGF-β superfamily members) in the Drosophila retina (Wells et al., 2017). Further, cell-based assays showed that promiscuous interactions between TGF-β superfamily ligands and their receptors enable cells to perceive information encoded by distinct ligand combinations (Antebi et al., 2017). Thus, discrete intracellular responses are dependent on ligand composition and concentration. We found that even though the intracellular responses downstream of SMA-6 are coordinated by the SMA-2/3/4 SMAD complex that represses expression of NLR-1, a neurexin-like cell adhesion receptor. Limiting NLR-1 expression is important as its inappropriate expression in the hypodermis causes defective neuronal migration and axon guidance, presumably because of aberrant extracellular adhesion and/or signaling. Together, our results imply that correct brain development requires tight regulation of Neurexin expression to ensure an optimal adhesive and architectural environment for navigating neurons. It will be interesting to determine if TGF-β signaling plays a similar function in vertebrate nervous systems.

**Limitations of the study**

Although we have shown that SMA-6 can physically interact with the TGF-β ligands (TIG-2, TIG-3, and UNC-129), additional experiments are needed to determine binding specificity, strength, and binding sites between these molecules. In addition, mechanistic understanding of how the three TGF-β ligands act together to control SMA-6 signaling and how SMA-6 controls SMAD activity without the TGF-β type II receptor DAF-4 requires further study. We found that SMA-6 limits expression of the NLR-1 neurexin-like cell adhesion receptor to enable faithful neuronal guidance. How mis-expressed NLR-1 causes neuronal guidance defects is unknown.

**STAR Methods**

Detailed methods are provided in the online version of this paper and include the following:
SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.103791.

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AUTHOR CONTRIBUTIONS
O.B., M.E.P., T.S., A.H., G.S., W.C., M.H., and R.P. performed experiments. S.A. analyzed the RNA sequencing data. R.P. supervised the research and wrote the manuscript.

DECLARATION OF INTERESTS
Authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Goat anti-Rabbit IgG (H+L) secondary antibody, HRP conjugate | Thermo Fisher Scientific | 65-6120 |
| Goat anti-Mouse IgG (H+L) secondary antibody, HRP conjugate | Thermo Fisher Scientific | 32430 |
| Mouse anti-FLAG/DYKDDDDK tag antibody | Cell Signaling | 2368 |
| Mouse anti-Histidine tag antibody | Bio-Rad | AD1.1.10 |
| Mouse anti-V5 tag antibody | Bio-Rad | SV5-Pk1 |
| Rabbit anti-Myc tag antibody | Abcam | ab9106 |
| **Bacterial and virus strains** |        |            |
| Bacteria Escherichia coli HT115 | CAENORHABDITIS GENETICS CENTER (CGC) | N/A |
| Bacteria Escherichia coli OP50 | CAENORHABDITIS GENETICS CENTER (CGC) | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| cOmplete™, EDTA-free Protease Inhibitor Cocktail | Merck | 11836170001 |
| Dynabeads Protein G | Thermo Fisher Scientific | 1003D |
| Lipofectamine 2000 transfection reagent | Thermo Fisher Scientific | 11668027 |
| Sodium azide | Sigma | S8032-25G |
| **Critical commercial assays** |        |            |
| Fast SYBR™ Green Master Mix | Thermo Fisher Scientific | 4385610 |
| ImProm-II™ Reverse Transcription System | Promega | A3800 |
| In-Fusion® HD Cloning Kit | Takara Bio | 102518 |
| Qubit DNA HS kit | Thermo Fisher Scientific | Q33230 |
| RNAeasy mini kit | Qiagen | 74104 |
| The LightCycler® 480 SYBR Green I Master | Roche | 4707516001 |
| TruSeq Stranded mRNA Sample Prep Kit | Illumina | 20020594 |
| **Deposited data** |        |            |
| RNA-seq data deposited into NCBI Gene Expression Omnibus | This paper | GEO: GSE63473 |
| **Experimental models: Cell lines** |        |            |
| HEK293T | N/A | N/A |
| **Experimental models: Organisms/strains** |        |            |
| *C. elegans*: Strain RJP1153: *daf-7(e1372); zds13[tph-1p::GFP]* | This study | RJP1153 |
| *C. elegans*: Strain RJP1154: *daf-1(m40); zds13[tph-1p::GFP]* | This study | RJP1154 |
| *C. elegans*: Strain RJP1330: *tig-2(ok3416); zds13[tph-1p::GFP]* | This study | RJP1330 |
| *C. elegans*: Strain RJP1334: *tig-3(tm2092); zds13[tph-1p::GFP]* | This study | RJP1334 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| C. elegans: Strain RJP1398: sma-4(e729); zdIs13[tph-1p::GFP] | This study | RJP1398 |
| C. elegans: Strain RJP1551: sma-6(wk7); zdIs13[tph-1p::GFP] | This study | RJP1551 |
| C. elegans: Strain RJP1553: sma-6(wk7); zdIs13[tph-1p::GFP]; yxEx615[sma-6p::sma-6; unc-122::GFP] | This study | RJP1553 |
| C. elegans: Strain RJP1554: sma-2(e502); zdIs13[tph-1p::GFP] | This study | RJP1554 |
| C. elegans: Strain RJP1555: daf-14(m77); mgIs71[tph-1p::GFP] | This study | RJP1555 |
| C. elegans: Strain RJP1611: daf-8(e1393); zdIs13[tph-1p::GFP] | This study | RJP1611 |
| C. elegans: Strain RJP1917: tig-2(ok3416); tig-3(tm2092); zdIs13[tph-1p::GFP] | This study | RJP1917 |
| C. elegans: Strain RJP2018: sma-6(e1482); zdIs13[tph-1p::GFP] | This study | RJP2018 |
| C. elegans: Strain RJP3203: mgIs71[tph-1p::GFP] (original strain GR1333 - backcrossed to N2) | Sze et al., 2000 | RJP3203 |
| C. elegans: Strain RJP3333: sma-3(wk30); zdIs13[tph-1p::GFP] | This study | RJP3333 |
| C. elegans: Strain RJP3393: zdIs13[tph-1p::GFP] (original strain SK4013 - backcrossed to N2) | Clark and Chiu., 2003 | RJP3393 |
| C. elegans: Strain RJP3519: daf-4(e1364); zdIs13[tph-1p::GFP] | This study | RJP3519 |
| C. elegans: Strain RJP3520: daf-4(ok828); zdIs13[tph-1p::GFP] | This study | RJP3520 |
| C. elegans: Strain RJP3603: sma-6(wk7); tig-2(ok3416); zdIs13[tph-1p::GFP] | This study | RJP3603 |
| C. elegans: Strain RJP3755: sma-6(ok2894); zdIs13[tph-1p::GFP] | This study | RJP3755 |
| C. elegans: Strain RJP3786: dbl-1(wk70); zdIs13[tph-1p::GFP] | This study | RJP3786 |
| C. elegans: Strain RJP3800: sma-6(wk7); rme-1(b1045); zdIs13[tph-1p::GFP] | This study | RJP3800 |
| C. elegans: Strain RJP3821: unc-129(ev554); rpEx6[tph-1p::gfp] | This study | RJP3821 |
| C. elegans: Strain RJP3837: sma-6(wk7); tig-3(tm2092); zdIs13[tph-1p::GFP] | This study | RJP3837 |
| C. elegans: Strain RJP3843: rme-1(b1045); zdIs13[tph-1p::GFP] | This study | RJP3843 |
| C. elegans: Strain RJP3871: arf-6(tm1447); mgIs71[tph-1p::GFP] | This study | RJP3871 |
| C. elegans: Strain RJP4047: daf-4(rp122); zdIs13[tph-1p::GFP] | This study | RJP4047 |
| C. elegans: Strain RJP4048: daf-4(rp123); zdIs13[tph-1p::GFP] | This study | RJP4048 |
| C. elegans: Strain RJP4070: sma-6(wk7); unc-129(ev554); rpEx6[tph-1p::GFP] | This study | RJP4070 |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| C. elegans: Strain RJP4158: sma-6(wk7); zdIs13[pph-1p::GFP]; yxEx765[ges-1p::sma-6; unc-122p::GFP] | This study | RJP4158 |
| C. elegans: Strain RJP4161: unc-129(ev554); tig-2(ok3416); rpEx6[pph-1p::GFP] | This study | RJP4161 |
| C. elegans: Strain RJP4162: unc-129(ev554); tig-3(tm2092); rpEx6[pph-1p::GFP] | This study | RJP4162 |
| C. elegans: Strain RJP4163: unc-129(ev554); tig-2(ok3416); tig-3(tm2092); rpEx6[pph-1p::GFP] | This study | RJP4163 |
| C. elegans: Strain RJP4167: daf-3(mgDf90); zdIs13[pph-1p::GFP] | This study | RJP4167 |
| C. elegans: Strain RJP4217: sma-6(wk7); zdIs13[pph-1p::GFP]; rpEx1792[pph-1p::sma-6 + myo-2p::mCherry] | This study | RJP4217 |
| C. elegans: Strain RJP4218: sma-6(wk7); zdIs13[pph-1p::GFP]; rpEx1793[pph-1p::sma-6 + myo-2p::mCherry] | This study | RJP4218 |
| C. elegans: Strain RJP4446: sma-6(wk7); zdIs13[pph-1p::GFP]; rpEx1904[elt-3p::sma-6::GFP + myo-2p::mCherry] | This study | RJP4446 |
| C. elegans: Strain RJP4509: sma-6(wk7); daf-3(mgDf90); zdIs13[pph-1p::GFP] | This study | RJP4509 |
| C. elegans: Strain RJP4570: zdIs13 [pph-1p::GFP]; rpEx2034[elt-3p::npr-1 cDNA + myo-2p::mCherry] | This study | RJP4570 |
| C. elegans: Strain RJP4571: zdIs13 [pph-1p::GFP]; rpEx2035[elt-3p::npr-1 cDNA + myo-2p::mCherry] | This study | RJP4571 |
| C. elegans: Strain RJP4572: zdIs13[pph-1p::GFP]; rpEx2036[elt-3p::npr-1 cDNA+ myo-2p::mCherry] | This study | RJP4572 |
| C. elegans: Strain RJP4655: tig-2(ok3416); zdIs13[pph-1p::GFP]; rpEx2079[imb-1p::tig-2 cDNA + myo-2p::mCherry] | This study | RJP4655 |
| C. elegans: Strain RJP4657: tig-2(ok3416); zdIs13[pph-1p::GFP]; rpEx2081[myo-3p::tig-2 cDNA + myo-2p::mCherry] | This study | RJP4657 |
| C. elegans: Strain RJP4660: tig-3(tm2092); zdIs13[pph-1p::GFP]; rpEx2084[imb-1p::tig-3 cDNA + myo-2p::mCherry] | This study | RJP4660 |
| C. elegans: Strain RJP4663: tig-3(tm2092); zdIs13[pph-1p::GFP]; rpEx2087[myo-3p::tig-3 cDNA + myo-2p::mCherry] | This study | RJP4663 |
| C. elegans: Strain RJP4666: unc-129(ev554); rpEx6[pph-1p::gfp]; rpEx2090[imb-1p::unc-129 cDNA + myo-2p::mCherry] | This study | RJP4666 |
| C. elegans: Strain RJP4669: unc-129(ev554); rpEx6[pph-1p::gfp]; rpEx2093[myo-3p::unc-129 cDNA + myo-2p::mCherry] | This study | RJP4669 |
| C. elegans: Strain RJP4672: zdIs13[pph-1p::GFP]; rpEx2096[ges-1p::nir-1 cDNA + myo-2p::mCherry] | This study | RJP4672 |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| C. elegans: Strain RJP4675: sma-3(wk30); zdIs13[ tph-1p::GFP]; rpEx2035[elt-3p::nir-1 cDNA+ myo-2p::mCherry] | This study | RJP4675 |
| C. elegans: Strain RJP4753: evIs82b[unc-129::GFP + dpy-20(+)l] | Colavita and Culotto., 1998 | RJP4753 |
| C. elegans: Strain RJP4825: sma-6(wk7); daf-14(m77); mgIs71[tph-1p::GFP] | This study | RJP4825 |
| C. elegans: Strain RJP5147: sma-6(ok2894); sma-3(wk30); zdIs13[ tph-1p::GFP] | This study | RJP5147 |
| C. elegans: Strain RJP5175: sma-3(wk30); zdIs13[ tph-1p::GFP]; qcIs55[vha-7p::gfp::sma-3 + rol-6] | This study | RJP5175 |
| C. elegans: Strain RJP5176: unc-129(ev554) evIs82b | This study | RJP5176 |
| C. elegans: Strain RJP5177: sma-6(wk7); evIs82b | This study | RJP5177 |
| C. elegans: Strain RJP5178: tig-2(ok3416); evIs82b | This study | RJP5178 |
| C. elegans: Strain RJP5179: tig-3(tm2092); evIs82b | This study | RJP5179 |
| C. elegans: Strain RJP5180: tig-2(ok3416); tig-3(tm2092); evIs82b | This study | RJP5180 |
| C. elegans: Strain RJP5181: tig-2(ok3416); unc-129(ev554) evIs82b | This study | RJP5181 |
| C. elegans: Strain RJP5182: tig-3(tm2092); unc-129(ev554) evIs82b | This study | RJP5182 |
| C. elegans: Strain RJP5183: tig-2(ok3416); tig-3(tm2092); unc-129(ev554) evIs82b | This study | RJP5183 |
| C. elegans: Strain ZC1334: sma-6(wk7); yxEx615[ sma-6p::sma-6; unc-122p::gfp] | Zhang and Zhang, 2012 | ZC1334 |
| C. elegans: Strain ZC1573: sma-6(wk7); yxEx765[ ges-1p::sma-6; unc-122p::gfp] | Zhang and Zhang, 2012 | ZC1573 |
| Oligonucleotides | | |
| Genotyping and cloning oligos available on request | | |
| Recombinant DNA | | |
| Plasmid: pRG62 elt-3p::sma-6::GFP | Rick Padgett | Gleason et al., 2014 |
| Plasmid: tph-1p::sma-6::GFP | This study | |
| Plasmid: elt-3p::nir-1 cDNA | This study | |
| Plasmid: ges-1p::nir-1 cDNA | This study | |
| Plasmid: myo-3p::tig-2 cDNA | This study | |
| Plasmid: myo-3p::tig-3 cDNA | This study | |
| Plasmid: myo-3p::unc-129 cDNA | This study | |
| Plasmid: rimb-1p::tig-2 cDNA | This study | Tomioka et al., 2016 (rimb-1 promoter) |
| Plasmid: rimb-1p::tig-3 cDNA | This study | Tomioka et al., 2016 (rimb-1 promoter) |
| Plasmid: rimb-1p::unc-129 cDNA | This study | Tomioka et al., 2016 (rimb-1 promoter) |
| Plasmid: pJKL964 Myc-sma-6 | Jun Liu | Tian et al., 2013 |
| Plasmid: pcDNA 3.1 (zeo) tig-2-FLAG | This study | |
| Plasmid: pcDNA 3.1 (zeo) tig-3-HA | This study | |
| Plasmid: pcDNA 3.2 unc-129-V5 | This study | |
| Software and algorithms | | |
| Prism | GraphPad | https://www.graphpad.com |
RESOURCE AVAILABILITY

Lead contact

- Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Roger Pocock (roger.pocock@monash.edu).

Materials availability

- Plasmids and Caenorhabditis elegans strains generated in this study are available from the lead contact on request.

Data and code availability

- RNA-seq data has been deposited into the NCBI Gene Expression Omnibus (GEO) under accession number GSE151035.
- This paper does not report original code.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Nematode strains and genetics

C. elegans strains were grown using standard conditions on NGM agar at 20°C on Escherichia coli OP50, unless otherwise stated (Brenner, 1974). All strains were backcrossed to wild-type (N2 Bristol) at least three times prior to scoring. mglS71 Is[tph-1p::gfp], zdi13 Is[tph-1p::gfp] or rpEx6[tph-1p::gfp] were used to score HSN anatomy and evls82b Is[unc-129p::gfp) was used to score DA/DB axon guidance. Detailed strain information can be found in Key Resources Table.

Cell culture

For in vitro studies, Human embryonic kidney (HEK293T) cells were cultured using DMEM with 10% FBS and 5 μM L-glutamine at 37°C. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol.

METHOD DETAILS

Microinjection and transgenic animal generation

Rescue and overexpression plasmids were delivered into wild-type or mutant hermaphrodites at 1–10 ng/μl concentration by microinjection (Mello et al., 1991). myo-2p::mCherry (5 ng/μl) was used as an injection marker.

Fluorescence microscopy

Animals were mounted on 5% agar pads, anesthetized with 20 μM sodium azide and examined using an automated fluorescence microscope (Zeiss, AXIO Imager M2) and ZEN software (version 3.1).

C. elegans plasmid generation

tph-1p::sma-6 rescue plasmid. tph-1p::sma-6 was generated by cloning the 2650 bp full-length sma-6 genomic sequence into the pPD49.26 plasmid (containing a 1748 bp tph-1 promoter) using NheI.

myo-3p::tig-2 cDNA rescue plasmid. myo-3p::tig-2 was generated by cloning the 1101 bp full-length tig-2 cDNA into the pPD95.86 plasmid (containing a 2385 bp myo-3 promoter) using NheI-KpnI.

myo-3p::tig-3 cDNA rescue plasmid. myo-3p::tig-3 was generated by cloning the 756 bp full-length unc-129 cDNA into the pPD95.86 plasmid (containing a 2385 bp myo-3 promoter) using NheI-KpnI.

myo-3p::unc-129 cDNA rescue plasmid. myo-3p::unc-129 was generated by cloning the 1224 bp full-length tig-3 cDNA into the pPD95.86 plasmid (containing a 2385 bp myo-3 promoter) using NheI-KpnI.
rimb-1p::tig-2 cDNA rescue plasmid. *rimb-1p::tig-2* was generated by cloning the 1101 bp full-length *tig-2* cDNA into the *rimb-1p::daf-2a* (containing a 2479 bp *rimb-1* promoter) using PCR fusion to replace the *daf-2a* coding sequence.

rimb-1p::tig-3 cDNA rescue plasmid. *rimb-1p::tig-3* was generated by cloning the 756 bp full-length *tig-3* cDNA into the *rimb-1p::daf-2a* (containing a 2479 bp *rimb-1* promoter) using PCR fusion to replace the *daf-2a* coding sequence.

rimb-1p::unc-129 cDNA rescue plasmid. *rimb-1p::unc-129* was generated by cloning the 1224 bp full-length *unc-129* cDNA into the *rimb-1p::daf-2a* (containing a 2479 bp *rimb-1* promoter) using PCR fusion to replace the *daf-2a* coding sequence.

e1t-3p::n1r-1 cDNA overexpression plasmid. *e1t-3p::n1r-1* was generated by cloning the 3543 bp full-length *n1r-1* cDNA into the pPD49.26 plasmid using NcoI. The 2057 bp elt-3 promoter was then cloned into this plasmid using PCR fusion.

ges-1p::n1r-1 cDNA overexpression plasmid. *ges-1p::n1r-1* was generated by cloning the 3543 bp full-length *n1r-1* cDNA into the pPD49.26-ges-1p plasmid using NheI-NcoI. This plasmid includes the 3272 bp ges-1 promoter.

tig-2-FLAG mammalian expression plasmid. *tig-2-FLAG* (C-terminal tag) was generated by cloning the 1098 bp full-length *tig-2* cDNA without the stop codon into the pcDNA3.1(zeo) mammalian expression plasmid using NheI-HindIII.

tig-3-HA mammalian expression plasmid. *tig-3-HA* (C-terminal tag) was generated by cloning the 753 bp full-length *tig-3* cDNA without the stop codon into the pcDNA3.1(zeo) mammalian expression plasmid using NheI-KpnI.

unc-129-V5 mammalian expression plasmid. *unc-129-V5* (C-terminal tag) was generated by cloning the 1224 bp full-length *unc-129* cDNA without the stop codon into the Wnt3A-V5 mammalian expression plasmid (Addgene, plasmid #43810) using PCR fusion to replace the Wnt3A coding sequence.

CRISPR-Cas9
daf-4 mutant alleles. Two single guide RNAs (sgRNA) targeting the first exon of *daf-4* were designed using a CRISPR design tool (http://crispr.mit.edu). The sgRNAs (GCCCCATTTCTATAAGGTGC and CTAC AAGAACCTTACAAGAG) were independently cloned into the Cas9 plasmid using In-Fusion HD Cloning Kit (Takara Bio, USA) and confirmed by Sanger sequencing. Plasmids were injected into wild-type hermaphrodites at a final concentration of 50 ng/μl per plasmid. The *daf-4* CRISPR-knockout candidates were individually selected on Sma and/or dauer phenotype, and incubated at 15°C to enable dauer recovery. CRISPR-Cas9-induced mutations were identified by Sanger sequencing using PCR products amplified from the *daf-4* gene. Two independent alleles were identified, *rp122* and *rp123*, which possess frameshift mutations resulting with premature stop codons. These mutant strains were backcrossed to wild-type males three times prior to analysis.

RNAi experiments
RNAi feeding experiments were conducted following the Ahringer lab protocol (Fraser et al., 2000). L4440 plasmids (with or without specific dsRNA) were transformed in Escherichia coli HT115 and selected with ampicillin and tetracycline. Four L4 hermaphrodites were moved into each RNAi bacteria seeded plate and progeny were scored after 4 days as young adults. For each knockdown strain, three different experiments were conducted on three independent days.

Co-immunoprecipitation and western blotting
Immunoprecipitation was performed using Dynabeads™ Protein G (10003D, Thermo Fisher Scientific). Briefly, cells were cultured in 6-well plates for one day to 70% confluency and transfected with *tig-2-FLAG*, *tig-3-HA* and *unc-129-V5* individually and in combination with *sma-6-Myc* to test the interactions. Cells were lysed with lysis buffer (Tris, NaCl, SDS, Triton X-100) after 24 hours of incubation. Lysates
were incubated with Dynabeads™ Protein and specific primary antibodies (see antibodies section). Immunoprecipitates were washed 3 times using lysis buffer and proteins were eluted with 2.5X LDS buffer. Proteins were separated on 12.5% sodium dodecyl sulfate polyacrylamide gels and screened by western blot using the appropriate primary and secondary antibodies.

**Antibodies**

The primary antibodies used in this study were rabbit anti-myc (ab9106, abcam), mouse anti-flag (2368, Cell Signaling), mouse anti-V5 (SV5-Pk1, Bio-Rad) and rabbit anti-HA (C29F4, Cell signaling). The secondary antibodies used in this study were goat anti-rabbit IgG (H+L), HRP conjugate (65-6120, Life Technologies) and goat anti-mouse IgG (H+L), HRP conjugate (32430, Life Technologies).

**RNA extraction**

Approximately 2000 non-starved, synchronized L2-stage animals were washed three times in M9 buffer and pelleted. 100 μl of M9 and 500 μl Trizol (Agilent) was added to each worm pellet and frozen at −80°C. Samples were freeze-thawed in liquid nitrogen and 37°C, a total of 7 times. The RNA was chloroform-extracted and purified using the RNAsasy Mini Kit (Qiagen), according to manufacturer’s protocol.

**RNA sequencing**

RNA sequencing was performed at Micromon Genomics (Monash University). mRNA samples were converted to indexed Illumina sequencing libraries using Illumina’s TruSeq Stranded mRNA Sample Prep Kit, employing oligo (dT)-conjugated beads to enrich for polyadenylated transcripts. Libraries were quantitated using a Qubit DNA HS kit (Invitrogen, Carlsbad CA., USA), sized using an AATI Fragment Analyzer (Advanced Analytical Technologies Inc., USA), and sequenced on an Illumina NextSeq500 configured to produce 75 nt paired-end reads. Fastq files were generated by bcl2fastq, trimming 3’ adapter sequences.

**Bioinformatics for RNA sequencing**

RNA-seq data was converted to a per-gene read-count matrix using RNAsik pipeline v1.5.0. Briefly, single-end reads were mapped to the C. elegans genome (WBcel235) using STAR (Dobin et al., 2013) in splice-aware mode, duplicates were marked using Picard Markduplicates, and reads were assigned to gene models (exonic only) from the WBcel235 annotation using featureCounts v1.5.2 (Liao et al., 2014). Genes were only considered “testable” if they had at least 5 read-counts in a sample. After applying this filter for testable genes, 16,259 of the 20,542 annotated protein-coding genes were retained. Differentially expressed genes between the different genotypes were identified using limma-voom (Law et al., 2014).

**qPCR assays**

Total RNA was isolated using the RNAeasy mini kit (Qiagen 74104), according to manufacturer’s instructions. Total cDNA was obtained using oligo(dT) primers and the ImProm-II™ Reverse Transcription System (A3800) followed by quantitative PCR using SYBR green (Thermo Scientific 4385610) and Light Cycler 480 (Roche). The cdc-42 reference gene was used as a control.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Neuronal phenotypes were analysed in 1-day adults using fluorescent reporter strains based on the position of cell bodies and axon trajectories. Axonal outgrowth and the position of cell bodies were scored as defective or normal in comparison with control animals. All phenotypic scoring was performed with minimum of two biological replicates, on independent days and at least eighty worms examined for each strain. Results were analysed using one-way ANOVA with Tukey’s Multiple Comparison Test or t test on GraphPad Prism version 7, where applicable. Values were presented as mean ± SEM (standard error of the mean) and were considered statistically significant when p value is lower than 0.05: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s. not significant. All of the statistical details of the experiments can be found in the figure legends.