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Marco Gallo · Allan K. Mah · Robert C. Johnsen
Ann M. Rose · David L. Baillie

# *Caenorhabditis elegans dpy-14*: an essential collagen gene with unique expression profile and physiological roles in early development

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**Abstract** We describe the molecular characterisation of *Caenorhabditis elegans dpy-14*, a gene encoding an essential cuticular collagen annotated as *col-59*. Expression of *dpy-14* starts at the 16 E cell stage, making it the earliest-expressing collagen reported to date. SAGE data and *dpy-14* promoter::GFP reporter constructs indicate that the gene is transcribed mainly during embryogenesis, specifically in ciliated neurons and hypoderm. Water permeability assays and lectin staining showed that a mutation in the DPY-14 collagen results in defects in the channels of the amphids, which are a class of ciliated neuron, while the amphids appear morphologically normal by dye filling methods. Behavioural assays showed that the ciliated neurons expressing the gene are functional in *dpy-14* mutants. All together, our data suggest that ciliated neurons and their hypodermal support cells collaborate in the transcription and synthesis of DPY-14, which then becomes a component of the amphid channels but not of the amphids proper. Interestingly, seam cells of *dpy-14* mutants do not properly fuse to form a syncytium. This novel phenotype due to collagen mutations further stresses that *dpy-14* plays a fundamental role in *C. elegans* physiology, since it is required for the proper development of the hypoderm.

**Keywords** Collagen · Hypodermis · Ciliated neurons · *dpy-14* · *col-59*

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M. Gallo · A. K. Mah · R. C. Johnsen · D. L. Baillie (✉)
Department of Molecular Biology and Biochemistry,
Simon Fraser Univervsity, 8888 University Drive,
V5A 1S6, Burnaby, BC, Canada
E-mail: baillie@sfu.ca
Tel.: +1-604-2914597

A. M. Rose
Department of Medical Genetics,
University of British Columbia, V6T 1Z3,
Vancouver, BC, Canada

## Introduction

The nematode collagen family contains about 180 members that encode peptides with a characteristic organization of their domains. Firstly, their N-terminal region contains a signal peptide (Kramer 1997) and an endoproteolytic processing site (Thacker et al. 2000). Secondly, two blocks of 8–10 Gly–X–Y and 40–42 Gly–X–Y repeats—where X and Y are usually Proline and Hydroxyproline, respectively (Johnstone 2000)—are fundamental for the interactions between collagen monomers that result in formation of collagen triple-helices (reviewed by Kramer 1994; Myllyharju and Kivirikko 2004). Thirdly, a short C-terminal domain (Kramer 1997) is present and, like the N-terminus, is proteolytically processed (Novelli et al. 2004).

Mutations in collagen genes affect the morphology of the nematodes. Mutants can thereby be categorized into four broad phenotypic classes according to their abnormal morphologies: dumpy (Dpy), short and fat; long (Lon), long and thin; roller (Rol), twisted in a left- or right-handed helix; and abnormal tail (Tal), shortened and malformed hermaphrodite tail (Kramer 1994).

Collagens are paramount components of the extracellular matrix (ECM). *Caenorhabditis elegans* possesses two types of ECMs: the cuticle and basement membranes (Kramer 1997).

The cuticle, the nematode exoskeleton, is fundamental for maintenance of morphology, protection from the external environment (Kramer 1997) and locomotion (Winter and Page 2000). It is a complex multi-layered structure and its composition is tightly regulated by differential expression of collagen genes prior to each ecdysis (Kramer 1997).

Cuticular structures are also present around the nematode ciliated neurons. Most ciliated neurons are part of sense organs or sensilla, each consisting of one or more ciliated nerve endings and two or three non-neuronal support cells, namely one sheath cell and one or two socket cells (Starich et al. 1995). In *C. elegans*,

ciliated neurons have chemosensory and mechanosensory roles (Ward et al. 1975; Ware et al. 1975), while in mammals they perform a wider range of functions. Previous studies have demonstrated that human ciliary dysfunctions may lead to genetic disorders, prominent among them Bardet–Biedl syndrome (Ansley et al. 2003) and primary ciliary dyskinesia (Meeks and Bush 2000). Although little is known about the molecular structure of mammalian axonemes (Zhang et al. 2002), much information has been accrued on the structure and cytological organization of *C. elegans* sensilla, namely the head amphids and the tail phasmids (Starich et al. 1995; Ward et al. 1975). *C. elegans* has two amphids, each one comprising a set of dendrites of 12 sensory neurons and two support cells, a sheath cell and a socket cell (Perkins et al. 1986). The sheath and socket cells form a channel that allows the amphids to reach the outside of the animal and respond to various chemical and mechanosensory signals. Nevertheless, the mechanisms underlying the partnership between ciliated neurons and their support cells enjoyed little attention. In the present work, we show that *dpy-14* is a mediator of this partnership.

*dpy-14(e188)* animals were originally isolated by Brenner (1974), in his EMS mutagenesis screen, and mapped to chromosome I. Unlike other collagen mutations, the recovered allele alters the morphology of early larval worms. Furthermore, *e188* is temperature sensitive lethal indicating that this gene is essential for proper *C. elegans* development.

Here we show that *col-59* is the molecular transcript of the *dpy-14* gene, and that two classes of cells cooperate in the synthesis and deposition of DPY-14 collagen around the sensilla.

## Materials and methods

### Nematode strains

We maintained strains of *C. elegans* as described (Brenner 1974). We employed the following strains: CB188 [*dpy-14(e188)I*], CB907 [*dpy-5(e907)I*], BC14109 (Ex[*bbs-1*p::GFP; *dpy-5*(+)]). In addition, we generated three independent transgenic lines carrying the *dpy-14*p::GFP construct in extrachromosomal arrays (BC13559, Ex[*dpy-5(e907)*; *dpy-14*p::GFP; *dpy-5*(+)]; BC6830, Ex[*dpy-5(e907)*; *dpy-4*p::GFP-PEST; *dpy-5*(+)]; BC6831 Ex[*dpy-5(e907)*; *dpy-14*p::GFP-PEST; *dpy-5*(+)) and one stable integrand (BC13560, [*dpy-5(e907)*; *dpy-14*p::GFP; *dpy-5*(+)]). The strain carrying the COL-19::GFP construct was kindly provided by A.P. Page.

### Generation of *dpy-14*p::GFP fusion constructs

Promoter::GFP fusion constructs were generated by "stitching PCR" (see Hobert 2002). Polymerase chain reactions (PCRs) employed the PCR machine DNA Engine Dyad, MJ Research. All PCR reactions made use of the Expand Long Template PCR System (Roche). The whole intergenic region between *dpy-14* (H27M09.4) and the immediately upstream gene was fused to GFP. Primer sequences are available upon request.

### Generation of transgenic lines

BC907 [*dpy-5(e907)I*] worms were microinjected with a Zeiss axioscope at 160× magnification. The injection mix contained *dpy-5*(+) rescue plasmid (pCeh361, kindly provided by C. Thacker and A. Rose) at a concentration of 86 ng/µl and *dpy-14*p::GFP construct at a concentration of ~30 ng/µl.

For the generation of strain BC13560, integration was achieved by irradiating BC13559 transgenic worms with 15 grey (1500 R) of X-rays.

### Analysis of SAGE data

SAGE data were obtained from the Genome BC *C. elegans* Gene Expression Consortium (http://www.elegans.bcgsc.bc.ca/; McKay et al. 2003).

### Functional and structural assessment of amphid channels

Assays for water permeability were performed on N2, *dpy-5(e907)* and *dpy-14(e188)* animals. Worms on OP50 plates were washed with 1 ml of autoclaved distilled water, and then left in water at room temperature for 40 min.

Lectin staining was performed as described (Ko and Chow 2000), adding 50 µl of fluorescein isothiocyanate (FITC)-lectin (Sigma Aldrich) to worms in 50 µl of M9 buffer. Dpy-14 worms were grown at 25°C. FITC-lectin fluorescence was assessed on a Zeiss axioscope employing the GFP filter.

### Microscopy

A Zeiss axioscope equipped with a QImaging camera was employed for GFP expression analysis and differential interference contrast (DIC) microscopy. Slides were usually prepared by washing worms in M9 buffer and immobilizing them by $NaN_3$ (500 mM) treatment. For water permeability experiments, levamisole (1 M in M9 buffer) was used instead of azide.

### Assessment of sensitivity to alkaline solution

A drop of alkaline hypochlorite solution (10 ml of 6% Javex Bleach, 25.0 ml of NaOH, 15.0 ml of distilled

water) was deposited on 5-cm OP50 plates, away from the bacterial medium. Ten worms were then placed directly in the drop. When embryos were used, as many embryos as possible were scooped up with a metal pick and deposited in the solution.

### Chemotaxis to lysine

Assays were performed as described (Vowels and Thomas 1994). Minimal media were prepared with 0.005 M Potassium Phosphate, 0.001 M $CaCl_2$, 0.001 M $MgSO_4$ and agar in distilled water. The experimental spot was prepared by making a 0.5 M Lys solution in minimal medium. Worms were grown at 15°C.

### Osmolarity assays

Assays were performed as described (Culotti and Russell 1978). Worms were grown at 15°C.

## Results

### The Dpy-14 phenotype arises from a mutation in a collagen gene

Dpy-14(I) phenotype was partially rescued by cosmids that cover several genes, including one designated *col-59* (Prasad et al. 1993). Inhibition of expression of *col-59* using double-stranded RNA (RNAi) resulted in embryonic lethality, first larval stage (L1) lethality and a small phenotype (Maeda et al. 2001), phenotypes reminiscent of Dpy-14. In order to prove the molecular identity of *dpy-14*, genomic DNA from a strain homozygous for the *dpy-14* canonical allele, CB188 [*dpy-14(e188)I*], was extracted and amplified by PCR. DNA sequencing revealed a G → A transition within the third exon of the collagen gene. Additional sequencing verified that the observed nucleotide substitution was not an artefact of the PCR amplification, because it was confirmed by independently sequencing two overlapping regions on the coding strand, as well as by sequencing the complementary sequence on the minus strand.

The mutation in *e188* alters the codon GGA (Gly) to AGA (Arg). This G139R mutation affects a Gly in the first collagen-like Gly–X–Y domain. The Gly–X–Y repeats are thought to be indispensable for the trimerization of the procollagen monomers that leads to the formation of the collagen triple-helix (Bella et al. 1994). Such collagen abnormality could explain the morphological mutant phenotype observed in Dpy-14 animals.

Alignment of the amino acid sequences of DPY-14, COL-165 and ROL-8 shows a consistent pattern formed by three blocks of conserved Cys residues (Fig. 1). These conserved residues allow a categorization of *C. elegans* collagens (Johnstone 2000) and classify DPY-14 and its cognate procollagens COL-165 and ROL-8 as members of Group 2.

As a further step in the molecular characterisation of *dpy-14*, we successfully rescued the Dpy-14 phenotype with a full-length construct of the collagen gene (data not shown).

### Study of *dpy-14* expression pattern

Our approach to the study of the expression pattern of *dpy-14* involved analysis of SAGE data and construction of *dpy-14* promoter::GFP (*dpy-14*p::GFP) reporter fusions. SAGE libraries, for different tissues, are obtained by isolating specific classes of cells with fluorescence-activated cell sorting techniques (McKay et al. 2003) and are made public by the *C. elegans* Gene Expression Consortium. SAGE data provide a reliable and high-throughput method to gain insight into the level of transcription of a gene in a specific tissue or at a specific time in development. SAGE data for *dpy-14* revealed a peculiar expression profile for a collagen gene (Fig. 2). First, *dpy-14* was almost exclusively expressed during embryogenesis (Fig. 2a). Second, not only was *dpy-14* strongly expressed in the hypodermis (278 tags)—as would be expected for a collagen gene giving rise to a Dpy phenotype—but the gene also showed surprisingly strong expression in the ciliated neurons (138 tags; Fig. 2b). This embryonic hypodermal and neural expression profile makes *dpy-14* unique amongst *C. elegans* collagens.

We sought corroboration of the SAGE data by construction of a transgenic line carrying the *dpy-14*p::GFP fusion construct. Because extrachromosomal arrays are sometimes lost during mitotic cell division (Stinchcomb et al. 1985; Mello et al. 1991), and the resulting mosaicism could affect the observed reporter expression pattern, we created a transgenic line (BC13560) with a stably inherited *dpy-14*p::GFP construct putatively integrated into the genome. The stable transgenic line was examined using GFP microscopy. We found that *dpy-14* expression starts at the "16 E cell period", a stage when the embryo consists of 224–500 cells and 4.25–5.5 h have elapsed since fertilization (von Ehrenstein and Schierenberg 1980; Fig. 3a–c).

As embryogenesis progresses, expression of *dpy-14* becomes clearly hypodermal and is mainly localized to hyp7 (Fig. 3d–f), a large syncytial cell that eventually envelops most of the body of the adult *C. elegans* (Yochem et al. 1998).

In addition to embryonic expression, as expected from SAGE data, we observed some GFP fluorescence in the hyp7 syncytium of L1 worms, but not at later larval stages or in adults (Fig. 3g–i). This L1 expression is probably an artefact due to the relatively long half-life of GFP (Inouye and Tsuji 1994; Li et al. 1998). This makes *dpy-14* the earliest-expressing *C. elegans* collagen gene reported to date (Gilleard et al. 1997).

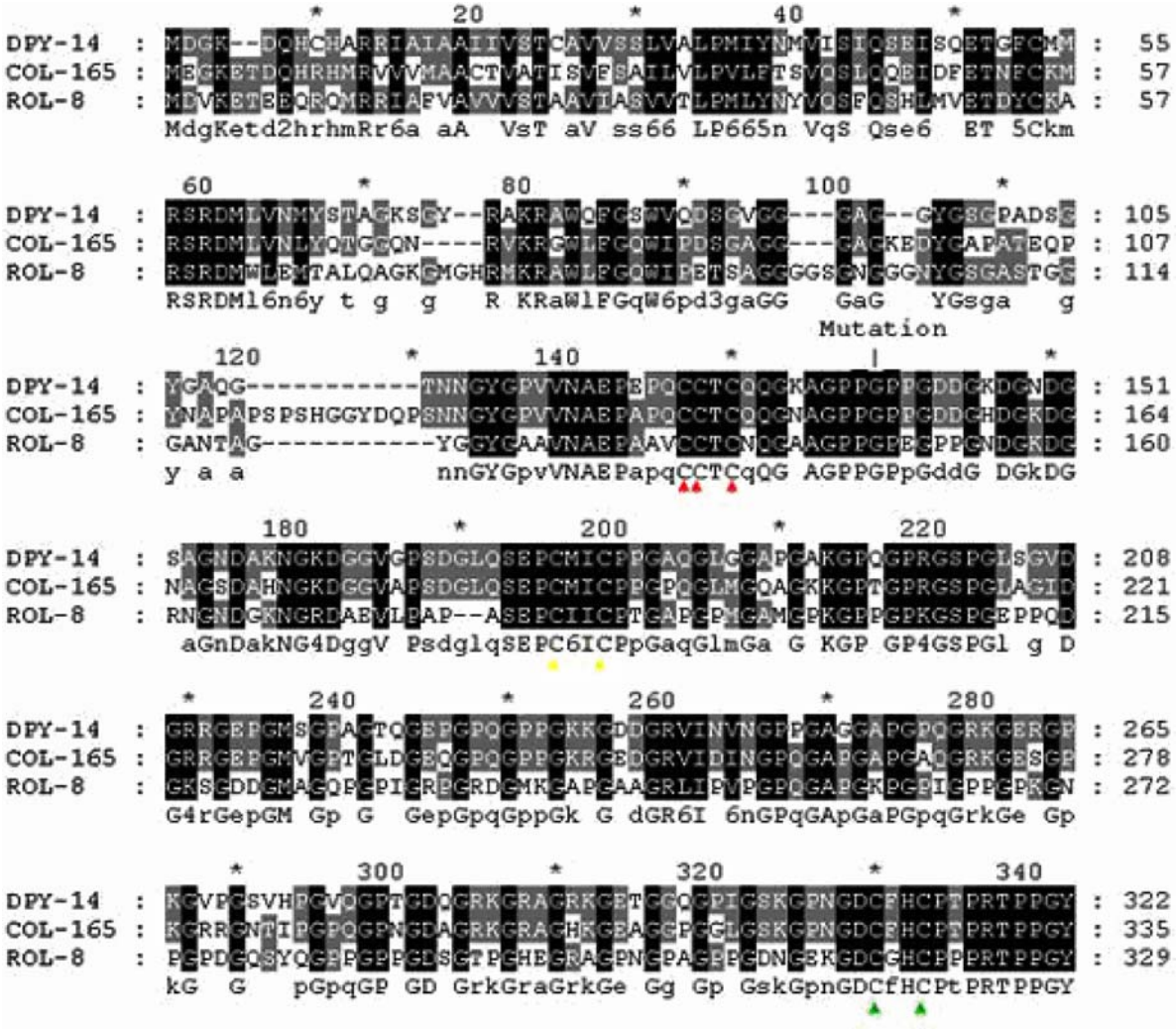


**Fig. 1** The Gly mutated in *dpy-14(e188)* is highly conserved among *C. elegans* collagens. Alignment of the amino acid sequence of DPY-14 and its two most closely related *C. elegans* collagens, COL-165 and ROL-8, is shown. The mutated Gly at position 139 in DPY-14 is indicated. *Arrows* point to conserved Cys residues, which can be used to assign collagens to specific classes. *Red arrows* show three conserved Cys in domain I (N-terminal of the first Gly–X–Y domain); *yellow arrows* point to two Cys in domain II (between the two Gly–X–Y domains); *green arrows* point to the last block of two conserved Cys in domain III (C-terminal to the second Gly–X–Y domain). This pattern of conserved Cys residues enables classification of DPY-14 as a Group 2 procollagen

We performed a detailed analysis of the specific cells that express *dpy-14* during embryo development. We particularly looked at embryos at the ''lima bean stage'', which occurs between 5.50 and 6.25 h after fertilization at 20°C, and at embryos at the ''plum stage'', which occurs 7.00–7.25 h after fertilization (von Ehrenstein and Schierenberg 1980). At the lima bean stage the neural and hypodermal tissues are not fully differentiated yet, while at the plum stage differentiation is complete (Sulston et al. 1983). SAGE data indicated predominant expression of the *dpy-14* transcript in ciliated neurons and hypodermis (Fig. 2). GFP expression of *dpy-14* at the lima bean stage was mainly localized to cells of AB lineage that may be grouped into two categories based on their potential to differentiate into hypodermal cells only (AB.alaa, AB.alap, AB.arpa, AB.arap, AB.arpp and AB.alpa), or into either hypodermal cells or ciliated neurons (AB.praa, AB.prap, AB.plpp, AB.plpa and AB.alpp; Sulston et al. 1983). Most of these hypodermal cells are ciliated neuron support cells (Table 1).

At the plum stage, expression was localized to hyp7 nuclei and to some amphids (ADFL, AFDL, ASEL, ASHL, ASJL, ASKL and RIAL) and amphid support cells (IlshL and OLQsoVL; Table 1).

The expression profile, shown by GFP reporter expression, was therefore compatible with the SAGE data, and was consistent in all four transgenic lines we generated.

## DPY-14 mutations affect amphid channel permeability and L1 cuticle in a temperature-dependent fashion

We observed expression of the GFP reporter construct in ciliated neuron support cell progenitors as well as in

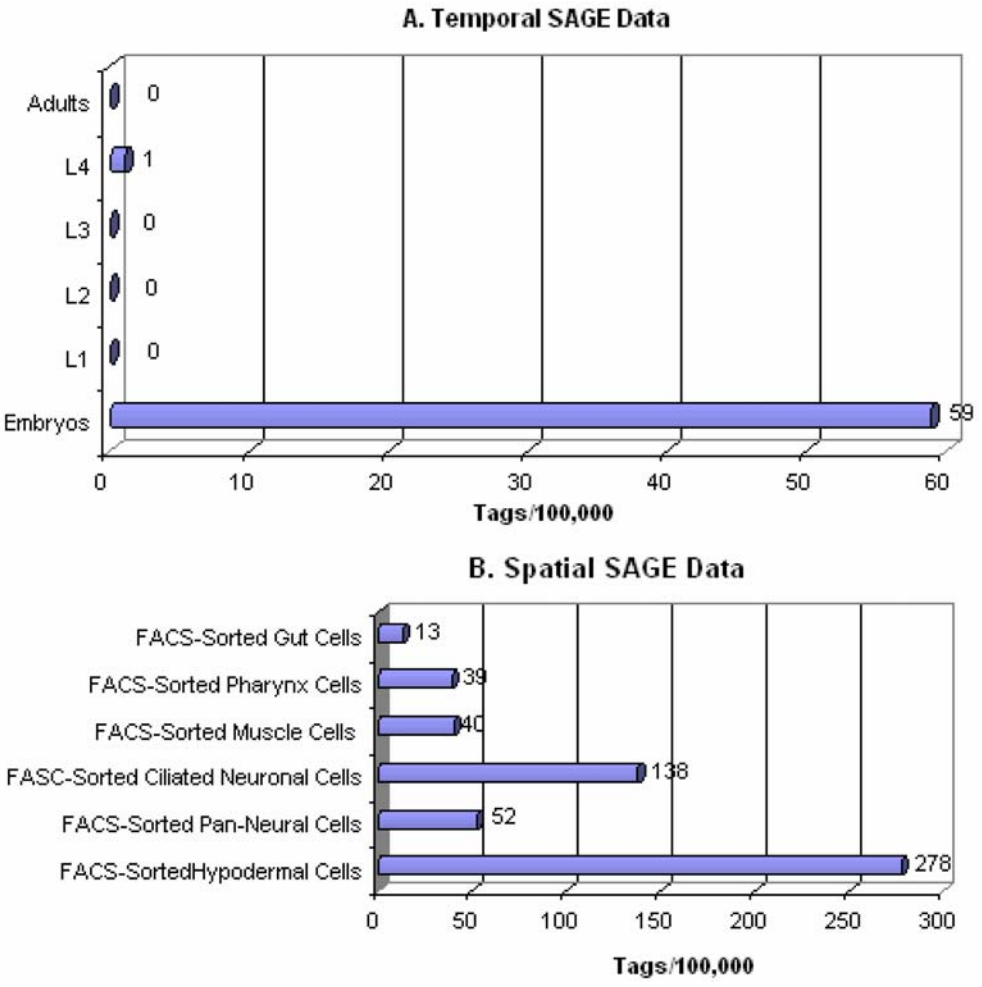


**Fig. 2** SAGE data describing the *dpy-14* expression profile. **a** Temporal expression profile. **b** Spatial expression profile. Tag counts for *dpy-14* were normalized to 100,000 tags, and only SAGE tag #1 was taken into account for the construction of the histograms

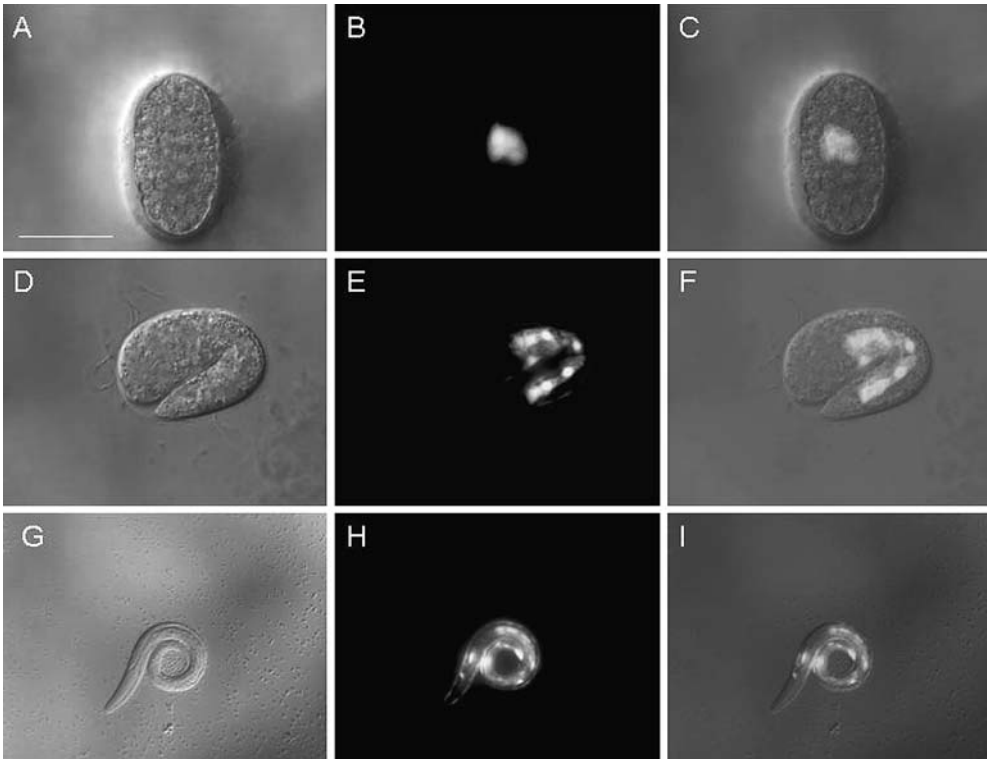


**Fig. 3** Expression pattern for *dpy-14*. **a** DIC image, **b** GFP image and **c** overlay of an embryo at the 16 E cell period expressing the *dpy-14*p::GFP construct. This constitutes the earliest expression point for *dpy-14*. **d** DIC image, **e** GFP image and **f** overlay of a transgenic embryo at late plum stage. **g** DIC image, **h** GFP image and **i** overlay of an L1 animal. Expression at late plum stage and L1 is localized to the nuclei of the hyp7 syncytium. L1 is the latest developmental stage when transgene expression is observable

fully differentiated support cells (Table 1). These cells are responsible for the integrity of the amphid channel. We therefore investigated the possibility that the mutation in *dpy-14* results in functionally defective amphid channels by assessing their water permeability. We placed *dpy-14(e188)* worms grown at 20 and 25°C, along with wild-type (N2) and *dpy-5(e907)* control animals, in distilled water for 40 min. DIC microscopy was used to examine animals immobilized with levamisole. We observed that azide caused water permeability, probably due to its ionic nature, and thus was not used for these experiments. Levamisole is a cholinergic agonist that induces spastic immobilization of the animals (Harrow and Gration 1985) but avoids the osmotic effects produced by azide.

Our results show that Dpy-14 adults grown at 20°C did not exhibit increased morphological defects due to osmotic pressure, relative to controls (Fig. 4). However, Dpy-14 adults temperature shifted to 25°C showed increased permeability to water in the region of the amphid channels. On the other hand, L1 Dpy-14 animals displayed water permeability in the amphid channels when raised either at 20 or 25°C, but the phenotype at the latter temperature was the most severe. Water leakage in the amphid channels of Dpy-14 animals is therefore a phenotype consistently observed at different stages of animal development and increases in severity in a temperature-dependent fashion.

In the water permeability assay, Dpy-5 controls gave similar results to wild-type worms. Water permeability observed in the amphid channel region of Dpy-14 animals is therefore a characteristic phenotype of these mutants resulting from defective DPY-14 collagen, and not a general consequence of cuticular defects resulting in Dpy phenotypes.

The phenotype of *dpy-14(e188)* is temperature sensitive. At 15°C individuals are wild-type in appearance, whereas they are Dumpy and lethal (developmentally arrested and sterile) at 25°C and Dumpy at intermediate temperatures. By temperature-shifting adult *dpy-14* mutants, we in fact observed a progressive increase in the severity of the phenotype of their progeny at increasing temperatures. Prominent among the morphological defects were vulval protrusions, inability to move and an egg-laying defective (Egl) phenotype. Growth curves for 15° and 25° are presented in Fig. 5. Adult worms were transferred from 15° to 25° 24 h prior to setting up $P_0$s for the 25° growth curve. Only nine of twenty, randomly chosen, $F_1$s matured and the growth curve is based on them. These nine gave some progeny (between 3 and 15). The worms grown at 25° matured when they were approximately 0.6 mm and they average

**Table 1** AB cell lineage that show *dpy-14*p::GFP reporter expression at the lima bean stage and cells expressing the gene at the plum stage (adapted from Sulston et al. 1983)

| | Progenitor cell | Ciliated neuron | Hypodermal cell |
|---|---|---|---|
| Lima bean stag embryo | AB.alaa | | CEPsoVR, ILshL, ILsoL, IlshR, ILsoR, ILshDL, ILshDR, OLQsoVR |
| | AB.alap | | CEPsoDL, CEPsoDR, OLLsoL, OLLsoR |
| | AB.arpa | | OLQshDL, OLQsoDL, OLQshDR, OLQsoDR, CEPshDL, CEPshDR |
| | AB.arap | | ILshVR, IlsoVR |
| | AB.praa | ADFR, ADLR, AFDR, AIBR, ASER, ASGR, ASIR, ASJR, ASKR | hyp3, hyp4, hyp6, hyp7 |
| | AB.arpp | | ADEshL |
| | AB.prap | AIZR | hyp7 |
| | AB.plpp | AIAL, PHAL | PHshL |
| | AB.plpa | AIYL | AMsoL, AMshL, CEPshVL |
| | AB.alpp | ADFL, ADLL, AFDL, ASER | CEPsoVL, ILshVL, ILsoVL, OLLshVL, OLQshVL, OLQsoVL |
| | AB.alpa | | hyp1, hyp2 |

| | Cell | Cell type |
|---|---|---|
| Plum stage embryo | Hyp7 | Hypodermal |
| | OLQsoVL | Hypodermal, amphid socket cell |
| | IlshL | Hypodermal, amphid sheath cell |
| | OLQVL | Neuron, outer labial quadrant sensilla |
| | RIAL | Ring interneuron |
| | AFDL | Amphid neuron |
| | ADFL | Amphid neuron |
| | ASEL | Amphid neuron |
| | ASHL | Amphid neuron |
| | ASJL | Amphid neuron |
| | ASKL | Amphid neuron |

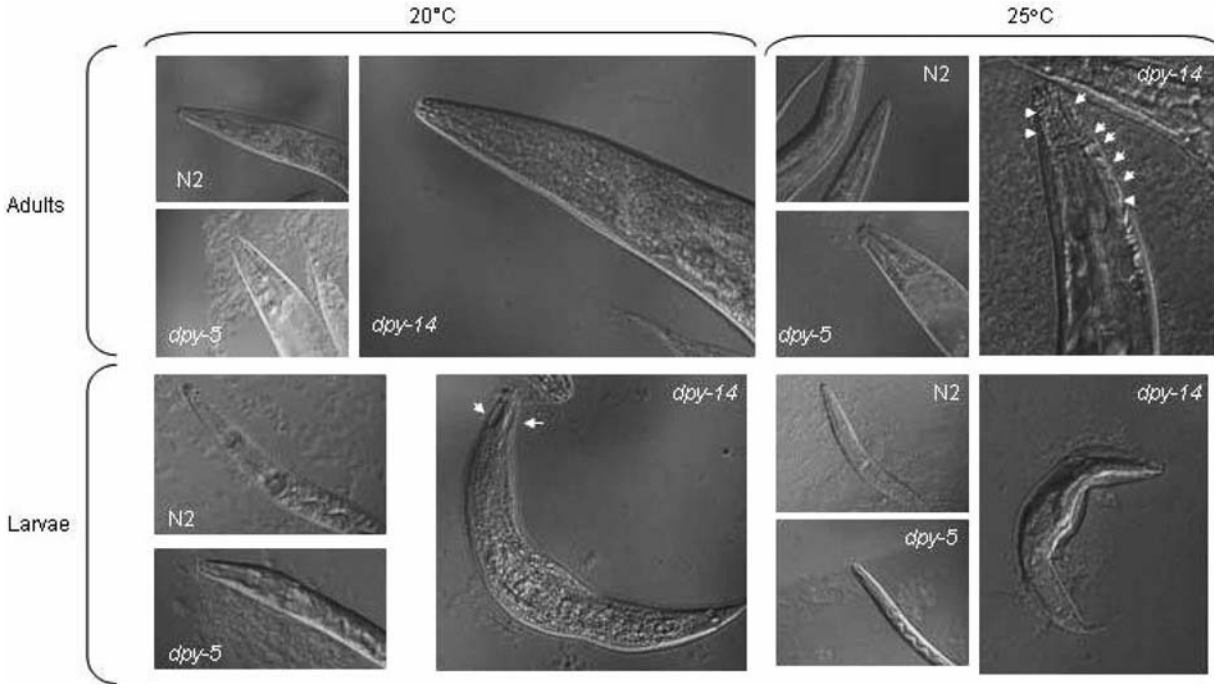


**Fig. 4** Effects of osmotic pressure on animals grown at 20 and 25°C. While Dpy-14 adults grown at 20°C do not show increased permeability to water with respect to controls, Dpy-14 larvae undergo water leakage, particularly in the amphid region, as indicated by the *arrows*. Also Dpy-14 adults grown at 25°C show water permeability in a region corresponding to the amphid channels (as indicated by the *arrows*). The osmotic effects on Dpy-14 larvae, raised at 25°C, were more pronounced than in adults, suggesting that DPY-14 is a quantitatively more important constituent of the cuticle of L1 larvae than of adult animals

0.95 mm final length. The worms grown at 15° matured when they were approximately 0.9 mm and reached an average final length of 1.15 mm. Dpy-14 grown at 25° matured at the same rate as wild-type (see Wood 1988b) which is approximately one-third of the time that it takes Dpy-14 to mature at 15°. From this we categorized worm length at maturity as either long ($>0.8$ mm) or short ($\leq 0.8$ mm), and fully fertile ($>50$ $F_1$ progeny). We did temperature shift experiments to try to determine the time in development at which DPY-14 is essential and determines length. For worms laid at 15° and transferred to 25° the critical time for length is between 5 and 8 h after being laid. At 5 h 75% of $F_1$s are short whereas at 8 h, 83% are long (at 6 and 7 h there were about equal numbers of long and short). At 8 h 75% of the long worms were fully fertile whereas 14% of long worms transferred at earlier times (5–7 h inclusive) were fully fertile. As for the short worms, none proved to be fertile (0/12 shifted at 5 h, 0/6 shifted at 8 h or later). Worms grown at 25° develop approximately three times as fast as worms grown at 15° (see Fig. 5). Therefore we did transfers from 25° to 15° at 20-min intervals (0–200 min). Effectively all $F_1$s transferred at 80 min (and earlier) matured into fully fertile (50/53), long (52/53) worms. Between 100 and 160 min inclusive 4/21 (20%) were short and not fertile while 15/21 were long and fully fertile (the other two longs were not fertile). At 180 min 3/4 were short and not fertile while one was long and fertile. At 200 min 4/5 were short and not fertile, one was long and not fertile. We tested whether or not the lethality is a consequence of thermal instability of the DPY-14 collagen. We reasoned that since *dpy-14* expression is mainly embryonic, the encoded collagen should be a major component of only L1 cuticle.

We therefore tested *dpy-14* worms with alkaline hypochlorite wash, a solution that normally dissolves the cuticle of adult animals, while leaving their eggs intact, which hatch to produce a synchronous population.

Dpy-14 and N2 worms were grown for at least three generations at 15°C, after which ten gravid worms were placed in a drop of alkaline hypochlorite solution at different dilutions. Plates were then placed at either 15 or 25°C. Surprisingly, the alkaline hypochlorite solution was lethal to Dpy-14 embryos in a temperature-dependent manner (Table 2). At 15°C an 80% concentration resulted in lethality, whereas at 25°C a 20% concentration was sufficient. We repeated the experiment described above by directly placing Dpy-14 and N2 embryos into drops of alkaline hypochlorite solution of different concentrations. The results fully reproduced the ones obtained with adults (data not shown).

## *dpy-14* is required for formation of cuticular structures in the amphid channels and proper development of the seam cells

Previous work (Link et al. 1988, 1992) showed that *C. elegans* mutants with aberrant cuticles expose antigens

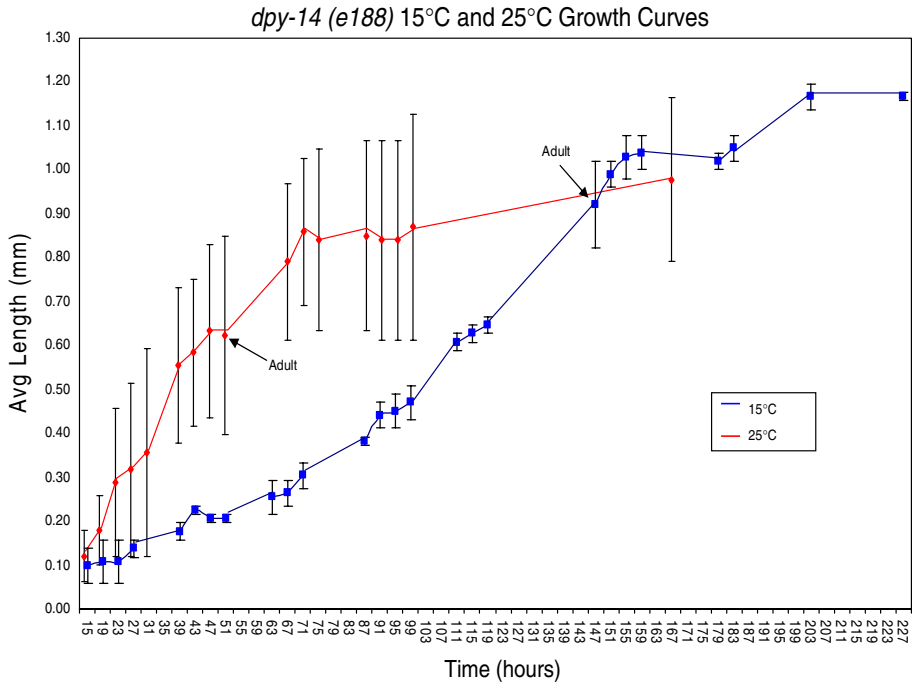


**Fig. 5** *dpy-14(e188)* growth curves at 15 and 25°C. Dpy-14 has been reported to be a temperature sensitive (TS) lethal at 25°C (see Wood 1988a). The 15° growth curve was generated from six randomly selected nematodes. The *bars* represent the size range of those nematodes. For the 25° curve 15 $P_0$s plates with a total of 206 $P_0$s were set up for 1.5 h. The numbers of $F_1$ eggs laid were counted (89 or 0.43 eggs/$P_0$). 20 $F_1$s were chosen at random. Nine of those matured and laid eggs (between 3 and 15)—these are the ones used for the growth curve. The other 11 died without giving progeny: five died without hatching while six hatched but did not mature to adulthood. In addition to those 20, we set up a further 30 to check for mature progeny. Out of the 50 set up 14 died w/o hatching, 16 hatched but did not mature, while 20 matured and gave $F_1$ progeny. None of the $F_1$ gave $F_2$ progeny. Dpy-14 does not appear to affect rate of maturation relative to N2 (wild-type) worm. N2 grown at 25° mature in approximately 46–47 h after being laid (see Wood 1988b)

**Table 2** Test to determine the concentration of alkaline hypochlorite (AH) solution that is lethal to *dpy-14(e188)* animals at different temperatures

| Determination of alkaline hypochlorite lethal concentration | | | |
|---|---|---|---|
| Temperature (°C) | Alkaline solution dilution | Dpy-14 growth | N2 growth |
| 15 | No solution | + | + |
| | 1/5 | + | + |
| | 2/5 | + | + |
| | 3/5 | + | + |
| | 4/5 | – | + |
| | No dilution | – | + |
| 25 | No solution | + | + |
| | 1/5 | + | + |
| | 2/5 | – | + |
| | 3/5 | – | + |
| | 4/5 | – | + |
| | No dilution | – | + |

The original concentration of AH solution was 1 M. Results showed that at 15°C, temperature at which mutant animals have wild-type phenotype, a concentration above 4/5 (or 80% of the original 1 M concentration) is required to cause lethality. At 25°C, which is the restrictive temperature for animals carrying the *e188* allele, a dilution to less that 1/5 (or 20%) of the original 1 M concentration is enough to impede animal development. + signs indicate growth, – signs indicate no growth on the plates examined

that are bound by lectins. We therefore performed staining of Dpy-14 animals with FITC-conjugated lectins in order to visualize structural abnormalities of their cuticle. Dpy-14 worms grown at 25°C showed abnormal staining of their amphid channels (Fig. 6). This finding confirmed that the cuticular lining of the amphid channels is defective in Dpy-14 mutants, and corroborates the hypothesis that these defects are responsible for the abnormal levels of water leakage observed in the head region of Dpy-14 animals (Fig. 4).

In order to assess other cuticular defects caused by the *dpy-14* mutation, we crossed the COL-19::GFP fusion construct (kindly provided by A.P. Page, UK) into *dpy-14(e188)* animals. COL-19 is an effective marker for adult cuticle morphology (Thein et al. 2003). Our results show that, while in wild-type worms seam cells fuse at mid-L4 (fourth larval stage) to form two continuous lateral syncytia (Podbilewicz and White 1994), in Dpy-14 worms not all seam cells fuse (Fig. 7, arrows). Moreover, annuli are irregularly spaced and misaligned, compared to wild-type. We also observed that GFP expression is absent from some areas of the cuticle, suggesting additional cuticular abnormalities (Fig. 7, arrowhead). Thein et al. (2003) defined five classes of adult cuticle collagen disruption observable with the COL-19::GFP marker, but the defect in seam

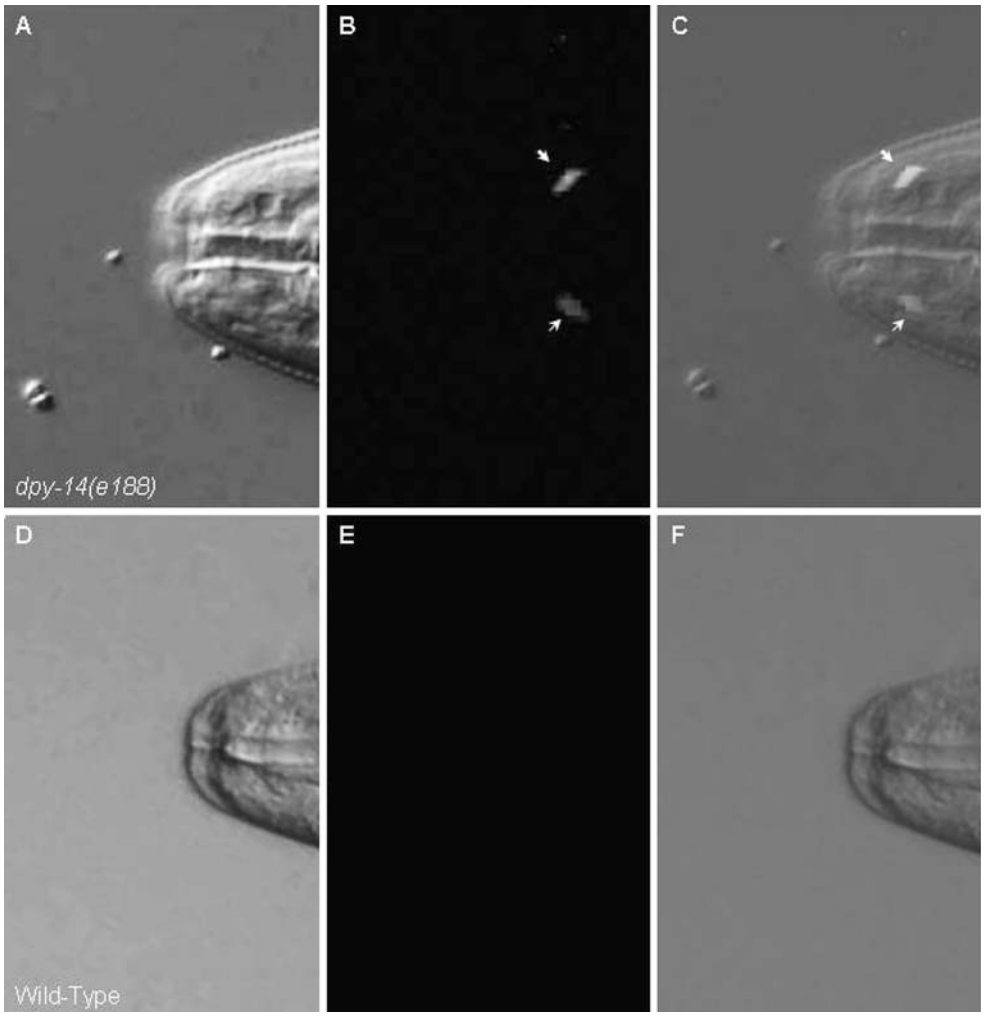


**Fig. 6** Mutation in *dpy-14* results in structural abnormalities in the amphid channels. Lectin staining allows visualization of abnormalities in the *C. elegans* cuticle. The results indicate that *dpy-14(e188)* animals raised at 25°C stain at the tip of their amphid channels. N2 wild-type worms did not stain. **a** DIC image, **b** lectin staining image and **c** overlay picture of a Dpy-14 animal. **d** DIC image, **e** lectin staining image and **f** overlay of a wild-type animal. Lectin staining images were taken with a GFP filter, since FITC, in this experiment, conjugated to lectins has similar spectral properties to GFP

cell fusion observed in *dpy-14* worms was not reported. The ''unfused seam cells'' phenotype therefore adds a sixth class of cuticular defects caused by collagen mutations.

## Mutation in *dpy-14* does not compromise amphid structure and function

We examined the morphology of the amphids, a subclass of ciliated neuron. In fact SAGE data indicate *dpy-14* expression in the ciliated neurons, and our *dpy-14*p::GFP constructs corroborated expression of this gene in some head amphids and in seven of their precursor cells of AB lineage (Table 1). Because amphids are open to the environment, they have the exploitable property of being stainable by dye filling (Starich et al. 1995). We therefore performed amphid staining with DiI filling dye and compared amphid morphology of animals grown at 20 and 25°C.

DiI staining indicated that amphids of Dpy-14 animals are morphologically indistinguishable from N2 wild-type and Dpy-5 controls. This was true for both adults and larvae grown either at permissive or restrictive temperatures (data not shown). Thus, we conclude that DPY-14 is not a structural component of the amphids.

*dpy-14* expression was observed in AB progenitor cells with the potential to differentiate into ASER, ASGR, ASIR and ASKR amphids, and in fully differentiated ASEL and ASKL, all of which are responsible for chemotaxis to lysine (Bargmann and Horvitz 1991; Pierce-Shimomura et al. 2001). It was determined that in a gradient of chemoattractant, worms move towards and accumulate at the peak of the gradient (Ward et al. 1975). Screens for defects in chemotactic responses to volatile (Ansley et al. 2003) or non-volatile compounds (Dusenberry et al. 1975; Bargmann et al. 1993) have proven to be effective for identifying mutants with compromised chemotaxis. We therefore performed assays for chemotaxis to Lys by adapting a previously developed assay for chemotaxis to non-volatile compounds (Vowels et al. 1994). Wild-type N2 worms were used as a control. Results indicated that differences in chemotaxis index (CI) between Dpy-14 animals ($CI = 0.375$, $SD = 0.177$, $n = 52$) and N2 worms

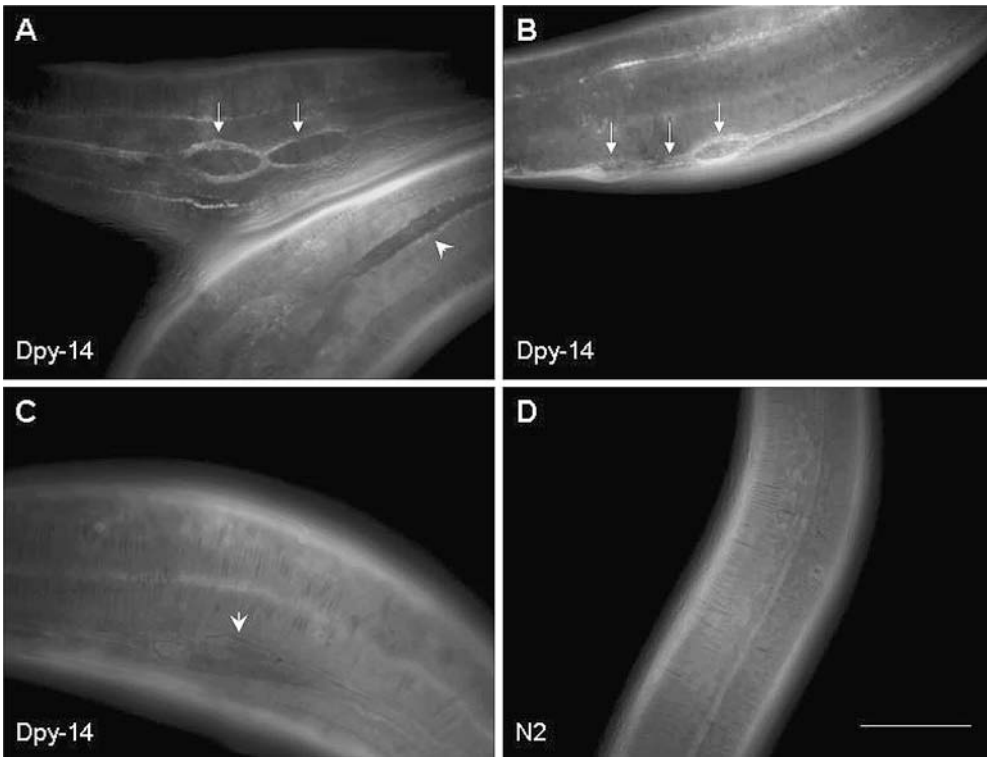


**Fig. 7** **a**–**c** Dpy-14 animals. **d** N2 animal. The COL-19::GFP translational fusion construct was crossed in *dpy-14(e188)* mutants. GFP expression allowed to visualize the extent of morphological defects in these animals. The most obvious defect is that not all seam cells properly fuse (*arrows*). Cuticular defects are identified by patches with no GFP expression (*arrowheads*) and by irregular spacing and orientation of annuli. *Bar* indicates 50 μm

(CI = 0.278, SD = 0.204, $n = 139$) were not statistically relevant, thereby showing that chemotaxis to Lys was not compromised in Dpy-14 mutants.

Dpy-14 animals were also tested for their ability to avoid solutions of high-osmolarity, also a diagnostic feature of functional amphids (Culotti and Russell 1978). None of the Dpy-14 animals tested displayed abnormal behaviour ($n = 30$). This further confirms that amphids of Dpy-14 animals are functional.

## Discussion

We show that *dpy-14* of *C. elegans* encodes a cuticular collagen. It is the earliest-expressing collagen gene and is localized to embryonic precursors of hypoderm and ciliated neurons and in some fully differentiated amphids. One might expect DPY-14 to be a major component of early larval cuticle due to its embryonic expression. However our results indicate DPY-14 is also part of the ECM that surrounds the head amphids. This is reinforced by the fact that the cuticle in the amphid channels is structurally abnormal, as indicated by lectin staining. It has been proposed that lectin binds to nematodes because they lack a cortical cuticular layer that masks endogenous carbohydrates (Link et al. 1992). It is therefore plausible that lectin binding to the amphid channels of Dpy-14 animals is due to absence or reduced levels of DPY-14 collagen. This hypothesis is compatible with the notion that replacement of Gly residues in collagen Gly–X–Y domains, as per *dpy-14(e188)*, inhibits formation of proper triple-helices (Kramer 1994). The consequent misfolding results in abnormal modification and degradation of most of the mutant collagens and, if involved in heterotrimer formation, any other collagen associated with it (Prockop and Kirivikko 1995). Degradation of misfolded collagen homo- or heterotrimers, due to the presence of DPY-14, could result in absence of cuticular components and thus exposure of antigens that can be bound by lectins.

Lectin staining suggests that DPY-14 localizes to the ECM of the amphid channels. This extracellular structure, if defective, could fail to provide the animals with an appropriate sealing from the external aqueous environment at all developmental stages, thus explaining the severe water leakage observed in *dpy-14* worms.

Since *dpy-14* is expressed in the amphids, we tested these neural structures for structural or functional abnormalities using dye filling staining. Dpy-14 neural structures were indistinguishable from wild-type, thereby indicating that DPY-14 is not a major structural component of the amphids.

A subset of the ciliated neurons that express *dpy-14* is responsible for positive chemotaxis to Lys. We hence tested whether these amphids are functional in Dpy-14 animals. The results indicate that both wild-type and Dpy-14 worms chemotax to Lys at equivalent rates. Moreover, both wild-type and Dpy-14 animals avoid swimming through high-osmolarity solutions. Normal levels of positive chemotaxis to Lys and normal responses to high-osmolarity solutions indicate that the amphids of *dpy-14(e188)* animals are functionally unaffected.

For the behavioural assays we performed, test and control animals were grown at 15°C, the permissive temperature for Dpy-14 worms. At this temperature, mutants are phenotypically close to wild-type and move on agar plates at the same rate as wild-type worms. On the contrary, Dpy-14 animals shifted (when young) to or grown at the 25°C restrictive temperature are usually strong Dpy and move much slower than wild-type animals. Mutants grown at the restrictive temperature could not therefore be used for chemotaxis assays. Hence we cannot exclude the possibility that higher temperatures prejudice the chemotactic responses of Dpy-14 individuals.

*dpy-14* is a unique collagen gene in that it is expressed not only in the hypoderm but also in the ciliated neurons, as showed by the strong SAGE data and GFP reporter expression. The fact that *dpy-14* is expressed in the amphids but does not seem to be one of their structural components seems paradoxical. To resolve this we hypothesize that *dpy-14* expression represents an example of functional cooperation between ciliated neurons and their support cells. Microscopy reconstructions of the intimate relationship between ciliated neurons and their support cells have been done (Ward et al. 1975; Wright 1980). We propose that belt junctions are used by the sheath cells to recruit DPY-14 from the ciliated neurons and from the socket cells, and that this collagen is then exocytosed by these support cells to become part of the amphid channel lining. Alternatively, it is possible that there are other means for the ciliated neurons to exocytose DPY-14 into their surrounding matrix without the mediation of the support cells. Either way, the end result is that ciliated neurons and their support cells transcribe and synthesise the DPY-14 collagen and deposit it into the amphid channels. This represents an example of functional cooperation between neural and hypodermal cells in the creation of structures required for proper embryo development. The rationale behind such cooperation could be that DPY-14 is a component of the ECM required for the proper topological organization of the ciliated neurons and for their protection from external stresses. Previous work demonstrated that the collagen MEC-5 is important for mechanosensation and mutations that affect its Gly–X–Y domains cause touch insensitivity (Du et al. 1996). Subsequent research demonstrated that precise organization of ECM collagens is necessary for appropriate mechanosensory responses (Emtage et al. 2004). It is therefore plausible that DPY-14 is a component of the ECM surrounding the amphids and provides shielding from external stresses to the animals. Our results indicate that DPY-14 functions in (1) prevention of water leakage into the amphid channels, and (2) disruption of neuronal transduction by ionic substances. These tasks are accomplished by sealing the amphid channels from their external environment. These roles for DPY-14 would provide a rationale for cooperative and concerted synthesis of this collagen by ciliated neurons and their hypodermal support cells.

Inability of Dpy-14 individuals to sustain environmental stresses seems to be dependent on temperature, as exemplified by their increased sensitivity to alkaline hypochlorite wash and water leakage at the 25°C restrictive temperature. These findings suggest that weakness of the collagenous cuticle is caused by thermal instability of mutant DPY-14. Sequencing of the *dpy-14(e188)* allele showed the presence of a G139R substitution in the first Gly–X–Y collagen-like domain. Crystallographic studies have shown that collagen triple-helices are formed by N--H (Gly) $\cdots$ O = C (X position) interactions between Gly–X–Y repeats of two different monomers (Bella et al. 1994), and that these interactions are mediated by water molecules (Kramer et al. 1999). We propose that at 15°C the local disruption of the triple-helical structure caused by the G139R substitution (Bella et al. 1994) does not fully compromise DPY-14 functionality, since the mutation is in a peripheral position within the trimerization domain. This would explain the nearly wild-type phenotype of *dpy-14(e188)* animals at the permissive temperature. However, at higher temperatures, a combination of steric hindrance and increased atomic thermal motion of the Arg side chain might disrupt the ability of water molecules to mediate interactions between collagen monomers. This would explain the increased severity of the Dpy-14 phenotype observed above 15°C. Increased instability of the cuticle in a temperature-dependent fashion could decrease the ability of *dpy-14(e188)* animals to sustain environmental stresses, thereby providing an explanation for the lethality of the allele at the restrictive temperature.

An important question to be answered is why *dpy-14*, a cuticular collagen expressed almost exclusively during embryogenesis, affects the morphology of animals at all stages. If in fact a defective cuticle is the cause of the Dpy phenotype, animals should be phenotypically wild-type from L2 (second larval stage) onwards. Our work shows that *dpy-14* mutants have abnormal seam cell syncytia. Therefore, mutation in this collagen gene not only does affect the L1 cuticle, but also induces subtle morphogenetic aberrations in these epithelial cells that result in their defective fusion. Abnormal epithelial cells could be the ultimate cause of the Dpy-14 phenotype, highlighting the importance of this gene during animal development. Importantly, this unfused seam cells phenotype was not previously observed in other collagen mutants using the COL-19::GFP marker (Thein et al. 2003), and it adds new elements to our knowledge of how collagens can affect animal development.

In conclusion, *dpy-14* occupies a unique place in the *C. elegans* collagen family, since its gene product is not merely a cuticular component, but it has profound implications on development of ciliated neurons and seam cells.

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