Analysis of PHA-1 Reveals a Limited Role in Pharyngeal Development and Novel Functions in Other Tissues

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ABSTRACT

PHA-1 encodes a cytoplasmic protein that is required for embryonic morphogenesis and attachment of the foregut (pharynx) to the mouth (buccal capsule). Previous reports have in some cases suggested that PHA-1 is essential for the differentiation of most or all pharyngeal cell types. By performing mosaic analysis with a recently acquired pha-1 null mutation (tm3671), we found that PHA-1 is not required within most or all pharyngeal cells for their proper specification, differentiation, or function. Rather, our evidence suggests that PHA-1 acts in the arcade or anterior epithelial cells of the pharynx to promote attachment of the pharynx to the future buccal capsule. In addition, PHA-1 appears to be required in the epidermis for embryonic morphogenesis, in the excretory system for osmoregulation, and in the somatic gonad for normal ovulation and fertility. PHA-1 activity is also required within at least a subset of intestinal cells for viability. To better understand the role of PHA-1 in the epidermis, we analyzed several apical junction markers in pha-1(tm3671) homozygous embryos. PHA-1 regulates the expression of several components of two apical junction complexes including AJM-1–DLG-1/discs large complex and the classical cadherin–catenin complex, which may account for the role of PHA-1 in embryonic morphogenesis.

Mutations in the pha-1 locus were first described ~25 years ago (Schnabel and Schnabel 1990; Granato et al. 1994a). Recessive pha-1 loss-of-function (LOF) mutations confer arrest as embryos or L1-stage larvae that display gross defects in pharyngeal development and body morphogenesis. Subsequent studies have shown that PHA-1 is part of a regulatory network that includes two Zn-finger proteins, SUP-35/ZTF-21 and SUP-37/ZTF-12, and a Skp1-related protein, SUP-36 (Schnabel et al. 1991; Mani and Fay 2009; Fay et al. 2012; Polley et al. 2014). Genetically, SUP-35–37 act at the same step, possibly within the context of a multiprotein complex (Polley et al. 2014). Notably, LOF mutations in sup-35, sup-36, or sup-37 completely suppress the lethality of pha-1 null and partial-LOF mutations, and overexpression of SUP-35 and SUP-36 can phenocopy pha-1 LOF (Mani and Fay 2009; Fay et al. 2012; Polley et al. 2014). These observations have led to the model that SUP-35–37 function in a manner that opposes or is antagonistic to PHA-1. Other regulators of this network include LIN-35, the Caenorhabditis elegans retinoblastoma protein (pRb) ortholog, and HCF-1, an ortholog of human host cell factor 1 (HCF1) (Fay et al. 2004; Mani and Fay 2009). LIN-35 and HCF-1 are positive and negative regulators of sup-35 transcription, respectively. In addition, both SUP-35 and SUP-36 are negatively regulated post-translationally by a conserved E2–E3 complex composed of UBC-18/UBCH7 and ARI-1/HHARI, which promotes ubiquitin-mediated proteolysis (Qiu and Fay 2006; Mani and Fay 2009; Fay et al. 2003, 2012; Polley et al. 2014).

pha-1 encodes a novel cytoplasmic protein that contains a DUF1114 domain of unknown function (Granato et al. 1994a; Fay et al. 2004). Although multiple PHA-1-related proteins are present in C. elegans and several closely related species (i.e., members of the elegans and Japonica super groups), orthologs of PHA-1 are not detected in most nematodes or in other metazoa. Notably, pha-1 is among the small minority (~15%) of C. elegans genes whose functions are required for viability under laboratory conditions (Schnabel and Schnabel 1990; Kemphues 2005). Thus,
**Materials and Methods**

**Strains and maintenance**

*C. elegans* strains were maintained according to standard methods (Stiernagle 2006). Strains used in this study include GE24* [pha-1(e2123) III],* WY847* [pha-1(tm3671) III; fdEx181 (pBX *pha-1*–wild-type genomic locus; sur-5::GFP)],* WY848* [pha-1(tm3671) III; fdEx182 (pBX; sur-5::GFP)], WY849* [pha-1(tm3671) III; fdEx183 (pBX; sur-5::GFP)], MH1384* [kuls46 (AJM-1::GFP; unc-119 + X)], MH1385* [kuls47 (AJM-1::GFP; unc-119 + II)], WY924* [pha-1(tm3671) III; kuls47 II; fdEx182], WY876* [pha-1(tm3671) III; kuls46 X; fdEx182], SU159* [ajm-1(ok160); jcEx44(AJM-1::GFP)], SM481* [Is (*pha-4::GFP-mem + prF4)], WY871* [pha-1(tm3671) III, Is (*pha-4::GFP-mem + prF4; fdEx182)], WY872* [pha-1(e2123) III, Is (*pha-4::GFP-mem + prF4; fdEx182)], SU295* [jcIs25 (pPE103 (JAC-1::GFP; prF4))] (Pettitt et al. 2003), WY997* [pha-1(tm3671) III; jcIs25; fdEx182], FT250* [unc-119(ed3) III; xls96 (pN455 (HMR-1::GFP) + unc-119+) (Achilleos et al. 2010)], WY996* [pha-1(tm3671) III; xls96; fdEx182], TH502* [unc-119(ed3) III; ddls290 (SAX-7::GFP + unc-119+) (Sarov et al. 2012)], WY995* [pha-1(tm3671) III; ddls290; fdEx182], SU265* [jcIs17 (HMP-1::GFP + DLG-1::dsRed + prF4)] (Raich et al. 1999), and WY990* [pha-1(tm3671) III; jcIs17; fdEx182].

**Mosaic analysis**

Mosaic analysis was carried out using strains WY847, WY848, and WY849 following established protocols (Yochem et al. 2000; Yochem 2006).

**Microscopy**

GFP and dsRed fluorescence images were collected using a Nikon Eclipse epifluorescence microscope and OpenLab software, and quantification was carried out using ImageJ. Averages of the mean fluorescence of the entire embryo were calculated to compare expression levels and backgrounds were subtracted using identically sized areas from an empty region of the slide. Fluorescence intensities provided in the figures are in arbitrary units. P-values were determined using a two-tailed Student’s t-test.

**Results**

**PHA-1 is required for pharyngeal cell reorientation and the stable attachment of arcade cells to pharyngeal epithelial cells**

The *C. elegans* pharynx (foregut) is an organ derived from 95 primordial cells that differentiate into seven distinct cell types (arcade, epithelial, muscle, gland, marginal, valve, and neural) (Albertson and Thomson 1976; Mango 2007, 2009). A principal function of the pharynx is to ingest food, to mechanically process it, and to deposit it into the intestine. Studies of pharyngeal morphogenesis by Portereiko and Mango (2001) defined three temporally separated steps (Figure 1, A and B). In the first step, termed reorientation, anterior pharyngeal epithelial cells are converted from a radial configuration into two parallel rows through changes in cell shape, location, and apicobasal polarity. In the second step, termed epithelialization, reoriented pharyngeal cells attach to the neighboring arcade cells, which are anterior to the pharynx, to form a contiguous epithelial tube connecting the nascent buccal cavity (mouth) to the intestine. In the third step, termed contraction, localized forces pull the arcade and pharyngeal epithelial cells closer together, and the pharynx shifts *en masse* slightly toward the anterior. This initiates the conversion of the primordial pharynx from a ball of cells into an elongated bilobed tube connecting the mouth to the intestine (Figures 1, B and C). Notably, the first step, reorientation, is essential for all subsequent steps of morphogenesis to occur.

Previous phenotypic analyses of *pha-1* employed what are now known to be partial-LOF alleles (e.g., *pha-1(e2123)* (referred to as *pha-1(ts)*), which may differ from the phenotypes displayed by null mutants (Schnabel and Schnabel 1990; Granato et al. 1994a; Fay et al. 2004; Mani and Fay 2009). We therefore examined pharyngeal morphogenesis using strains homozygous for the *pha-1(tm3671) (pha-1(0))* null deletion, which contain a 203-bp deletion that removes the N-terminal portion of the second *pha-1* exon and creates a premature stop codon (Fay et al. 2012). Cell-shape changes during pharyngeal morphogenesis were visualized using a *pha-4*-driven plasma membrane–GFP reporter that is expressed exclusively within cells of the pharyngeal primordium, intestine, and arcade cells (Portereiko and Mango 2001; Fay et al. 2003).
was often abnormally constricted along the dorsal–ventral axis (Figure 1C). A role for PHA-1 in reorientation is also consistent with reorientation defects observed in lin-35; pha-1(fd1) and lin-35; ubc-18 double mutants (Fay et al. 2003, 2004). We also note that equivalent levels of expression of the Ppha-4::membrane–GFP reporter in wild type, pha-1(ts) and pha-1(0) mutants indicates that a reduction or loss of PHA-1 activity does not affect the specification of the pharyngeal primordium (Figure 1).

The highly penetrant pharynx unattached (Pun) phenotype of pha-1(ts) mutants indicates that PHA-1 is also required for the maintenance of pharyngeal attachment to the buccal capsule at later stages of development. Furthermore, on the basis of the arcade cell marker, bath-15::GFP (Shaw et al. 2010), we found that the buccal capsule in pha-1(ts) mutants is typically severed or discontinuous, with the break occurring at the junction of the arcade and pharyngeal epithelial cells (Supporting Information, Figure S1). In more subtle cases, a morphologically abnormal X-shaped (Chi) structure was detected at the junction of the arcade and pharyngeal cells (Figure S1), which may interfere with the ability of these animals to feed, thereby leading to larval arrest. Taken together, our findings demonstrate that PHA-1 is critical for both the initiation and maintenance of pharyngeal epithelial cell attachment to the arcade cells.

Several epithelial apical junction markers are misregulated in pha-1 mutants

To further characterize pharyngeal defects in pha-1(0) mutants, we made use of a fluorescence reporter for AJM-1, an apical junction protein that is broadly required for morphogenesis and the integrity of epithelia in C. elegans (Koppen et al. 2001; McMahon et al. 2001; Lynch and Hardin 2009). For these assays, AJM-1::GFP served as a marker of epithelialization for both the pharynx and arcade cells (Portereiko and Mango 2001). Consistent with previous reports, AJM-1::GFP is first detected in wild-type embryos beginning at the lima-bean stage (~350 min postfertilization) in the central region of the primordial pharynx (Figure 2). Expression of AJM-1::GFP is also observed in the region corresponding to the arcade cells beginning at around the comma stage (~400 min), coincident with the onset of reorientation. Expression of AJM-1::GFP increases throughout the 1.5- and 2.0-fold stages of embryogenesis in both the pharynx and arcade regions of wild-type embryos (~430–450 min; Figure 2).

Although pha-1(ts) mutants at 25° displayed a similar pattern of AJM-1::GFP to that of wild-type embryos, expression was typically weaker and less uniform than expression in wild type at corresponding stages (Figure 2). In contrast, AJM-1::GFP expression was dramatically reduced in pha-1(0) mutants with little or no detectable expression in the region of the primordial pharynx through the 2.0-fold stage (Figure 2). In addition, pha-1(0) mutants that failed to undergo reorientation never expressed AJM-1::GFP in the region of the arcade cells, consistent with the model that epithelialization of the
arcade cells strictly follows integration with the anterior pharynx (Portereiko and Mango 2001). Although some 2.0-fold \textit{pha-1}(0) embryos did not express AJM-1::GFP, expression was always observed in late-stage, terminally arrested \textit{pha-1}(0) embryos, although levels were strongly reduced as compared with wild-type or \textit{pha-1(ts)} embryos (Figure S2). To see if our observation was specific to AJM-1 or reflected a more generalized defect in the epithelia of \textit{pha-1}(0) mutants, we examined expression patterns of several markers associated with adherens junctions and integrity in 1.5-fold \textit{pha-1}(0) embryos. Two well-characterized junctional complexes in \textit{C. elegans} include a classical cadherin–catenin complex (HMR-1/cadherin, JAC-1/p120-catenin, HMP-1/α-catenin and HMP-2/β-catenin) and the more basal AJM-1 and DLG-1/discs large complex (Labouesse 2006; Lynch and Hardin 2009). DLG-1, a binding partner of AJM-1, is essential for proper localization of AJM-1 (Koppen et al. 2001), whereas SAX-7 has been proposed to function as the transmembrane component of the complex (Chen et al. 2001). Loss of \textit{pha-1} resulted in a strong reduction in AJM-1::GFP in the pharynx and intestine (4.6-fold), and lateral epithelial cells (5.4-fold; Figure 3B). In addition, a modest increase in DLG-1::dsRed expression (1.5- to 1.7-fold) was observed in both the epidermis and gastrointestinal tract of \textit{pha-1}(0) mutants, whereas no obvious changes were detected with SAX-7::GFP (Figure 3B). In the case of the cadherin–catenin complex, expression of HMR-1::GFP was reduced ~1.9-fold in both the pharynx and epidermis of \textit{pha-1}(0) mutants, whereas JAC-1 expression was enhanced 1.4- to 1.6-fold in the pharynx and epidermis (Figure 3C). In contrast, HMP-1::GFP showed no change in \textit{pha-1}(0) mutants (Figure 3C). Our results indicate that components of two major junctional complexes are misregulated in epithelial cells of \textit{pha-1}(0) mutants, consistent with the role of PHA-1 in regulating epidermal morphogenesis.

**PHA-1 is not required autonomously for the differentiation or function of most pharyngeal cells**

To better understand the role of PHA-1 in pharyngeal development and to more broadly identify tissues in which PHA-1 performs important functions, we carried out a mosaic analysis using the \textit{pha-1}(0) allele strain. These strains have a mitotically unstable extrachromosomal array containing wild-type \textit{pha-1} [\textit{pha-1(+)}] rescuing sequences along with a broadly expressed visible marker, SUR-5::GFP (Yochem et al. 1998). These strains produce animals containing a mixture of wild-type and \textit{pha-1}(0) mutant cells that can be distinguishable by the presence or absence of nuclear GFP. The pattern of array inheritance was used to determine the cell lineages in which the array was retained.
or lost during development. On the basis of the phenotype of individual worms and the inheritance profile of the array, it was possible to infer tissue-specific requirements for \textit{pha-1} and to correlate losses within particular lineages with specific phenotypes. A schematic summary of the mosaic analysis is shown in Figure 4A.

Intriguingly, in view of previous reports suggesting a role for \textit{PHA-1} in pharyngeal cell specification and differentiation, our data strongly indicate that \textit{PHA-1} was not required autonomously for the specification, differentiation, or function of most pharyngeal cells. Evidence includes our isolation of three adult animals in which \textit{pha-1(+)} was missing.

Figure 3 Loss of \textit{PHA-1} affects normal expression of epithelial junction markers. (A) Schematic representation of the proposed organization of epithelial junctions in \textit{C. elegans}. Arrows show the direction of expression changes in the \textit{pha-1(0)} background. (B and C) Quantification of fluorescence intensities of members of the DLG-1/AJM-1 complex (B) and cadherin–catenin complex (C) in wild-type and \textit{pha-1(0)} mutant backgrounds. Also shown are representative fluorescence images of 1.5-fold-stage embryos. Fluorescence is expressed relative to wild type, which was arbitrarily set to 1.0. Error bars indicate 95% confidence intervals. Statistical analysis was performed using Student’s \textit{t}-test; **\textit{P} < 0.001.
Arrested L1 larvae but contained pharynxes with relatively normal morphologies, suggesting that pha-1 is not required within most ABa-derived pharyngeal cells for pharyngeal differentiation or morphogenesis. Nevertheless, such animals typically contained elongated pharynxes with relatively normal morphologies, suggesting that pha-1 is not required within most ABa-derived pharyngeal cells for pharyngeal differentiation or morphogenesis. We also identified one ABa-minus ABp-plus embryo in which pharyngeal cell reorientation had failed (Figure 4, H and I). This finding suggested that although pha-1 may not be autonomously required within most pharyngeal cells, there is

![Figure 4](image-url)

**Figure 4** pha-1(0) mosaic analysis. (A) Early embryonic lineages are indicated along with major cell types produced by each lineage. Solid lines indicate lineages where pha-1 plays an essential function. Numbers indicate viable L4 larvae or adults that were isolated with losses at the indicated cell divisions. (B–K) DIC, showing B, D, F, H, and I and corresponding fluorescence C, E, G, I, and K images of pha-1(0) mosaic animals carrying the fde182 extrachromosomal array, which expresses both wild type pha-1 and the SUR-5::GFP marker. Anterior is to the left. (B and C) L4-stage larva in which pha-1 is absent from the MS lineage. Note the normal appearance of the posterior pharyngeal bulb and isthmus (dashed outline) despite the absence of pha-1 activity. (D and E) Adult ABa-mosaic animal with a normal pharynx in which pha-1 is present only in the left ventral tripartite epidermal section (pm2, e1, e2; indicated by arrows in E) derived from ABal. (F and G) P1-minus arrested L1 larva. Note the presence of a normal-appearing pharynx; solid arrows indicate the pharyngeal lumen in F. Also note normal body morphology. (H and I) Embryo (1.5-fold stage) that is missing pha-1 in all cells derived from ABa. Dashed line indicates the boundary of the pharynx. Note the absence of any anterior pharyngeal extension. (J and K) Arrested L1 larvae in which pha-1 is missing within the E lineage. Solid arrows in J indicate pharyngeal lumen; bracket in J, posterior bulb of pharynx (MS-plus); dashed line encircles the intestine (GFP-minus). (L) A clear (Clr) fluid-filled adult containing multiple vacuoles (v) that was missing pha-1 in the excretory cell. The solid and open dashed lines denote the outlines of the intestine and gonad, respectively. Bar in B, 10 μm (for B, C, F–K) and bar in D, 10 μm (for D, E, and L).
nevertheless a requirement for \textit{pha-1} within the 
ABa lineage for pharyngeal morphogenesis to occur.
Taken together, these findings suggest a requirement
for \textit{PHA-1} within a subset of arcade cells, but because one or
more pharyngeal epithelial cells is always close to
one or more arcade cells in the ABa part of the cell
lineage (Sulston et al. 1983), the possibility has not
been excluded that \textit{PHA-1} acts within a subset of
the ABa-derived pharyngeal cells (e.g., e2) or
that \textit{PHA-1} may be required in some combination of
arcade and anterior pharyngeal cells. In addition, it is possible
that \textit{PHA-1} also acts in other cells that are close to the
pharyngeal epithelium and arcade cells in the cell
lineage, such as the marginal cells.

\textbf{\textit{PHA-1} performs essential functions in tissues outside
of the pharynx}\n
Interestingly, our mosaic analysis with the null allele also
revealed several previously unknown tissue requirements
for \textit{PHA-1}. Most notably, we failed to observe viable larvae
or adults in which \textit{pha-1} was missing entirely from the
intestinal lineage (n > 10,000). Rather, our data indicate that
the absence of \textit{pha-1} within the intestine leads to L1
larval arrest. Consistent with this, we identified 19 arrested L1
larvae in which the \textit{pha-1}-rescuing array was lost in either
the P1 (5), EMS (7), or E (7) lineages (Figure 4, J and K).
These animals failed to display gross morphological
defects of their intestines, and their intestinal cells appeared to
contain microvilli (data not shown). Nevertheless, it is possible
that these intestines contained subtle structural defects that
we did not detect. It is also possible that these larvae
arrested for reasons unrelated to intestinal defects, although
their gross morphologies, including the pharynx, appeared
normal (Figure 4, F and J). Moreover, the complete absence
of viable intestine-minus mosaics strongly indicates that
\textit{PHA-1} is required in this tissue. Somewhat surprisingly,
we identified several adults that were missing \textit{pha-1} in the
majority of intestinal cells but were nevertheless viable. This
included one sick, slow-growing adult that contained only
four \textit{PHA-1}-positive posterior gut nuclei (out of a total of
20 binucleate intestinal cells in the adult). Taken together, our
data suggest that \textit{pha-1} is autonomously required for some
aspect of intestinal cell function but that this activity is not
strictly required in all intestinal cells for growth and
development to proceed.

We also observed a small percentage of mosaic animals
(\textasciitilde1\%) that became clear, vacuolated, and filled with fluid
(the \textit{Clr} phenotype), a defect associated with abnormal
osmotic regulation (Nelson and Riddle 1984). The \textit{Clr}
phenotype did not become evident until \textasciitilde2–3 days into
adulthood, at which time clear fluid-filled animals containing
multiple vacuoles could be detected (Figure 4L). Osmotic
regulation in \textit{C. elegans} is controlled by four cells
that comprise the excretory system, a primitive analog of
the renal system of vertebrates (Nelson et al. 1983). We
examined the excretory system for the presence of the
\textit{pha-1} array in \textit{Clr} adults. Notably, 21 of 24 \textit{Clr}
adults lacked detectable GFP expression in the excretory cell
(one of four cells within the excretory system). In contrast, array
expression in the excretory cell was detected in 25 of 25 adults
that did not show the \textit{Clr} phenotype. Furthermore, in the three
\textit{Clr} adults that showed GFP expression in the excretory cell,
expression was not detected in the anatomically and lineally
connected excretory duct cell. Taken together, our observations
suggested a role for \textit{PHA-1} in the excretory system, in
particular the excretory cell. We note, however, that corre-
lating GFP expression with specific excretory cells using DIC
optics was difficult, and the poor cellular morphology of the
\textit{Clr} adults further compounded this problem. Moreover,
given the relatively late onset of the \textit{Clr} phenotype, the role
of \textit{PHA-1} in the excretory system may be subtle.

Although, as noted above, MS-minus animals grew
normally to become viable adults, abnormal-looking proximal
gonads were often observed, especially in older adult
hermaphrodites. Specifically, \textit{PHA-1} appears to promote
normal spermatogenesis, ovulation, or both. This is notable
because MS gives rise to both Z1 (through MSp) and Z4
(through MSa), which produce the large majority of anterior
and posterior somatic gonad cells, respectively (Figure 4A).
Gonadal defects correlated with the reduction or absence of
\textit{PHA-1} expression within individual gonad arms; MSa-
minus/MSp-plus (Z1+/Z4−) mosaics showed defects
specifically in the posterior gonad, whereas MSpa/MSp-minus
(Z1−/Z4+) mosaics showed defects in the anterior gonad
(Figure 5, A–D). Gonad arms deficient for \textit{pha-1} produced
endoderm effects (Figure 5, C and D), which arise under
conditions where oocytes fail to undergo timely fertilization
(McCarter et al. 1997, 1999). We further identified 12
animals that were missing \textit{pha-1} within sublineages of MS
that give rise to the sheath and spermathecal cells of the somatic
gonad (SS lineage). Although the patterns of array inheritance
in these animals were often complex, we observed a clear
 correlation between the absence of \textit{pha-1} expression within
somatic gonad arms and the incidence of fertilization defects
(Figure 5, E–J). This led to a reduction in the number of sperm
and to the abnormal persistence of residual bodies, which are
anucleate byproducts of spermatogenesis (IHernault 2006).
In addition, MS-minus animals often contained abnormal
embryos, oocytes, or oocyte fragments within the uterus
(Figure 5K). Consistent with these data, MS-mosaic animals had
reduced fecundity. Whereas nonmosaic controls had an average
brood size of 264 (SD = 15.1; n = 3), animals that contained
one positive and one negative gonad arm had an average brood
size of 144 (SD = 34.4, n = 4). Nevertheless the complete
absence of \textit{PHA-1} in the MS lineage did not preclude the pro-
duction of progeny as we observed a single MS-minus animal
with a brood size of 201. Collectively, these findings indicate an
autonomous role for \textit{PHA-1} in the somatic gonad. In contrast,
we found no essential role for \textit{PHA-1} within the developing
germline (Figure 4). F1 progeny from germline-minus adults
were, as expected, embryonic lethal, and displayed a phenotype
identical to that of \textit{pha-1(0)} animals that failed to inherit the
\textit{pha-1(+) }array in the parental strain (data not shown).
rhythmic egg laying. PHA-1 may play a minor role in some aspect of vulval development or in the neurons or muscles that are required for PHA-1 minus sperm. Although bloated, these Egl animals had vulvae that retained abnormal numbers of eggs (Egl; data not shown). Finally, we occasionally observed adult hermaphrodites that appeared normal and were able to lay eggs. Therefore, PHA-1 may play a minor role in some aspect of vulval development or in the neurons or muscles that are required for rhythmic egg laying.

**Discussion**

The role of PHA-1 in *C. elegans* development has been difficult to assess, given the previous lack of an available null mutation, the absence of any conserved functional domains, the broad pattern of cytoplasmic expression, and the somewhat contradictory nature of published reports. Here we demonstrate that PHA-1 does not have an autonomous role in the differentiation, morphogenesis, or function of most or all pharyngeal cells (Figure 4). Rather, the role of PHA-1 in pharyngeal morphogenesis may reflect a requirement within a subset of arcade cells, a subset of pharyngeal epithelial cells, or both (Figure 1). At this time it is not feasible to distinguish between these possibilities because promoters are not available for expressing *pha-1* specifically within either the arcade or anterior pharyngeal epithelial cells in early embryos. Furthermore, there may be some plasticity in the requirement for PHA-1 within arcade and/or pharyngeal epithelial cell types as opposed to an absolute requirement within any single cell type. Our general findings for PHA-1 contrast with published reports for the transcriptional regulator PHA-4/FOXO, which directly regulates the differentiation of all pharyngeal cell types (Mango et al. 1994; Horner et al. 1998; Gaudet and Mango 2002; Mango 2009).

Our mosaic analysis of the *pha-1(0)* mutant also implicates several new functions for PHA-1, including roles in the intestine, somatic gonad, and excretory system (Figure 4). Notably, these defects are not observed in homozygous *pha-1(ts)* strains shifted to the nonpermissive temperature after embryogenesis (Schnabel and Schnabel 1990; Granato et al. 1994a) (data not shown). How defects in these distinct tissues might be connected to a single cellular or molecular function is currently unclear. However, the ability of mutations in *sup-35*, *sup-36*, and *sup-37* to suppress all of the *pha-1*-associated defects uncovered by our mosaic analysis suggests that a common underlying molecular mechanism may account for the phenotype of different tissues. This is also consistent with a mosaic analysis demonstrating a role for *SUP-37* in both the somatic gonad and intestine (Fay et al. 2012), two tissues implicated in our mosaic analysis of *pha-1*. More generally, we can conclude that the functional interactions of *SUP-35–37* with *PHA-1* are not limited to those associated with foregut morphogenesis.

We also show that PHA-1 is required in AB-derived lineages, which contribute to the large majority of the epidermis,
for normal morphogenesis. This is consistent with morphological abnormalities observed in pha-1 mutant embryos and larvae. Moreover, the role of PHA-1 in the epidermis is supported by our analysis of apical junction markers in the epithelium of pha-1(0) mutants (Figure 2 and Figure 3). Namely, expression levels of AJM-1 and HMR-1/cadherin were reduced, whereas levels of DLG-1/discs large and JAC-1/p120-catenin were elevated. Although the precise meaning behind the specific pattern of apical marker deregulation in pha-1(0) mutants is unclear, these changes clearly implicate PHA-1 in the normal function of the epithelium, possibly by regulating cell polarity, differentiation, or epithelial junctions. We note that although the strongly reduced expression of AJM-1 may account, at least in part, for the body morphogenesis defects observed in pha-1 mutants (Schnabel and Schnabel 1990; Granato et al. 1994a; Fay et al. 2004), it cannot explain defects in pharyngeal development, as ajm-1(0ki160) null mutants did not display defects in pharyngeal morphogenesis (data not shown). Interestingly, a reduction in hmr-1 levels by RNAi has been reported to result in a Pun phenotype (Ferrier et al. 2011), consistent with the possibility that the unattached pharynx in pha-1 mutants is related to reduced HMR-1 levels. We also raise the possibility that initial attachment of the pharynx and the ensuing biomechanical tension experienced by pharyngeal cells may in part promote pharyngeal cell differentiation as has been shown for various cell types in other systems (Benjamin and Hillen 2003; Mamimoto et al. 2012; Franze 2013; Janmey et al. 2013). Whether this phenomenon occurs in C. elegans, however, is unknown.

PHA-1 family members have expanded and rapidly evolved within the elegans and Japanica super groups. Our findings suggest that PHA-1 family members may regulate numerous aspects of nematode development in these species. It is worth noting that one of our identified downstream targets of PHA-1, the apical junction protein AJM-1, is also not well conserved outside of the nematode phylum. Thus, pha-1 and sup-35–37 may represent a regulatory module that has coevolved with ajm-1 within the Caenorhabditis lineage.

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Analysis of PHA-1 Reveals a Limited Role in Pharyngeal Development and Novel Functions in Other Tissues

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Figure S1  The *pha-1*(*ts*) mutant shows defects in the arcade–pharynx junction. (A–H) DIC (A,C,E,G) and corresponding fluorescence (B,D,F,H) images of wild-type (A,B) and *pha-1*(*ts*) mutant larvae (C–H) expressing the *bath-15*::GFP arcade cell reporter, which marks the anterior half of the buccal capsule. Anterior is to the left. Arcade (a) and pharyngeal (p) contributions to the buccal capsule (white lines) are indicated. Arrow indicates region of separation or aberrant morphology at the division between the arcade- and pharynx-derived buccal capsule. The *bath-15*::GFP reporter marks both the cell bodies (posterior and round) and processes (anterior and posterior, variably shaped) of the arcade cells. Note that the anterior portion of the buccal capsule in mutant animals where the capsule has been severed coincides with the location of anterior *bath-15* expression, consistent with breakage occurring at the junction of the arcade and epithelial cells. (I,J) *pha-1*(*ts*) larva with a severed buccal capsule (I) and abnormal arcade cell morphology (J), as indicated by arrowheads. I and J depict different focal plains of the same larva. Size bar in A represents 10 µm for A–J.
Figure S2  AJM-1::GFP expression in various strain backgrounds. (A–J) DIC (A,C,E,G, I) and corresponding AJM-1::GFP fluorescence (B,D,F,H,J) images. Anterior is to the left, with genotypes indicated within panels. Arrows indicate pharyngeal lumen. Note that expression of AJM-1::GFP in terminally arrested pha-1(0) embryos (A–D) is weaker and disorganized as compared with a recently hatched wild-type L1 larva of similar chronological age (E,F). In addition, AJM-1::GFP expression is largely missing in the region between the non-elongated pharynx (dashed yellow line, A–D) and the developing buccal cavity (arrowhead). (G,H) Expression of AJM-1::GFP in a pha-1(0) L1 larva that has been rescued by wild-type pha-1 genomic sequences present on an extrachromosomal array (fdEx182). Additional fluorescence in nuclei in panel H is due to the expression of SUR-5::GFP by the fdEx182 array. (I,J) AJM-1::GFP expression is restored in pha-1(0) mutants suppressed by sup-35(RNAi). Size bar in A represents 10 µm for A–J.