**PROS-1/Prospero Is a Major Regulator of the Glia-Specific Secretome Controlling Sensory-Neuron Shape and Function in *C. elegans***

**Highlights**
- *C. elegans* glia expression profiling identifies many post-embryonically secreted proteins
- The transcription factor *pros-1*/Prospero functions post-developmentally in glia
- *pros-1* controls expression of genes encoding secreted/membrane proteins
- *pros-1* acting through its targets controls morphology/function of associated neurons

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**In Brief**
Wallace et al. find a post-developmental role for the transcription factor *pros-1*/Prospero in regulating expression of glia-secreted proteins and controlling the morphology and function of glia-associated neurons. *pros-1* homologs are expressed in differentiated glia in vertebrates and may play similar roles in regulating glia-neuron interactions.

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PROS-1/Prospero Is a Major Regulator of the Glia-Specific Secretome Controlling Sensory-Neuron Shape and Function in *C. elegans*

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SUMMARY

Sensory neurons are an animal’s gateway to the world, and their receptive endings, the sites of sensory signal transduction, are often associated with glia. Although glia are known to promote sensory-neuron functions, the molecular bases of these interactions are poorly explored. Here, we describe a post-developmental glial role for the PROS-1/Prospero/PROX1 homeodomain protein in sensory-neuron function in *C. elegans*. Using glia expression profiling, we demonstrate that, unlike previously characterized cell fate roles, PROS-1 functions post-embryonically to control sense-organ glia-specific secretome expression. PROS-1 functions cell autonomously to regulate glial secretion and membrane structure, and non-cell autonomously to control the shape and function of the receptive endings of sensory neurons. Known glial genes controlling sensory-neuron function are PROS-1 targets, and we identify additional PROS-1-dependent genes required for neuron attributes. *Drosophila* Prospero and vertebrate PROX1 are expressed in post-mitotic sense-organ glia and astrocytes, suggesting conserved roles for this class of transcription factors.

INTRODUCTION

Animals use specialized sense organs to obtain information from their environment. Sense organs, in general, consist of sensory neurons or receptor cells, the primary mediators of sensory signal transduction, and glia-like support cells, which have been defined anatomically for decades but have been relatively poorly studied (Cuscheri and Bannister, 1975; Engström, 1967; Murray, 1993). Glia-like cells in the mammalian taste bud, olfactory epithelium, cochlea, and retina, classified as type I cells, sustentacular cells, Deiters’ cells, and RPE (retinal pigment epithelial) cells, respectively, show a number of functions typically associated with glia. These include regulating neurotransmitter signaling (Bartel et al., 2006; Dooley et al., 2011; Hegg et al., 2009; Lawton et al., 2000; Matsunobu et al., 2001), buffering extracellular potassium levels (Boettger et al., 2002; Dvoryanchikov et al., 2009; Trotier, 1998), and releasing neurotrophic factors and neuromodulators (Breunig et al., 2010; Hansel et al., 2001; Strauss, 2005). Thus, functional similarities between sense-organ and CNS glia, and similarities between sensory-neuron receptive endings and synapses (Shaham, 2010), suggest that understanding sensory neuron-glia interactions may have broad relevance.

*C. elegans* is an attractive model system for studying glia-neuron interactions (Oikonomou and Shaham, 2011; Shaham, 2006, 2015). Its simple anatomy, facile genetics, optical transparency, and well-characterized behavioral responses allow for single-cell-resolution in vivo analyses of the contributions that glial cells make to nervous system function. The *C. elegans* amphids are a pair of bilaterally symmetric head sense organs that mediate responses to multiple sensory inputs (Bargmann et al., 1993; Bargmann and Horvitz, 1991; Kimura et al., 2004; Ward, 1973). Each amphid consists of 12 sensory neurons, possessing specialized receptive endings that respond to distinct cues. These neurons associate with two glial cells, the amphid sheath (AMsh) and amphid socket (AMso) glia, that together form a matrix-filled channel through which sensory cilia of eight neurons are exposed to the external environment (Perkins et al., 1986). The remaining four neurons have elaborately structured receptive endings that are ensheathed by the AMsh glial cell. These glia-ensheathed receptive endings include the branched wing-like cilia of the odor-sensing neurons AWA, AWB, and AWC, as well as the microvilli-like fingers of the AFD thermosensory neuron (Doroquez et al., 2014; Perkins et al., 1986) (Figure 1A).

We previously showed that post-embryonic ablation of AMsh glia, after the amphid had formed, results in defects in the associated sensory neurons (Bacaj et al., 2008). AMsh glia are required for the proper morphology of the AWA, AWB, and AFD sensory neurons and for dye uptake by cilia of channel neurons, which serves to report on neuronal integrity and access to the environment. Glial ablation also results in defects in behaviors mediated by these neurons. However, the molecular mechanisms controlling these interactions were not well understood.

Here, we characterize the molecular basis for the post-embryonic regulation of sensory-neuron morphology and function by AMsh glia. We developed a larval glial cell isolation method using fluorescence-activated cell sorting (FACS), followed by RNA sequencing (RNA-seq), to identify 598 AMsh glia-enriched...
transcripts. Approximately 90% of highly enriched AMsh genes encode secreted or transmembrane proteins, consistent with a role in regulating neuronal properties. A post-embryonic RNAi screen of a subset of these genes led to the identification of the homeodomain transcription factor **pros-1** as a glial regulator of neuronal morphology and function. Expression studies, cell-specific RNAi experiments, and mosaic rescue experiments confirm that **pros-1** acts in glia to control sensory-neuron shape and function. To characterize the mechanisms mediating **pros-1** functions, we identified **pros-1** target genes using differential gene-expression analyses of AMsh glia isolated from **pros-1** RNAi-treated and control animals. Approximately one-third of AMsh-enriched genes encoding secreted or transmembrane proteins require PROS-1 for their expression, revealing a key function for this gene in regulating expression of the glia-specific secretome. PROS-1 targets include genes with known roles in amphid morphology and function, and the RNAi screens we performed identify new genes that contribute to **pros-1** function. Thus, PROS-1 is a key transcriptional regulator governing the expression of secreted and membrane proteins in AMsh glia.

**Prospero**, the *Drosophila* homolog of **pros-1**, plays well-known roles in the establishment of cell fate during nervous system development (Doe et al., 1991; Freeman and Doe, 2001). Importantly, we found that while post-embryonic inactivation of **pros-1** by RNAi promotes profound defects in sensory-neuron function, general aspects of glial cell fate are unperturbed, thus revealing functions for this classical cell fate gene in differentiated glia. Prospero homologs are expressed in glia associated with sense organs in many species, including sheath cells in *Drosophila* external sense organs (Manning and Doe, 1999), mechanosensory support cells in fish neuromasts (Pistocchi et al., 2009), and Deiters’ cells in the mammalian ear (Bermingham-McDonogh et al., 2006), and are also expressed in astrocytes in *Drosophila* and mammals (Peco et al., 2016) (Zhang et al., 2014). Thus, our studies of **pros-1** function in *C. elegans* provide general insights into the regulation of neuronal function by glia.

**RESULTS**

**Identification of Transcripts Enriched in Post-embryonic AMsh Glia**

To identify glial genes controlling sensory neuron function, and not development, we isolated AMsh glia from late-stage *C. elegans* larvae and performed gene expression analysis (see
Cells expressing the AMsh glia-specific reporter F16F9.3::dsRed were sorted by FACS and subjected to RNA extraction and purification. mRNA was amplified and used to generate a cDNA library for sequencing. Quantitative gene expression profiling was carried out to identify genes enriched in AMsh glia, by comparing AMsh glia to all other cell types not expressing dsRed. This approach led to the identification of 598 candidate AMsh glia-enriched genes (fold enrichment > 3.5, p-adj < 0.05; see Experimental Procedures for statistical analysis and cutoff rationale). Of these 598 genes, 383 (64%) encode proteins with signal peptides or transmembrane domains (Table S1; Figure 1B). This is significantly higher than the prevalence of such proteins in the C. elegans genome (45%) (Suh and Hutter, 2012). Strikingly, of 177 highly enriched AMsh genes (fold enrichment > 10), 155 genes (88%) encode proteins with signal peptides or transmembrane domains (Figure 1B). To validate this gene list, we checked the expression levels of six known AMsh glia-specific genes (Bacaj et al., 2008). All six genes showed strong enrichment in AMsh glia in our analysis (Figure 1C), confirming that we successfully isolated larval AMsh glial transcripts. We also made transcriptional reporters with upstream regulatory regions for two genes, K02E11.4 and R11D1.3, both of which are highly enriched in AMsh glia in our analysis, but not previously studied. Transcriptional reporters for both genes showed specific expression in AMsh glia (Figure 1D), as well as in the analogous phasmid sheath glia in the tail, confirming that we successfully identified AMsh glial genes.

Glia-Enriched pros-1/Prospero Is Required for Sensory-Neuron Function

To uncover the molecular pathways through which AMsh glia regulate functional properties of associated sensory neurons, we screened animals for sensory deficits following post-embryonic RNAi against 140 of the 598 AMsh glia-enriched genes. Following 3 days of RNAi treatment, first-day adults were assessed for defects in chemosensation, using standard chemotaxis and avoidance assays. RNAi targeting mRNAs encoding the Prospero-related transcription factor, PROS-1, results in defects in all chemosensory behaviors tested, including chemotaxis to benzaldehyde, methylpyrazine, and sodium chloride, attractants sensed by AWC, AWA, and ASE sensory neurons, respectively. Avoidance of 1-octanol, a repellant sensed by ADL and ASH sensory neurons is also perturbed (Bargmann, 2006; Bargmann et al., 1993; Bargmann and Horvitz, 1991; Chao et al., 2004) (Figures 2A–2D). pros-1 RNAi animals also exhibit defective thermotaxis behavior (Figure 2E), suggestive of functional defects in the AFD thermosensory neuron. Post-embryonic loss of pros-1, therefore, phenocopies ablation of AMsh glia (Bacaj et al., 2008).
2008), which also results in functional defects of associated sensory neurons.

**PROS-1/Prospero Functions Post-embryonically and Does Not Affect Glial Cell Fate**

In *Drosophila*, Prospero regulates the decision between glial and neuronal fates during external sense-organ formation. Prospero mutants fail to produce glia, and Prospero overexpression results in overproduction of glia and of cells inappropriately expressing both glial and neuronal markers (Manning and Doe, 1999). *C. elegans* pros-1 is also expressed in glial cells and their precursors during embryogenesis (expression patterns in *Caenorhabditis* [EPIC], http://epic.gs.washington.edu/); however, we specifically carried out our RNAi studies post-embryonically, after glial and neuronal fate had been established. Thus, the pros-1(RNAi) defects we observe may not result from a loss of glial cell fate. Indeed, we found that AMsh glia continue to express glial markers following post-embryonic pros-1 RNAi, including the AMsh-specific marker F16F9.3 and the pan-glial marker mir-228 (Figure 3). Furthermore, pros-1(RNAi) AMsh glia do not show inappropriate expression of markers specific to their sister neurons. The cholinergic neuron URB, the sister cell of the AMsh glial cell, expresses unc-17/vAChT, a specific marker for cholinergic neurons, as well as rab-3, a pan-neuronal marker (Alfonso et al., 1993; Duerr et al., 2008). Neither is expressed in AMsh glia of pros-1(RNAi) animals, nor are AMsh reporters expressed in URB (Figure 3). Finally, AMsh glia of pros-1(RNAi) animals are grossly normal in morphology.

Figure 3. Post-embryonic pros-1 RNAi Does Not Affect AMsh Glia Fate
(A) Animals expressing F16F9.3::dsRed, an AMsh glia reporter, and unc-17::GFP, a cholinergic neuron reporter.
(B) Animals expressing the pan-glial marker mir-228::GFP and the pan-neuronal marker rab-3::mCherry. White arrowheads, AMsh glia cell body. Scale bar represents 10 µm.
(see below). These results indicate that the defects we observed following pros-1 RNAi are not a result of general cell-fate abnormalities. Thus, pros-1 controls specific post-developmental aspects of glial function.

Animals Lacking PROS-1/Prospero Exhibit Defects in Sensory Neuron Receptive-Ending Structure and Microenvironment

To uncover the cellular basis for the behavioral deficits exhibited by pros-1(RNAi) animals, we assessed the morphology of sensory-neuron receptive endings and the structure of the AMsh glial membranes surrounding these receptive endings using fluorescence and electron microscopy. The AWC odor-sensing neuron and the AFD thermosensory neuron both have elaborate receptive endings that are ensheathed by AMsh glia (Figure 1A). Following pros-1 RNAi, both neurons extend dendrites normally to the tip of the nose, but their receptive endings are largely absent. AWC neurons lack the wing-like cilium found in wild-type adults (Figures 4A and 4F), while AFD neurons lack the microvilli-like fingers found in wild-type adults (Figures 4B, 4C, and 4F).

In contrast to neurons with glia-ensheathed receptive endings, the ASE channel neuron does not show morphological defects following pros-1 RNAi, with an apparently normal cilium at the tip of the dendrite (Figures 4D and 4F). However, unlike wild-type animals, ASE and other channel neuron cilia are not exposed to the environment following pros-1 RNAI treatment. While the overall morphology of the AMsh glial cell appears normal when assessed by fluorescence microscopy (Figures 4E and 4F), electron microscopy reveals striking defects in the glial channel housing sensory-neuron receptive endings (Figure 4G). In wild-type animals, neurons with simple cilia penetrate through the AMsh glial cell and enter an extracellular channel, generated by these glia. This channel is filled with an electron-dense extracellular matrix, secreted by AMsh glia, and is thought to be important for sensory function (Perkins et al., 1986) (Bacaj et al., 2008). The channel formed by the AMsh glial cell is continuous with an opening through the cuticle formed by the anteriorly positioned AMso glial cell (Figures 1A and 4G). Animals treated with pros-1 RNAi show a striking defect in amphid channel architecture. At the anterior position, where cilia normally enter the glial channel, individual cilia of pros-1(RNAi) animals are surrounded by the AMsh cell, but the channel is indistinct and glia-secreted matrix material is absent (Figure 4G, bottom). More anteriorly, cilia that normally access the environment are blocked by an electron-dense deposit (Figure 4G, top). To confirm these structural defects, we assessed the accessibility of channel-neuron cilia to the environment by carrying out dye-filling assays. In wild-type animals, a subset of exposed channel neurons can take up dye. Consistent with the channel defects we observed by electron microscopy, pros-1(RNAi) animals are unable to take up dye (Figure 4H).

In summary, pros-1 is required post-embryonically for maintenance of the glial channel through which channel neurons are normally exposed to the environment, for secretion of the extracellular matrix surrounding channel neurons, and for the proper morphology of the receptive endings of other glia-ensheathed neurons. Defects in these functions are consistent with the behavioral deficits we observed in pros-1(RNAi) animals and suggest a role for pros-1 in regulating aspects of glial membrane structure and secretion.

PROS-1/Prospero Functions in Glia to Control Neuron Structure and Function

We identified pros-1 as a candidate glial regulator of neuronal function based on AMsh glia expression profiling. To confirm that pros-1 expression is indeed prominent in these glia, we examined animals carrying a recombinerated fosmid expressing a C-terminally tagged PROS-1::GFP reporter under the control of pros-1 genomic regulatory elements. PROS-1::GFP is detected in the nuclei of AMsh glia (Figure 5A), confirming our expression profiling data. Importantly, PROS-1::GFP is not detected in amphid sensory neurons, identified by specific markers for AWC and AFD glia-ensheathed neurons and by Dil-staining for channel neurons (Figures 5B–5D). PROS-1::GFP is also not detected in the AMso glia (Figure 5E). PROS-1::GFP is detected in many other sense organ-associated glia in the head, including the astrocyte-like CEP sheath glia, and the glia of the inner and outer labial sensilla (Figure S1), suggesting that pros-1 may play conserved roles in regulating sensory function across sense organs.

The expression of pros-1 in AMsh glia, but not in amphid sensory neurons, is consistent with the hypothesis that pros-1 functions within glia, controlling glial channel morphology and extracellular matrix secretion cell autonomously and sensory-neuron receptive-ending shapes and functions through a non-cell-autonomous mechanism. To test this idea, we used two approaches. First, we generated a transgenic strain for glia-specific RNAi by restoring expression of the Argonaute protein RDE-1 specifically in AMsh glia in an rde-1(ne219) mutant background. rde-1 is essential for RNAi induced by exogenous double-stranded RNA (dsRNA) and is required for incorporation of processed small interfering RNAs (siRNAs) into silencing complexes but is not required for the intercellular transport of dsRNA precursors (Parnell and Fire, 2001; Tabara et al., 1999). rde-1(ne219) mutant animals therefore take up dsRNA but cannot carry out RNAi. Expression of rde-1 under an AMsh glia-specific promoter thus results in AMsh glia-specific gene silencing after dsRNA feeding. Importantly, AMsh glia-specific pros-1 RNAi results in the same defects observed using systemic pros-1 RNAi, including amphid channel neuron dye-filling, sodium chloride chemotaxis, and benzaldehyde chemotaxis defects (Figures 5F–5H), albeit with reduced penetrance. This can be explained, at least in part, by our finding that AMsh glia RNAi efficiency is reduced in this transgenic strain (S.W.W. and S.S., unpublished data).

A Genetic Lesion in pros-1 Resembles pros-1(RNAi)

To further confirm that pros-1 expression is required in AMsh glia for sensory neuron function, we characterized a pros-1 deletion mutant, tm258, provided by the NBP (National Biosciences Project) as a pros-1(tm258)/ht2 balanced strain. tm258 is a large deletion and is predicted to be a null allele. pros-1(tm258) mutant animals show greatly reduced larval viability, characterized by slow larval development and larval lethality. On rare occasions, pros-1(tm258) mutants survive to adulthood but usually fail to
Figure 4. *pros-1* Regulates the Morphology of Glia-Ensheathed Neuronal Receptive Endings and the Amphid Channel Structure

Scale bar represents 10 μm, unless stated otherwise.

(A) AWC neuronal receptive endings (NREs), visualized with *odr-1::YFP.*

(B) AFD NREs, visualized with *srtx-1::GFP.* Scale bar represents 5 μm.

(C) Electron micrographs confirm loss of AFD microvilli after *pros-1* RNAi (asterisk, AFD dendrite; arrowheads, AFD microvilli). Scale bar represents 0.5 μm.

(D) ASE NREs, visualized with *gcy-5::GFP.*

(E) AMsh glia, visualized with *F16F9.3::dsRed.*

(F) Quantification of morphologies assessed by fluorescence microscopy in (A) (lack of wing-like cilium), (B) (lack of microvilli-like fingers), (D) (normal channel cilium), and (E) (normal sheath membrane). Error bars represent SEM. AWC n > 100, AFD n > 70, ASE n > 20, and AMsh n > 50; ****p < 0.0001 (z test).

(G) Left: amphid channel schematic (green, AMsh glia; red, channel neuron cilia; blue, glial-secreted extracellular matrix; gray, AMso glia). Right: corresponding electron micrographs at two sections through the amphid. Scale bar represents 0.5 μm.

(H) Top: *pros-1* RNAi amphid neuron dye-filling defect. Bottom: quantification of dye-filling defect. Error bars represent SEM, n = 150; ****p < 0.0001 (z test).
give progeny. pros-1(tm258) larvae show highly penetrant amphid neuron dye-filling defects (Figure 5I). Larval viability and dye-filling defects are not seen in pros-1(tm258)/hT2 heterozygotes, indicating that a single copy of pros-1 is sufficient for pros-1 function. Larval viability and amphid neuron dye-filling defects in pros-1(tm258) mutants can be rescued with a fosmid covering the pros-1 locus, indicating that these defects are a specific consequence of pros-1 loss (Figure 5I).

The larval growth defects precluded direct analysis of pros-1(tm258) adults. To overcome this, we took advantage of the mosaic expression pattern of extrachromosomal arrays and analyzed pros-1(tm258) adults that contain a rescuing pros-1 fosmid as an unstable array but lack expression of the array in AMsh glia, based on a glia-specific GFP co-injection marker. Such mosaic transgenic pros-1(tm258) animals showed normal larval development, indicating that the larval-development defects are not a result of pros-1 loss in AMsh glia. Importantly, mosaic adults lacking pros-1 expression in AMsh glia showed the same amphid neuron dye-filling defects and benzaldehyde chemotaxis defects observed following pros-1 RNAi, while control animals with the pros-1 rescuing fosmid present in AMsh glia showed normal responses (Figures 5I and 5J). These results confirm the specificity of the RNAi phenotype observed and support our conclusion that pros-1 expression is required in AMsh glia for sensory function.

Many PROS-1 Target Genes Encode Secreted Proteins

pros-1 encodes a homeodomain transcription factor. To identify target genes that mediate pros-1 activity, we first looked at
Figure 6. *pros-1* Target Genes in AMsh Glia

(A) AMsh glia-specific gene expression with *pros-1* RNAi.

(B) AMsh glia secreted/transmembrane gene enrichment correlates with *pros-1*-dependency. Genes with indicated value or higher enrichment are scored for being PROS-1 targets. Red numbers indicate the number of genes in each data point. For very high glial enrichment levels, gene numbers are low.

(C) *pros-1*-dependent genes discussed in the text.

(D) *fig-1(ns306)* dye-filling defects (error bars represent SEM, n > 80; ****p < 0.0001, z test) and 1-octanol avoidance defects (n = 5, mean ± SD; ****p < 0.0001, Student’s t test).

(E) LIT-1C localization in AMsh glia. Scale bar represents 10 μm. Right: error bar represents SEM, n > 300; ****p < 0.0001 (z test).

(legend continued on next page)
expression of known AMsh glia genes. Animals treated with pros-1 RNAi showed a strong reduction in expression of the glial genes vap-1, F11C7.2, and fig-1, as determined by analysis of transcriptional reporters (Figure 6A). vap-1 encodes a secreted protein with conserved cysteine-rich secretory protein, antigen 5, and pathogenesis-related 1 (CAP) domains, found in cysteine-rich secretory proteins that play a role in regulating extracellular matrix remodeling, whereas F11C7.2 and fig-1 encode secreted proteins containing conserved TSP-1 (thrombospondin type 1) domains, found in extracellular matrix proteins including thrombospondins, known to be involved in glia-neuron interactions (Christopher et al., 2005; Gibbs et al., 2008).

To identify additional pros-1-dependent genes, we isolated AMsh glia using FACS and carried out gene expression profiling from pros-1 RNAi-treated and control animals. Cells were isolated from late-stage larvae (L3 and L4) grown post-embryonically for 36–42 hr in liquid cultures of E. coli HT115 for RNAi by feeding. This time point was chosen because time-course experiments showed that pros-1(RNAi) defects of animals treated from the first larval stage (L1) become apparent at L3/4 (Figure S2), most likely enriching for primary transcriptional targets. Effectiveness of RNAi by feeding when animals are grown in liquid culture was verified with dye-filing assays (Figure S2).

Homeodomain transcription factors, including the pros-1 homolog Prospero, can act as both positive and negative regulators of transcription (Choksi et al., 2009), and, indeed, we found glial genes with either decreased or increased expression levels following pros-1 RNAI. 439 genes show a significant reduction in expression (Table S2), while 634 genes show a significant increase (Table S3; see Experimental Procedures for statistical analysis). Comparison of AMsh-enriched genes encoding secreted or transmembrane proteins with pros-1-dependent genes revealed a positive correlation between the degree of enrichment in AMsh glia and likelihood of being a pros-1 target (Figure 6B). Strikingly, of the 155 highly enriched (>10×) AMsh glia genes that encode proteins with signal peptides or transmembrane domains (Figure 1), 49 genes (32%) showed a significant reduction in expression following pros-1 RNAI. Thus, pros-1 controls the expression of a large portion of the AMsh glia-specific secretome (along with other genes; Figure S3), consistent with the defects in extracellular matrix secretion and membrane abnormalities we observed in pros-1 mutants.

To validate the candidate pros-1 target genes we identified, we assessed the expression levels of transcriptional reporters for two genes, R11D1.3, encoding a secreted protein of unknown function, and K02E11.4, encoding a secreted protein containing a conserved uridine 5′-diphosphate (UDP)-glucoronyltransferase domain, found in enzymes that act in the olfactory epithelium to modulate chemosensory cues (Leclerc et al., 2002). These were identified in our RNA-seq studies as having 18-fold and 4.3-fold decreased expression in pros-1 RNAI animals (Figure 6C). Importantly, both reporters show clear reductions in AMsh glia GFP expression in animals treated with pros-1 RNAI (Figure 6A). The reduction in expression is particularly strong for R11D1.3::GFP, which correlates with the increased pros-1 dependence of this gene seen in our expression profiling data. These results confirm that we identified pros-1-dependent genes in our expression profiling experiments.

Of the genes we identified as pros-1 dependent based on transcriptional reporters (vap-1, fig-1, and F11C7.2; Figure 6A), only F11C7.2 also showed pros-1 dependence in our expression profiling (Figure 6C). One explanation for this difference may be in the timing of the experiments. Reduction in transcriptional reporter expression was observed in adults after 3 days of RNAI treatment, while we isolated AMsh glia from late-stage larvae at 36–42 hr of RNAI treatment. Thus, fig-1 and vap-1 may represent secondary targets for PROS-1.

Taken together, our expression profiling studies, coupled with phenotypic analysis of pros-1 mutants, reveal that PROS-1 is a major regulator of the AMsh glia secretome.

**PROS-1 Target Genes Encoding Membrane and Secreted Proteins Account for Neuronal Dye-Filling Defects in pros-1 Mutants**

To identify functionally important genes whose expression changes contribute to the defects observed in pros-1 mutants, we searched for known regulators of AMsh glial function among our list of candidate pros-1-dependent genes and carried out additional RNAI screens.

As described above, expression of the fig-1 gene is strongly dependent on pros-1. fig-1 encodes a secreted thrombospondin-domain-containing extracellular matrix protein necessary for amphid neuron dye-filling and avoidance of 1-octanol (Figure 6D) (Baca et al., 2008). Thus, pros-1 acts in glia to control neuronal function and to regulate the amphid channel extracellular microenvironment partly by promoting expression of FIG-1 protein.

We also found that expression of the Patched-related gene daf-6, a negative regulator of amphid channel expansion, increases significantly following pros-1 RNAI (4.2-fold increase), a result we confirmed by analysis of a daf-6::GFP expression reporter (Figure 6A). DAF-6 is a membrane protein that restricts amphid channel size, and daf-6 mutants have abnormally expanded channels. Thus, overexpression of DAF-6 may contribute to the loss of amphid channel membrane following pros-1 RNAI. Supporting these observations, DAF-6 functions by antagonizing the activity of lit-1, a Nemo-like kinase that localizes to the amphid channel membrane and promotes channel expansion (Oikonomou et al., 2011). Although lit-1 expression level was not affected in pros-1(RNAI) animals, LIT-1 protein, which normally lines the amphid channel, showed only diffuse localization (Figure 6E). Together, these results suggest that pros-1 acts, at least in part, to regulate amphid channel size by inhibiting expression of daf-6 to support lit-1 function.

See also Figures S2 and S3 and Tables S2 and S3.
Finally, to identify additional genes mediating pros-1 function, we screened candidate pros-1 target genes by RNAi for defects in amphid neuron dye filling. To compensate for possible redundancy among pros-1 target genes, we carried out these experiments in pros-1(tm258)/hT2 heterozygous animals, which show normal dye filling (Figure 5I) but which we predict to have reduced levels of pros-1 expression and may in turn have reduced expression of redundantly acting target genes and therefore provide a sensitized background for detecting functional defects. Indeed, pros-1(tm258)/hT2 heterozygotes treated with RNAi against the gene rdy-2, previously identified as required for dye filling (Liégeois et al., 2007), show increased defects compared to rdy-2(RNAi) animals alone (56% defective versus 16%). This screen identified five candidate pros-1 target genes that exhibit low, but statistically significant, dye-filling defects and thus may represent functional targets of pros-1 in AMsh glia (Figure 6F). Notably, all are predicted to encode proteins with transmembrane domains.

DISCUSSION

Differences between Embryonic and Post-embryonic Glial Transcriptomes

The amphid sense organ of C. elegans is an attractive model system for studying interactions between glia and neurons because of its relatively simple anatomy and because the functional properties of amphid sensory neurons have been extensively studied (Shaham, 2006, 2015). To characterize the molecular mechanisms through which AMsh glia regulate neuronal function, and not development, we took advantage of recently developed larval cell isolation techniques and adapted these to identify AMsh glia post-embryonic transcripts by RNA-seq (Spencer et al., 2014; Zhang and Kuhn, 2013). This approach led to the identification of 598 candidate AMsh glial genes. We previously identified 298 transcripts enriched in embryonic AMsh glia (Bacaj et al., 2008). Of the 598 post-embryonic transcripts identified in this study, only 72 were also detected in our previous study. While the large number of newly identified transcripts can be partly explained by differences in transcript analysis and statistical methods used (RNA-seq versus microarray), this difference highlights the advantage of using larval cell isolation for identification of genes with post-embryonic functions and reveals distinct transcriptomes for these stages.

PROS-1 Is a Regulator of the Glial Secretome

Here, we demonstrate that RNAi against pros-1 results in similar defects to AMsh glia ablation, including defective morphology of glia-ensheathed sensory-neuron receptive endings and defective functions of channel neurons. pros-1 was previously noted to affect larval survival and amphid neuron dye filling (Kolotuiev et al., 2013), and its role in excretory canal formation was characterized in detail, but the nature of the amphid neuron dye-filling defect was not previously explored. Importantly, in this study, we show that pros-1 is expressed in AMsh glia, as well as glia of other sense organs, but not in amphid neurons, and is required for maintenance of the matrix-filled amphid channel, through which channel neurons are exposed to the external environment, and for sensory behaviors controlled by these neurons. Additionally, we have shown that pros-1 is required for proper morphology of the glia-ensheathed receptive endings of odor- and thermosensory neurons and that pros-1 loss of function results in defects in the corresponding chemotaxis and thermotaxis behaviors. pros-1 thus acts cell autonomously to maintain amphid channel structure and non-cell autonomously to maintain the receptive endings of ensheathed sensory neurons, perhaps through glia-to-neuron signaling. Both glia-neuron communication and amphid channel shape and content are predicted to require secreted and/or transmembrane proteins. Consistent with this idea, AMsh glia show a striking over-representation of genes encoding secreted or transmembrane proteins, of which approximately one-third are pros-1 dependent. PROS-1 is, therefore, a key regulator of the AMsh glia secretome. Of note, our expression profiling experiments also reveal that pros-1 regulates expression of non-secreted genes, suggesting pros-1 has additional functions.

Some pros-1 targets we identified, such as fig-1 and daf-6, may partly explain the defects we observe in pros-1 mutants (Figure 6G), as these components have known independent roles in AMsh glia. The large number of pros-1 target genes we identified suggests that other targets may act redundantly, making the identification of functionally important target genes challenging. Remarkably, the robustness of amphid neuron dye filling in wild-type animals allowed us to identify weak but statistically significant defects in five candidate pros-1 target genes. Of these, F41H10.5 encodes a transmembrane protein with a conserved TLC (TRAM, LAG1, and CLN8) domain, which have general roles in lipid metabolism and in mammals regulate calcium channel activity (Papanayotou et al., 2013; Winter and Ponting, 2002), and F31D5.2 encodes a transmembrane protein with a major facilitator superfamily domain, a conserved domain found in solute transporters (Yan, 2013). While the remaining three genes encode proteins of unknown function, it is notable that all three encode predicted transmembrane proteins. While further work is required to assess the roles of these genes in pros-1-mediated functions, these observations are consistent with roles in glia-neuron interactions.

PROS-1/Prospero Has Cell-Fate-Independent Functions

pros-1 encodes a Prospero-related homeodomain transcription factor. Prospero has well-known roles in determining cell-fate decisions during nervous system development in Drosophila (Doe et al., 1991). When neuroblasts divide, Prospero is distributed asymmetrically between daughter cells and promotes differentiation of ganglion mother cells into neurons and glia by inhibiting expression of cell-cycle genes and promoting expression of genes for terminal differentiation (Choksi et al., 2006; Freeman and Doe, 2001). During the final round of cell divisions generating external sense organs in Drosophila, a precursor cell divides to produce a glia-like sheath cell and a neuron. Prospero mutants produce neither, while Prospero overexpression results in overproduction of glia and causes a partial neuron-to-glia fate conversion (Manning and Doe, 1999). Prospero expression is downregulated in neurons after the final cell division but is retained in glia. Notably, in Prospero mutants that do generate neurons, these exhibit defective axon and dendrite morphology. Although this defect might reflect a role
for the transient expression of Prospero in neurons, our results raise the possibility that the defects may originate from the glia-like sheath cell.

In addition to sheath cells in the sense organs of the Drosophila wing, Prospero is also expressed in the glia-like support cells of the antenna-maxillary complex (AMC), the larval chemosensory organ (Balakireva et al., 2000). Expression profiling to identify Prospero-dependent genes in whole-AMC extracts was performed (Guennin et al., 2010). However, Prospero is expressed in neurons and glia-like cells of the AMC, and reported targets are mainly involved in neuronal axon growth and synaptic function and therefore likely to represent Prospero targets in neurons and not support cells.

Vertebrate homologs of Prospero are expressed throughout the nervous system. In the mammalian brain, Prox1 is expressed in the hippocampus, is involved in neurogenesis, and likely has a role in establishing cell fate (Galeeva et al., 2007; Elsir et al., 2012). Prox1 is also expressed in differentiated glia-like support cells in sense organs, including support cells of the mammalian cochlea and the zebrafish neuromast (Ber-mingham-McDonogh et al., 2006; Pistocchi et al., 2009). Prox1 is expressed in progenitor cells during cochlea development, but post-embryonically, its expression is restricted to glia-like cells, including Deiters’ cells and pillar cells (Berming-ham-McDonogh et al., 2006). Prox1 loss in neuromast support cells results in defective uptake of dye by associated mechanosensory hair cells, suggestive of functional defects (Pistocchi et al., 2009). These observations show that expression of Prospero homologs is a conserved feature of sense-organ-associated glia. Importantly, our results suggest that Prospero is likely to be a key factor maintaining the functions of these organs well past embryonic development.

EXPERIMENTAL PROCEDURES

Strains and Plasmids
C. elegans strains were maintained using standard methods (Stiernagle, 2006). Wild-type strain is Bristol N2. See the Supplemental Experimental Procedures for details of alleles and transgenic strains.

RNAi
Standard RNAi was carried out as described previously (Kamath and Ahringer, 2003). Overnight cultures of E. coli HT115 carrying pL4440 expression vectors targeting specific genes (Ahringer Library and Vidal Library) were seeded into nematode growth medium plates supplemented with carbenicillin (25 μg/ml) and isopropyl β-D-1-thiogalactopyranoside (1 mM). After 1–2 days of incubation at room temperature, embryos obtained from bleaching gravid adults were added to the RNAi plates. Unless stated otherwise, animals were scored 3 days later, as first-day adults.

Behavioral Assays
All behavior assays were performed on first-day adults. Chemotaxis assays, avoidance assays, and thermotaxis assays were carried out using standard procedures (Bargmann et al., 1993; Chao et al., 2004; Ryu and Samuel, 2002; Ward, 1973). See the Supplemental Experimental Procedures for details. Statistical analysis was performed with an unpaired Student’s t test (GraphPad).

Dye-Filling Assays
DiI (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) (Sigma) stock solution was made at 5 mg/ml in N,N-dimethylformamide. Animals were stained with Dil (stock solution diluted 1:1000 in M9 buffer) for 30–60 min, followed by three M9 washes to remove excess dye. Dye filling was scored as % animals that show amphid neuron dye uptake, with statistical analysis performed with a two-proportion z test (in silico).

Fluorescence and Electron Microscopy
Fluorescence and electron micrographs were obtained using standard procedures. See the Supplemental Experimental Procedures for details. Quantification of morphological defects assessed by fluorescence microscopy was carried out by scoring individual animals for wild-type versus defective morphology, with statistical analysis performed with a two-proportion z test (in silico).

Cell Isolation and FACS Analysis
Briefly, synchronized larvae were grown from L1 in liquid culture of E. coli HT115 for RNAi treatment. After growth for 36–42 hr, cells were isolated from late-stage larvae (L3 and L4) by protease treatment and mechanical disruption, as described previously (Zhang and Kuhn, 2013), with modifications (Menachem Katz, personal communications). AMsh glia expressing a dsRed transgene (nsIs143) were sorted using a BD FACSAria sorter (Rockefeller University Flow Cytometry Resource Center). See the Supplemental Experimental Procedures for details.

RNA Isolation and Sequencing
RNA was extracted from TRizol LS (Ambion)-treated cells by phase separation, following the manufacturer’s guidelines. RNA was purified using Picopure RNA isolation kit (Artcurus). 1–5 ng purified total RNA was obtained per sample. All subsequent steps were performed by the Rockefeller University Genomics Resource Center. RNA quality was verified to make sure sample degradation had not occurred (Agilent Bioanalyzer). mRNA amplification and cDNA preparation were performed using the SMARTer mRNA amplification kit (Clontech). Labeled samples were sequenced using an Illumina HiSeq 2000 sequencer.

RNA-Seq Quality Assessment and Differential Gene Expression Analysis
Sequence quality was assessed and gene alignment was carried out as described in the Supplemental Experimental Procedures. Three independent replicates were obtained from control animals, and four independent replicates were obtained for pros-1 RNAi animals. Two different statistical methods were used for differential gene expression analysis, DESeq and voom. For DEseq analysis, DESeq2 was applied to normalize count matrix, and voom (Law et al., 2014) was applied for gene differentiation analysis. Significant genes from both analyses were combined.

To identify transcripts enriched in AMsh glia compared to other cells (control AMsh versus control non-AMsh), we used a fold change of 3.5 and an adjusted p value threshold of <0.05. This fold change cutoff was determined as follows. We sought genes that are >4-fold enriched in AMsh glia, as these changes can be easily assayed in vivo using GFP reporters. For genes showing pan-glial expression, a true enrichment of R should correspond to an RNA-seq-detected enrichment of 95%/46% of the total number of genes, assuming each animal has 959 total cells and 50 glial cells. For R = 4, RNA-seq enrichment is 3.5 x. To identify genes whose expression is regulated by pros-1 (pros-1 RNAi AMsh versus control AMsh), we used a less stringent threshold to take into account possible variation in RNAi efficiency between replicates (fold change > 2, adjusted p value < 0.1). To identify putative secreted or transmembrane genes, we used Phobius, a combined membrane topology and signal peptide predictor (Stockholm Bioinformatics Center; http://phobius.abc.su.se).

ACCESSION NUMBERS
The accession number for the RNA-seq data generated in this study is RNA: PRJEB12954.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.03.051.

AUTHOR CONTRIBUTIONS

S.W.W. and S.S. conceived of the studies, designed experiments, interpreted data, and wrote the paper. A.S. performed the studies on AFD neuron (thermotaxis and morphology). Y. Liang performed most of the RNA-seq analysis. Y. Lu performed the electron microscopy studies. S.W.W. performed all the other studies.

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