Opposing Activities of LIT-1/NLK and DAF-6/Patched-Related Direct Sensory Compartment Morphogenesis in C. elegans

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Abstract

Glia cells surround neuronal endings to create enclosed compartments required for neuronal function. This architecture is seen at excitatory synapses and at sensory neuron receptive endings. Despite the prevalence and importance of these compartments, how they form is not known. We used the main sensory organ of C. elegans, the amphid, to investigate this issue. daf-6/Patched-related is a glia-expressed gene previously implicated in amphib sensory compartment morphogenesis. By comparing time series of electron-microscopy (EM) reconstructions of wild-type and daf-6 mutant embryos, we show that daf-6 acts to restrict compartment size. From a genetic screen, we found that mutations in the gene lit-1/Nemo-like kinase (NLK) suppress daf-6. EM and genetic studies demonstrate that lit-1 acts within glia, in counterbalance to daf-6, to promote sensory compartment expansion. Although LIT-1 has been shown to regulate Wnt signaling, our genetic studies demonstrate a novel, Wnt-independent role for LIT-1 in sensory compartment size control. The LIT-1 activator MOM-4/TAK1 is also important for compartment morphogenesis and both proteins line the glial sensory compartment. LIT-1 compartment localization is important for its function and requires neuronal signals. Furthermore, the conserved LIT-1 C-terminus is necessary and sufficient for this localization. Two-hybrid and co-immunoprecipitation studies demonstrate that the LIT-1 C-terminus binds both actin and the Wiskott-Aldrich syndrome protein (WASP), an actin regulator. We use fluorescence light microscopy and fluorescence EM methodology to show that actin is highly enriched around the amphid sensory compartment. Finally, our genetic studies demonstrate that WASP is important for compartment expansion and functions in the same pathway as LIT-1. The studies presented here uncover a novel, Wnt-independent role for the conserved Nemo-like kinase LIT-1 in controlling cell morphogenesis in conjunction with the actin cytoskeleton. Our results suggest that the opposing daf-6 and lit-1 glial pathways act together to control sensory compartment size.

Introduction

Sensory organs are the gates through which information flows into the nervous system. In many sensory organs, specialized glial cells form a chemically isolated compartment around neuronal receptive endings [1,2]. For example, in the skin, the mechanosensory Pacinian corpuscles consist of an unmyelinated nerve ending that is surrounded by lamellae formed by a modified Schwann glial cell [3]. In the olfactory epithelium, sensory neurons are ensheathed by glia-like sustentacular cells [4,5]. In the inner ear, hair cells are surrounded by Deiter’s cells, which express the glial marker glial fibrillary acidic protein (GFAP) [6]; and in the vertebrate eye, retinal pigmented epithelial cells contact photoreceptor cell cilia [7]. At least in some cases, the integrity of the glial compartment is essential for proper sensory neuron function [8]. Glial compartments also enclose excitatory neuronal synapses in the cerebellum and hippocampus [9,10], and are thought to be important for synaptic function through limiting neurotransmitter diffusion, and regulating levels of synaptic effectors. Despite the prevalence of such glial compartments, little is known about their development.

To determine how such compartments form, we turned to the major sense organ of the nematode Caenorhabditis elegans, the amphid. C. elegans has two bilaterally symmetric amphids located in the head [11]. Each amphid consists of 12 sensory neurons, which mediate many of the behavioral responses of the animal, and two glial cells, the sheath and socket glia (Figure 1A, top). Amphid neurons are bipolar, projecting an axon into the nerve ring (the main neuropil of the animal) and extending a dendrite anteriorly to the tip of the nose. The two amphid glia also extend anterior processes collateral to the dendrites. At the nose tip, sheath and socket glia form discrete single-cell tubular channels
Author Summary
The nervous system of most animals consists of two related cell types, neurons and glia. A striking property of glia is their ability to ensheathe neuronal cells, which can help increase the efficiency of synaptic communication between neurons. Sensory neuron receptive endings in the periphery, as well as excitatory synapses in the central nervous system, often lie within specialized compartments formed by glial processes. Despite the prevalence of these compartments, and their importance for neuronal function and signal transmission, little is known about how they form. We have used the amphid, the main sensory organ of the worm Caenorhabditis elegans, to investigate glial sensory compartment morphogenesis. We demonstrate that the glia-expressed gene daf-6/Patched-related acts to restrict the size of the sensory compartment, while the Nemo-like kinase lit-1 acts within glia in the opposite direction, to promote sensory compartment expansion. We show that LIT-1 localizes to the sensory compartment through a highly conserved domain. This domain can interact both with actin, which outlines the compartment, and with the regulator of actin polymerization WASP, which acts in the same pathway as lit-1. We postulate that Nemo-like kinases could have broader roles as regulators of cellular morphogenesis, in addition to their traditional role in regulating the Wnt signaling pathway.

Results

daf-6/Patched-Related Inhibits Amphid Sensory Channel Growth
The amphid sheath glial cell forms a compartment that surrounds the ciliated endings of amphid sensory neurons, constraining them into a tight bundle (Figure 1A–C). Within this bundle, 10 sensory cilia are stereotypically arranged in three successive columns containing 3, 4, and 5 cilia, respectively (Figure 1C; [11]). We previously reported the cloning and characterization of daf-6, a Patched-related gene required for amphid channel morphogenesis [14]. In daf-6 mutant adults, the amphid channel is grossly enlarged, the socket and sheath glia channels are not continuous, and distal portions of sensory cilia are neither bundled nor exposed to the environment (Figure 1D and 1E).

At least two interpretations of this phenotype are possible: First, daf-6 might act to open the sheath glia channel at its anterior end. Thus in daf-6 mutants, the channel pocket would form but would remain scaled, and would continuously enlarge as matrix material is deposited. Second, daf-6 might act to constrain the luminal diameter of the sheath glia channel. Thus, in daf-6 mutants, the sheath and socket glia would properly align and form an open compartment, yet without lateral constraints on its size, the sheath channel would expand circumferentially. In this latter model, loss of the sheath-socket junction would be a later secondary defect.

To discriminate between these possibilities, we used electron microscopy (EM) to follow the development of amphid sensory compartments in wild-type and daf-6(e1377) mutant embryos. We used high-pressure freezing to fix embryos at several time points between 300 and 450 min post-fertilization, the time period during which the amphid is generated [19], collected serial sections, and assessed channel morphology.

By 300 min, sensory dendrites that have not yet formed cilia are evident in wild-type embryos. The tips of these dendrites are laterally ensheathed by the sheath glial cell, but the sheath cell also forms a cap blocking the anterior portion of the compartment and preventing access of neuronal processes to the socket (Figure S1).

By 400 min, a well-defined amphid primordium is formed in wild-type embryos (Figures 1F and S1). The sheath glia cap is gone and the open channel is continuous with the socket glia channel. At this stage, the socket channel is devoid of neuronal processes as dendritic tips have yet to extend cilia. Instead, a dense arrangement of filaments traverses the socket channel and forms a link between the tips of the sensory dendrites and the outside of the embryo (asterisk in Figure 1F). These filaments are consistent with an extracellular matrix proposed to anchor dendrites during retrograde extension [20]. Although cilia have not yet formed, structures resembling basal bodies (the initial sites of cilia construction) are visible at dendrite endings (arrow in Figure 1F).

In daf-6 mutant embryos, the initial stages of amphid development are unperturbed (n = 3). By 400 min, the sheath and socket channels are aligned and open. Dendrites lacking cilia, but containing basal body-like structures, reside within the sheath channel, while filaments emanating from the dendrite tips and traversing the sheath and socket channels are seen (Figure 1H). However, only slightly later, at 420 min and before cilia have formed, bloating of the amphid sheath channel is apparent, and dendrites begin to unbundle (Figure 1I, compare to Figure 1G).

These studies indicate that daf-6 is not required for aligning the sheath and socket channels or for opening the amphid sensory compartment. Rather, daf-6 seems to function in restricting compartment diameter.
Figure 1. daf-6 restricts amphid sensory compartment size. In longitudinal sections and diagrams (A, B, D, F, and H) anterior is left. White scale bars, 10 μm. Black scale bars, 1 μm. (A) Schematic of the C. elegans amphid. Top: Each amphid consists of 12 neurons (only one is depicted here) and two glial cells, the sheath and the socket. Bottom: Detail of the anterior tip of the amphid. Matrix is secreted by the Golgi apparatus. tj, tight junction. Adapted from [13]. (B, D) The ASER neuron and the amphid sheath glia visualized, respectively, with mCherry (red; driven by the gcy-5 promoter) and GFP (green; driven by the T02B11.3 amphid sheath promoter [32] in a wild-type (B) or daf-6(e1377) (D) animal (transgenes nsEx2766 and nsEx2752, respectively)). The ASER neuron extends a single cilium through the length of the amphid channel in the wild type (arrow). In the mutant, the cilium is bent and not exposed to the environment, and the amphid pocket is bloated (asterisk). (C, E) Electron micrograph of a cross-section through the anterior portion of the amphid sheath glia channel in an adult wild-type animal (C) or a daf-6(e1377) adult mutant (E). Arrow in (C), sensory cilium.
Loss of lit-1/NLK Restores Amphid Sensory Compartment Morphology and Function to daf-6 Mutants

The abnormal expansion of the amphid sensory compartment in daf-6 mutants suggests that active processes promote compartment expansion and that these processes are balanced by daf-6 activity during development. We surmised that mutations in genes promoting compartment expansion might, therefore, counteract the loss of daf-6 and restore compartment size and function.

To identify such genes, we screened for mutants able to generate a normal compartment in the absence of daf-6 function, taking advantage of an easily scored daf-6 mutant defect: the inability to form dauer larvae. Dauer is an alternative developmental state induced by starvation and perception of high concentration of dauer pheromone. Dauer animals are highly resistant to environmental insults and can survive in the presence of 1% sodium-laurylsulfate (SDS) [21]. daf-6 mutants fail to become dauer larvae, presumably due to their sensory deficits [22], and are thus killed by exposure to SDS. We therefore randomly mutagenized animals homozygous for the strong loss-of-function daf-6(e1377) allele [14] using ethyl methanesulfonate (EMS), allowed F2 animals to starve, and treated them with SDS. Resistant animals could have suppressed the daf-6 amphid sensory compartment defects or could have constitutively activated a more downstream step in dauer formation. To distinguish between these mutant classes, we examined the ability of amphid sensory neurons to fill with dye provided in the medium. When exposed to a solution of the lipophilic dye DiI, wild-type animals readily take up dye, whereas daf-6 mutant animals fail to do so, presumably due to their defective amphid sensory compartments (Figure 2A) [13,23].

From a screen of 60,000 mutagenized genomes we identified seven mutants that survived SDS treatment and that dye filled properly. We further characterized one of these daf-6 suppressors, given the allele designation ns132. As shown in Figure 2A, approximately 40% of ns132; daf-6(e1377) animals are able to take up dye in at least one amphid. Likewise, the ns132 allele was able to suppress amphid channel defects in another daf-6 mutant, n1543, supporting the notion that ns132 is a bypass suppressor (Figure 2A).

To further confirm the rescue of the daf-6 amphid defects in ns132; daf-6(e1377) animals, we examined amphid sensory compartments using fluorescence microscopy. We found that cilia in these double mutants projected through a compartment of normal appearance (Figure 2B, compare to Figure 1D). In addition, ns132; daf-6(e1377) individuals that displayed normal dye filling in one of the two amphids had one amphid channel that resembled a wild-type channel by EM serial reconstruction (Figure 2C; n = 5). Interestingly, even in rescued amphids, cilia packing was more variable compared to the regular 3:4:3 packing observed in wild-type animals, and the amphid sensory compartment was somewhat wider than normal (Figure 2C, compare to Figure 1C), perhaps reflecting a partial suppression of the daf-6 defects.

We used single nucleotide polymorphism (SNP) mapping and transgenic rescue methods (Figure 2D) to identify the gene defective in ns132 animals as lit-1. lit-1 encodes a Ser/Thr MAP kinase that is highly conserved from C. elegans to mammals. Supporting this assignment, a genomic region containing lit-1 restored dye-filling defects to ns132; daf-6(e1377) animals (Figures 2A and S2E), as did a transgene in which the lit-1 promoter region (2.5 kb upstream of the lit-1 start codon) drives the lit-1 cDNA (Figure 2A). Furthermore, a temperature-sensitive mutation in lit-1, ts132, also suppressed the dye-filling defects of daf-6(n1543) mutants (Figure 2A). Finally, we found that animals containing the ns132 allele have a C-to-T mutation in the coding region of lit-1, converting codon 437, encoding glutamine, to a stop codon. This mutation is predicted to result in a truncated LIT-1 protein (Figure 2D) lacking the last 26 amino acids of the highly conserved carboxy-terminal (C-terminal) domain.

LIT-1 Functions in Amphid Glia During Compartment Formation

To determine in which cells lit-1 functions to regulate compartment development, we first examined its expression pattern by generating animals harboring a transgene in which the lit-1 promoter drives expression of a nuclearly localized dsRed fluorescent protein (NLS-RFP). We found that lit-1 is expressed in amphid sheath glia (Figure 3A), among other cells. In addition, the expression pattern of this reporter partially overlaps with that of pnr-10 (Figure 3B), a gene expressed in ensheathing glia of other sensory organs [24], suggesting that lit-1 could act in compartment formation in other C. elegans sensory structures as well.

Next, we pursued cell-specific rescue experiments to determine in which cells lit-1 can act to regulate compartment morphogenesis. We generated lit-1(ns132); daf-6(e1377) animals containing a transgene in which a lin-26 promoter fragment drives expression of the lit-1 cDNA in glia, but not neurons, of embryos at the time of amphid sensory compartment formation [25]. We found that transgenic animals were rescued (Figure 3C), supporting the notion that lit-1 can act in glia to regulate compartment morphology. Importantly, expression of the lit-1 cDNA in amphid sensory neurons during the time of amphid morphogenesis (using the dyf-7 promoter; [20]) failed to rescue lit-1(ns132); daf-6(e1377) animals (Figure 3C).

To determine whether lit-1 can control amphid sensory compartment structure after compartment formation is complete, we examined lit-1(ns132); daf-6(e1377) animals expressing the lit-1 cDNA under the control of the sheath glia-specific vap-1 promoter. vap-1 expression begins in late embryos [14], after the compartment has formed. We found that these transgenic animals were not rescued (Figure 3C), supporting the conclusion that lit-1 is required within amphid sheath glia at the time of amphid morphogenesis to influence compartment formation.

Finally, to ascertain whether the kinase activity of LIT-1 is required, we generated a mutant lit-1 cDNA that disrupts the ATP binding domain [VALK]K to VALGK and which has been shown to eliminate LIT-1 kinase activity in vitro [17], lit-1(ns132); daf-6(e1377) animals carrying a lin-26 promoter::LIT-1(K97G) cDNA transgene still displayed 30% dye filling, similar to controls, suggesting that LIT-1 kinase activity is indeed required for glial compartment morphogenesis (Figure 3C). None of the transgenes used in Figure 3C had an effect on the dye filling of wild-type animals (n>100).
lit-1 Promotes Amphid Sensory Compartment Expansion

Since daf-6 normally acts to restrict amphid sensory compartment expansion, the observation that lit-1 mutations suppress daf-6 suggests that lit-1 may normally promote compartment growth. Consistent with this idea, the lit-1(ns132) allele enhances the dye-filling defects of che-14(ok193) mutants (Figure 4A). CHE-14 protein is similar to the Drosophila and mammalian protein Dispatched, and is important for apical secretion and amphid sensory compartment morphogenesis [16], suggesting a role in lumen expansion. The enhancement of che-14 defects by lit-1(ns132) suggests that both genes may be involved in this process.

To further test the idea that lit-1 promotes compartment expansion, we examined lit-1(ns132) single mutants for dye-filling abnormalities; however, no defects were observed (Figure 4B), suggesting that amphid morphology in these animals may be normal. However, two observations suggest that ns132 is a weak...
allele of lit-1. First, the ns132 lesion truncates only 26 amino acids from the C-terminus of the LIT-1 protein and leaves the kinase domain intact (Figure 2D). Second, null alleles of \(\text{lit-1}\) are embryonic lethal [17,26], whereas ns132 mutants are fully viable.

To examine the consequences of more severe defects in \(\text{lit-1}\) function, we turned to animals homozygous for the \(\text{lit-1}(t1512)\) temperature-sensitive allele. \(\text{lit-1}(t1512)\) animals grow nearly normally at 15°C, but exhibit early embryonic lethality at 25°C [26]. At 20°C, some \(\text{lit-1}(t1512)\) embryos escape lethality and grow to adulthood. We reasoned that in some of these escapers, LIT-1 activity could be low enough to allow us to discern defects in amphib morphogenesis. Indeed, as shown in Figure 4B, nearly 50% of \(\text{lit-1}(t1512)\) adults grown at 20°C exhibit defects in a sensitized amphib dye-filling assay (this assay was developed to detect weak defects in dye filling; see Experimental Procedures). These results suggest that amphib structure, and perhaps compartment morphogenesis, has been perturbed in these mutants.

To assess whether compartment morphology is indeed perturbed, we performed serial-section EM on dye-filling defective adult \(\text{lit-1}(t1512)\) animals raised at 20°C (\(n=3\)). Whereas in wild-type animals a cross-section through the sheath channel immediately posterior to the socket-sheath boundary (yellow line in Figure 4C) reveals the stereotypical 3:4:3 arrangement of the 10 channel cilia, in \(\text{lit-1}(t1512)\) mutants (Figure 4D), the amphib sensory compartment has a smaller diameter and contains fewer cilia. Fewer cilia are also found in the socket channel in \(\text{lit-1}(t1512)\) animals (unpublished data). Furthermore, in wild-type animals, cross-sections roughly 1 µm posterior to the sheath-socket junction (blue line in Figure 4C) reveal a less packed arrangement of cilia that are loosely surrounded by the sheath glia membrane; by contrast, in \(\text{lit-1}(t1512)\) animals the sheath glia is tightly wrapped around individual cilia (arrowheads in Figure 4D), consistent with the idea that compartment diameter is reduced. Importantly, despite the posterior displacement of some cilia in \(\text{lit-1}(t1512)\) animals, the total number of cilia is normal (blue section in Figure 4D).

Taken together, the che-14, dye-filling, and EM studies suggest that \(\text{lit-1}\) opposes \(\text{daf-6}\) by promoting channel expansion during amphib morphogenesis.

**Mutation of the MAP Kinase Kinase Kinase mom-4/TAK1 Also Suppresses the Compartment Defects of daf-6 Mutants**

The kinase activity of LIT-1 was previously shown to depend on MOM-4/TAK1, a MAP kinase kinase. MOM-4 increases LIT-1 kinase activity in vitro and mutations in \(\text{mom-4}\) interact genetically with mutations in \(\text{lit-1}\) during anterior/posterior polarity establishment in early embryos [27]. We therefore tested whether mutations in \(\text{mom-4}\) could also suppress the dye-filling defects of \(\text{daf-6}\) mutants. While complete loss of \(\text{mom-4}\), like loss of \(\text{lit-1}\), leads to early embryonic lethality, some animals homozygous for a temperature-sensitive allele of \(\text{mom-4}\), \(\text{ne1539ts}\), can escape lethality. We found that whereas only 1% of \(\text{mom-4}(\text{ne1539ts})\); \(\text{daf-6}(e1377)\) double-mutant escapers grown at 15°C exhibit suppression of the \(\text{daf-6}\) dye-filling defect, 18% of surviving animals grown at 20°C can take up dye (\(p<10^{-6}\), Chi-squared test; Figure 5A). This observation suggests that \(\text{mom-4}\) acts similarly to \(\text{lit-1}\) in compartment expansion.

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**Figure 3. Suppression of daf-6 mutations requires loss of lit-1 in glia.** (A) Image of an amphid sheath cell body expressing \(\text{lit-1p::NLS-RFP}\) (red; in nucleus) and \(\text{vap-1p::GFP}\) (green) (transgene \(\text{msEx2308}\)). Yellow, overlapping expression. Left is anterior. Scale bar, 10 µm. (B) Image of an adult (head) expressing \(\text{lit-1p::GFP}\) (green) and \(\text{ptr-10p::NLS-RFP}\) (red) (transgene \(\text{msEx2159}\)). Arrows, cells with overlapping expression. Left is anterior. Scale bar, 10 µm. (C) Dye-filling assay for indicated genotypes (\(n=90\)). None of the transgenes had an effect on the dye filling of wild type animals (\(n>100\), unpublished data). Error bars, SEM, \(p\) value calculated using Chi-squared test.

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Figure 4. **LIT-1 is required for amphid sensory compartment morphogenesis.** (A, B) Dye filling in animals carrying the indicated mutations ($n \geq 100$). Error bars, SEM. In (B) a sensitized dye-filling assay was used (see Experimental Procedures). (C) Left: Schematic of the arrangement of the cilia (red) and the sheath glial channel (green) in a wild-type adult animal. Not all cilia are depicted. Right: electron micrograph of cross-sections of the amphid channel. Section outlined in yellow is just below the socket-sheath junction; blue outlined section is approximately one micron posterior. Scale bars, 1 μm. (D) Same as in (C), but for a dye-filling defective lit-1(t1512) adult animal. The panel arrangement is a reflection of the one in (C). Arrowheads, tight ensheathment of individual cilia by the sheath glia. Scale bars, 1 μm.

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To test whether \textit{mom-4}, like \textit{lit-1}, acts within glia to regulate amphid morphogenesis, we constructed \textit{mom-4(ne1539s); daf-6(e1377)} double mutants expressing a \textit{lin-26} promoter::GFP::\textit{mom-4} cDNA transgene. When these animals were grown at 20°C, only 7% were filled with dye (Figure 5A), consistent with the hypothesis that \textit{mom-4} acts within glia during early amphid morphogenesis, similar to \textit{lit-1}.

To assess whether \textit{mom-4} and \textit{lit-1} function in the same pathway to promote channel expansion, we examined dye filling in \textit{daf-6} mutants that were also homozygous for both \textit{lit-1(ne132)} and \textit{mom-4(ne1539s)} alleles. We found that the \textit{mom-4; lit-1; daf-6} triple mutant is viable at both 15°C and 20°C and is not suppressed to a greater extent than \textit{lit-1; daf-6} double mutants at either temperature (Figure 5A). This result is consistent with the idea that \textit{lit-1} and \textit{mom-4} function in the same pathway to control channel expansion, similar to their established roles in embryonic cell polarity.

The roles of \textit{lit-1} and \textit{mom-4} in Wnt signaling in \textit{C. elegans} have been extensively studied [28,29]. In this context, MOM-4 activates LIT-1, which then forms a complex with the \textit{LIT-1/WRM-1} complex phosphorylates the \textit{LIT-1}, which then forms a complex with the \textit{LIT-1} to promote channel expansion, similar to their established roles in embryonic cell polarity.

To determine where within the amphid sheath glia \textit{LIT-1} and \textit{MOM-4} localize, we first examined localization of the \textit{GFP::LIT-1} fusion protein in animals harboring a loss-of-function mutation in \textit{daf-6} (Table S1). Strikingly, we found that both fusion proteins were tightly associated with the amphid sensory compartment (Figure 6A and 6B).

To determine whether \textit{LIT-1} localization requires functional \textit{lit-1}, we examined localization of the \textit{GFP::LIT-1} fusion protein in \textit{lit-1(ne1539s)} single mutants at 20°C. GFP::\textit{LIT-1} was properly localized in all animals we observed (n = 44), suggesting that \textit{LIT-1} localizes to the sheath channel independently of its regulator.

Although we cannot eliminate the possibility that multiple redundant Wnt pathways contribute to channel formation and that these operate through \textit{LIT-1} targets other than \textit{POP-1}, the most parsimonious interpretation of our data is that the \textit{MOM-4/\textit{LIT-1}} kinase module operates independently of Wnt signaling to promote expansion of the amphid glial compartment.

**Figure 5.** \textit{mom-4;TAK1} mutations suppress the loss of \textit{daf-6}. (A) Dye filling in animals of the indicated genotypes (n=90). The alleles used are: \textit{daf-6(e1543), lit-1(ne132), mom-4(ne1539), daf-6} is marked with \textit{unc-3(e151)} in all strains except for \textit{mom-4; daf-6}. \textit{unc-3(e151)} does not affect dye filling (unpublished data). Error bars, SEM. p value calculated using Chi-squared test. (B) Schematic of Wnt signaling during endoderm specification in \textit{C. elegans} in contrast to the LIT-1 MAPK module (red), Wnt signaling does not appear to be involved in amphid sheath channel formation (see text and Table S1). doi:10.1371/journal.pbio.1001121.g005
The C-Terminus of LIT-1 Is Necessary and Sufficient for Amphid Sensory Compartment Localization

The channel localization of LIT-1 raised the possibility that in lit-1(ns132) mutants, LIT-1 localization might be disrupted. To test this, we expressed GFP-tagged LIT-1(Q437Stop) (the mutation corresponding to ns132) in wild-type animals and examined its localization. While GFP::LIT-1 reproducibly lines the amphid sensory compartment, GFP::LIT-1(Q437Stop) fails to localize in about one-third of animals and is instead diffusely distributed throughout the cell (Figure 6D and 6G). This result suggests that the highly conserved C-terminal region of LIT-1 may be required for compartment localization. In addition, the fraction of animals in which GFP::LIT-1(Q437Stop) is mislocalized (31%, Figure 6G) mirrors the fraction of daf-6 mutants suppressed by the lit-1(ns132) allele (Figure 2A), raising the possibility that mislocalization may account for the suppression we observed.

The observation that GFP::LIT-1(Q437Stop) still localizes to the amphid channel in some animals raised the possibility that the C-terminal 26 amino acids may represent only a portion of the full targeting domain. To test this idea, we generated animals expressing a GFP::LIT-1ΔCt fusion protein in which all sequences downstream of the kinase domain are deleted. We found that in these animals LIT-1 never accumulated at the amphid sensory compartment, and was diffusely distributed throughout the cell (Figure 6E and 6G), demonstrating that the C-terminal domain is necessary for LIT-1 compartment localization.

To determine whether the C-terminal domain of LIT-1 is sufficient for channel localization, we generated animals express-
ing a GFP::LIT-1 C-terminal domain fusion protein. Remarkably, we found that this fusion protein accumulated at the amphid sensory compartment in a pattern identical to that of full-length LIT-1 (Figure 6F and 6G).

Previous work showed that LIT-1 also localizes to the cell nucleus [30,33,34], and we found this to be the case for amphid sheath glia as well (Figure S8). However, disruption of the C-terminal domain of LIT-1 does not result in its exclusion from the nucleus (Figure S8), suggesting that nuclear functions of LIT-1 may not be abrogated in lit-1(ns132) mutants.

Although the C-terminal domain of LIT-1 is highly conserved from C. elegans to mammals, its function is not well studied. Our studies demonstrate that this domain is both necessary and sufficient for LIT-1 localization to the amphid sensory compartment, and suggest that proper localization is important for LIT-1 function in compartment formation.

ACT-4 Interacts with the C-terminal Domain of LIT-1 and Is Enriched around the Amphid Sensory Compartment

Because of the importance of the LIT-1 C-terminal domain in compartment localization, we used this domain as bait in a yeast two-hybrid screen with the aim of identifying proteins that interact with LIT-1.

From a screen of approximately 10^8 clones, we identified 26 positive clones (Table S2, Figure 7A). While some clones were isolated multiple times, others were found only once, suggesting that our screen was not saturated. We were intrigued that 4 of the 26 interacting clones identified encoded the C. elegans actin protein ACT-4. EM studies of the amphid sheath glia channel had previously shown that the channel is lined by an electron dense subcortical layer (red arrowheads in Figure 1C) [15]. A similar layer can be seen in other highly secreting cells such as pancreatic acinar cells and adrenal chromaffin cells. In these cells, this electron dense layer has been demonstrated to be enriched in actin [35,36].

To determine whether ACT-4 might be part of the electron-dense subcortical layer near the amphid sensory compartment, we examined animals expressing a GFP::ACT-4 fusion protein in amphid sheath glia. Strikingly, we found that although GFP::ACT-4 was seen throughout the cell, it was highly enriched at the amphid sensory compartment (Figure 7B). We wondered whether other actin proteins also accumulate at the channel and, therefore, generated animals expressing a protein fusion of GFP to ACT-1. Again, we found increased channel localization (unpublished data), suggesting that actin filaments may be components of the subcortical density.

To examine the localization pattern of ACT-4 at higher resolution, we used scanning EM coupled with photo-activated localization microscopy (PALM). In this method, serial sections are imaged by scanning EM and using single-molecule fluorescence of mEos::ACT-4 [37]. Images are then superimposed, using fiduciary markers (fluorescent gold beads), to reveal the subcellular localization of fluorescent proteins. As shown in Figure 7C, at the anterior portion of the amphid channel, where an electron dense subcortical region has been described, mEos::ACT-4 is localized near the sensory compartment membrane (blue trace). mEos::ACT-4 does not localize to the sensory compartment in more posterior sections (Figure 7D, 2 μm posterior to 7C), which should lack the subcortical electron density. These observations support the notion that actin is intimately associated with the glial sensory compartment and that the subcortical density may be composed at least in part of actin.

We also found that GFP::ACT-4 was properly localized in lit-1(ns132) mutants (n = 50), suggesting that actin accumulates around the sensory compartment independently of lit-1, and consistent with the possibility that actin may recruit LIT-1. To test this possibility we tried to disturb GFP::ACT-4 localization by treating the animals with an inhibitor of actin polymerization, cytochalasin D. After a 2 h incubation with 1 mM of the drug, the cell bodies of the sheath glia assumed a rounded morphology, indicative of breakdown of the actin cytoskeleton. However, the sensory compartment localization of neither GFP::ACT-4 nor GFP::LIT-1 was disturbed (unpublished data). This result suggests that the subcortical actin around the amphid channel could be part of a stable structure with a lower turnover rate than the rest of the actin cytoskeleton.

Similarly, LIT-1, MOM-4, and ACT-4 all localized to the sensory compartment in daf-6(n1543) mutants (Figure S4), suggesting that DAF-6 is not involved in recruiting these proteins.

The Actin Regulator WASP Binds LIT-1 and Is Required for Sensory Compartment Expansion in daf-6 Mutants

In addition to actin, our two-hybrid studies suggested that the LIT-1 C-terminal domain can also bind to the proline-rich region of WASP-1, the C. elegans homolog of the Wiskott-Aldrich Syndrome Protein (WASP) [32, Figure 7A]. Furthermore, we could immunoprecipitate the LIT-1 C-terminal domain using WASP-1 from cultured Drosophila S2 cells co-expressing both proteins (Figure 7H), suggesting that LIT-1 and WASP-1 can interact. Although GFP::WSP-1 expressed in amphid sheath glia is diffusely localized (unpublished data), co-expression with mCherry::LIT-1 revealed partial co-localization (Figure 7E-G), supporting the notion that LIT-1 and WASP-1 may interact in vivo.

To determine whether wsp-1 plays a role in amphid morphogenesis, we examined wsp-1(n324) mutants, which, unlike actin mutants, are viable [38]. We did not find any defects in dye filling in the single mutant. However, wsp-1(n324) suppresses the daf-6(n1543) dye-filling defects (Figure 7E). Furthermore, daf-6 mutants homozygous for both lit-1(ns132) and wsp-1(n324) were as dye-filling defective as lit-1(n132); daf-6(n1543) mutants alone, consistent with the hypothesis that LIT-1 and WASP-1 act in the same pathway.

Interestingly, we found that overexpression of a GFP::LIT-1 fusion protein results in abnormal glial morphology (Figure S5B, compare to Figure S5A) and distorted sensory compartment morphology (Figure S5C, compare to Figure 6A). This result, together with the genetic and physical interactions between LIT-1 and actin and LIT-1 and WASP, are consistent with the possibility that LIT-1 facilitates glial morphogenesis by regulating actin dynamics.

Discussion

lit-1 Regulates the Morphogenesis of a Subcellular Structure

LIT-1 is the C. elegans homolog of Nemo-like kinase (NLK) [39], a Serine/Threonine kinase originally described in Drosophila [40]. In C. elegans, lit-1 (loss of intestine) was first identified for its role in endoderm specification during early embryogenesis [26]. Subsequent work established lit-1 as a component of the Wnt/B-catenin asymmetry pathway that directs many cell fate decisions in C. elegans [28,29]. NLK also plays roles in control of the Wnt [41,42], TGFB [43], and Notch [44] signaling pathways in vertebrates.

Although LIT-1/NLK has been implicated in cell fate determination, we identified lit-1 mutations as suppressors of lesions in daf-6, a gene that affects morphogenesis of the amphid glial sensory compartment, but not glial cell fate. Indeed, lit-1 single mutants seem to have well-specified amphid components.
Furthermore, despite an established connection between lit-1 and the Wnt/β-catenin asymmetry pathway (a major regulator of cell fate decisions in *C. elegans*), we found no evidence linking Wnt signaling to amphid morphogenesis (Table S1). These observations are consistent with the idea that the role of lit-1 in sensory organ morphogenesis does not involve cell fate decisions, but instead reflects a novel function in cellular morphogenesis.

Within the context of cell fate decisions, LIT-1/NLK often acts by impinging upon the activity of nuclear transcription factors [30,43,44]. It is unclear whether the role of lit-1 in sensory organ morphogenesis might also involve transcriptional regulation. The C-terminal domain of LIT-1 is required for its role in amphib sensory morphogenesis and for its amphid channel localization, but it is not essential for the ability of LIT-1 to enter the nucleus. This suggests that LIT-1 may exert its primary influence on channel morphogenesis at the channel itself. However, LIT-1 C-terminus can interact not only with cytoskeletal proteins (actin and WASP) but also with the transcription factors ZTF-16 and MEP-1 (Table S2). Thus, while it is likely that sensory compartment localization is important for LIT-1 function, we cannot rule out the possibility that LIT-1 has independent relevant functions in the nucleus.

### Opposing Activities of *lit-1* and *daf-6* Direct Sensory Compartment Morphogenesis

Our results suggest that *daf-6* and *lit-1* diverge in the morphogenesis of the sheath glia sensory compartment by exerting opposing influences. In *daf-6* mutants, neurons and glia form an amphib primordium in which all components are initially linked and aligned; however, the sensory compartment expands abnormally. Conversely, in *lit-1* mutants, the sensory compartment is too narrow. Mutations in *lit-1* can correct for the loss of *daf-6*, thus, *lit-1*; *daf-6* double mutants have relatively normal glial channels. A situation that mimics *lit-1*; *daf-6* double mutants arises in animals with mutations in genes controlling neuronal cilia development. In these animals, channel localization of LIT-1, as well as DAF-6, is perturbed. Consistent with the *lit-1*; *daf-6* phenotype, channel formation is only mildly defective in these mutants [14].

The observation that *lit-1* loss-of-function mutations suppress *daf-6* null alleles argues that *lit-1* cannot function solely upstream of *daf-6* in a linear pathway leading to channel formation. Our data, however, are consistent with the possibility that *daf-6* functions upstream of *lit-1* to inhibit *lit-1* activity. Alternatively, *lit-1* and *daf-6* may act in parallel. Our studies do not currently allow us to distinguish between these models.

### Vesicles, the Actin Cytoskeleton, and Sensory Compartment Morphogenesis

How might DAF-6 restrict the size of glial sensory compartments? Electron micrographs of the *C. elegans* amphib reveal the presence of highly organized Golgi stacks near the amphid channel. These images also show vesicles, containing extracellular matrix, that appear to be released by the sheath glia into the channel (Figure 1A) [11]. These studies suggest that vesicular secretion may play a role in channel morphogenesis. Interestingly, DAF-6 is related to Patched, a protein implicated in endocytosis of the Hedgehog ligand, and the *C. elegans* Patched gene *ptc-1* is proposed to regulate vesicle dynamics during germ-cell cytokinesis [45]. Furthermore, DAF-6 can be seen in punctate structures, which may be vesicles [14], and DAF-6 and CHE-14/Dispatched function together in tubulogenesis [14,16], a process hypothesized to require specialized vesicular transport. Together these observations raise the possibility that DAF-6 may restrict amphib sensory compartment expansion by regulating vesicle dynamics in the sheath glia [14].

If indeed DAF-6 controls membrane dynamics, it is possible that LIT-1, which localizes to and functions at the sheath glia channel, also interacts with such processes. How might LIT-1 localize to the glial sensory compartment and control vesicle dynamics? Previous studies suggest that cortical localization of LIT-1 requires it to stably interact with WRM-1/β-catenin [33,34]. In the sheath glia, however, we found that *wrm-1* is not required for sensory compartment morphogenesis or for LIT-1 localization and that LIT-1 and WRM-1 do not co-localize to the amphib sensory compartment (unpublished data). Instead, we found that LIT-1 physically interacts with actin and that actin is highly enriched around the amphib sensory compartment. Thus, actin might serve as a docking site for LIT-1. The interaction between LIT-1 and actin may not be passive. Indeed, we showed that LIT-1 also binds to WASP, and mutations in *wsp-1*/WASP suppress *daf-6* similarly to mutations in *lit-1*. Furthermore, WASP activity is stimulated by phosphorylation of Serines 483 and 484 [46], suggesting that LIT-1, a Ser/Thr kinase, could activate WASP to promote actin remodeling.

Remodeling of the cortical actin cytoskeleton plays important roles in several aspects of membrane dynamics [47]. For example, WASP-dependent actin polymerization has a well-established role in promoting vesicle assembly during clathrin-mediated endocytosis [48]. Recent work has demonstrated positive roles for actin polymerization in exocytosis as well [49,50]. In pancreatic acinar cells, secretory granules become coated with actin prior to membrane fusion [51], and in neuroendocrine cells, actin polymerization driven by WASP stimulates secretion [32]. During *Drosophila* myoblast fusion, actin polymerization, dependent on WASP and WASP interacting protein (WIP), is required for targeted exocytosis of prefusion vesicles [53], and antibodies against WASP inhibit fusion of purified yeast vacuoles [54]. An attractive possibility, therefore, is that LIT-1 might regulate sensory compartment morphogenesis by altering vesicle trafficking through WASP-dependent actin polymerization.

Glial ensheathment is a feature of many animal sensory organs and synapses, and LIT-1 and WASP are highly conserved, suggesting that our studies may be broadly relevant. Interestingly,
LIT-1 was recently shown to be required for cell invasion through basement membranes in C. elegans and in metastatic carcinoma cells [55], processes that require extensive remodeling of the actin cytoskeleton. Our results may, thus, represent a general mechanism for regulating cell shape changes using localized interactions of LIT-1/NLK with cytoskeletal proteins.

Materials and Methods

Strains, Plasmid Construction, and lit-1 Mapping and Cloning

See Supporting Information.

Dye-Filling Assay

Animals were washed off NGM plates with M9 buffer, resuspended in a solution of 10 μg/mL of DiI (1,1’-dioctadecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate) (Invitrogen D282), and rotated in the dark for 1.5 h at room temperature. Animals were then transferred to a fresh NGM plate, anaesthetized with 20 mM sodium azide, and observed using a dissecting microscope equipped with epifluorescence. Animals in which none of the amphid neurons filled with dye were scored as dye-filling defective (Dyf). For the sensitized dye-filling assay, 1 μg/mL of DiI was used, and the incubation time was 15 min. Animals were scored as dye filling defective (Dyf) if either one or two amphids failed to fill.

Transmission Electron Microscopy and Fluorescence Electron Microscopy (fEM)

See Supporting Information and [37].

Fluorescence Microscopy and Image Analysis

Images were acquired using a DeltaVision Image Restoration Microscope (Applied Precision) equipped with a 60×/NA 1.42 Plan Apo N oil immersion objective (Olympus) and a Photometrics CoolSnap camera (Roper Scientific), or an Upright Axioplan LSM 510 laser scanning confocal microscope (Zeiss) equipped with a C-Apochromat 40×/NA 1.2 objective. Acquisition, deconvolution, and analysis of images from the DeltaVision system were performed with Softworx (Applied Precision); images from the confocal microscope were acquired and analyzed using LSM 510 (Zeiss).

Yeast Two-Hybrid Screen

LexA::LIT-1Ct was used as bait in a Y2H screen using the Drosophila LexA::LIT-1Ct was used as bait in a Y2H screen using the D. melanogaster wingless protein as prey. 

Cloning

Disruption of the LIT-1 carboxy-terminal domain. (A–C) Fluorescence images of the indicated genotypes (B) lit-1(ns132); daf-6(e1377); and (C) lit-1(ns132); daf-6(e1377) animals after incubation for 1.5 h in 10 μg/mL of DiI (red). Scale bars, 50 μm. (D) Using SNP mapping (see Supplemental Materials and Methods, Text S1), ns132 was mapped to the right end of chromosome III, distal to the SNP F54F12:17329 at genetic position +20.72. The cosmids ZK520, ZK525, W96F12, and K08E3 were used for the construction of transgenic strains (see panel E). (E) Dye-filling in animals of the indicated genotypes (n≥90). The alleles used were daf-6(e1377) and lit-1(ns132). lit-1 genomic and lit-1(ns132) genomic correspond to constructs pG01 and pG02, respectively (see Supplemental Materials and Methods, Text S1).

Supporting Information

Figure S1 Amphid sensory compartment morphogenesis in wild-type embryos. Electron micrographs of cross-sections through the amphid primordium in wild-type animals. Top: At approximately 380 min after fertilization, the amphid pocket is blocked anteriorly by a cap formed by the sheath glia (left). More posteriorly (middle and right), the sheath wraps around the dendrites of the amphid neurons. Bottom: At approximately 400 min after fertilization, the amphid channel is open, with filaments (asterisk) visible at the level of the socket (left; arrow indicates socket self junction). More posteriorly (middle and right), the sheath glia wraps around the dendrites of the amphid neurons. Filaments (asterisk) can be seen in the middle section.

(TIF)

Figure S2 Dye-filling assay and lit-1(ns132) mapping and cloning. (A–C) Fluorescence images of (A) wt, (B) daf-6(e1377), and (C) lit-1(ns132); daf-6(e1377) animals after incubation for 1.5 h in 10 μg/mL of DiI (red). Scale bars, 50 μm. (D) Using SNP mapping (see Supplemental Materials and Methods, Text S1), ns132 was mapped to the right end of chromosome III, distal to the SNP F54F12:17329 at genetic position +20.72. The cosmids ZK520, ZK525, W96F12, and K08E3 were used for the construction of transgenic strains (see panel E). (E) Dye-filling in animals of the indicated genotypes (n≥90). The alleles used were daf-6(e1377) and lit-1(ns132). lit-1 genomic and lit-1(ns132) genomic correspond to constructs pG01 and pG02, respectively (see Supplemental Materials and Methods, Text S1).

(TIF)

Figure S3 Nuclear localization of LIT-1 is not abrogated by disruption of the LIT-1 carboxy-terminal domain. (A–C) Fluorescence images of sheath glia cell body and nucleus in animals transgenic for the indicated GFP::LIT-1 fusion protein. Transgenes depicted: nsEx2606 (A), nsEx2609 (B), nsEx2747 (C). Arrow, cell nucleus. Scale bar, 10 μm. The T02B11.3 promoter was used to drive all constructs.

(TIF)

Figure S4 Sensory compartment localization of LIT-1, MOM-4, and ACT-4 are independent of daf-6. (A–C) Fluorescence images of adult daf-6(n1343) animals expressing the indicated GFP fusion proteins. The T02B11.3 amphid sheath promoter was used to drive all constructs. Transgenes depicted: nsEx2606 (A), nsEx2840 (B), nsEx2876 (C). Arrow, cell nucleus. Scale bars, 10 μm.

(TIF)

Figure S5 Overexpression of LIT-1 within the sheath glia disrupts cellular morphology. (A) Fluorescence projection image of the sheath glia promoter F16F9.3 driving dsRed (transgene nsEx3272). (B) Fluorescence projection image of a transgenic animal carrying a high copy number of the T02B11.3 amphid sheath promoter driving GFP::LIT-1 (transgene nsEx2619). Compare the extensive branching of the sheath glia process with...
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20. Page 79.
21. Shaping Glial Tubes

Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: GO EAP EMJ SS. Performed the experiments: GO EAP SW YL. Analyzed the data: GO EAP SW EMJ SS.

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Clones identified from a yeast-two-hybrid screen for proteins that interact with the carboxy-terminal domain of LIT-1.

Text S1

Tables S1 and S2

Table S1 Components of the Wnt signaling pathway do not affect amphid morphogenesis.

Table S2 Clones identified from a yeast-two-hybrid screen for proteins that interact with the carboxy-terminal domain of LIT-1.

Text S1

Supplemental Materials and Methods.

Author Contributions

Author(s) have made the following declarations about their contributions: Conceived and designed the experiments: GO EAP EMJ SS. Performed the experiments: GO EAP SW YL. Analyzed the data: GO EAP SW EMJ SS.

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