The HLH-6 Transcription Factor Regulates C. elegans Pharyngeal Gland Development and Function

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Abstract

The Caenorhabditis elegans pharynx (or foregut) functions as a pump that draws in food (bacteria) from the environment. While the "organ identity factor" PHA-4 is critical for formation of the C. elegans pharynx as a whole, little is known about the specification of distinct cell types within the pharynx. Here, we use a combination of bioinformatics, molecular biology, and genetics to identify a helix-loop-helix transcription factor (HLH-6) as a critical regulator of pharyngeal gland development. HLH-6 is required for expression of a number of gland-specific genes, acting through a discrete cis-regulatory element named PGM1 (Pharyngeal Gland Motif 1). hlh-6 mutants exhibit a frequent loss of a subset of glands, while the remaining glands have impaired activity, indicating a role for hlh-6 in both gland development and function. Interestingly, hlh-6 mutants are also feeding defective, ascribing a biological function for the glands. Pharyngeal pumping in hlh-6 mutants is normal, but hlh-6 mutants lack expression of a class of mucin-related proteins that are normally secreted by pharyngeal glands and line the pharyngeal cuticle. An interesting possibility is that one function of pharyngeal glands is to secrete a pharyngeal lining that ensures efficient transport of food along the pharyngeal lumen.

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Introduction

An important question in the study of organ development is how different cells are instructed to become part of a common structure and yet are also specified to have a distinct identity within that structure. This problem is well-illustrated in the pharynx of the nematode C. elegans. The pharynx is a small (80 cells) neuromuscular organ that pumps food (bacteria) in from the environment and initiates digestion (Figure 1A). It contains five different cell types (muscles, epithelia, neurons, marginal cells and glands) that are not restricted by their lineal origins. Recruitment of cells to the pharynx involves the "organ identity factor" PHA-4 (the C. elegans FoxA ortholog), which is required for cells to adopt a pharyngeal identity [1–3]. Available data supports a model in which PHA-4 directly regulates most or all genes that are expressed in the pharynx [4]. However, PHA-4 alone cannot be responsible for all aspects of organ development and must function with other factors to control the various sub-programs of pharyngeal organogenesis, such as specification of the distinct cell types. Aside from the involvement of PHA-4, little is known about the specification and development of any of the distinct pharyngeal cell types, though regulators of pharyngeal muscle development have been identified [5–8].

In this work, we chose to examine development of the pharyngeal glands, one of five cell types in the pharynx [9]. We chose this cell type for three reasons: first, nothing is known about regulation of gland gene expression nor about the specification of the glands (aside from the general involvement of PHA-4). Second, the function of the glands in C. elegans is poorly understood, although proposed roles include initiation of digestion, molting of the pharyngeal cuticle and resistance to pathogenic bacteria [9–12] and the digestive tract glands of parasitic nematodes are known to play crucial roles in host-parasite interactions (reviewed in [13]). Third, several genes with gland-specific expression have been identified, based on a combination of microarray and in situ hybridization data [14–16].

The pharyngeal glands are five cells in the posterior bulb of the pharynx with cellular projections that open into the pharyngeal lumen at discrete points along the length of the pharynx [9] (Figure 1A). The glands are further divided into two sub-groups, g1 and g2, based on their appearance in electron micrographs, though the significance of these sub-types is not known.

Given recent advances in computational biology and genomics, one powerful approach to exploring the issue of cell type specification is to identify a group of co-expressed (and presumably co-regulated) genes and from this group identify shared regulatory elements. These elements can then be used as tools for determining and characterizing the relevant trans-acting factor(s).

Here we identify both a cis-acting regulatory element (PGM1) and the corresponding trans-acting factor (HLH-6) that are together necessary and sufficient for pharyngeal gland-specific gene expression. We further show that elimination of HLH-6 results in the loss of a subset of pharyngeal glands, disrupted function of the remaining glands and defects in feeding that lead to partial starvation. Based on our analysis of hlh-6 mutants, we propose that one function of pharyngeal glands is to assist in the...
transport of food through the pharyngeal lumen. The glands secrete mucin-like proteins that line the pharyngeal lumen, which possibly lubricate the tract to ensure efficient passage of bacteria. These results not only demonstrate an important function of the pharyngeal glands, but also illustrate evolutionary conservation of the glands in ensuring efficient transport of food through the front end of the digestive tract.

Results

Identification of a Candidate cis-Acting Element in Pharyngeal Gland-Expressed Genes

To investigate regulation of pharyngeal gland development, we first searched for cis-regulatory elements in the promoters of gland-expressed genes. Co-expressed genes often share common cis-acting regulatory elements, and identification of elements required for gland expression could lead to the identification of the corresponding trans-acting factors. We began with a list of fourteen confirmed and probable gland-specific genes, based on previous work [14] (Table 1). Twelve of these fourteen genes are predicted to encode proteins whose only recognizable features are a signal peptide and multiple copies of the ShK motif, a cysteine-rich sequence first described in metridin toxin from the sea anemone [18]. Proteins containing only ShK motifs appear to be gland-specific, while proteins containing ShK motifs in the presence of other recognizable domains (such as aminotransferases in FYR-2) are not gland-specific [14]. We modified the original list of fourteen genes by excluding one gene (C14C6.5) that contains motifs in addition to ShK and also lacks supporting expression data. We also added one gene (T10B10.6) that encodes an ShK protein and is expressed solely in pharyngeal glands according to available in situ hybridization data [15,16] (Table 1). We will refer to ShK-encoding genes with confirmed gland-specific expression as phat genes, for pharyngeal gland toxin-related.

To verify the quality of the list of fourteen genes, we constructed GFP or YFP reporters for four of the genes (two of which were previously reported; [14]) and found that all four were expressed specifically in pharyngeal glands (Figure 1 and Table 1). Of the four genes, three (B0507.1, phat-1, and phat-3) were expressed in all five glands (Figure 1C,E,I), while phat-5 was only expressed in the two anterior-most glands, the left and right g1A cells (g1AR and g1AL; Figure 1G). Previous reports have suggested that the g1AR and g1P cells are fused [9], yet we see no expression of phat-5-expressed YFP from g1AR to g1P, suggesting that YFP is restricted from diffusing between these cells or that the two cells are not fused.

By searching the upstream 500 bp (relative to the ATG) of the fourteen gland genes using the Improbizer program [14] for shared sequence motifs, we identified one candidate gland-specific cis-acting element, which we named PG1M (for Pharyngeal Gland Motif 1; Figure 1B). This size of promoter was justified because many of the gland genes have neighboring genes within 500 bp upstream, consistent with the observation that C. elegans promoters are generally small [19,20]. PG1M was the only motif identified by Improbizer that had a position weight matrix score higher than any of the motifs generated in control runs (See Materials and Methods), suggesting that it might be a functional regulatory element. In addition, PG1M appeared to be enriched in the promoters of gland-expressed genes, as these promoters were four times more likely to contain significant occurrences of PG1M (12/14 = 86%) than a control set of promoters from pharyngeal (but not gland-specific) genes (20/96 = 21%) (Table S1).

PG1M Is Necessary for Expression in Pharyngeal Glands

Analysis of PG1M in the context of pharyngeal gland-specific promoters demonstrated that PG1M was required for expression. Site-directed mutations in PG1M sequences eliminated expression of phat-1 and phat-3 reporters, and greatly reduced expression of B0507.1 and phat-5 reporters (Figure 1C,J). The promoter of phat-5 has one other potential occurrence of PG1M that could account for its residual activity (at −118 bp; Figure S1). The B0507.1 promoter has no other apparent PG1M sequences, suggesting that the remainder of its expression is dependent on an as yet unidentified cis-regulatory motif. Together, these results suggested that PG1M is necessary for the high level expression of a subset of genes in pharyngeal glands. We queried other gland-expressed genes to determine whether they also required PG1M for expression. We analyzed the expression of two genes that were not part of our original data set, but that were reported to be expressed in glands: pqm-8 and lys-8 [21,22]. The pqm-8 reporter was expressed exclusively in pharyngeal glands whereas the lys-8 reporter was expressed in pharyngeal glands and the intestine, as reported (Figure 1K,M). Mutation of a PG1M sequence in the pqm-8 promoter completely abolished expression (Figure 1K–L). The lys-8 promoter had three potential PG1M sites at −180, −452 and −581 bp relative to the ATG (Figure S1). Two of these sequences (at −180 and −452) are not required for expression in pharyngeal glands (data not shown), while mutation of the third site (−581 bp) resulted in a loss of expression (Figure 1M–N).

Not all pharyngeal gland genes contain identifiable PG1M sequences. In a search for additional pharyngeal gland genes based on in situ hybridization data [15,16], we identified Y8A9A.2 as a probable gland-expressed gene that does not contain a PG1M sequence in its promoter. Expression in pharyngeal glands was verified with a transcriptional Y8A9A.2::GFP reporter containing 2000 bp of upstream sequence (relative to the ATG) (Figure S2). This reporter does not contain any sequence that resembles a PG1M site, suggesting that its expression is PG1M independent or that there is an occurrence of PG1M that is too divergent to be recognizable. Based on further analysis (below), Y8A9A.2::GFP expression is likely to be PG1M-independent.

Author Summary

To make an organ, cells must be instructed to be part of a common structure yet must also be assigned specific roles or identities within that structure. For example, the stomach contains a variety of different kinds of cells, including muscles, nerves, and glands. This same complexity is seen even in relatively simple organs, like the pharynx (foregut) of the nematode C. elegans. The pharynx is a neuromuscular organ that pumps in food (bacteria) from the environment. This organ is relatively simple (containing only 80 cells) yet contains five distinct kinds of cells. How these different cells are specified is unclear but likely involves combinations of developmental regulators known as transcription factors. Here, we examine one cell type, the pharyngeal glands, and identify a key regulator of their development, the transcription factor HLH-6. Interestingly, HLH-6 is closely related to a mammalian transcription factor, Sgn1, which is involved in development of mammalian salivary glands, suggesting that C. elegans pharyngeal glands are evolutionarily related to mammalian salivary glands. A further connection is that the pharyngeal glands of C. elegans appear to be required for efficient feeding, possibly by secreting mucin-like proteins that ensure the smooth passage of food along the digestive tract.
Closer examination of PGM1 revealed that it contains an E-box (CAnnTG), the consensus binding site for basic helix-loop-helix (bHLH) transcription factors [23]. Mutations that specifically disrupt the E-box sequence eliminate PGM1 activity (Figure 1). However, the E-box is not sufficient for PGM1 activity: mutation of sequence flanking the E-box in the \textit{phat-1} reporter resulted in a significant loss of expression (data not shown), suggesting that an extended sequence is required for activity. Alignment of the functionally defined PGM1 sequences revealed an extended consensus of CAnvTGhdYMAAY (where V = A, C or G, H = A, C or T, D = A, G or T, M = A or C, and Y = C or T; Figure 2A). This extended consensus is present in all 12 of the 14 genes in our initial list that contained PGM1 (Figure 2A). The functionally defined consensus may represent either an extended binding preference for the relevant \textit{trans}-acting factor or the juxtaposition of binding sites for two (or more) distinct factors.

Figure 1. PGM1 is required for expression of some pharyngeal gland genes. (A) Diagram of pharynx, highlighting the pharyngeal glands, modified from [9]. (B) WebLogo [80] of computationally identified PGM1. (C–N) Fluorescence micrographs of gland-expressed GFP or YFP reporters with wild-type promoter sequence (left column) or promoter sequence in which PGM1 is mutated (right column). In wild-type sequences (left) the E-box is underlined and in mutant sequences (right) the mutation is underlined. Anterior is at left and the pharynx is outlined. Scale bars represent 10 \(\mu\)m.

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The Extended PGM1 Is Necessary and Sufficient for Expression in Pharyngeal Glands

Given that PGM1 is necessary for expression of many genes in pharyngeal glands, we next asked whether PGM1 was also sufficient for gland expression. Indeed, three tandem copies of the PGM1 sequence from phat-3 placed upstream of a “promoter-less” reporter (to make the “3×PGM1” construct) was sufficient to activate pharyngeal gland expression in 78% (31/40) of transgenic animals (Figure 2B–C). A fraction of these animals (7/31) also showed weak expression in the I3 pharyngeal neuron, a sister cell of the g1P gland [24]. These results indicate that PGM1 is a pharyngeal gland-specific enhancer element, and further suggests that PGM1 is a binding site for one or more transcription factors that function in pharyngeal glands.

Given the apparent extended consensus sequence for PGM1, we performed additional enhancer tests to determine what portions of PGM1 were required for its activity. We first tested a version of the 3×PGM1 plasmid in which all three copies of the E-box were changed from CAnnTG to AAnnTG. This construct showed no expression in transgenics, indicating that the E-box was required for PGM1 activity (Figure 2D).

We next tested an enhancer in which sequence flanking the E-box were altered (3×PGM1ΔE) and found that this sequence was also required for PGM1 activity (Figure 2E), demonstrating that the E-box is necessary but not sufficient for PGM1 activity.

HLH-6 Functions through PGM1

Since PGM1 activity is dependent on an E-box sequence, our search for the relevant trans-acting factor(s) began with bHLH proteins. bHLH proteins typically bind to DNA as heterodimers, composed of a ubiquitous “Class I” subunit and a tissue-restricted “Class II” partner (reviewed in [25]). In C. elegans, the sole Class I bHLH is encoded by hlh-2 [26], which is expressed in many cells throughout development, including the glands. To identify the relevant Class II bHLH, we examined data from microarray experiments that identified candidate pharynx-expressed genes [4,27], including three Class II bHLHs: hlh-3, hlh-6 and hlh-8. Both hlh-3 and hlh-8 are expressed exclusively in non-pharyngeal tissue (in neurons and muscles, respectively; [28]) suggesting that they are false positives with respect to the microarray data and are thus unlikely to function through PGM1. At the time of our analysis, hlh-6 was uncharacterized and was therefore a candidate PGM1 trans-acting factor.

To examine the involvement of hlh-6 in PGM1 activity, we first determined the expression of a transcriptional reporter that included almost all intergenic sequence (1175 bp of 1190 bp) between hlh-6 and its nearest upstream neighbour, T15H9.2. We found that hlh-6::GFP was expressed strongly and specifically in the pharyngeal glands (98% of transgenics), with occasional (12%), weak expression in the pharyngeal neuron I3 (Figure 3). Expression was first detectable shortly after the terminal cell division that gives rise to pharyngeal glands (bean stage embryos) and persisted throughout the life cycle in all five pharyngeal glands. Because PGM1 and hlh-6 both appear to be active in pharyngeal glands and because PGM1 contains a bHLH binding site, we hypothesized that HLH-6 is the cognate trans-acting factor for PGM1.

We determined that HLH-6 is required for PGM1 activity by demonstrating that PGM1-dependent reporters were not expressed in hlh-6(tm299) mutants. The deletion mutant hlh-6(tm299) (generously provided by S. Mitani; [29]) is a probable null, as it removes 595 bp from hlh-6, including all but one nucleotide from the second intron, resulting in a frameshift (Figure 3A). The mutation is homozygous viable (see Materials and Methods), which allowed us to examine gland reporter expression in these mutants. We found that expression of 6/6 gland reporters (phat-1, phat-3, phat-5, B0507.1, pnm-8 and lys-8) was significantly reduced in hlh-6 animals (Figure 4A,C; Figure S2). For example, only 26% of hlh-6 mutants had visible phat-1::GFP expression (n = 65), and this expression was significantly weaker than the expression seen in 100% of wild type animals. Four of the other gland reporters showed a similar loss of expression in hlh-6 mutants. Expression of
the B0507.1 reporter was less affected than the others, consistent with it being only partially PGM1 dependent. Likewise, expression remained unaffected in hlh-6 mutants (Figure S2). There is thus a perfect correlation between PGM1-dependent gene expression and hlh-6-dependent gene expression, implying that HLH-6 is acting directly on the reporters rather than earlier in the pathway of gland specification.

To confirm that loss of reporter expression was due to the hlh-6 mutation, we performed transgenic rescue with either genomic or a "minigene". The genomic fragment contains 1860 bp of the upstream promoter (including 840 bp of the upstream neighbour, T15H9.2) and 60 bp downstream of the predicted stop codon. The minigene construct consists of 568 bp of the genomic region containing the hlh-6 gene, including 541 bp of the 5'UTR and 26 bp of the first exon. The "minigene" reporter is expressed only in hlh-6 mutants (Figure 3). The 568 bp promoter fragment is only active in pharyngeal glands. Both genomic and minigene versions of the hlh-6::YFP reporter were either missing or failed to express all gland reporters (Figure 4B). This finding was verified with a nuclear-localized fluorescent reporter (data not shown). Based on the position and morphology of expressing cells, it appeared that the three g1 glands (g1AR, g1AL and g1P) were present, while the two g2 cells were either missing or failed to express all gland reporters (hlh-6::YFP, phat-1::YFP, B0507.1::GFP, et al.).

The apparent absence of g2 glands in hlh-6 mutants could be explained by three possibilities: first, the g2 glands may undergo apoptosis; second, the cells may be mis-specified and adopt an alternate fate; third, the cells may persist as undifferentiated cells. The sister cells of the g2 glands undergo apoptosis in normal development [24] and so we tested whether blocking apoptosis would restore g2 glands. Strong loss-of-function mutations in col-3 result in the survival of all cells that normally undergo programmed cell death [31,32]. However, only 9% of hlh-6; col-3 double mutants (n = 32) expressed the hlh-6::YFP reporter in g2 cells, comparable to the expression in hlh-6 mutants (Figure 4C). This finding was verified with a nuclear-localized fluorescent reporter (data not shown). Based on the position and morphology of expressing cells, it appeared that the three g1 glands (g1AR, g1AL and g1P) were present, while the two g2 cells were either missing or failed to express all gland reporters (hlh-6::YFP, phat-1::YFP, B0507.1::GFP, et al.).

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To address the possibility that g2 glands adopt an alternate cell fate, we performed nuclear counts in the back half of the posterior pharyngeal bulb where the g2 cells are normally located using a pha-4 reporter, which is expressed in all pharyngeal nuclei except...
for some pharyngeal neurons [3]. There are 11 pharyngeal cells in this region (four muscles, three marginal cells, three glands and one neuron), 10–11 of which express pha-4 post-embryonically (expression in the pharyngeal neuron in the posterior bulb is variable). We expected that hlh-6 mutants would either have a wild type number of PHA-4-expressing cells or an average loss of $1.6$ such cells (because $80\%$ of hlh-6 mutants do not have visible g2 cells). There was a significant decrease in pha-4::GFP::HIS2B expressing cells between wild type and hlh-6 mutants (9.1 vs. 7.8, respectively, $p<0.05$), suggesting that either the presumptive g2 cells do not express pha-4::GFP::HIS2B or the cells are not present.

Consistent with these cells not having a pharyngeal identity, we did not observe an increase in the numbers of other pharyngeal cell types, demonstrating that the presumptive g2 cells have not adopted an alternate pharyngeal identity (Figure S3). In the course of these nuclear counts, we also observed that the numbers of other types of pharyngeal nuclei were not affected in hlh-6 mutants. In particular, pm6 cells, which are lineally-related to the g2 glands, were present and expressed the correct markers (data not shown). This suggests that the hlh-6 mutation specifically affects glands and does not act in the differentiation of other pharyngeal cell types, as expected given the expression pattern of hlh-6.

The failure of the presumptive g2 cells to express any tested pharyngeal reporters implies that these cells were not present in hlh-6 mutants. To explore this possibility, we followed the lineages that give rise to g2 in hlh-6 mutant animals. In eight cases (73%), the immediate precursor to the g2 cell (MSnapapa) failed to undergo its terminal division, but remained in its usual position within the embryo (Figure 5). In one case, the grandmother of g2 failed to divide. Such a lineage defect would prevent formation of one of the pm6 muscles, though we do not see a loss of pm6 cells in hlh-6 mutants. In the remaining two cases (18%), the g2 precursor underwent its normal division. Thus, in 82% of cases, the g2 cell failed to be generated, consistent with our observation that 84% of hlh-6 mutants do not express hlh-6 in g2 cells. Interestingly, PHA-4 expression is lost in the arrested g2 precursors, based on our counts of pha-4::GFP::HIS2B nuclei, yet PHA-4 must be normally expressed earlier in this lineage (i.e., in the g2 grandmother MSnapap), as no other pharyngeal cells (e.g., pm6 cells, which are cousins of the g2s) were missing. Formally, this result indicates that hlh-6 is required for maintenance of pha-4 expression in g2 cells, though the nature of this regulation is unclear.

hlh-6 Mutants Are Feeding Defective

In addition to a loss of gland gene expression and defects in gland development, hlh-6 animals display a variety of characteristics that indicate a starvation phenotype: partially penetrant larval arrest, slow growth, smaller body size and decreased brood size among those surviving to adulthood. On average, 32%
HLH-6 Regulates Pharyngeal Gland Development

The g2 glands are not generated in hlh-6 mutants. The lineages of the g2 glands in wild-type and hlh-6 mutants. MSn is used because both the M5a and M5p cell give rise to a g2 cell. If n = a, the g2L cell is made (as well as pm6VL and vpi2DL) and if n = p, the g2R cell is made (and pm6VR and vpi2DR). The sister cell of g2 cell undergoes apoptosis (X) in wild-type animals.

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Genetic Ablation of Glands Phenotypes hlh-6

Because some gland genes are expressed independently of hlh-6, hlh-6 mutants might be only partially impaired with respect to gland activity. To examine the effect of complete loss of pharyngeal glands, we genetically ablated the glands using an hlh-6::egl-1 transgene, which activates expression of the proapoptotic gene egl-1 in pharyngeal glands [43]. Induction of egl-1 is sufficient to induce apoptosis in other cells, such as pharyngeal neurons [44]. We assayed the presence or absence of glands using an integrated phtat-1::mTomato reporter and followed the presence of the hlh-6::egl-1 transgene with an intestine-specific mTomato marker [45]. Transgenic animals that lacked pharyngeal glands were viable but showed delayed growth and development, with 39% (n = 23) larval arrest, comparable to hlh-6 mutants (data not shown). These results suggest that the pharyngeal glands of C. elegans are primarily involved in efficient feeding and that in the absence of hlh-6, glands are entirely nonfunctional with respect to growth and fecundity.

Pharyngeal Glands Secrete Mucin-Related Proteins that Line the Pharyngeal Cuticle

By analogy to foregut glands in other organisms, we postulated that pharyngeal glands could function in feeding by one of three ways: first, glands may secrete digestive enzymes required for efficient feeding; second, glands may produce secretions that coat food to ensure its passage along the lumen; third, glands may produce secretions that line the lumen and prevent adhesion of food. The first possibility, that the glands produce digestive enzymes, was suggested in part by the fact that the gland-expressed gene lys-8 is predicted to encode a hydrolase [22]. However, the ability of HB101 bacteria to rescue the starvation phenotype of hlh-6 animals suggests that glands are not required for digestion of food.

The other two possibilities, in which the glands lubricate the pharyngeal lumen, were suggested by the ability of a less sticky food source (HB101) to rescue hlh-6 starvation. As noted, the majority of known gland-expressed genes are predicted to encode secreted proteins that contain multiple copies of the ShK domain.

Mutations that affect feeding generally do so by affecting the rhythmic contractions of pharyngeal muscle, resulting in decreased or arrhythmic pharyngeal pumping and therefore “inefficient” feeding. Such mutations affect either pharyngeal muscle morphology and/or function (e.g., pha-2, eat-2; [33,40]) or the neurons that innervate the muscles (e.g., eat-4 and cxh-28; [41,42]). hlh-6 differs from other genes involved in feeding as hlh-6 functions in pharyngeal glands. Consistent with hlh-6 not acting in either pharyngeal muscle or neurons, we find that hlh-6 mutants had normal pharyngeal pumping with respect to both rate and rhythm of the muscle. Control animals (rol-6 unc-4) had an average of 169 ± 29 pumps per minute (n = 20) and hlh-6 mutants (rol-6 hlh-6 unc-4) had an average of 156 ± 42 pumps per minute (n = 19). Likewise, peristaltic contractions of the pharyngeal isthmus were also normal, with both control and mutant strains showing an average of one isthmus contraction per four pharyngeal pumps. These findings indicate that hlh-6 mutants are defective in some other aspect of food transport for which the glands are required.

hlh-6 mutants grown on HB101 were not starved, exhibiting wild type growth rates and a suppression of larval arrest (Figure 6D and data not shown). Two factors that affect the ability of different food sources to rescue eat mutants are bacterial cell size and the relative “stickiness” of the cells [39]. HB101 and OP50 cells are the same size (2.8 ± 0.7 μm and 3.0 ± 0.4 μm, respectively), but OP50 are more adhesive compared to HB101 [39].

Figure 5. The g2 glands are not generated in hlh-6 mutants. The lineages of the g2 glands in wild-type and hlh-6 mutants. MSn is used because both the M5a and M5p cell give rise to a g2 cell. If n = a, the g2L cell is made (as well as pm6VL and vpi2DL) and if n = p, the g2R cell is made (and pm6VR and vpi2DR). The sister cell of g2 cell undergoes apoptosis (X) in wild-type animals.
Interestingly, this family of proteins is similar to a group of secreted mucins from the parasitic nematode *Toxocara canis* [46–48]. The *T. canis* mucins are defined by multiple copies of the ShK domain (sometimes referred to as the SXC domain), a signal sequence and stretches of Ser/Thr-rich (probable sites of glycosylation). We find that, like the *T. canis* proteins, the PHAT proteins contain stretches of Ser/Thr-rich sequence between their ShK domains (Figure S4) and many of these Ser/Thr sites are predicted to be sites for O-linked glycosylation [49]. The PHAT proteins may therefore function as mucin-like proteins.

We found that a representative PHAT protein, PHAT-5, lines the pharyngeal lumen, consistent with the protein having a mucin-like function. We examined the subcellular location of PHAT-5 using a phat-5::mCherry fusion expressed under the control of the *hlh-6* promoter. The PHAT-5::mCherry fusion protein was visible in discrete puncta throughout the cell bodies of the glands, as well as along their extensions (Figure 8A). In live animals, these

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**Figure 6. Phenotypic analysis of *hlh-6* mutants.** (A–B) The stuffed pharynx phenotype of *hlh-6* mutants grown on OP50-GFP bacteria. (A) NDIC image, (B) merged NDIC and fluorescence image. Anterior is at left and scale bars represent 10 μm. (C–E) Assays for growth defects in wild-type, *hlh-6* mutants and *hlh-6* mutants rescued by either the *hlh-6* genomic fragment, the *hlh-6* minigene or by using the HB101 strain of *E. coli*. (C) Graph of body length versus time, (D) time to reach adulthood and (E) brood sizes. For the *hlh-6* mutants the L1 arrested animals are omitted. Error bars represent one standard deviation.
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**Figure 7. Staining of intestinal fat stores of *hlh-6* mutants.** (A–D) Fluorescence images of animals grown in the presence of Nile Red. (A) wild type, (B) daf-16(RNAi), (C) *hlh-6* and (D) *hlh-6*; daf-16(RNAi). Anterior is at left and the pharynx is outlined. Scale bars represent 10 μm.
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**Figure 8. Localization of PHAT-5.** (A) Representative PHAT protein, PHAT-5, lines the pharyngeal lumen, consistent with the protein having a mucin-like function. We examined the subcellular location of PHAT-5 using a phat-5::mCherry fusion expressed under the control of the *hlh-6* promoter. The PHAT-5::mCherry fusion protein was visible in discrete puncta throughout the cell bodies of the glands, as well as along their extensions (Figure 8A). In live animals, these
puncta could be seen to traffic along the extensions, suggesting that the protein had been packaged into secretory vesicles. More importantly, the PHAT-5::MCHERRY fusion protein was found along the lumen of the pharynx, indicating that the protein had been secreted from the glands (Figure 8A–C). The fusion protein had a discrete anterior boundary, extending as far as the cheilostom groove in the buccal cavity (Figure 8B,C), the boundary between the epidermal cuticle and the pharyngeal cuticle [50], suggesting that PHAT-5 is specifically associated with pharyngeal cuticle. In addition, PHAT-5 fusion protein remained associated with shed pharyngeal cuticle, arguing that the protein forms part of the lining of the pharyngeal lumen (Figure S6). No protein was seen to co-localize with bacteria in the pharynx lumen, suggesting that PHAT-5 does not coat food particles.

To investigate whether the glands of \textit{hlh-6} mutants are functionally impaired, we examined whether PHAT-5::MCHERRY could be secreted by the glands of \textit{hlh-6} mutants. \textit{phat} gene expression is absent from \textit{hlh-6} animals, so we expressed the PHAT-5 fusion under the control of the \textit{hlh-6} promoter, which remains active in \textit{hlh-6} mutants. The \textit{hlh-6::phat-5::mCherry} construct was expressed in pharyngeal glands, but no protein was seen at the pharyngeal lumen, likely reflecting a functional

Figure 8. PHAT-5::MCHERRY localization in wild type and \textit{hlh-6} mutants. Fluorescence and NDIC images of (A–C) wild-type and (D–F) \textit{hlh-6} animals expressing the \textit{hlh-6::phat-5::mCherry} translational fusion construct. (B) and (C) are close-ups of animal shown in (A). (E) (F) are close-ups of (D). Fluorescence and NDIC images of (G) wild-type and (K) \textit{hlh-6} animals expressing the \textit{myo-2::phat-5::mCherry} translational fusion construct with corresponding close-ups in (H–I) and (L–M). Arrowheads indicate the pharyngeal lumen, arrows mark the processes of the g1 glands and triangles mark the boundary of the pharyngeal cuticle. PHAT-5::MCHERRY is not found in the intestinal lumen of wild type animals (J) but is present in the intestinal lumen of \textit{hlh-6} mutants (N), indicated by carats. Anterior is at left and the pharynx is outlined. Scale bars represent 10 \textmu m.

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defect in the hkh-6 glands (Figure 8D–F). No rescue of the hkh-6 phenotype by hkh-6;phat-5::mCherry was observed. Punctate signal was observed in the gland ducts and in live animals these puncta appeared to migrate along the ducts as in wild type, suggesting that vesicles were still present and capable of being transported within the glands. The hkh-6 mutants are therefore defective either in secretion of the PHAT-5 protein or in retention of this protein at the pharyngeal lumen. To distinguish between these possibilities we expressed PHAT-5::MCHERRY in pharyngeal muscles (using the myo-2 promoter; [51]) to investigate the localization of PHAT-5 independent of gland function. In wild type animals, pharyngeal muscle could secrete PHAT-5::MCHERRY. Signal was seen lining the pharyngeal cuticle in addition to puncta throughout the muscles (Figure 8G–I). In hkh-6 mutants, some signal was visible on the luminal surface, but we also observed significant signal in the intestinal lumen (though not associated with cell surfaces), which was not observed in wild type animals (Figure 8J–M). This result suggests that while PHAT-5::MCHERRY can associate with the pharyngeal cuticle in hkh-6 animals, this association is less stable, resulting in the movement of the fusion protein along the digestive tract. This observation is consistent with the hypothesis that the pharyngeal lining is defective in hkh-6 mutants, likely due to the absence of other gland-secreted proteins, including the other PHAT proteins. No rescue of the hkh-6 phenotype by myo-2::phat-5::mCherry was observed.

Discussion

Based on our findings, we propose that HLH-6 regulates a battery of pharyngeal gland-expressed genes in C. elegans and is required for both differentiation and function of the glands. While some glands are present in hkh-6 mutants, they are non-functional, as the removal of pharyngeal glands phenocopies the loss of hkh-6. The pharyngeal glands are essential for efficient feeding and appear to play a role in facilitating the transport of bacteria along the pharyngeal lumen, though they are not involved in regulation of pharyngeal pumping. These findings illustrate a previously unknown role for the pharyngeal glands in efficient feeding and demonstrate that aspects of both foregut gland development and function are evolutionarily conserved.

PGM1 and HLH-6 Are Required for Gene Expression in Pharyngeal Glands

We identified both a cis-regulatory element and trans-acting factor that are required for expression in pharyngeal glands, though it is presently not known whether the two components interact directly. There are two lines of evidence that support the hypothesis that HLH-6 interacts directly with PGM1. First, the PGM1 motif contains a functional E-box (Figure 1), and bHLH proteins (like HLH-6) bind to E-boxes. Second, PGM1 activity requires HLH-6 (Figure 4). A formal possibility is that HLH-6 acts upon a second bHLH that in turn binds to PGM1, as seen with the cascades of neurogenic and myogenic bHLH factors [52,53]. However, no other C. elegans class II bHLH is known to be expressed in pharyngeal glands, though some hkh-6 mutants have multiple defects in gland differentiation, yet still produce g1 (and occasionally g2) gland-like cells and express at up to but not beyond three bases outside of the E-box [54,55], while the YMAAY sequence extends beyond this limit. Second, ectopic expression of HLH-6 (≥HLH-2) is not sufficient to activate ectopic expression of a gland-expressed marker (data not shown), suggesting that an additional factor is required to induce target gene expression. Third, we tested whether HLH-6 (≥HLH-2) could bind to PGM1 in vitro using electrophoretic mobility shift assays (EMSA), but were unable to detect an interaction (Text S1 and Figure S5), though we are able to detect interactions between other bHLH dimers and E-box-containing sequences. Thus, the YMAAY sequence likely represents a binding site for an additional factor. This factor may be limiting with respect to activation of gland genes in viv and binding to PGM1 in vitro. Precedence for such a model comes from studies of mammalian Mash1, which must form a complex with the POU domain transcription factor Brn2 in order to bind to specific target sequences [56]. Similarly, the pancreatic determinant PTF1 is a complex of the bHLH Pitf1 with a ubiquitous Class I bHLH and the mammalian Su(H) ortholog RBP-J [57]; the PTF1 complex binds to a composite DNA sequence consisting of an E-box and a Su(H) site [58].

Involvement of an additional factor may explain the specificity of PGM1 activity. A general question in transcription factor biology is how specificity of response is achieved. For example, the E-box of PGM1 could be recognized by any of the numerous bHLH factors expressed in the various tissues of C. elegans, yet it is only activated in pharyngeal glands (Figure 2). One solution to this problem is that related transcription factors distinguish between different binding sites based on subtle differences within the core DNA sequence. For example, different MyoD-containing bHLH dimers have well-characterized binding site preferences [59,60], as do the C. elegans bHLH factor Twist/HLH-8 [29,61] and the Drosophila bHLHs atonal and scute [62]. However, given that binding of bHLH factors to E-boxes may be somewhat promiscuous in vitro, an additional approach to ensure specific response is the involvement of spatially restricted co-factors. Tertiary interactions between bHLH dimers and non-bHLH co-factors are known to affect dimerization and activity [63,64]. In our case, a cofactor may recognize the YMAAY portion of PGM1 and be required for transcriptional activation of target genes.

HLH-6 and the Pharyngeal Gene Network

The FoxA transcription factor PHA-4 is required for specification of pharyngeal cells, including glands [1]. One question, then, is the regulatory relationship between PHA-4 and the HLH-6 gene battery. We have shown in other work that HLH-6 is a probable direct target of PHA-4, so PGM1-dependent genes are at least indirectly regulated by PHA-4 [30]. However, previous work suggested that most or all pharyngeal genes are directly regulated by PHA-4 [4]. Consistent with this idea, we find candidate PHA-4 binding sites in the regulatory regions of all seven gland genes analyzed in addition to the PGM1 motif (Figure S1). Furthermore, a deletion of the phat-1 promoter that removes a predicted PHA-4 binding site drastically reduces but does not eliminate reporter expression and does not affect the pattern of expression (data not shown). Similar results are seen with the PHA-4 sites in other promoters (e.g. myo-2; [4]). PHA-4 may regulate gland-specific gene expression both directly and indirectly, consistent with the proposed model of PHA-4 action. This type of feed-forward transcriptional regulation is also observed in other developmental pathways, such as the myogenic cascade of bHLH transcription factors [53].

Other Factors Required for Gland Development

hkh-6 mutants have multiple defects in gland differentiation, yet still produce g1 (and occasionally g2) gland-like cells and express at
least some gland-specific markers (such as B0507.1 and Y8A9A.2; Figure 4, Figure S2). Therefore, different factors activate expression of different gene batteries in pharyngeal glands, as occurs in body wall muscles and in the excretory cell of C. elegans [65,66]. It will be interesting to identify more HLH-6-independent genes to determine whether the function of that gene battery is distinct from the role of the HLH-6-dependent gene battery; that is, are the different functions of the cells parsed out in an interpretable manner?

Previous work suggested a role for pharyngeal glands in feeding, based on analysis of the kel-1 gene [11]. KEL-1 is detected in pharyngeal glands and kel-1 mutants arrest as early larvae and fail to reach adulthood, in contrast to hlh-6 mutants and gland-ablated animals which are starved but viable. One possible explanation for the difference in phenotypes is that kel-1 function is not limited to pharyngeal glands. In fact, available in situ hybridization data for kel-1 indicates that the message is broadly expressed throughout embryogenesis, with no apparent enrichment in glands [15,16]. Thus, loss of kel-1 likely affects cells in addition to the pharyngeal glands.

**Evolutionary Conservation of Foregut Gland HLH Genes**

An interesting finding is that both the regulation (by hHLH factors) and function (feeding of foregut glands appears to be evolutionarily conserved. The closest mammalian homolog of HLH-6 is Sgn1, a bHLH required for normal salivary gland development in the mammalian foregut [68]. In addition, development of salivary glands in the Drosophila foregut depends on the combined activity of forkhead (the ortholog of PHA-4) and sage (a salivary gland expressed bHLH) [69], although sage is not the closest homolog to hlh-6. Database searches have found other genes encoding proteins with high similarity to HLH-6, including the Ash2 gene, which is expressed in the digestive tract glands of the jellyfish P. carnea [70], and related sequences from the genomes of parasitic nematodes. Gland function in parasitic nematodes is critical for parasitism, suggesting a conserved function of foregut glands in the processing or passage of food [46,71]. Targeting gland development or function may offer a new strategy for controlling these parasitic species.

**Materials and Methods**

**Worm Strains**

Standard nematode handling conditions were used [72]. The hlh-6(tm299) II allele was kindly provided by S. Mitani [29]. Presence of the tm299 deletion was followed by genomic PCR with oligonucleotides oGD65 (5’ CATAACCGGTATACAGCATATTTACTCGAAT 3’) and oGD97 (5’ TTATACATTTGA-GAATGGGGTCTACTCGAC 3’). The original hlh-6(tm299)-bearing chromosome contains a linked larval lethal mutation (let-x) to the left of hlh-6. hlh-6 was outcrossed five times and the arms of LG II were replaced by selecting appropriate recombinants tested for the presence of hlh-6(tm299) by PCR. First, we placed unc-4 in cis with let-x hlh-6 and then selected Rol non-Daf recombinants from let hlh-6 unc-4/rol-6(e187) daf-19(m86) to obtain +rol-6 hlh-6 unc-4. Because this strain is Rol Unc, in all subsequent functional assays a rol-6 unc-4 strain was used as a control.

**Construction of Plasmids**

All transcriptional reporters were made by PCR amplification of promoter fragments from genomic DNA, followed by cloning into either the pPD95.77 or pPD95.77-YFP vectors (gifts from A. Fire), which contain the coding sequences for gfp and yfp, respectively. Mutations in occurrences of PGM1 in the promoters were subsequently made by PCR-based site-directed mutagenesis [73].
Enhancer constructs were built using synthetic oligonucleotides that were cloned into pPD95.77. Use of this vector for enhancer assays was established previously [67].

The 750 base pair phat-5 cDNA was amplified from a cDNA library provided by R. Barstead using primers sGD570 (5’ aagagaccATGTGACGAAGGGCGAG 3’) and sGD571 (5’ cccgacctTTAATGTCAGGTCGATGCC 3’). The product was digested with enzymes KpnI and EcoRI (restriction sites are underlined), and cloned in-frame to YFP or mCherry [45]. The phat-5::YFP fusion was placed under the control of the lys-8 promoter, while the hkh-6 minimal promoter was sub-cloned from min-hkh-6::YFP [30] in front of the phat-5::mCherry fusion to create the hkh-6::phat-5::mCherry construct. The nvo-2::phat-5::mCherry plasmid was cloned using the nvo-2 promoter from plasmid pSESM744 [4]. All clones were verified by restriction digests and sequencing. Details of plasmids and cloning strategies are available upon request.

For rescue of hkh-6 mutants, we subcloned a 3398 bp PstI-XhoI fragment of fosmid WRM066cG05 that contains hkh-6(+) into pBlueScriptII(SK+). The “mini-gene” construct was created by amplification and subcloning of the hkh-6 cDNA from a library provided by R. Barstead. The cDNA was ligated to a 568 bp fragment of the hkh-6 promoter that is active in pharyngeal glands [30]. A synthetic intron was cloned in to a blunt-ended 9 bp fragment of fosmid WRM066cG05 that contains the hkh-6::egl-1 promoter, while the hkh-6 promoter is 9 bp from genomic N2 DNA using primers oGD531 (5’ Ggtactgaaaatttaaatagtcg 3’) and oGD532 (5’ ttcagccgctacgtggtctg 3’). The amplified product was digested with AgeI and EagI and cloned downstream of a 747 bp fragment of the hkh-6 promoter. Design of this construct was based on previous work [44].

Construction of Transgenic Lines

Reporter DNA was injected at 5–30 ng/µL together with 50 ng/µL pRF4 (rol-6(sa11006)), which confers a dominant Roller phenotype [74], and 20–45 ng/µL pBS II (SK+) to a total DNA concentration of 100 ng/µL. For some analyses, we included 20 ng/µL of an intestine specific reporter (elt-2::GFP::LacZ ges-1::mKRP::Hsp2B or elt-2::mTomato::HIS2B) that served as an independent marker for transgenic arrays when scoring expression [75]. For injections with enhancer constructs, 50 ng/µL of the construct was injected with 50 ng/µL pRF4 into N2 animals. For hkh-6::phat-5::mCherry, 40 ng/µL was injected while nvo-2::phat-5::mCherry was injected at 5 ng/µL, because the nvo-2 promoter is very strong and can be toxic at higher concentrations. Except where noted, a minimum of two independent transgenic lines were analyzed for each construct.

The integrated hkh-6 reporter isIs10 [hkh-6::YFP ges-1::mKRP::Hsp2B rol-6(sa11006)] and integrated phat-1::YFP reporter isIs12 [phat-1::YFP elt-2::GFP::LacZ rol-6(sa11006)] were generated by germline-induced integration of extrachromosomal arrays carried in a wild-type background [76]. The pha-4::GFP::HIS2B reporter was provided by Dr. Susan Mango as an integrated array (SM496), which was crossed into the GD211 strain.

To induce cell death in glands, the hkh-6::egl-1 construct was injected at 20 ng/µL with 30 ng/µL elt-2::mTomato::HIS2B and 50 ng/µL pBS II (SK+) into a strain carrying an integrated phat-1::YFP reporter (GD139 isIs12; see above). Doubly transgenic animals were identified based on the Rol phenotype of GD139 (100%) and the presence of red intestinal fluorescence. Animals lacking visible YFP expression (indicating a loss of glands) were then analyzed for survival and growth.

For rescue of hkh-6, both the genomic fragment and the mini-gene were injected at 50 ng/µL with 30 ng/µL of phat-1::YFP and 20 ng/µL elt-2::GFP::LacZ into N2 animals. These arrays were subsequently crossed into GD211.

Motif Searches using Improbizer

We used the Improbizer program [14]; available at http://www.soe.ucsc.edu/~kent/improbizer/ to search for possible gland-specific regulatory elements. We initially searched for motifs occurring once per sequence, using the input sequence as background. The motif presented here (PGM1) was obtained with a search for a motif size of six. Searches for motifs of larger sizes (8–20 bases) recurrently found variations of PGM1. Other parameters of Improbizer were used at their default settings. We also performed control runs in which the input gene sequence was randomized and searched and found that only PGM1 obtained an Improbizer score greater than the scores of ten or more control runs.

To find probable occurrences of PGM1 in other promoters (as in ppg-8 and lys-8), we used the Improbizer sister program, Motif Matcher (www. http://www.cse.ucsc.edu/~kent/improbizer/motifMatcher.html), which searches for top-scoring matches to the Improbizer-generated position weight matrix.

Cell Lineage Analysis

Lineages of embryos from hkh-6/mC6g heterozygotes or hkh-6 homozygotes were examined using a 4D-microscope [77]. The genotype of hkh-6/mC6g progeny was determined after recording by the presence or absence of GFP, which marks the mC6g balancer chromosome. The identities of cells was determined by lineaging backwards using the data base SIMI Biocell.

Growth Assays

All animals were grown on OP50 except for the OP50-GFP bacteria used to visualize the stuffed pharynx and the HB101 bacteria used to rescue the hkh-6 mutant; all bacterial strains were provided by the Caenorhabditis Genetics Center. OP50-GFP was grown on NGM plates containing 100 µg/mL ampicillin and HB101 was grown on NGM plates containing 200 µg/mL streptomycin.

For measurement of body length, embryos laid over a one hour period by gravid adults were collected from and grown at 25°C. Larvae were removed from plates and transferred to slides at the indicated times. Pictures were taken at 400x magnification and the lengths of the animals were measured using ImageJ (http://rsb.info.nih.gov/ij/) as described previously [34]. Greater than twenty animals were analyzed for each genotype at each time-point. For measuring time to reach adulthood, single eggs were placed on plates and followed at 24 hour intervals until the animal reached adulthood. For brood sizes the number of eggs laid was counted throughout the lifetime of each animal.

The intestinal fat stores of the hkh-6 mutants were measured using the dye Nile Red (Sigma N-3013) as described [35]. Briefly, L4 animals of the indicated genotype were transferred to plates with 0.05 µg/mL Nile Red and allowed to grow for 24 hours before being scored using conventional fluorescence microscopy. At least fifteen animals were observed for each genotype and one animal that represents the average level of fluorescence per each genotype is shown. daf-16(RNAi) was performed by “feeding RNAi” using an available daf-16 dsRNA-expressing bacterial strain [78,79]. Adults were placed on the RNAi plates and their progeny were transferred back onto RNAi-Nile Red plates for scoring.
For pharyngeal pumping assays, L4 animals were transferred to fresh plates and grown for 24 hours before scoring. Pumping was counted under a dissecting microscope at 100× magnification.

Supporting Information

Figure S1 Scale diagrams of the analyzed gland-specific promoters. Triangles indicate candidate PHA-4 binding sites (TARTTKRY) and black rectangles indicate occurrences of PGM1. The grey rectangle represents a weak occurrence of a functional PGM1.

Found at: doi:10.1371/journal.pgen.1000222.s001 (0.56 MB TIF)

Figure S2 Representative images of the (A) phat-3, (B) phat-5, (C) B0570.1, (D) phm-8 and (E) lys-8 reporters in hlh-6 mutant animals. Expression of Y246A4.2::GFP in (F) wild type and (G) hlh-6 mutants. Anterior is at left and the pharynx is outlined. Scale bars represent 10 μm.

Found at: doi:10.1371/journal.pgen.1000222.s002 (1.10 MB TIF)

Figure S3 Pharyngeal cell type-specific markers were examined to determine if the g2 cells had adopted an alternate pharyngeal cell fate. We used the pan-neuronal rgef-1::GFP marker to count pharyngeal neurons (expect 7 in wild type), myo-2::GFP::His2B to count pharyngeal muscle nuclei (expect 4 in wild type) and pax-1::GFP::His2B to count pharyngeal marginal cell nuclei (expect 4 in wild type, as pax-1::GFP is also expressed in the pm6 muscle) [51,81,82]. We saw no change in the number of these three markers in wild-type animals and hlh-6 mutants (6.6 vs. 7.0 neurons, 3.6 vs. 3.7 muscles and 3.7 vs. 3.6 marginal cells, respectively). Error bars are standard deviation.

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Figure S4 (A) C. elegans PHAT-1 and T. canis MUC-5 [47] protein sequences showing predicted signal sequence (highlighted in yellow), ShK motifs (red) and Ala/Ser/Thr-rich tracts predicted to contain O-glycosylation sites (underlined). Signal sequences predicted using SignalP 3.0 [83]. (B) PHAT-1 contains numerous predicted O-glycosylation sites that lie between the ShK motifs. Generated using the NetOGlyc 3.1 server [49].

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Figure S5 Electrophoretic mobility shift assay of PGM1. Lane 1 is free probe, Lane 2 is probe with unprogrammed reticulocyte lysate. Lanes 6 and 8 are reactions in which the two proteins were independently transcribed and translated. Lanes 7 and 9 are reactions in which the two proteins were co-translated. Other lanes are as indicated. Open arrow indicates free probe, thin arrow indicates non-specific shift obtained with reticulocyte lysate alone, black arrow indicates HLH-2+HLH-3 shift. No HLH-2+HLH-6 shift is observed, though expression of both proteins has been verified by 35S-Met labeling (not shown).

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Figure S6 Expression of phat-5::mCherry constructs in wildtype animals. (A) Expression of hlh-6::phat-5::mCherry that is weaker than that shown in Figure 8a for a comparison with the decreased levels in the hlh-6 mutant. The pharyngeal lumen is indicated by arrowheads. (B,C) Expression of lys-8::phat-5::YFP with a random loss of the reporter in subsets of glands. (D) Loss of the reporter in g1P, g2L and g2R so that only g1AL and g1AR express the fusion construct. (E) Loss of the reporter in g1AL, g1AR, g2L and g2R so that only g1P expresses the construct. Arrows indicate the boundary of PHAT-5::YFP attachment to the pharyngeal lumen. (F,E) Expression of lys-8::phat-5::YFP during the L1 to L2 molt. The expelled buccal cavity cuticle is indicated by an asterisk and the boundary of the new buccal cuticle is indicated by triangles.

Found at: doi:10.1371/journal.pgen.1000222.s006 (1.08 MB TIF)

Table S1 Lists of gland and pharyngeal (non-gland) genes and their associated Motif Matcher score.

Found at: doi:10.1371/journal.pgen.1000222.s007 (0.04 MB DOC)

Text S1 Supplemental materials.

Found at: doi:10.1371/journal.pgen.1000222.s008 (0.02 MB DOC)

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Author Contributions

Conceived and designed the experiments: RBS JG. Performed the experiments: RBS RS. Analyzed the data: RBS RS JG. Wrote the paper: RBS JG.

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