Cell Architecture: Surrounding Muscle Cells 
Shape Gland Cell Morphology 
in the Caenorhabditis elegans Pharynx

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ABSTRACT The acquisition and maintenance of shape is critical for the normal function of most cells. Here we investigate the 
morphology of the pharyngeal glands of Caenorhabditis elegans. These unicellular glands have long cellular processes that extend 
discrete lengths through the pharyngeal musculature and terminate at ducts connected to the pharyngeal lumen. From a genetic 
screen we identified several mutants that affect pharyngeal gland morphology. The most severe such mutant is an allele of sma-1, 
which encodes a β-spectrin required for embryonic elongation, including elongation of the pharynx. In sma-1 mutants, gland 
projections form normally but become increasingly abnormal over time, acquiring additional branches, outgrowths, and swelling, 
suggestive of hypertrophy. Rather than acting in pharyngeal glands, sma-1 functions in the surrounding musculature, suggesting that 
pharyngeal muscles play a critical role in maintenance of gland morphology by restricting their growth, and analysis of other mutants 
known to affect pharyngeal muscles supports this hypothesis. We suggest that gland morphology is maintained by a balance of forces 
from the muscles and the glands.

A CQUISITION of shape is an important step in the mat-
uration and function of cells, the maxim “form follows function” being as much a principle of biology as in other 
disciplines. In some cases, morphology is a largely intrinsic 
property of the cell necessary for function, as for example the biconcave disk shape of mammalian erythrocytes or the 
capacity of neurons grown in culture to develop axons. In 
other cases, cell morphology results from interactions be-
tween a cell and its surroundings, including other cells 
and the extracellular matrix (ECM). Such interactions are 
particularly evident in the formation of complex organ struc-
tures, in which a variety of different cell types must interact 
to form a mature, functioning structure. 

The Caenorhabditis elegans pharynx serves as a useful model for organ development: morphogenetic events can 
be analyzed both at the level of the whole organ and at 
the level of individual cells. The usual advantages of model 
organism genetics and the transparency of the nematode 
apply to study of the pharynx, as does the relative simplicity 
of the organ (95 cells in total) (Mango 2009; Kormish et al. 
2010). However, the pharynx also exhibits features of more 
complex organs, in particular, the diverse lineal histories of 
pharyngeal cells and the presence of multiple different cell 
types within the organ (Sulston et al. 1983). Previous stud-
ies have elucidated some aspects of pharyngeal morphogen-
esis, such as the attachment of the pharynx to the buccal 
cavity (or mouth) and the intricate formation of two toroid 
cells (pm8 and vpi1) that connect the pharynx to the in-
testine (Portereiko and Mango 2001; Rasmussen et al. 
2008). In the case of pharyngeal attachment, changes in cell 
shape and orientation at the anterior end of the pharynx 
lead to epithelial connections that anchor the pharynx to 
the mouth of the animal; this connection is ultimately re-
quired for the proper elongation of the organ as a whole. In 
pharynx unattached (Pun) mutants, the organ generally 
lacks the distinct bilobed shape that results from differential 
elongation of pharyngeal cells (Fay et al. 2004; Qiu and Fay 
2006; Mani and Fay 2009). The formation of the pm8 and 

vpi1 cells at the posterior end of the pharynx is likewise
a study of cell shape arising from a combination of intrinsic cellular properties and interactions with neighboring cells. Careful analysis of pm8 development revealed a dorsal-to-ventral movement of the cell, akin to a falling curtain, during which movement the cell drapes itself around extensions from a neighboring support cell, the mc3 marginal cell (Rasmussen et al. 2008). Following this movement, the cell self-fuses to produce the terminal toroid shape of pm8.

Other cells within the pharynx have unique and interesting morphologies, such as the pharyngeal glands (Albertson and Thomson 1976), which are the focus of this article. The five pharyngeal glands are single-celled structures, whose cell bodies reside in the posterior bulb of the pharynx (Figure 1A). Each of the glands connects to the pharyngeal lumen via a cellular extension that differs in length and connectivity depending on the cell's subtype. The longest projection is made by g1P and extends a distance of ~125 μm in adults, terminating at a duct near the anterior end of the pharynx. The projections of the two g1A cells extend ~50 μm in adults and form ducts at the posterior end of the anterior pharyngeal bulb. Finally, the two g2 cells have relatively short extensions (<10 μm) that connect to the lumen within the posterior bulb. Along their length, these extensions are flanked by pairs of pharyngeal muscle cells that ultimately fuse to form binucleate cells that surround the gland extension (Figure 1B). Formation of the gland projections has been suggested to occur by a process referred to either as the "fishing line model" or "retrograde extension" (Pilon 2008; Heiman and Shaham 2009). In this model, the glands attach to the pharyngeal lumen at the site of their birth and migrate posteriorly, forming their projections as a result of moving away from the anchor point of the duct. This model was first suggested by Sulston in his landmark article describing the C. elegans embryonic lineage (Sulston et al. 1983) and has since been demonstrated to occur in the formation of projections of several other C. elegans cells, including pharyngeal neurons, amphid neurons, and body wall muscles, as well as cells in higher organisms (including granule cells in the mammalian cerebellum) (Morck et al. 2003; Dixon and Roy 2005; Heiman and Shaham 2009). However, whether the pharyngeal glands use such a mechanism has not been established.

Postembryonically, the gland projections grow roughly twofold, in conjunction with growth of the pharynx. During this time, the glands retain their original points of connection to the lumen and maintain the morphology of the projections. Thus, another question is how the size and shape of the gland projections is regulated during growth of the entire animal. This problem is referred to as "scaling." In neurons, scaling can occur in response to tension along the axon as a result of organismal growth (Bray 1984) or by ongoing signaling between neurons and neighboring tissue during growth (Parrish et al. 2009).

To investigate morphogenesis of C. elegans glands and maintenance of their projections, we conducted a forward genetic screen to identify mutants with abnormal glands. Our collection includes mutants in which the gland projections appear to be "overgrown," exhibiting swelling, branching, and apparent hypertrophy. This overgrowth generally worsens with age, suggesting inappropriate maintenance of gland size and structure during growth. Cloning of one such mutant revealed it to be an allele of the β-spectrin-encoding gene sma-1. Consistent with previous reports, we show that sma-1 is expressed in the myoepithelial cells surrounding the glands, but not in the glands themselves, suggesting that the pharyngeal muscles play a role in maintenance of gland morphology. Accordingly, we find that several previously characterized mutants with defective or impaired pharyngeal muscles exhibit similar gland defects and we further provide evidence that the muscles normally maintain the shape of the gland projections. The results suggest a model in which maintenance of gland morphology and scaling of projections results from a balance of forces between ongoing gland growth and restraint by pharyngeal muscles.

Materials and Methods

Worm strains

Standard nematode handling conditions were used (Brenner 1974). All strains were grown at 20°C, except where noted. Genes and alleles used were:

LG I: unc-89(e1460), let-502(sb118), dpy-5(e61), and let-381(gk302) /hT2[bli-4(e937) let-?(q782) qIs48] (I;III).
LG II: ivls12 [phat-1::YFP elt-2::GFP rol-6(su1006)] and rol-6(e187) hh-6(tm299) unc-4(e120).
LG III: sma-2(e502) and sma-3(e491).
LG IV: jcs1[ajm-1::GFP rol-6(su1006)], dpy-13(e184), and let-60(sy101sy127).
LG V: sma-1(e30).
LG X: pha-2(ad472).

The integrated strain GD139 (hhl-6::YFP, elt-2::LacZ::GFP, rol-6(su1006)) was generated by gamma irradiation as described (Evans 2006) and was outcrossed to the wild-type N2 four times. Mutagenesis of GD139 was performed with ethylmethanesulfonate (EMS) as previously described (Brenner 1974). Individual L4 animals were picked from the mutagenized population to fresh plates and grown at 20°C. Individual F1 progeny were then picked and transferred to new plates and F2 progeny screened under a rol-6(su1006) ethylmethanesulfonate (EMS) as previously described (Evans 2006) and was outcrossed to the wild-type in wild-type animals and have not observed significant gland abnormalities, possibly representing normal variation in gland morphology or a minor effect caused by transgenes. We typically observe infrequent and minor defects in older adults that may result from age-related decline (Garigan et al. 2002). However, these effects are relatively small, allowing us to readily detect alterations in gland morphology associated with a number of different genetic backgrounds. We have generated numerous transgenic lines with the phat-1 reporter in wild-type animals and have not observed significant gland defects associated with the presence of this (or other) transgenes. Additionally, extrachromosomal transgenes always faithfully recapitulate the expression pattern seen in ivls12.

In the course of mapping one of the mutants obtained in the screen (allele iv52), we discovered that the original strain carrying the integrated transgenes (GD139) has a probable inversion on the right arm of LG II. SNP mapping of iv52 (and of iv56) revealed a lack of recombination between N2 and CB4856 (Hawaiian) chromosomes along the right arm of LG II from +4 to +22, suggestive of a chromosomal rearrangement. We therefore examined the original (unmutagenized) strain, GD139, and discovered the same recombination on LG II, suggesting that the rearrangement exists in the parent strain (Supporting Information, Figure S1). This rearrangement likely resulted from the integration of the phat-1::YFP transgene, which maps to the same region of the genome. The rearrangement is most likely an inversion (rather than a transposition), as no other region of the genome showed suppression of recombination (data not shown). Importantly, the parent strain has normal glands and appears healthy, indicating that the rearrangement did not result in a gland phenotype. However, during our subsequent analysis of mutants, we were conscious of the possibility that the rearrangement might have a subtle influence on mutant phenotypes (e.g., might act as a modifier), and where possible, gland phenotypes were examined using a second, unintegrated reporter. Because the integration event appears to substantially suppress recombination along the right arm of LG II, this strain may be useful as a balancer for this region of the genome.

### Table 1 Gland projection defects in different mutants

| Strain | Stage | Transgene | WT (%) | Abnormal (%) | N |
|-------|-------|-----------|--------|--------------|---|
| ivls12 | L1    | ivls12    | 100    | 0            | 57|
| sma-1(e30) | L1 | ivls12  | 13     | 87           | 132b|
| rol-6(su1006) | L1 | Ex    | 98     | 2            | 40 |
| pyr-1(RNAi inj) | L1 | ivls12 | 38     | 62           | 39b|
| unc-52(RNAi inj) | L1 | ivls12 | 25     | 75           | 48b|
| elt-2(RNAi feed) | L1 | ivls12 | 0      | 100          | 37b|
| elt-2(RNAi feed) | L1 | ivls12 | 0      | 100          | 42 |
| ivls12 | Adult | ivls12 | 96     | 4            | 50 |
| ivls12 | Adult Ex | ivls12 | 43     | 57           | 61b|
| ivls12 | Adult | ivls12 | 23     | 71           | 66b|
| ivls12 | Adult | ivls12 | 1      | 99           | 77b|
| unc-89(e1460) | Adult | ivls12 | 53     | 47           | 109b|
| elt-2(RNAi inj) | Adult | ivls12 | 37     | 63           | 51b|
| elt-2(RNAi inj) | Adult | ivls12 | 0      | 100          | 52b|
| ivls12 | Adult Ex | ivls12 | 93     | 7            | 81 |
| ivls12 | Adult | ivls12 | 89     | 11           | 71 |
| elt-2(RNAi inj) | Adult | ivls12 | 95     | 5            | 42 |
| elt-2(RNAi inj) | Adult | ivls12 | 94     | 6            | 72 |
| elt-2(RNAi feed) | Adult | ivls12 | 94     | 6            | 128b|

To visualize glands, animals carried either an integrated phat-1::YFP transgene (ivls12) or an extrachromosomal array (Ex) generated by microinjection of DNA into the mutant strain. Use of different transgenic arrays (extrachromosomal vs. integrated) did not appear to have any influence on gland morphology. The ztf-11 (ok8546) strain produces 16% L1 lethals; all of the animals scored here were L1 lethal. RNAi was performed either by injection (inj) or by feeding (feed); see Materials and Methods for details.

a Apparent background levels of gland “abnormalities,” possibly representing normal variation in gland morphology or a minor effect caused by transgenes. We typically observe infrequent and minor defects in older adults that may result from age-related decline of animals.

b The frequency of defects was significantly greater (P < 0.001, χ² goodness-of-fit, 2 d.f.) compared to wild type. In all other cases, the frequency of defects was not significantly different than wild type (P > 0.3, χ² goodness-of-fit, 2 d.f.).

### Construction of plasmids

GFP reporters (ceh-2::GFP, dlg-1::GFP, and sma-1::GFP) were made by PCR amplification from genomic DNA followed by cloning of fragments into the GFP expression vector pPD95.77. Oligonucleotides used to amplify genomic DNA were made by PCR amplification from genomic DNA followed by cloning of fragments into the GFP expression vector pPD95.77.
fragments are listed below; lowercase sequence indicates engineered ends to facilitate cloning.

dehydrated in ethanol and infiltrated with Polybed 812 resin (Polysciences). Polymerization was performed at 60°C for 48 hr. Silver-gray sections were cut with an ultramicrotome (Leica) equipped with a diamond knife, and sections were stained with uranyl acetate and lead citrate and examined in a H-700 Hitachi electron microscope.

Laser microsurgery
L2-adult stage hermaphrodites were mounted in 1 μl of 10 mM muscimol (Sigma M1523) in M9 on a 5% agarose pad under a coverslip. GFP expressing g1P gland cells were imaged with a Microradiance 2000 confocal microscope using a Nikon 60X, 1.4 NA lens. Gland cell processes were cut using a 440-nm MicroPoint Laser System from Photonic Instruments. After surgery, animals were recovered to an agar plate seeded with HB101, and remounted for confocal imaging 1–3 days postsurgery. Regeneration was scored in nine animals in which successful cuts were made at the L2 stage.

Results
Genetic screening identifies a variety of gland defects
Gland projections are proposed to develop during embryogenesis, as gland cells migrate from their site of birth to their final position in the pharynx (Sulston et al. 1983). Glands may therefore form attachments to the pharyngeal lumen at the site of their birth, with the gland projection being drawn out as the gland cell migrates posteriorly. To investigate development of pharyngeal glands and their extensions, we

Additional details of plasmids and cloning strategies are available upon request.

Construction of transgenic lines
Transgenes were injected at 10–20 ng/μl together with 50 ng/μl pRF4 [rol-6(su1006)] (Kramer et al. 1990; Mello et al. 1991) and pBSII SK+ to a total DNA concentration of 100 ng/μl.

RNAi
RNAi was performed by injection of dsRNA prepared from Ahringer library clones, as previously described (Fire et al. 1998; Kamath et al. 2003). dsRNA was injected at 1 μg/μl, except where noted. In the case of elt-2, we performed RNAi by feeding, as previously described (Timmons and Fire 1998), as elt-2(RNAi) gives a highly penetrant (~100%) L1 lethality by feeding (McGhee et al. 2009).

Volume measurements
To estimate gland cell volumes, images from adult transgenic animals were collected as a Z series at 0.8-μm intervals using a Zeiss AxioCam and ApoTome, supported by Axiovision 4.8 software. For each optical section, we measured the area of the GFP+ regions using ImageJ (Rasband 1997–2010). Area measurements were then summed for each structure to provide an estimate of GFP+ volumes.

Transmission electron microscopy
For electron microscopy, worms were anesthetized in 5 mM levamisole and were decapitated with a 27 gauge × 1/2 Monoject needle. Heads were fixed in 3% glutaraldehyde in Millonig’s phosphate buffer for 1 hr at room temperature. Postfixation was in 2% OsO₄ for 20 min. Samples were

Figure 2 Representative images of wild-type parent strain (GD139) and mutants with gland defects. In all images, the open dashed line indicates the outline of the pharynx, and anterior is at left. (A) GD139 ivs12 [phat-1::YFP elt-2::GFP] larvae. (B) Expression of the phat-1 reporter is lost in hhl-6 (iv52) animals but elt-2::GFP expression (to mark the presence of the transgene) in the intestine is unaffected (arrowhead). (C) Cell body of g1P gland (arrowhead) is found anterior of its normal position and g2 glands are not visible in iv50 homozygotes. (D) iv37 mutants show abnormal swelling of gland projections in the anterior pharyngeal bulb (arrowheads). (E) Abnormal swelling of gland projections in the anterior pharynx of iv50 homozygote (arrowheads). (F) sma-1(iv38) mutants exhibit severe swelling and occasional branching of gland projections, and the pharynx is incompletely elongated.
performed a genetic screen for animals with gland defects. We screened animals carrying an integrated, gland-specific YFP reporter (phat-1::YFP) for any alterations to gland cell number or morphology. A screen of ~5000 haploid genomes identified five recessive mutants (Figure 2). Of these five mutants, one (iv52) lacked observable gland reporter expression, one (iv36) lacked expression in a subset of glands and showed apparent defects in g1P migration, and three (iv37, iv38, and iv50) had wild-type numbers of expressing cells with abnormal morphology of the gland extensions.

**Isolation of a new allele of hlh-6**

By genetic mapping, complementation, and allele sequencing, we find that the mutant lacking observable reporter expression (Figure 2B), iv52, is an allele of the previously characterized gene hlh-6, which encodes a bHLH transcription factor that is required for expression of many gland genes, including phat-1 (Raharjo and Gaudet 2007; Smit et al. 2008). Homozygous iv52 animals have the same phenotype as hlh-6(tm299) homozygotes, which carry a deletion allele predicted to be a molecular null (Smit et al. 2008), and iv52 fails to complement hlh-6(tm299). Sequencing of hlh-6 from iv52 homozygotes identified a single nucleotide change in the coding region, 544G > A (position is relative to the start of the cDNA), which results in a Glu-to-Lys (E182K) missense mutation in the predicted product (Figure S2). This mutation lies in the predicted DNA binding domain of HILH-6 and affects a glutamate residue that, in other bHLH proteins, makes direct contact with the E-box, the DNA binding site for bHLH proteins (Ma et al. 1994). Molecularly, this alteration is predicted to be a strong loss of function, which is consistent with our observation that iv52 animals are phenotypically indistinguishable from tm299 animals.

**Isolation of mutant affecting gland cell body position**

Another mutant, iv36, lacks observable expression of phat-1::YFP only in the g2 glands, while all three g1 glands are visible. Interestingly, the g1P cell body occupies a position anterior to its wild-type location, yet the gland projections of all three g1 cells appeared normal (Figure 2C). This phenotype supports the retrograde extension model for formation of the g1P gland extension in that the mutant appears to be defective for migration of g1P. However, there may be other explanations for this phenotype that we are currently investigating. This mutant mapped to the right arm of chromosome II, but further mapping was prevented by the probable inversion in this region that likely resulted from the integration of the phat-1::YFP transgene (see Materials and Methods). Whole-genome sequencing or isolation of other alleles (using a different transgenic strain lacking the inversion on II) will allow identification of the relevant gene.

**Mutants affecting morphology of the gland projection**

The remaining three mutants, iv37, iv38, and iv50, all affect gland morphology to varying degrees (Figure 2, D–F). SNP mapping placed these mutants at three different map positions, indicating that each mutation affects a different gene. Two of these mutations (iv37 and iv50) result in variable defects in the gland extensions. In iv37 homozygotes, defects are restricted to the anterior bulb region of the pharynx, with 57% (n = 61) of animals showing defects in the g1P and/or the g1A cells. Initial BSA-SNP mapping placed iv37 on the right arm of LG III. In iv50 mutants, defects are not restricted to the anterior bulb region, but are instead visible along the anterior half of the pharynx in 71% (n = 66) of animals. BSA-SNP mapping placed iv50 in the middle of LG III.

The final mutation, iv38, resulted in striking gland defects in 100% of examined animals. Animals homozygous for iv38 have normal gland cell bodies and all glands appear to connect with the pharyngeal lumen at their normal positions. However, the gland projections have a variety of abnormalities, including additional branching, swelling, and thickening, and abnormal trajectories (Figure S3). The gland defects are not the result of general defects in pharyngeal cellular morphology, as the pharyngeal neuron I3 (which lies in direct contact with the anterior portion of the g1P projection) appears normal in iv38 animals. We examined iv38 animals with a ceh-2::GFP marker that is expressed in I3 (and other neurons) (Aspock et al. 2003). Of 35 iv38; ceh-2::GFP animals examined, 89% had normal-looking I3 neurons, while 11% showed minor “blebs” along the axon. We scored iv38 animals with a different, unintegrated reporter (hlh-6::mTomato). These animals also showed 100% penetrance of gland defects (data not shown), arguing that the phenotype is specific to iv38 and is not caused or enhanced by the inversion associated with iv52. Given the severity and 100% penetrance of the gland phenotype in these mutants, we chose to examine iv38 in more detail.

**iv38 is an allele of sma-1**

In addition to their gland defects, iv38 homozygotes are smaller (Sma) than wild-type animals of the same developmental stage and are somewhat dumpy (Dpy) in appearance. However, mutations in other dpy genes (that result in smaller body size; dpy-5 and dpy-13; Table 1) have no effect on gland morphology, suggesting that the gland defects are not simply a byproduct of defects in body size. SNP mapping placed iv38 in a region on LG V between +0.5 and +5.6. We searched this region for genes associated with a Dpy or Sma phenotype, either by mutation or RNAi, and identified sma-1 as a candidate gene. We determined that iv38 is an allele of sma-1 by three criteria. First, we examined pharyngeal glands in sma-1(e30) mutants and found that these animals exhibited the same defects observed in iv38 homozygotes (Figure 3, D and E). Second, we found that e30 and iv38 failed to complement each other with respect to both the gland phenotype and the Sma phenotype. Third, we sequenced sma-1 in iv38 homozygotes and found a single mutation at position 5253 of the cDNA (5253G > A), which changes a Trp codon to a stop codon (W1751Stop; Figure 3B).
Glands lack expression of epithelial markers. (A and B) Expression of hlh-6::mTomato in pharyngeal glands. Arrows indicate the anterior ends/ducts of g1P (A) and one of the g1A cells (B). (C and D) Expression of a rescuing AJM-1::GFP translational fusion in the same regions as A and B; arrowheads indicate the location of the gland ducts. (E and F) Merge of hlh-6::mTomato and AJM-1::GFP expression; there is no apparent overlap of expression in the gland ducts. (G) Expression of a dlg-1::GFP reporter shows a lack of expression in the pharyngeal glands (arrows).

Since glands appear to lack sma-1 expression, one possible explanation for the sma-1 gland phenotype is that pharyngeal muscles normally function to constrain growth of the glands and that, in sma-1 mutants, the defective muscles are unable to adequately restrain gland growth. Consistent with a model in which muscles constrain growth of the glands, we observed that the gland phenotype becomes progressively more severe as animals develop. In newly hatched sma-1 larvae (L1s), 67% had relatively mild defects in gland morphology and 20% had moderate-to-severe defects (the remaining 13% appeared normal; n = 132). In contrast, 100% of young sma-1 adults exhibited moderate-to-severe defects (n = 86) (Table 1). One possibility is that, as expression of components of the adherens junctions, which are markers of epithelial identity. Previous reports suggested that the gland duct, which connects the gland projection to the pharyngeal lumen, contains adherens junctions (Albertson and Thomson 1976). However, our results suggest that the glands do not express at least two of the components of the adherens junctions, AJM-1 and DLG-1 (Mohler et al. 1998; McMahon et al. 2001). Neither a rescuing AJM-1::GFP translational fusion nor a dlg-1 transcriptional reporter are visible in gland cells (Figure 4), suggesting that while the duct may be associated with adherens junctions, the glands themselves must express some other molecule(s) that facilitate connection to the pharyngeal lumen.

The sma-1 gene encodes a β1H-spectrin that is expressed in C. elegans epithelial cells and is a component of the membrane cytoskeleton (McKeown et al. 1998; Norman and Moerman 2002; Praitis et al. 2005). Mutations affecting sma-1 result in a smaller body size due to defects in embryonic elongation, consistent with its expression in epithelial cells. SMA-1/β1H-spectrin forms part of the apical membrane cytoskeleton and is thought to play a role in stabilizing interactions between this network and the actin cytoskeleton during morphogenesis. Gland defects in sma-1 mutants could result either from structural defects in the gland themselves or from defects in the surrounding pharyngeal muscles. Previous reports, using both reporter transgenes and α–SMA-1 immunostaining, do not indicate SMA-1 expression in glands, a finding that we verified using a sma-1 transcriptional reporter, which includes the entire upstream intergenic region and first intron of the sma-1a isoform (which would include any and all upstream sequence of the sma-1b isoform). We observed expression of sma-1::GFP in all epithelial cells, as expected (hypodermis, excretory canal cell, intestine, pharyngeal muscles, and pharyngeal marginal cells) (McKeown et al. 1998) but saw no observable expression in gland cells (Figure 3C). Likewise, use of a nuclear-localized sma-1::GFP::HIS2B reporter did not indicate any expression in gland nuclei (Figure 3D).

This finding raised the possibility that gland defects in sma-1 animals are a consequence of defects in the surrounding musculature and further suggests that glands lack epithelial characteristics. Accordingly, glands also lack gland morphology and 20% had moderate-to-severe defects (the remaining 13% appeared normal; n = 132). In contrast, 100% of young sma-1 adults exhibited moderate-to-severe defects (n = 86) (Table 1). One possibility is that, as expression of components of the adherens junctions, which are markers of epithelial identity. Previous reports suggested that the gland duct, which connects the gland projection to the pharyngeal lumen, contains adherens junctions (Albertson and Thomson 1976). However, our results suggest that the glands do not express at least two of the components of the adherens junctions, AJM-1 and DLG-1 (Mohler et al. 1998; McMahon et al. 2001). Neither a rescuing AJM-1::GFP translational fusion nor a dlg-1 transcriptional reporter are visible in gland cells (Figure 4), suggesting that while the duct may be associated with adherens junctions, the glands themselves must express some other molecule(s) that facilitate connection to the pharyngeal lumen.

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animals grow, growth of glands is counterbalanced by forces exerted by the surrounding musculature. If this force is weakened, as in *sma-1* mutants, then growth of glands during development would be relatively less constrained, resulting in the types of overgrowth defects seen here.

An alternate possibility, which cannot be ruled out from these studies, is that *SMA-1* is present and functional in pharyngeal glands, but not detected by our reporter or by previous analyses. An ideal approach would be to test rescue of the gland phenotype by expressing *sma-1* under the control of tissue-specific promoters (e.g., gland-specific or muscle-specific promoters). Unfortunately, the large size of the *sma-1* gene (>12 kb cDNA) proved challenging with respect to the cloning of such rescuing constructs. Therefore, to test the hypothesis that pharyngeal muscle integrity influences gland morphology, we chose to examine several other mutants with impaired pharyngeal muscle structure and/or function.

**Defects in pharyngeal muscles result in abnormal gland morphology**

To test the general hypothesis that the pharyngeal muscles are important in maintenance of gland morphology, we examined glands in two classes of mutants: those in which pharynx size was reduced but in which pharyngeal muscles were otherwise normal and those in which pharyngeal elongation and/or muscle structure were impaired. In the experiments that follow, we scored either young adults (if mutants were viable) or arrested embryos and/or L1 larva (if mutants were lethal).

One possible explanation for the gland defects in *sma-1* mutants is that the glands are growing to normal size/volume while contained in a smaller pharynx. We therefore examined other mutants with reduced pharynx size, namely *sma-2* and *sma-3*, which encode downstream signaling components of a TGFβ signaling pathway required for normal *C. elegans* size (Savage et al. 1996). Unlike *sma-1* mutants, *sma-2* and *sma-3* mutants undergo normal elongation and have normal pharyngeal morphology, but are smaller than wild type due to reduced postembryonic growth of cells. Although not previously reported, we find that the average pharynx length in *sma-2* and *sma-3* adult animals is significantly smaller than that of wild-type animals of the same age (Table 2). However, the glands of *sma-2* and *sma-3* mutants do not show any apparent defects (Figure 5A, Table 1). Thus, one interpretation is that the gland defect in *sma-1* mutants is not due to the smaller pharynx size, but rather due to defects in elongation and/or structure of the pharyngeal muscles. An alternate interpretation is that glands are normal in *sma-2* and *sma-3* mutants because all cells (including glands) are smaller in these mutants. However, on the basis of our analyses of glands in other mutants (below), we propose that glands in *sma-1* mutants are defective because of defects in pharyngeal muscle integrity rather than the smaller size of the pharynx.

One aspect of the *sma-1* mutant phenotype is incomplete elongation of both the embryo and the pharynx. Embryonic elongation occurs as a result of contractions of the actin cytoskeleton of the lateral epidermal cells (seam cells) (Priess and Hirsh 1986; Chisholm and Hardin 2005; Gally et al. 2009). These contractions are regulated by a network of genes including the Rho kinase encoded by *let-502*. Depletion of maternal and zygotic *let-502* results in arrest after hatching as unelongated larvae (Wissmann et al. 1997). Using the temperature-sensitive mutation *let-502(sb118)* (P. Mains, personal communication), we found that glands in *let-502* mutants raised at the restrictive temperature (25º) showed the same types of defects present in *sma-1* hatchlings (Figure 5B, Table 1), while glands in animals raised at the permissive temperature (15º) were normal, suggesting that defects in elongation lead to defects in gland morphology.

Loss of function of other genes required for elongation resulted in consistent gland defects resembling those seen in *sma-1* mutants. RNA-mediated interference (RNAi) of *pyr-1*,
which encodes a CAD protein with three enzymatic activities (Carbamoyl phosphate synthetase, Aspartate transcarbamoylase, and Dihydroorotase) that is involved in pyrimidine synthesis (Franks et al. 2006), causes an incompletely penetrant early arrest, in which animals have unelongated or incompletely elongated pharynges (as well as other morphological defects). pyr-1(RNAi) animals display gland defects that correlate with the severity of the pharyngeal phenotype. For example, viable pyr-1(RNAi) progeny have apparently normal glands while arrested larvae with pharyngeal defects have abnormal glands (Figure 5C). A similar result was obtained by RNAi against unc-52, which encodes the basement membrane component perlecan; disruption of unc-52 results in embryonic arrest midway through elongation (paralyzed arrest at twofold, Pat, phenotype) (Rogalski et al. 1993). unc-52(RNAi) resulted in 100% embryonic arrest, accompanied by frequent gland defects similar to those seen in sma-1 embryos and L1 larvae. We conclude that the gland defects in the above mutants specifically result from defects in elongation rather than being merely a consequence of embryonic or larval arrest, as other lethal mutations that we have tested do not result in defects in gland projections (let-381, let-60, ztf-11, and elt-2; Figure 5D, Table 1) (Han and Sternberg 1990; Maeda et al. 2001; McGhee et al. 2009; Amin et al. 2010).

We also examined mutants in which pharyngeal elongation was normal but where muscle structure was impaired. We examined the effects of mutation in unc-89, which encodes an M-line–specific component of the actin-myosin contractile apparatus (Benian et al. 1996), and RNAi-mediated knockdown of two pharyngeal troponin T genes, tnt-3 and tnt-4. These mutants are expected to affect integrity of the musculature but not elongation of the pharynx, in contrast to sma-1 mutants. Importantly, the genes being tested are known to be expressed and act specifically in muscles (either pharyngeal muscle only or in both pharyngeal and nonpharyngeal muscles) and not in pharyngeal glands. The unc-89 (e1460) mutation is a hypomorphic allele in which UNC-89 product is produced but leads to defects in muscle fiber organization with little or no M-line (Waterston et al. 1980; Benian et al. 1996); this allele, unlike the null, is viable and moves normally. Mutants have normal pharynx morphology, but gland projections are frequently defective, with 47% of mutants exhibiting swelling of projections or outgrowths along their length (Figure 5F). While this phenotype is less severe than that of sma-1, it is likely to reflect the hypomorphic nature of the unc-89(e1460) allele.

Removal of a pharynx muscle-specific troponin T (tnt-4) results in gland defects like those seen in sma-1 (and other) mutants. C. elegans contains four predicted troponin T genes, tnt-1–4, two of which (tnt-3 and tnt-4) are expressed in pharyngeal muscles (Kohara 2001a,b; Amin et al. 2007). RNAi against tnt-3 had little effect (Table 1). In contrast, RNAi against tnt-4 resulted in L1 arrest, presumably due to a reduction in pharyngeal pumping and subsequent starvation. Overall pharyngeal morphology of tnt-4(RNAi) animals was unaffected, suggesting that TNT-4 is not required for pharyngeal elongation, but rather for pharyngeal function. However, gland morphology was abnormal in all tnt-4 (RNAi) animals, consistent with a role for muscle in constraining growth of gland extensions (Figure 5G). We also tested a sublethal dose of tnt-4 dsRNA. The resulting adults had normal pharyngeal morphology and size but showed frequent defects in gland morphology (Figure 5H, Table 1).

Finally, we examined a mutant (pha-2) in which only a subset of pharyngeal muscles is defective. In pha-2 mutants, the pharynx is incompletely elongated, exhibiting a shortened pharyngeal isthmus as a result of defects in the differentiation of the pm5 muscles (Avery 1993; Morck et al. 2004). Accordingly, pha-2 is expressed in a subset of pharyngeal muscle cells, including pm4 and pm5, but is absent from the pharyngeal glands (Morck et al. 2004, 2006). In pha-2 mutants, the gland projections display severe abnormalities like those seen in sma-1 mutants (Figure 6A). As with sma-1, the gland phenotype becomes more severe as pha-2 animals develop, with late larvae and adults having grossly swollen gland projections. Interestingly, the defects in the gland projections are largely confined to the posterior pharynx (Figure 6B). The anterior portion of the longest projection from g1P is normal; this projection is surrounded by the pm2 and pm3 muscles, which are unaffected in pha-2 mutants. Dramatically, the posterior portion of the g1P projection and the whole of the g1A projections are defective. These projections are surrounded by the pm4 and pm5 muscles, where pha-2 functions. The localized nature of the gland defects in pha-2 mutants, corresponding to

![Figure 6](image_url)
the region where pha-2 results in muscle defects, strongly supports the hypothesis that the gland projection defects result from defects in the immediately surrounding musculature.

Analysis of gland projections by transmission electron microscopy

Taken together, the results from the various mutants argue that the morphology of the gland projections is influenced by the integrity of the surrounding pharyngeal musculature. The implication of this model is that the muscles and glands exert balancing pressure on one another, with a decrease in the pressure exerted by muscles resulting in apparent overgrowth of the glands. To consider this possibility in more detail, we examined pharyngeal cross-sections by transmission electron microscopy (TEM) of wild-type and sma-1 (iv38) animals. Consistent with the observations with the phat-1::YFP reporter, the gland projection in sma-1 animals is considerably overgrown compared with wild type (Figure 7, A and B). We also note that although the sma-1 gland is considerably larger, in both mutant and wild type there are no significant gaps or spaces between cells. This observation is consistent with a model in which gland growth is normally physically constrained by the surrounding musculature. When the muscles are impaired or defective, gland projections exert sufficient force to expand within the space between adjacent muscles. As a corollary to this model, we predict that a decrease in the force of gland growth would result in greater musculature inhibition of gland projections.

An alternative explanation for the overgrowth of the glands is that defects in pharyngeal muscle structure results in hollow spaces between adjacent cells, and that the glands simply grow to occupy preexisting intercellular gaps. To test this possibility, we examined the structure of pharyngeal muscles when the glands had been removed, to determine whether any such spaces would occur between glands.

Gland-specific apoptosis can be induced by expressing the proapoptotic gene egl-1 under the control of the hih-6 promoter (hih-6::egl-1) (Smits et al. 2008). TEM of hih-6::egl-1 revealed that, in the absence of glands or gland projections, the pharyngeal muscles enclose any space where the glands would have been, i.e., there are no intercellular gaps into which the glands might grow. Thus, the overgrowth of glands is more likely to occur because of an imbalance in forces exerted by the glands and muscles, with the glands and muscles effectively “pushing” against one another.

Increased volume of gland projections in sma-1 mutants

The gland projections in sma-1 (and other) mutants appear to be larger than those of wild-type animals, both by live imaging with a cytoplasmic GFP and in TEM cross-sections. To estimate the volume of the gland cell bodies and of the g1P projection, we measured GFP+ areas in optical sections from wild type and sma-1 mutants. For these measurements, we examined the subset of sma-1 mutants with relatively severe g1P defects, as assessed using a fluorescence dissecting microscope. Because of significant overlap of gland cell bodies, we cannot reliably distinguish the boundaries of the cells and instead measured total cell body volume (i.e., total volume of all five gland bodies). Nonetheless, the average volume of the cell bodies (in arbitrary units) is not significantly different between sma-1 mutants and wild-type animals (44,060 ± 7730 and 40,190 ± 1170, respectively; n = 3 of each genotype, P > 0.05, Student’s t-test). In contrast, the estimated volume of the g1P projection was ~1.5-fold larger in sma-1 mutants than in wild type (10,690 ± 200 and 7120 ± 710, respectively; n = 3 of each genotype, P < 0.005, Student’s t-test), even though the length of the projection is ~70% that of wild type. The estimated volume measurement is consistent with measurements of EM photomicrographs, in which the cross-sectional area of the g1P projection is ~2.5-fold larger in sma-1 compared to WT (Figure 7 and data not shown). Thus it is not the case that the increase of gland cell projection volume resulted in a redistribution of cytoplasm or a uniform increase in gland cell volume. These results suggest that in wild-type animals, the pharyngeal muscles not only shape the gland projection but also restrict its growth.

Gland projections exhibit limited growth following laser severing

If glands are normally constrained by pharyngeal muscles, then a decrease in the pressure exerted by the glands (or an increase in the pressure exerted by the muscles) would be expected to prevent their growth. To test this prediction, we performed laser microsurgery on gland projections in larvae in which the gland projections were fully formed and examined the gland projections in subsequent development. Normally, glands continue to grow throughout the life of the animal, keeping pace with growth of the pharynx and the rest of the body. We imagined three possible outcomes following laser severing, depending on the capacity of the gland to grow and the relationship between the glands and the
muscles (Figure 8). First, growth of glands may be dependent on their attachment to the pharyngeal lumen. For example, growth of the pharynx may place tension on the gland projection to stimulate growth; loss of this attachment by severing could result in a failure of the gland projection to grow or even in a retraction of the projection during subsequent growth of the pharynx. Thus, the length of the gland projection would remain constant or would shrink following surgery, with the distance between the duct and point of surgery increasing as the pharynx grows. The second possibility is that, following severing, the gland projection might fully regenerate, as occurs in C. elegans neurons following similar laser severing (“axotomy”) (Yanik et al. 2004). Regeneration would only occur if the gland projection was able to penetrate past the pharyngeal muscles. Thus a third possibility is that, following severing, pharyngeal muscles would press in on the severed portion of the gland projection, ultimately preventing further growth of the cell into this region. However, the intact portion of the projection could continue to grow.

We found that, following laser surgery, gland projections maintained the distance between the distal end of the projection where severing occurred and the original site of the duct (n = 9). This is consistent with the third possibility described above, in which glands continue to grow, keeping pace with pharyngeal growth but do not regenerate past the point where they were severed. This result suggests that muscle and gland growth are counterbalanced: where the gland projection remains intact, the gland is able to continue growing in scale with the rest of the pharynx; where the gland projection has been eliminated as a result of severing, the pharyngeal muscles “close in” and prevent further invasion by the glands. While this result is consistent with a model in which glands and muscles exert pressure on one another, an alternate interpretation is that gland projections adhere to pharyngeal muscles along their length and that this adhesion stimulates gland growth, pulling it along as the muscle lengthen. We attempted to distinguish between these two interpretations by performing laser ablation of muscles in animals, to see whether glands grew into the area of ablated cells. While such laser treatment did appear to result in swelling of glands in the area of surgery (data not shown), the animals were badly damaged and did not survive long after surgery, preventing appropriate analysis.

Discussion

The long cellular projections of the pharyngeal glands extend to discrete points along the length of the pharynx, allowing the glands to secrete material into the pharyngeal lumen. At least one function of the glands is to secrete mucin-like PHAT proteins that line the pharyngeal lumen, and these secretions may aid in the passage of food along the pharynx (Smit et al. 2008). Complete coverage of the pharyngeal lumen by these secretions may thus be achieved in part by having multiple points of secretion along the length of the organ, at the sites of the gland ducts. Here we have investigated how the gland projections are maintained during development and growth of C. elegans. Following the establishment of these long cellular projections, their shape in subsequent growth and development is constrained by the surrounding pharyngeal muscles. Thus, the shape of the gland projections is not strictly an inherent property of the cell, but results instead from interactions between the glands and the organ environment in which they develop. Notably, in a variety of mutants with impaired pharyngeal muscles, gland projections become abnormally swollen and/or branched, apparently as a result of overgrowth that is not properly constrained by the pharyngeal muscles.

While we have tested multiple mutants affecting pharyngeal muscle integrity, the effects of these mutations on gland morphology may not occur via a single mechanism. For example, mutants in which pharyngeal elongation and hence muscle morphology is abnormal (such as sma-1 and let-502) may result in gland hypertrophy through loss of one regulatory mechanism, while mutants affecting pharyngeal muscle ultrastructure and/or function (such as tnt-4 and unc-89) may have a different effect on gland growth. Interestingly, our genetic screen identified other mutants with localized defects in the pharyngeal gland projections, but apparently normal pharynx morphology. Such mutants may specifically affect the ultrastructure of specific subsets of pharyngeal muscles, resulting in regional defects in gland projections. For example, the gland defects in iv37 mutants may result from specific defects in the muscles of the anterior bulb (pm4 muscles), rather than some defect localized to a specific region of the gland projection.
One interesting problem faced by cells with long cellular projections, like the glands, is the issue of “scaling,” i.e., maintaining an appropriate length in relation to the rest of the organism during growth. How do the gland projections scale during growth of the pharynx? One simple expectation was that gland projections are anchored by the ducts at one end and by the cell bodies at the other end. In other systems, tension-induced growth may be responsible for scaling of long projections (Bray 1984). However, gland projections continue to grow following laser surgery that separates the gland projection from the duct. This result suggests that scaling of the gland projections does not require a single “anchor” like the duct. Instead, the gland projection may adhere to the pharyngeal muscles along its length, thus providing tension in response to muscle growth. Alternatively, the glands may neither adhere to the muscles nor require tension for growth, and may simply be opportunistic in their growth, held back largely by the force of the surrounding muscles. While we cannot currently distinguish between these models, the behavior of the glands in sma-1 (and other) mutants suggests that gland projections are not subject to pulling forces, but rather push outwards, with the muscles normally functioning to constrain the projection and prevent overgrowth.

Establishment or maintenance of cell morphology by mechanical constraint from surrounding tissue is not unique to the pharyngeal glands. In many situations, cellular morphology is critically influenced by interactions with other cells and/or components of the ECM. For example, branching of mammalian salivary glands (to which the pharyngeal glands are likely related; Smit et al. 2008) depends on forces generated by surrounding tissue. Salivary gland branching is initiated by budding of the early gland, which begins as a sac-like ball of cells. This budding results not from intrinsic forces in the gland epithelia, but rather due to localized pressure from surrounding mesenchyme and ECM (Hsu and Yamada 2010). Regulation of mammary gland branching and growth also bears interesting similarities to the regulation of pharyngeal gland morphology. In mammary glands, new glandular branches are formed by growth and proliferation of luminal epithelial cells, but this branching and growth is counterbalanced by the surrounding by myoepithelial cells. Growth of new branches occurs at sites where luminal epithelia is not surrounded by myoepithelial cells. Growth is eventually blocked by the myoepithelial cells reestablishing coverage of the nascent luminal branch, suggesting some form of growth constraint, much as growth of the pharyngeal glands appears to be constrained by surrounding myoepithelial cells (Ewald et al. 2008). It is interesting to note that some of the pharyngeal gland defects present as branching of the gland projection; one can imagine the development of more elaborate branched structures by alterations in local mechanical constraint. Mechanical forces are also thought to regulate growth in the case of “cell competition” in the Drosophila wing disc, in which slower growing clones of cells are outcompeted by faster growing neighbors, ultimately leading to apoptosis of the smaller clones, thereby ensuring uniform growth of the wing disc (Shraiman 2005). More generally, the shape of individual cells in an epithelial layer is a consequence of their interactions with neighbors and the resulting forces exerted by cells on one another (Gibson and Gibson 2009). A key difference in the case of the pharyngeal glands is that neither the glands nor the surrounding musculature are proliferative, and any growth of the tissue is strictly due to an increase in cell size rather than cell number. Thus, in this case, mechanical constraint by pharyngeal muscles acts solely to regulate expansion of the gland cells. How such mechanotransduction works at a molecular level in the glands is unclear, just as it is unclear whether or how glands are connected to neighboring muscles. Study of additional mutants from our screen, together with testing of candidate growth control genes, should provide insight into the underlying mechanism(s) that act to control pharyngeal gland growth and maintain the morphology of the long cellular projections.

Interestingly, the pharyngeal glands (also called esophageal glands) of other nematodes generally resemble those of C. elegans, with cell bodies in the posterior of the pharynx and long cellular extensions that reach to discrete points along the length of the pharynx (Chitwood and Chitwood 1950). In most cases, the dorsal gland (comparable to g1P) extends to near the end of the pharynx, while the subventral glands (comparable to g1A) extend roughly halfway along the length of the pharynx, though there are some exceptions with respect to both cell body position and length of projections. Notably, there is some diversity in the relative size of the gland projections, with some glands having relatively thin projections like those in C. elegans and others having projections that are roughly as wide as the cell body itself. Our results suggest that one way in which gland morphology can be affected through evolution is by alterations in the relative force exerted by the pharyngeal muscles on the gland projections. Thus, the variations in gland morphology among different nematode species may reflect differences in muscle structure or architecture as well as or instead of differences in the glands themselves.

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Cell Architecture: Surrounding Muscle Cells Shape Gland Cell Morphology in the *Caenorhabditis elegans* Pharynx

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Figure S1  Suppression of recombination along the right arm of LG II in strain GD139 ivls12 [phat-1::YFP  elt-2::GFP rol-6]. BSA-SNP analysis shows no detectable recombination at three SNPs along the right arm of LG II. Progeny from +/ivls12 mothers collected in bulk are: ‘n’ = non-Roller, non-transgenic (+/+) and ‘R’ = Roller, transgenic (+/ivls12 and ivls12 homozygotes). At each SNP, non-Rollers show only the Hawaiian (CB4856) form of the SNP. A fourth SNP (Y6D1A at +4 m.u.) also shows only the Hawaiian form in non-transgenics (not shown).
Figure S2  Diagram of *hlh-6* gene, indicating the previously described *tm299* deletion allele and the newly isolated missense mutation, *iv52*. 

**Figure S3** Additional examples of gland defects in *sma-1*(iv38) animals. (A) Branching of glP visible in anterior pharynx, as well as swelling and blebbing of glA projection. (B) Swelling of both glP and glA projections. (C) Extensive branching and swelling of all projections. (D) Branching and swelling of glA projections; anterior glP projection is out-of-plane.