Genes to Cells

Caenorhabditis elegans homologue of Prox1/Prospero is expressed in the glia and is required for sensory behavior and cold tolerance

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The *Caenorhabditis elegans* (*C. elegans*) amphid sensory organ contains only 4 glia-like cells and 24 sensory neurons, providing a simple model for analyzing glia or neuron-glia interactions. To better characterize glial development and function, we carried out RNA interference screening for transcription factors that regulate the expression of an amphid sheath glial cell marker and identified *pros-1*, which encodes a homeodomain transcription factor homologous to *Drosophila prospero*/mammalian *Prox1*, as a positive regulator. The functional PROS-1::EGFP fusion protein was localized in the nuclei of the glia and the excretory cell but not in the amphid sensory neurons. *pros-1* deletion mutants exhibited larval lethality, and rescue experiments showed that *pros-1* and human *Prox1* transgenes were able to rescue the larval lethal phenotype, suggesting that *pros-1* is a functional homologue of mammalian *Prox1*, at least partially. We further found that the structure and functions of sensory neurons, such as the morphology of sensory endings, sensory behavior and sensory-mediated cold tolerance, appeared to be affected by the *pros-1* RNAi. Together, our results show that the *C. elegans* PROS-1 is a transcriptional regulator in the glia but is involved not only in sensory behavior but also in sensory-mediated physiological tolerance.

Introduction

Glial cells control many aspects of the nervous system, including the regulation of survival, differentiation and interconnection of neurons, in both vertebrates and invertebrates. In mammals, secreted and cell-surface signals, for example, thrombospondin (TSP) family members and integrin-like proteins from glia, control synaptogenesis and synaptic activities (Christopherson *et al.* 2005; Eroglu & Barres 2010). In *Drosophila*, several lines of evidence have elucidated the mechanisms of neuron/glial cell fate determination and glial control of neuronal survival. Asymmetric expression of the glia cells missing (*gcm*) gene, which encodes a transcription factor, determines cell fate; cells that are *gcm* positive develop as glia and cells that are *gcm* negative develop as neurons (Hosoya *et al.* 1995). Prospero, a homeodomain transcription factor, regulates *gcm* asymmetric expression to induce glial cell fate (Hirata *et al.* 1995). *gcm* mutants or toxin-mediated cell killing of the glia result in apoptosis of neurons, whose axons are associated with the glia (Booth *et al.* 2000).

In *Caenorhabditis elegans*, the nervous system is composed of 302 neurons and 56 glial cells (Ward *et al.* 1975; White *et al.* 1986). Of these, 50 glial cells associate with sensory neurons. The cephalic sheath (CEPsh) glial cells, which are associated with CEP sensory dendrites and the nerve ring, have been shown to regulate extension of CEP sensory dendrites and axon guidance/branching within the nerve ring (Yoshimura *et al.* 2008). The amphid sensory organ contains 12 pairs of

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sensory neurons and 2 pairs of glial cells called amphid sheath cells and socket cells. Several studies have suggested that amphid sheath glia alter neuronal activity, for example, the glial Degenerin/epithelial Na^+ channels (DEG/ENaCs) ACD-1, together with the neuronal DEG/ENaC DEG-1, is necessary for acid avoidance and attraction to lysine (Wang *et al.* 2008, 2012), *hlh-17* expressed in glia is involved in dopamine (DA) signaling (Felton & Johnson 2011), and the glial expression of *swip-10* regulates DA neuron excitability and DA-dependent behavior (Hardaway *et al.* 2015). Ablation of the amphid sheath glia, which is tightly associated with the 12 pairs of sensory neurons, results in morphological and functional disruption of the amphid sensory neurons (Bacaj *et al.* 2008). In addition, *fig-1*, which encodes a TSP1 thrombospondin domain-containing protein, has been shown to be exclusively expressed in the amphid and phasmid sheath glia and is required for neuronal dye filling. *fig-1* is, however, required for neither proper neural morphology nor behavior toward most stimuli tested (Bacaj *et al.* 2008). Thus, the molecular mechanisms underlying glial regulation of the development and function of *C. elegans* sensory neurons remain largely unclear.

We previously showed that a *C. elegans* H^+/myo-inositol transporter (HMIT) gene, *hmit-1.2,* is expressed in the amphid sheath glia (Kage-Nakadai *et al.* 2011). To elucidate the transcriptional mechanisms in glial cells, we screened for transcription factors that regulate *hmit-1.2* expression in the amphid sheath glia. In the current study, we identified *pros-1*, which encodes a *C. elegans* homologue of *Drosophila prospero*/mammalian *Prox1*, which are required for the early determination of cell fates, as a positive regulator of *hmit-1.2* expression. We showed that *pros-1* was expressed in the glia and was required for the morphology and function of the sensory neurons.

Results

pros-1 is required for normal gene expression in amphid sheath glia

We previously reported that a HMIT gene, *hmit-1.2,* was selectively expressed in the sheath glia and the excretory cell, a fluid regulatory cell in *C. elegans* (Kage-Nakadai *et al.* 2011). To identify transcription factor(s) that regulate glial development and/or function, we carried out an RNA interference (RNAi) screen for clone(s) that regulate *hmit-1.2* expression in the glia using *hmit-1.2p::egfp* transgenic animals from a sublibrary for transcription factors (727 clones, listed in Table S1 in Supporting Information) that were prepared by the Ahringer Library. Because *hmit-1.2* expression was greatly induced by osmotic stress, we carried out RNAi under the hyperosmotic conditions with a high concentration of NaCl to enhance the expression of EGFP. Of all 727 clones, we excluded 18 clones that did not grow repeatedly and screened 709 clones that cover approximately 75% of all 934 transcription factors (Reece-Hoyes *et al.* 2007). As a result, we found that the postembryonic *pros-1* (*K12H4.1*) RNAi dramatically reduced the expression of *hmit-1.2p::egfp* in the amphid sheath glia (Fig. 1A,B). In contrast, the *hmit-1.2p::egfp* expression in the excretory cell was not decreased (Fig. 1A,B).

To determine whether other glial cell markers are transcriptionally regulated by *pros-1*, we examined the expression of *fig-1*, which encodes a TSP1 thrombospondin domain-containing protein and is exclusively expressed in amphid and phasmid sheath cells (Bacaj *et al.* 2008). It was observed that *fig-1* expression was also severely disturbed by *pros-1* RNAi (Fig. 1C, D). In contrast, *pros-1* RNAi did not affect the expression of *vha-8*, a glial cell marker, which encodes a v-ATPase and is expressed in the amphid sheath glia and the excretory cell (Fig. 1E,F), and increased the expression of *daf-6*, which encodes a patched-related protein, in both the amphid sheath glia and the excretory cell (Fig. 1G,H). These results suggest that *pros-1* positively regulates the glial expression of *hmit-1.2* and *fig-1* genes and negatively regulates the *daf-6* expression. It was also noted that *pros-1* knockdown did not ablate or disrupt glial cells (see Fig. 1H).

Expression analyses showed that PROS-1::EGFP was localized in the nuclei of the amphid sheath glia, the phasmid sheath glia and the excretory cell but not of the sensory neurons (Fig. 2A). These results support the idea that PROS-1 regulates gene expression in the sheath glia and in the excretory cell as a transcription factor.

pros-1 larval lethality was rescued by human *Prox1*

pros-1 encodes a homeodomain transcription factor homologous to *Drosophila* Prospero/mammalian Prox1 that regulates the cell fate of glia/neurons and lymphatic/vascular endothelial cells, respectively (Bürglin 1994). To determine the *pros-1* function in *C. elegans*, we generated the *pros-1* deletion mutant, *tm258* (Fig. 2B). The deletion caused a frame-shift and stop codon before the homeodomain and prospero domain, which are essential for the Prospero/Prox1 function, suggesting that *pros-1*(*tm258*) is a functionally null allele. The *pros-1*(*tm258*) mutants exhibited larval lethality

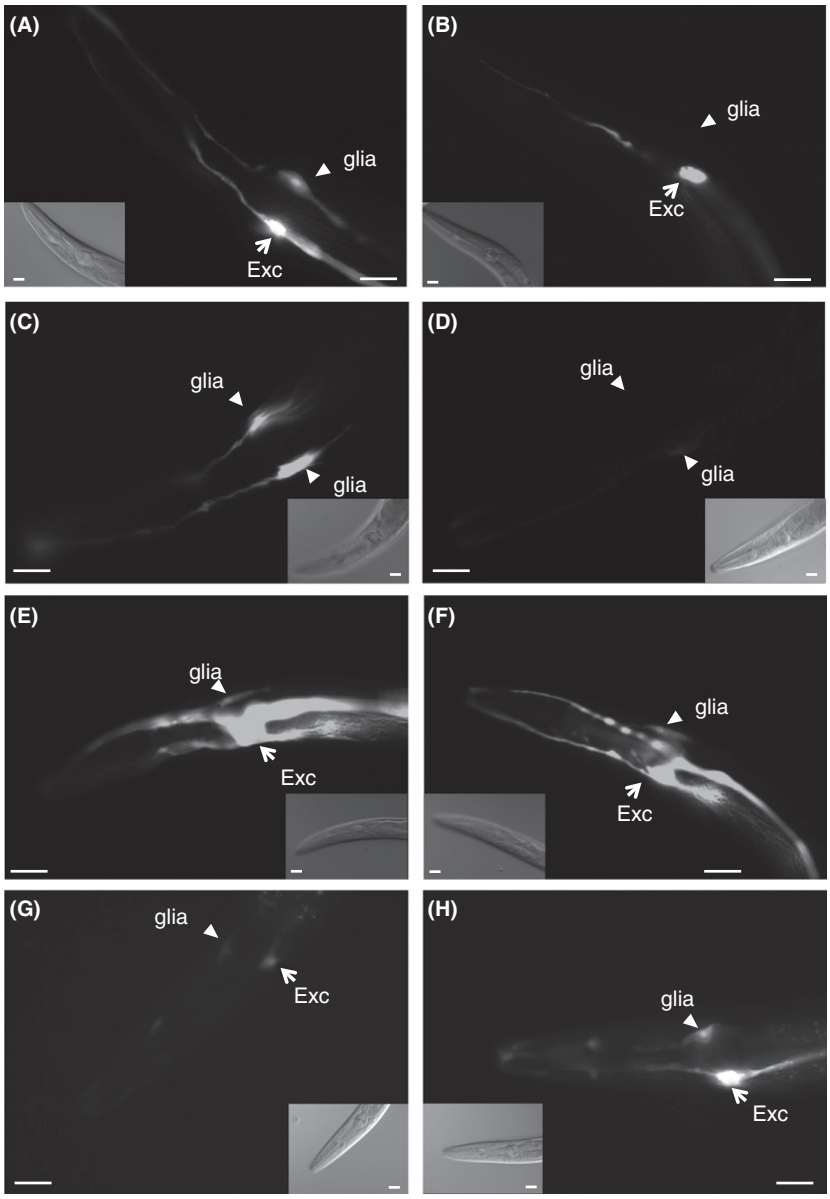

Figure 1 Gene expression in the amphid sheath glia was affected by *pros-1* RNA interference (RNAi). (A,B) *hmit-1.2p::egfp* expression with the control RNAi (A) and *pros-1* RNAi (B). (C,D) *fig-1p::egfp* expression with the control RNAi (C) and *pros-1* RNAi (D). (E,F) *vha-8p::egfp* expression with the control RNAi (E) and *pros-1* RNAi (F). (G,H) *daf-6p::cfp* expression with the control RNAi (G) and *pros-1* RNAi (H). Arrowheads indicate the amphid sheath glia, and arrows indicate the excretory cells. Insets show differential interference contrast images. Young adult hermaphrodites were photographed. Scale bars = 20 μm. Exc, excretory cell.

(100%, n = 115) (Fig. 2C). The lethal phenotype was rescued by the *pros-1* transgene (Fig. 2C). In addition, the human *Prox1* transgene also restored the larval lethality, although it did not rescue the fertility (Fig. 2C). These results suggest that the *C. elegans pros-1* gene is a functional homologue of human *Prox1*, at least partially.

pros-1 lethality was likely to be caused by dysfunction of the excretory cell

Caenorhabditis elegans pros-1 is essential for excretory cell canal formation and osmoregulatory function (Kolotuev *et al.* 2013). The excretory cell is crucial for survival (Liégeois *et al.* 2007). Meanwhile, the

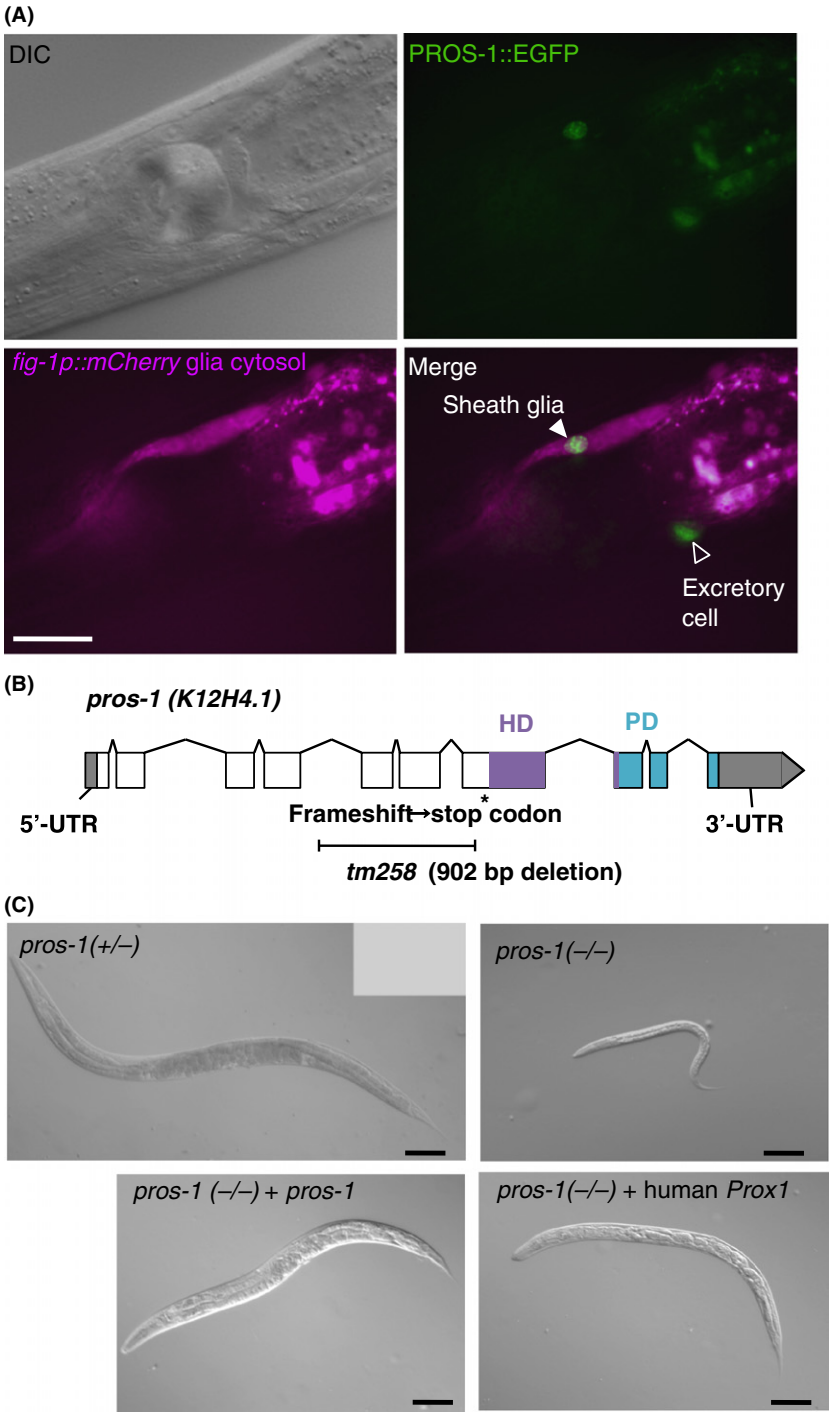

Figure 2 The larval lethal phenotype of the *pros-1* deletion mutant was rescued by the *pros-1* transgene and the human *Prox1* transgene. (A) PROS-1::EGFP was localized in the nucleus of the amphid sheath glia expressing cytosolic mCherry. PROS-1::EGFP was also localized in the nucleus of the excretory cell. Scale bar = 20 μm. (B) Schematic illustration of the *pros-1* gene structure and deletion. HD and PD indicate homeodomain and prospero domain, respectively. (C) *pros-1* homozygotic mutants exhibited larval lethality (right upper panel). *pros-1p::pros-1::egfp* rescued the larval lethal phenotype of *pros-1*(*tm258*). A fertile adult hermaphrodite was photographed (left lower panel). *pros-1p::humanProx1::egfp* rescued the larval lethal phenotype of *pros-1*(*tm258*). Adult hermaphrodites were sterile, although eggs were observed inside the body of the hermaphrodite (right lower panel). Scale bars = 100 μm. UTR, untranslated region.

amphid sheath glia are not essential for viability (Bacaj *et al.* 2008). To examine which tissue(s) are responsible for the lethal phenotype of *pros-1* mutants, we carried out rescue experiments using multicopy extrachromosomal (Ex) or single-copy integrated (SCI) *pros-1* transgenes with various lengths of the *pros-1* promoter region. As a result, the *pros-1* lethality was rescued under all the tested transgenes, including *pros-1p* (*1.5 kb*)*::egfp* SCI (see Fig. 3A homozygous adult), in which PROS-1::EGFP was observed in the excretory cell but not in the amphid sheath glia (Fig. 3A *pros-1::EGFP* expression). Taken together, the larval lethality of the *pros-1* deletion mutant was likely to be caused by the abnormality of the excretory cell.

pros-1 is required for dye filling of sensory neurons

Because amphid sheath glia physically interact with amphid sensory neurons, we hypothesized that the associated sensory neurons may be affected by the *pros-1* knockdown. To test this, we carried out DiI dye-filling assay. As a result, the percentages of animals whose amphid sensory neurons normally filled with the dye were significantly decreased by *pros-1* RNAi ($P < 0.01$, Mann–Whitney *U*-test) (Fig. 3B, C). We also confirmed that *pros-1*(*tm258*) animals were severely defective in dye filling of amphid sensory neurons (Fig. 3A dye filling). These results are comparable with a report showing that *pros-1/rdy-3* (*mc41*) mutant animals displayed DiO dye-filling defects of amphid sensory neurons (Liégeois *et al.* 2007). The RNAi and mutant studies indicated that *pros-1* is required for dye filling of sensory neurons.

Dye-filling defects of *pros-1* mutants were rescued by *pros-1* expression driven by the 9-kb promoter region

To determine the responsible tissue(s) for the defects in the dye filling of *pros-1* mutants, rescue activity was tested in a series of promoter truncated rescue strains. As a result, dye filling was rescued when the *pros-1* expression was driven by the 9-kb promoter region of *pros-1* but not by the 3-kb or less promoter region (see Fig. 3A dye filling), suggesting that the dye-filling activity requires the 3- to 9-kb upstream region of *pros-1*. However, abnormality in the excretory cell of *pros-1* mutants remained but was partially rescued by the 3-kb promoter region (Figs 3A exc. canal, S1 in Supporting Information). We did not see obvious differences between those expression patterns, as both *pros-1p* (*9 kb*)*::pros-1:egfp* and *pros-1p*(*3 kb*)*::pros-1::egfp* were expressed in the amphid sheath glia and the excretory cell from larval to adult stages. However, truncation of the 3- to 9-kb upstream region might affect the expression level of PROS-1, which could determine the susceptibility of dye filling. Indeed, we noticed that *pros-1p*(*3 kb*)*::pros-1:egfp* was expressed in both the amphid sheath glia and the excretory cell when it was introduced, along with the *fig-1p::mCherry* that is specifically expressed in the glial cell (see Fig. 2A), but absent in the amphid sheath glia along with the *hmit-1.2p* (*828 bp*)*::DsRed* that is predominantly expressed in the excretory cell (see Fig. S2 in Supporting Information). These observations were consistent across strains that were independently obtained. These results offer the possibility that the expression driven by the 3-kb upstream region may be essentially weak or unstable in the amphid sheath glia, and the lower expression of PROS-1 in glia might affect the dye-filling activity.

pros-1 is required for the proper morphology of the sensory endings and sensory behavior

Because dye-filling activity was severely affected by the inactivation of *pros-1*, we planned to determine whether the structure of sensory endings is affected or not. We visualized the sensory endings of the AWB neurons that sense repellent odorants and the ASE neurons that detect sodium chloride in the *pros-1* RNAi-treated group. The endings of AWB neurons were structurally disrupted—shortened (38% and 0%) or abnormally branched (27% and 6%) in *pros-1* RNAi-treated animals ($n = 26$) and control animals ($n = 31$), respectively (Fig. 4A). The ending of ASER neurons was occasionally creased by the *pros-1* RNAi (16%, $n = 19$) but not by the control RNAi (0%, $n = 26$) (Fig. 4A). These results suggest that *pros-1* is also involved in the normal morphology of the sensory neurons.

Next, we examined whether *pros-1* knockdown also affects sensory behavior, such as osmotic avoidance and chemotaxis. *C. elegans* is repelled by high osmolarity, and the response requires the ASH sensory neurons (Bargmann *et al.* 1990). We found that the osmotic avoidance behavior of *pros-1* RNAi-treated animals was significantly reduced when compared to that of control RNAi ($P < 0.01$, Mann–Whitney *U*-test) (Fig. 4B). We also found that chemotaxis to the volatile attractant diacetyl, which is sensed by the AWA neurons, was significantly decreased by the *pros-1* RNAi ($P < 0.01$, Student's *t*-test) (Fig. 4C). These results suggest that the function of the amphid

(A)

Allele	Homozygous adult	Dye-filling % animals (*n*)	Exc. canal % animals (*n*)	*pros-1::EGFP* expression Exc.	Sheath glia
Wild-type N2	+	100(34)	100(15)		
pros-1(tm258) m+z-	−	0(17)			
pros-1;Ex[pros-1p(9k)::pros-1::EGFP]	+	89(35)	87(15)	+	+
pros-1;Ex[pros-1p(9k)::pros-1]	+	97(29)			
pros-1;Is[pros-1p(9k)::pros-1SCI]	+	83(12)			
pros-1;Ex[pros-1p(3k)::pros-1::EGFP]	+	0(28)	47(15)	+	+
pros-1;Ex[pros-1p(1.5k)::pros-1::EGFP]	+	0(40)		+	+
pros-1;Is[pros-1p(1.5k)::pros-1::EGFP SCI]	+	0(32)		+	−

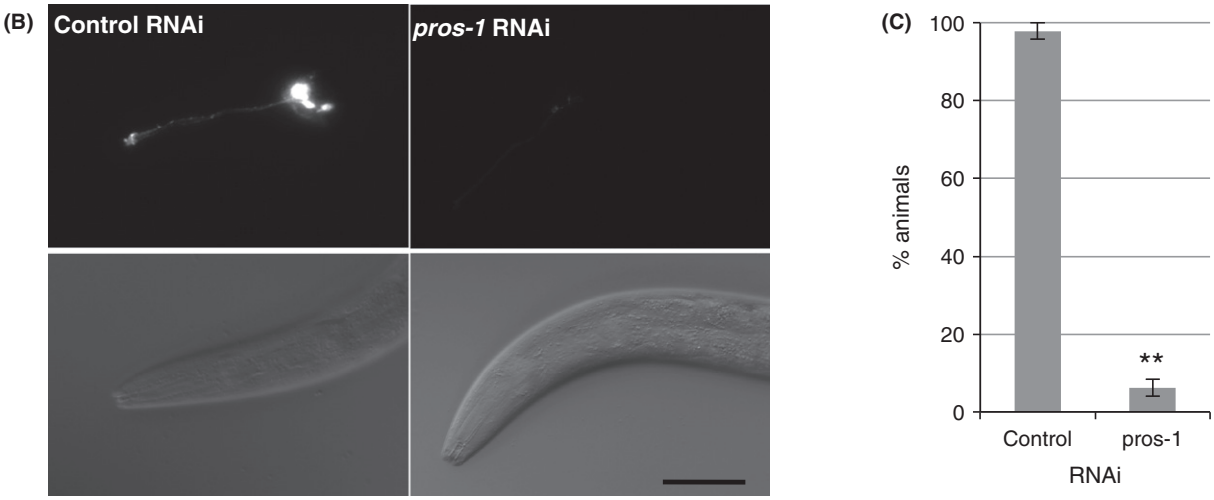

Figure 3 Phenotypes in *pros-1* knockdown, deleted and rescued animals. (A) Alleles, phenotypes and *pros-1::EGFP* expression patterns are described. Exc, excretory cell; SCI, single-copy integration. Homozygous adult: observed = +, not observed = −. Dye-filling % animals: percentages of animals whose amphid sensory neurons were dye filled. Exc. canal % animals: percentages of animals whose canals extend beyond the vulva. *pros-1::EGFP* expression: detected = +, undetected = −. (B) Amphid sensory neurons were stained by DiI fluorescent dye with the control RNAi (left upper panel) but not stained with *pros-1* RNA interference (RNAi) (right upper panel). Lower panels show differential interference contrast images. Scale bar = 50 μm. (C) Percentages of normally stained animals are indicated. A significant decrease was seen for the *pros-1* knockdown (**$P < 0.01$, Mann–Whitney *U*-test). Four independent experiments were carried out. Error bars indicate the standard error of the mean. Approximately 20 young adult hermaphrodite animals were tested in each experiment.

sensory neurons was altered by the *pros-1* knockdown.

pros-1 is involved in cold tolerance

Finally, we planned to determine whether *pros-1* knockdown affects not only sensory behavior but also sensory-mediated physiological responses, such as temperature experience-dependent cold tolerance. Worms cultivated at 15 °C, but not at higher temperatures such as 20 °C, can tolerate cold shock. The cold tolerance is mediated by ASJ amphid sensory neurons, which are originally known as light- and pheromone-sensing neurons, through temperature sensing and insulin secretion (Ohta *et al.* 2014). We found that *pros-1* knockdown animals showed

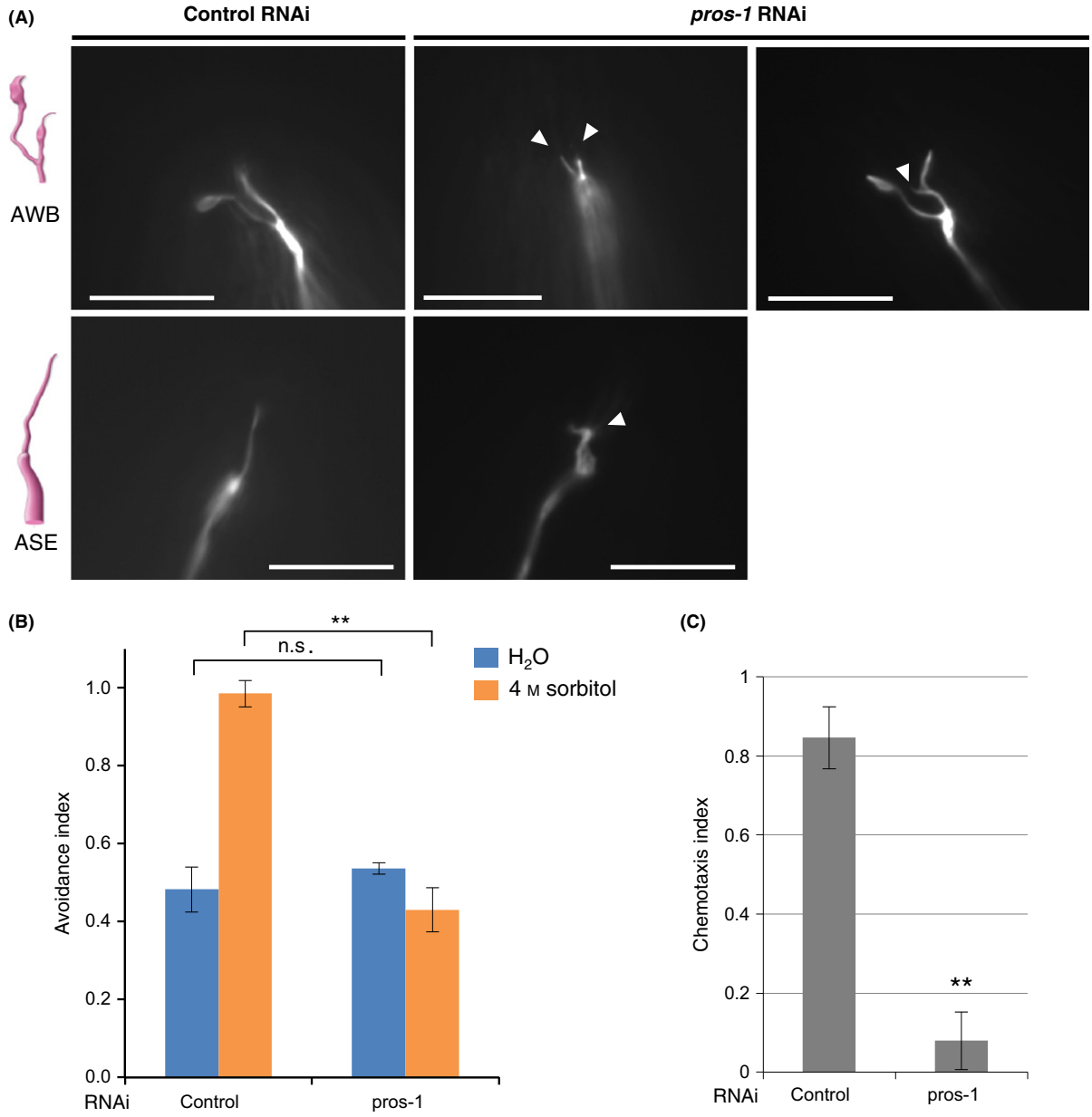

Figure 4 Sensory behaviors and morphology of the sensory endings were disturbed by the *pros-1* RNA interference (RNAi). (A) *kyIs104* [*str-1p::GFP*] expressing GFP in AWB neurons (upper panels) and *ntIs1* [*gcy-5p::GFP*], in which GFP is expressed in ASER in adult animals (lower panels), were treated by control RNAi (left panels) or *pros-1* RNAi (right panels). Arrowheads indicate shortened or additionally branched AWB sensory endings (upper panels) and an abnormal crease of the ASE ending (lower panel). Young adult hermaphrodites were photographed for fluorescence images. Schematic pictures of AWB and ASE sensory endings were modified from those found at www.wormatlas.org (Altun *et al.* 2002–2015). Scale bars = 10 μm. (B) Osmotic avoidance index of control RNAi- and *pros-1* RNAi-treated animals. A significant decrease was observed for 4 M sorbitol avoidance in *pros-1*-treated animals (**$P < 0.01$, Mann–Whitney *U*-test). There was no significant difference between control RNAi- and *pros-1* RNAi-treated animals for H_2O avoidance ($P = 0.468$, Mann–Whitney *U*-test). n.s., not significant. Error bars represent the standard error of the mean (SEM) for four independent replicates. Each replicate contained approximately 20–30 adult hermaphrodite animals that were tested. (C) Chemotaxis index of *pros-1* RNAi-treated animals was significantly decreased in comparison with that of control RNAi (**$P < 0.01$, Student's *t*-test). Error bars indicate the SEM for three independent replicates. Each replicate contained approximately 20 adult hermaphrodite animals that were tested.

abnormal cold tolerance. The survival rate of *pros-1* RNAi-treated animals at 2 °C after cultivation at 20 °C was significantly increased when compared to that of control RNAi-treated animals ($P < 0.001$, Mann–Whitney *U*-test) (Fig. 5A gray bars). This abnormal phenotype is similar to the phenotype of ASJ defective mutant animals. Unexpectedly, the survival rate of *pros-1* RNAi-treated animals at 2 °C after cultivation at 15 °C was significantly decreased in comparison with the control RNAi group ($P < 0.001$, Mann–Whitney *U*-test) (Fig. 5A, black bars). None of the mutations associated with neural or intestinal function have been reported to affect survival rate at 2 °C after cultivation at 15 °C (Ohta *et al.* 2014). To determine whether ASJ neurons respond to temperature stimuli, we carried out calcium imaging using a genetically encoded calcium indicator, cameleon. As a result, ASJ neurons in the *pros-1* knockdown animals responded normally to temperatures ranging from 17 to 23 °C (Fig. 5B). These results suggest that the *pros-1* gene is involved in cold tolerance but some unknown mechanisms are likely to underlie.

Discussion

pros-1 is likely to function in late differentiation and maintenance of glial cells in *C. elegans*

Drosophila prospero is one of the master genes for glial/neuron cell fate determination in which asymmetric segregation of Prospero into the glia-producing daughter cell is required (Hirata *et al.* 1995). Prospero also maintains the mitotic potential of glial precursors (Griffiths & Hidalgo 2004). Mammalian Prox1 is a master regulator of lymphatic endothelial cell differentiation in the embryo (Wigle & Oliver 1999). Recently, Kato *et al.* (2015) reported that Prox1 functions in the differentiation of the oligodendrocyte cell lineage in mice. Our results showed that not every glial cell marker tested was affected, and the morphology of the amphid sheath glia was not substantially disrupted under the embryonic RNAi of *pros-1*, suggesting that the glial cell fate was not essentially altered. In addition, we showed that postembryonic RNAi also decreased gene expression in the glia. Because amphid sheath glia complete the last mitosis at the late embryonic stage (Sulston *et al.* 1983), our data suggest that *pros-1* functions in postmitotic glia as a regulator of gene expression. Taken together, *pros-1* may function in late differentiation and maintenance of glial cells rather than in early cell fate determination in *C. elegans*, although we could not rule out the possibility that the RNAi was insufficient for effective knockdown at the embryonic stage.

PROS-1 regulates glial gene expression that could affect the morphology and function of the amphid sensory neurons

We showed that the structure and functions of sensory neurons, such as dye filling, ciliary morphology and sensory behavior, were affected by *pros-1* RNAi. Glial cell marker analyses showed that *pros-1* positively regulates the glial expression of *hmit-1.2* and *fig-1* genes and negatively regulates *daf-6* expression.

The dye-filling activity of *pros-1* in the sheath glia may be mediated by *fig-1* at least partially, because animals with deletions in *fig-1* have been reported to be defective in dye filling. The dye-filling defective phenotype caused by dysfunction of amphid sheath glia is also reminiscent of *daf-6* mutants (Herman 1987; Perens & Shaham 2005). *daf-6* encodes a patched-related protein that is localized to the luminal membrane of the amphid sheath channel and required for channel formation (Albert *et al.* 1981; Perens & Shaham 2005). Up-regulation of *daf-6* might contribute to the defective dye filling of the *pros-1* mutant.

pros-1 is required for proper morphology of sensory endings of ASE channel and AWB wing cilia. *daf-6* mutants are defective in the morphology of ASE channel cilia (Perens & Shaham 2005). Thus, proper expression of *daf-6* might be responsible for the *pros-1* activity in channel cilia morphology. Although *pros-1* knockdown also perturbed the morphology of wing cilia, *daf-6* is not associated with wing cilia morphology. *fig-1* mutants showed proper neural morphology (Bacaj *et al.* 2008), suggesting that *fig-1* is also independent here. Therefore, the *pros-1* activity in the morphology of wing cilia is likely to be mediated by other gene(s).

Our data suggest that *pros-1* is required for osmotic avoidance. As *daf-6* mutants are defective in sensory behavior that requires direct exposure of sensory endings to external environment (Albert *et al.* 1981), proper expression of *daf-6* might contribute to the *pros-1* function in osmotic avoidance. Although *pros-1* knockdown perturbed chemotaxis to volatile diacetyl, *daf-6* mutants normally respond to volatile odorants (Albert *et al.* 1981). We showed that *pros-1* is involved in sensory-mediated cold tolerance. *daf-6* mutations, however, do not

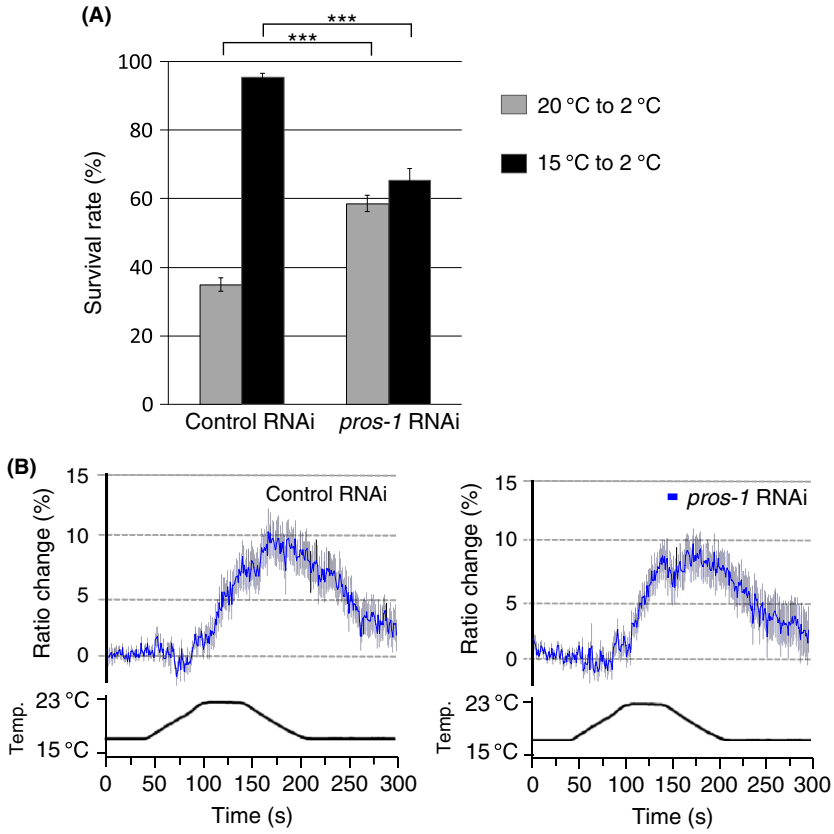

Figure 5 *pros-1* RNA interference (RNAi) affected cold tolerance but not ASJ neural responses. (A) *pros-1* knockdown animals showed abnormal cold tolerance after cultivation both at 20 °C and 15 °C. For each assay, $n \geq 12$. Each replicate contained approximately 30–130 animals. Error bars indicate the standard error of the mean. ***$P < 0.001$. Statistical significance was assessed by Mann–Whitney *U*-test. (B) Control RNAi- and *pros-1* RNAi-treated animals could respond to temperature stimuli. *eri-1;lin-15B* mutants expressing yellow cameleon driven by the *trx-1* promoter, *trx-1p::yc3.60*, were tested with calcium imaging. Each graph represents average response to temperature stimuli. For each graph, $n \geq 20$.

affect cold tolerance (Ohta *et al.* 2014). Therefore, the *pros-1* activities in sensory behavior to volatile diacetyl and cold tolerance likely involve unknown gene(s).

The involvement of *pros-1* in cold tolerance

Our study showed that the *pros-1* knockdown animals were tolerant to cold shock after cultivation at 20 °C. This phenotype is similar to the phenotype of ASJ defective mutant animals (Ohta *et al.* 2014), implying the *pros-1* function in ASJ neurons. However, ASJ neurons of the *pros-1* knockdown animals responded normally to temperatures, at least under the tested conditions ranging from 17 to 23 °C. These incompatible results suggest two possibilities. One is that *pros-1* may be required for ASJ intracellular signal transduction and/or insulin release but not for temperature sensation itself. Another possibility is that the *pros-1* function in tissue(s) other than ASJ neurons is involved in cold tolerance. Because the expression of *daf-28*, which is one of the insulin genes expressed in ASJ, was not decreased by *pros-1* knockdown (data not shown), the latter possibility may be more plausible.

We also showed that *pros-1* knockdown animals were sensitive to cold shock after cultivation at 15 °C. None of the mutants associated with neural or intestinal function showed such phenotype (Ohta *et al.* 2014), suggesting that unknown tissue(s) may be involved in this phenomenon. The excretory cell, where *pros-1* is expressed, might be the responsible tissue, although further studies are needed to test this hypothesis.

PROS-1 functions in the excretory cell and the sheath glia

pros-1 has been shown to be required for excretory canal formation. *pros-1*(*mc41*) mutant animals failed to extend the excretory canal and occasionally accumulated vacuoles in the excretory cell. In addition, *pros-1*(*mc41*) homozygotic animals were 100% larval lethal (Liégeois *et al.* 2007). These findings are compatible with those of *pros-1*(*tm258*). *pros-1* RNAi caused the accumulation of large vacuoles in the excretory cell and/or shortened canal; 5%–40% animals had a cystic canal (Kolotuev *et al.* 2013) and approximately 60% animals had a cystic canal and/or shortened canal (this study, Fig. S3 in Supporting Information). Although Liégeois *et al.* also reported that *pros-1*(*mc41*) mutants display severe dye-filling defects of sensory neurons, the expression and function of *pros-1* in the sheath glia were unknown. In the present study, we showed that *pros-1* is expressed in the sheath glia and is required for the structure and functions of sensory neurons, such as dye filling, ciliary morphology and sensory behavior. A similar observation of *pros-1* gene function has been published recently, during revision of our manuscript (Wallace *et al.* 2016). The *pros-1* RNAi and mutants will be useful to elucidate further mechanisms underlying canal formation, and the interaction between glia and sensory neurons. In addition, our results showed the involvement of *pros-1* not only in sensory behavior, but also in sensory-mediated physiological tolerance. Further studies showing where and how PROS-1 acts in the phenomena will be worthwhile.

Experimental procedures

Strains

Caenorhabditis elegans strains were cultured using standard techniques (Brenner 1974). The wild-type *C. elegans* strain used was Bristol strain N2. The following strains were obtained from the Caenorhabditis Genetics Center: JU486 *mfIs4* [*egl-17::YFP + daf-6::CFP + unc-119*(+)], CX3553 *lin-15*(*n765*); *kyIs104 X* [*str-1::GFP + lin-15*(+)], OH3192 *ntIs1* [*gcy-5p::GFP + lin-15*(+)] *V*, KP3948 *eri-1*(*mg366*) *IV; lin-15B*(*n744*) *X*. The *pros-1* deletion mutant strain was obtained from the UV/TMP mutagenized library, which has been previously described (Gengyo-Ando & Mitani 2000), and this strain was identified by polymerase chain reaction (PCR) amplification with primers spanning the deletion region of *pros-1*(*tm258*)*III*. The primers that were used for PCR genotyping were as follows: tm258_1stround, 5′-GAAGTCAACGGAAGGGATGA-3′, 5′-AAATCGGAG GCACAGAAAGT-3′; tm258_2ndround, 5′-TGAGACGGA AACGATGACAG-3′, 5′-GCACATTTTGAAATGGGGAC T-3′. The *tm258* mutants were balanced by *hT2*[*bli-4*(*e937*) *let-?*(*q782*) *qIs48*] (*I;III*).

Constructs and transgenic lines

hmit-1.2p::egfp, fig-1p::egfp and *vha-8p::egfp* were constructed by subcloning upstream genomic fragments of each gene into the BamHI/NotI sites of the pFX_EGFPT expression vector (Gengyo-Ando *et al.* 2006). To generate extrachromosomal (Ex) transgenic animals, these plasmids were injected into N2 with an injection marker *pRF-4*[*rol-6d*] at 100 ng/μL each. *tmIs807*[*hmit-1.2p::egfp*] was generated as previously described (Mitani 1995). *pros-1p*(*0.5 kb*)*::pros-1::egfp, pros-1p*(*1.5 kb*)*::pros-1::egfp, pros-1p*(*3 kb*)*::pros-1::egfp, pros-1p*(*6 kb*)*::pros-1::egfp,* and *pros-1p*(*9 kb*)*::pros-1::egfp* were constructed by subcloning *pros-1* genomic fragments that contain corresponding promoter and coding regions into pFX_EGFPT. *pros-1p*(*1.5 kb*)*::humanProx1::egfp* was constructed by subcloning *pros-1p*(*1.5 kb*) into BamHI/NotI site of pFX_EGFPT followed by subcloning human *Prox1* cDNA into NotI/BglII site. For expression analyses in the amphid sheath glia, *pros-1p::pros-1::egfp* was co-injected with *fig-1p::mCherry*. To generate Ex transgenic animals for rescue experiments, plasmids were injected into *pros-1*(*tm258*)*/hT2*[*bli-4*(*e937*) *let-?*(*q782*) *qIs48*]. SCI strains were generated as previously described (Kage-Nakadai *et al.* 2012, 2014).

Bacterial RNAi feeding

RNA interference was carried out by feeding animals dsRNA-producing bacteria, as previously described (Kamath *et al.* 2001), with some modifications. Briefly, P0 animals at the L4 stage were transferred to plates containing RNAi-bacteria grown on NGM containing 100 μg/mL ampicillin and 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and cultured at 20 °C (or 15 °C in cold tolerance assays) until the F1 animals developed into young adults. F1 animals were used for the following assays so that knockdown was effective from the embryonic stages (referred to as embryonic RNAi in the text). For feeding RNAi screening, postembryonic RNAi was simultaneously carried out. In this case, synchronized animals at L1–L2 stage were transferred to the feeding RNAi plates and cultured until the transferred animals became young adults. For the screening using *hmit-1.2p::egfp* transgenic animals, modified NGM plates that contained ampicillin, IPTG and 4fold NaCl (200 mM final) were used to induce EGFP expression. For cold tolerance assays, the *eri-1;lin-15B* strain was used to sensitize RNAi (Kennedy *et al.* 2004; Wang *et al.* 2005). The RNAi sublibrary (727 clones shown in Table S1 in Supporting Information) for transcription factors was prepared from the Ahringer Library using a list of the 934 putative transcription factor genes in *C. elegans* (Reece-Hoyes *et al.* 2007).

Microscopy

Differential interference contrast and fluorescence images were obtained using a BX51 microscope that was equipped with a DP30BW CCD camera (Olympus, Japan).

Odor chemotaxis assay and osmotic avoidance assay

Assays for chemotaxis to odorants were carried out as described (Bargmann *et al.* 1993), with some modifications. Animals at the young adult stage were collected in microfuge tubes and washed 3 times with M9 buffer (5 mM potassium phosphate, 1 mM $CaCl_2$, 1 mM $MgSO_4$ and 0.5 g/L gelatin). Approximately 20 animals were spotted at the center of 9-cm assay plates. Excess liquid was removed with paper towels, at the same time dispersing the animals along the midline of the plates. Assay plates were prepared as follows: 1 μL of 10^{-3} dilution of diacetyl and 0.5 μL of 1 M sodium azide as an anesthetic were placed at a spot 1 cm from the edge of the plate. One microliter of EtOH and sodium azide was spotted on the other side. Thirty min after placing the animals at the center of the plates, the animals were counted, and the chemotaxis index was calculated as [(number of animals within a 3-cm radius of odorant spot)–(number of animals within a 3-cm radius of control spot)]/(total number of animals). Osmotic avoidance assays with a 4-M sorbitol ring were carried out as previously described (Culotti & Russell 1978), with some modifications. We placed 20–30 animals inside a high osmotic ring (1.5 cm in diameter) made with 15 μL of 4 M sorbitol. The avoidance index was defined as the fraction of animals that remained inside the ring after 10 min.

Cold tolerance assay

Temperature experience-dependent cold tolerance assays were carried out as previously described (Ohta *et al.* 2014; Ujisawa *et al.* 2014). Briefly, adult animals (approximately 30–130 animals/plate) cultured at 20 °C or 15 °C were transferred to 2 °C in a refrigerated cabinet (CRB-41A, Hitachi, Tokyo, Japan). After 48 h, the plates were transferred to room temperature (22–24 °C), and the living or dead animals on the plate were counted.

In vivo calcium imaging

In vivo calcium imaging was carried out as previously described (Ohta *et al.* 2014; Ujisawa *et al.* 2014). RNAi-treated *eri-1;lin-15B* animals expressing yellow cameleon 3.60 driven by the *trx-1* promoter, *trx-1p::yc3.60* (pTOM13), were used for calcium imaging. Animals glued onto an agar pad on glass were placed onto a Peltier-based thermocontroller (Tokai Hit, Sizuoka, Japan) on the stage of an Olympus IX81 at the initial imaging temperature for 2 min, and fluorescence was introduced into a Dual-View optics system (Molecular Devices, Sunnyvale, CA, USA). Fluorescence images of donor and acceptor in yellow cameleon were simultaneously captured using an EM-CCD camera EVOLVE512 (Photonics, Oxford, MA, USA). Images were taken with a 100-ms exposure time with 1 × 1 binning. Fluorescence intensities were measured using the MetaMorph image analysis system (Molecular Device). Ratio changes in the acceptor/donor fluorescence were measured.

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References

Albert, P.S., Brown, S.J. & Riddle, D.L. (1981) Sensory control of dauer larva formation in *Caenorhabditis elegans*. *J. Comp. Neurol.* **198**, 435–451.

Bacaj, T., Tevlin, M., Lu, Y. & Shaham, S. (2008) Glia are essential for sensory organ function in *C. elegans*. *Science* **322**, 744–747.

Bargmann, C.I., Hartwieg, E. & Horvitz, H.R. (1993) Odorant-selective genes and neurons mediate olfaction in *C. elegans*. *Cell* **74**, 515–527.

Bargmann, C.I., Thomas, J.H. & Horvitz, H.R. (1990) Chemosensory cell function in the behavior and development of *Caenorhabditis elegans*. *Cold Spring Harb. Symp. Quant. Biol.* **55**, 529–538.

Booth, G.E., Kinrade, E.F. & Hidalgo, A. (2000) Glia maintain follower neuron survival during Drosophila CNS development. *Development* **127**, 237–244.

Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71–94.

Bürglin, T.R. (1994) A *Caenorhabditis elegans* prospero homologue defines a novel domain. *Trends Biochem. Sci.* **19**, 70–71.

Christopherson, K.S., Ullian, E.M., Stokes, C.C., Mullowney, C.E., Hell, J.W., Agah, A., Lawler, J., Mosher, D.F., Bornstein, P. & Barres, B.A. (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* **120**, 421–433.

Culotti, J.G. & Russell, R.L. (1978) Osmotic avoidance defective mutants of the nematode *Caenorhabditis elegans*. *Genetics* **90**, 243–256.

Eroglu, C. & Barres, B.A. (2010) Regulation of synaptic connectivity by glia. *Nature* **468**, 223–231.

Felton, C.M. & Johnson, C.M. (2011) Modulation of dopamine-dependent behaviors by the *Caenorhabditis elegans* Olig homolog HLH-17. *J. Neurosci. Res.* **89**, 1627–1636.

Gengyo-Ando, K. & Mitani, S. (2000) Characterization of mutations induced by ethyl methanesulfonate, UV, and trimethylpsoralen in the nematode *Caenorhabditis elegans*. *Biochem. Biophys. Res. Commun.* **269**, 64–69.

Gengyo-Ando, K., Yoshina, S., Inoue, H. & Mitani, S. (2006) An efficient transgenic system by TA cloning vectors and RNAi for *C. elegans*. *Biochem. Biophys. Res. Commun.* **349**, 1345–1350.

Griffiths, R.L. & Hidalgo, A. (2004) Prospero maintains the mitotic potential of glial precursors enabling them to respond to neurons. *EMBO J.* **23**, 2440–2450.

Hardaway, J.A., Sturgeon, S.M., Snarrenberg, C.L., Li, Z., Xu, X.Z., Bermingham, D.P., Odiase, P., Spencer, W.C., Miller, D.M., Carvelli, L., Hardie, S.L. & Blakely, R.D. (2015) Glial expression of the *Caenorhabditis elegans* gene swip-10 supports glutamate dependent control of extrasynaptic dopamine signaling. *J. Neurosci.* **35**, 9409–9423.

Herman, R.K. (1987) Mosaic analysis of two genes that affect nervous system structure in *Caenorhabditis elegans*. *Genetics* **116**, 377–388.

Hirata, J., Nakagoshi, H., Nabeshima, Y. & Matsuzaki, F. (1995) Asymmetric segregation of the homeodomain protein Prospero during Drosophila development. *Nature* **377**, 627–630.

Hosoya, T., Takizawa, K., Nitta, K. & Hotta, Y. (1995) Glial cells missing: a binary switch between neuronal and glial determination in Drosophila. *Cell* **82**, 1025–1036.

Kage-Nakadai, E., Imae, R., Yoshina, S. & Mitani, S. (2014) Methods for single/low-copy integration by ultraviolet and trimethylpsoralen treatment in *Caenorhabditis elegans*. *Methods* **68**, 397–402.

Kage-Nakadai, E., Kobuna, H., Funatsu, O., Otori, M., Gengyo-Ando, K., Yoshina, S., Hori, S. & Mitani, S. (2012) Single/low-copy integration of transgenes in *Caenorhabditis elegans* using an ultraviolet trimethylpsoralen method. *BMC Biotechnol.* **12**, 1.

Kage-Nakadai, E., Uehara, T. & Mitani, S. (2011) H+/myo-inositol transporter genes, hmit-1.1 and hmit-1.2, have roles in the osmoprotective response in *Caenorhabditis elegans*. *Biochem. Biophys. Res. Commun.* **410**, 471–477.

Kamath, R.S., Martinez-Campos, M., Zipperlen, P., Fraser, A.G. & Ahringer, J. (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol.* **2**, RESEARCH0002.

Kato, K., Konno, D., Berry, M., Matsuzaki, F., Logan, A. & Hidalgo, A. (2015) Prox1 inhibits proliferation and is required for differentiation of the oligodendrocyte cell lineage in the mouse. *PLoS ONE* **10**, e0145334.

Kennedy, S., Wang, D. & Ruvkun, G. (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* **427**, 645–649.

Kolotuev, I., Hyenne, V., Schwab, Y., Rodriguez, D. & Labouesse, M. (2013) A pathway for unicellular tube extension depending on the lymphatic vessel determinant Prox1 and on osmoregulation. *Nat. Cell Biol.* **15**, 157–168.

Liégeois, S., Benedetto, A., Michaux, G., Belliard, G. & Labouesse, M. (2007) Genes required for osmoregulation and apical secretion in *Caenorhabditis elegans*. *Genetics* **175**, 709–724.

Mitani, S. (1995) Genetic regulation of *mec-3* gene expression implicated in the specification of the mechanosensory neuron cell types in *Caenorhabditis elegans*. *Dev. Growth Differ.* **37**, 551–557.

Ohta, A., Ujisawa, T., Sonoda, S. & Kuhara, A. (2014) Light and pheromone-sensing neurons regulates cold habituation through insulin signalling in *Caenorhabditis elegans*. *Nat. Commun.* **5**, 4412.

Perens, E.A. & Shaham, S. (2005) *C. elegans* daf-6 encodes a patched-related protein required for lumen formation. *Dev. Cell* **8**, 893–906.

Reece-Hoyes, J.S., Shingles, J., Dupuy, D., Grove, C.A., Walhout, A.J., Vidal, M. & Hope, I.A. (2007) Insight into transcription factor gene duplication from *Caenorhabditis elegans* Promoterome-driven expression patterns. *BMC Genom.* **8**, 27.

Sulston, J.E., Schierenberg, E., White, J.G. & Thomson, J.N. (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**, 64–119.

Ujisawa, T., Ohta, A., Okahata, M., Sonoda, S. & Kuhara, A. (2014) Cold tolerance assay for studying cultivation-temperature-dependent cold habituation in *C. elegans*. *Protoc. Exch.* doi:10.1038/protex.2014.1032.

Wallace, S.W., Singhvi, A., Liang, Y., Lu, Y. & Shaham, S. (2016) PROS-1/Prospero is a major regulator of the glia-specific secretome controlling sensory-neuron shape and function in *C. elegans*. *Cell Rep.* **15**, 550–562.

Wang, D., Kennedy, S., Conte, D., Kim, J.K., Gabel, H.W., Kamath, R.S., Mello, C.C. & Ruvkun, G. (2005) Somatic misexpression of germline P granules and enhanced RNA interference in retinoblastoma pathway mutants. *Nature* **436**, 593–597.

Wang, Y., Apicella, A., Lee, S.K., Ezcurra, M., Slone, R.D., Goldmit, M., Schafer, W.R., Shaham, S., Driscoll, M. & Bianchi, L. (2008) A glial DEG/ENaC channel functions with neuronal channel DEG-1 to mediate specific sensory functions in *C. elegans*. *EMBO J.* **27**, 2388–2399.

Wang, Y., D'Urso, G. & Bianchi, L. (2012) Knockout of glial channel ACD-1 exacerbates sensory deficits in a *C. elegans*

mutant by regulating calcium levels of sensory neurons. *J. Neurophysiol.* **107**, 148–158.

Ward, S., Thomson, N., White, J.G. & Brenner, S. (1975) Electron microscopical reconstruction of the anterior sensory anatomy of the nematode *Caenorhabditis elegans*. *J. Comp. Neurol.* **160**, 313–337.

White, J.G., Southgate, E., Thomson, J.N. & Brenner, S. (1986) The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **314**, 1–340.

Wigle, J.T. & Oliver, G. (1999) Prox1 function is required for the development of the murine lymphatic system. *Cell* **98**, 769–778.

Yoshimura, S., Murray, J.I., Lu, Y., Waterston, R.H. & Shaham, S. (2008) mls-2 and vab-3 Control glia development, hlh-17/Olig expression and glia-dependent neurite extension in *C. elegans*. *Development* **135**, 2263–2275.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 DIC images showing the excretory canal.

Figure S2 *pros-1p(3 kb)::pros-1:egfp* was expressed in the excretory cell but absent in the amphid sheath glia when introduced along with the *hmit-1.2p(828 bp)::DsRed* that is predominantly expressed in the excretory cell.

Figure S3 *tmIs807*[*hmit-1.2p::egfp*] animals were treated by control (L440) RNAi (upper panels) or *pros-1* RNAi (lower panels).

Table S1 List of transcription factors included in Ahringer Library