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); jce44(AJM-1::GFP)], SM481 [Is (Ppha-4::GFP-mem + pRF4)], WY871 [pha-1(tm3671) III, Is (Ppha-4::GFP-mem + pRF4); fdEx182], WY872 [pha-1(e2123) III, Is (Ppha-4::GFP-mem + pRF4); fdEx182], SU295 [jcls25 (pPE103 (JAC-1::GFP); pRF4)] (Pettitt et al. 2003), WY997 [pha-1(tm3671) III; jcls25; 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; dds290 (SAX-7::GFP + unc-119+)] (Sarov et al. 2012), WY995 [pha-1(tm3671) III; dds290; fdEx182], SU265 [jcls17 (HMP-1::GFP + DLG-1::dsRed + pRF4)] (Raich et al. 1999), and WY990 [pha-1(tm3671) III; jcls17; 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).
Table: C. elegans pha-1

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 P*pha-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 severely 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 phar-
ynx (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 com-
pared with wild type (Figure S2). Similar results were also
observed using an independent array of AJM-1::GFP inte-
grated on a different chromosome (data not shown). Finally,
AJM-1::GFP expression defects in \textit{pha-1}(0) mutants were
fully rescued by an extrachromosomal array containing wild-
type copies of \textit{pha-1} or by inhibition of \textit{sup-35} by RNAi (Figure
S2). These findings indicate that PHA-1 plays an important
role in the timing and strength of AJM-1 expression and more
generally in the process of pharyngeal epithelialization. They
also demonstrated that suppression and rescue of \textit{pha-1}(0)
defects correlates with the normal expression of AJM-1.

In our studies examining the expression of AJM-1::GFP in
the embryonic pharynx, we noticed that this marker was
expressed at much lower levels in the developing embryonic
epidermis (formerly termed the hypodermis) of \textit{pha-1}(0) null
mutants as compared with wild-type or \textit{pha-1(ts)} embryos
(Figure 2 and Figure 3B). To see if our observation was spe-
cific to AJM-1 or reflected a more generalized defect in the
epithelia of \textit{pha-1}(0) mutants, we examined expression pat-
terns of several markers associated with adherens junc-
tions 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/\alpha-catenin and HMP-2/\beta-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 (Kop-
pen et al. 2001), whereas SAX-7 has been proposed to func-
tion 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 lat-
eral epithelial cells (5.4-fold; Figure 3B). In addition, a mod-
est 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.

\textbf{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 de-
velopment and to more broadly identify tissues in which
PHA-1 performs important functions, we carried out a mo-
saic analysis using the \textit{pha-1}(0) allele strain. These strains
have a mitotically unstable extrachromosomal array contain-
ing wild-type \textit{pha-1} [\textit{pha-1(+)}] rescuing sequences along
with a broadly expressed visible marker, SUR-5::GFP
(Yochem \textit{et al.} 1998). These strains produce animals con-
taining 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.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{AJM-1::GFP expression in \textit{pha-1} mutants. Representative
images of AJM-1::GFP (kuls46) in wild-type, \textit{pha-1(ts)} at 25°C, and \textit{pha-1}
(0) embryos at the indicated stages. Anterior is to the left, dorsal is up.
Location of the pharynx (p) is indicated with a solid white line, and arcade
cells (a) are indicated with a dashed white line (sensory depression, s).
Arrowheads indicate AJM-1 puncta in the developing epidermis. Bar,
5 \textmu m. Graph shows quantification of AJM-1::GFP expression patterns
for each genotype and stage. The number of embryos examined for each
stage and genotype ranged from 21 to 32.}
\end{figure}
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 PHA-1 in pharyngeal cell specification and differentiation, our data strongly indicate that 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}}

\textbf{Figure 3} Loss of 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 t-test; ** \textit{P} < 0.001.
entirely from the MS lineage (i.e., MS-minus), as well as three animals in which pha-1(+ ) was missing in one of the daughters of MS (MSa/p). These MS-mosaic animals developed at normal rates and had pharynxes with wild-type morphology (Figure 4, B and C). Given that about half of the cells comprising the pharynx are derived from MS (Sulston et al. 1983), it appears that PHA-1 is dispensable for the normal development and function of at least half of the cells of this organ. Furthermore, although we failed to isolate adults in which pha-1 was missing entirely from ABa or its daughters ABal and ABar, we did identify adults that had suffered multiple losses within the ABa sublineages that give rise to the pharynx. This included an adult in which only the left ventral tripartite epidermal section (pm2, e1, e2, and e3, which are derived from ABal and ABar) retained the array within the ABa-derived pharyngeal lineage (Figure 4, D and E). Taken together, these findings suggest that PHA-1 does not function autonomously to promote the differentiation, morphogenesis, or function of the large majority of pharyngeal cells.

To complement the above analysis, we also analyzed embryos and arrested L1 larvae that were mosaic for pha-1. We identified seven P1-minus mosaics, which uniformly arrested as L1 larvae but contained pharynxes with relatively normal morphology (Figure 4, F and G). We also observed one L1 in which pha-1 was missing within both the MS and ABar lineages. Although arrested, this animal had a pharynx with relatively normal morphology (data not shown). Given that the anteriormost pharyngeal epidermal cells, e1D, e1VL, and e1VR, are all derived from ABar, this would suggest that pha-1 is not required in the e1 lineage for normal morphogenesis. (For pharyngeal anatomy, D signifies dorsal, whereas VL VR, and DL indicate ventral left, ventral right and dorsal left respectively; ant and post indicate anterior and posterior.) It also suggests that arcade cells derived from ABal (post-VL, post-DL, ant-DL, and ant-V) could be a focus for pha-1. Additional arrested L1s with little or no detectable expression in ABa-derived pharyngeal lineages were also observed, although the patterns of array inheritance within these animals were often quite complex. 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
nevertheless a requirement for *pha-1* within the ABa lineage for pharyngeal morphogenesis to occur. Taken together, these findings suggest a requirement for *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 *PHA-1* acts within a subset of the ABa-derived pharyngeal cells (e.g., e2) or that *PHA-1* may be required in some combination of arcade and anterior pharyngeal cells. In addition, it is possible that *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.

**PHA-1 performs essential functions in tissues outside of the pharynx**

Interestingly, our mosaic analysis with the null allele also revealed several previously unknown tissue requirements for *PHA-1*. Most notably, we failed to observe viable larvae or adults in which *pha-1* was missing entirely from the intestinal lineage (n > 10,000). Rather, our data indicate that the absence of *pha-1* within the intestine leads to L1 larval arrest. Consistent with this, we identified 19 arrested L1 larvae in which the *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 *PHA-1* is required in this tissue. Somewhat surprisingly, we identified several adults that were missing *pha-1* in the majority of intestinal cells but were nevertheless viable. This included one sick, slow-growing adult that contained only four *PHA-1*-positive posterior gut nuclei (out of a total of 20 binucleate intestinal cells in the adult). Taken together, our data suggest that *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 (~1%) that became clear, vacuolated, and filled with fluid (the *Clr* phenotype), a defect associated with abnormal osmotic regulation (Nelson and Riddle 1984). The *Clr* phenotype did not become evident until ~2–3 days into adulthood, at which time clear fluid-filled animals containing multiple vacuoles could be detected (Figure 4L). Osmotic regulation in *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 *pha-1* array in *Clr* adults. Notably, 21 of 24 *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 *Clr* phenotype. Furthermore, in the three *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 *PHA-1* in the excretory system, in particular the excretory cell. We note, however, that correlating GFP expression with specific excretory cells using DIC optics was difficult, and the poor cellular morphology of the *Clr* adults further compounded this problem. Moreover, given the relatively late onset of the *Clr* phenotype, the role of *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, *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 *PHA-1* expression within individual gonad arms; MSa-minus/MSp-plus (*Z1*/–*Z4*/+) mosaics showed defects specifically in the posterior gonad, whereas MSa-plus/MSp-minus (*Z1*/–*Z4*/+) mosaics showed defects in the anterior gonad (Figure 5, A–D). Gonad arms deficient for *pha-1* produced endomitotic oocytes (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 *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 *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 (I.Hernault 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 *PHA-1* in the MS lineage did not preclude the production of progeny as we observed a single MS-minus animal with a brood size of 201. Collectively, these findings indicate an autonomous role for *PHA-1* in the somatic gonad. In contrast, we found no essential role for *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 *pha-1(0)* animals that failed to inherit the *pha-1(+)* array in the parental strain (data not shown).
rhythmic egg laying. 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 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 is strongly expected to arise when the array is absent within particular regions of the epidermis or possibly when the copy number of the array within the hypodermal syncytium is strongly reduced. Notably, loss of the array within either the ABA or ABp lineages, which are the major contributors to the epidermis, was never detected in hatched larvae (Figure 4). In contrast, loss within the C lineage, which contributes to the epidermis but to a lesser extent than ABA or ABp, was detected in morphologically normal viable adults (Figure 4). Finally, we occasionally observed adult hermaphrodites that retained abnormal numbers of eggs (Egl; data not shown). Although bloated, these Egl animals had vulvae 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(ts) 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(ok160)* 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; Mammoto 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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Literature Cited


Yochem, J. K., 2006 Nomarski images for learning the anatomy, with tips for mosaic analysis. WormBook pp. 1–47.

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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.