The *C. elegans* eyes absent ortholog EYA-1 is required for tissue differentiation and plays partially redundant roles with PAX-6

Miwa Furuya a, Hiroshi Qadota a,1, Andrew D. Chisholmb, Asako Sugimoto a,*

a Laboratory for Developmental Genomics, RIKEN Center for Developmental Biology, Kobe 650-0047, Japan
b Sinsheimer Laboratories, Department of Molecular, Cellular and Developmental Biology, University of California, Santa Cruz, CA 95064, USA

Received for publication 26 May 2005, revised 18 July 2005, accepted 5 August 2005
Available online 9 September 2005

Abstract
eyes absent/Eya is a conserved transcriptional coactivator involved in development of various tissues and organs in arthropods and vertebrates. In *Drosophila* eye development, *eya* functions as part of the transcriptional regulatory network along with *eyeless*/Pax6, *sine oculis*/Six and *dachshund*/Dach. Here, we present the first functional study of the *C. elegans* Eya homolog, EYA-1. Loss of EYA-1 function by RNAi and deletion mutations resulted in early larval lethality with incomplete penetrance, associated with defects of differentiation and morphogenesis of several tissues and organs. In late embryogenesis, morphological defect in the head region, pharyngeal malformation and excess cell deaths in the anterior region were observed. Consistently, EYA-1 was expressed in the nuclei of a subset of anterior cells including pharyngeal and body wall muscle cells, starting from the morphogenesis stage in embryogenesis. Interestingly, *eya-1* and *pax-6*/Pax6 mutants showed a strong genetic interaction for larval viability and embryonic anterior morphogenesis. Thus, *eya-1* appears to play a partially redundant role with *pax-6* during *C. elegans* embryogenesis.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Caenorhabditis elegans; eyes absent; Eya; Pax6; Transcriptional regulation; Morphogenesis

Introduction

The Eyes absent/Eya proteins are conserved transcriptional coactivators first identified in *Drosophila* as a gene required for eye development (Bonini et al., 1993). The Eya proteins are defined by a conserved 271 amino-acid C-terminal motif called Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997), which was shown to be involved in protein–protein interaction (Chen et al., 1997; Pignoni et al., 1997), and more recently to have a protein tyrosine phosphatase activity (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). Genetic analyses in *Drosophila* revealed that *eya* functions for eye specification as a component of a transcriptional regulatory network, which includes three other conserved nuclear protein families: *eyeless* (*ey*) (Quiring et al., 1994) and *twin of eyeless* (*toy*) (Czerny et al., 1999) that belong to the Pax6 family of paired and homeodomain transcription factor; *sine oculis* (*so*) (Cheyette et al., 1994) that belongs to the SIX family of homeodomain transcription factor; and *dachshund* (*dac*) (Mardon et al., 1994), the DACH family of transcriptional cofactor. ey is required for the expression of *eya* and *so* which cooperatively activate *dac* (Halder et al., 1998). Although eya itself does not directly bind to DNA, it physically interacts with DNA binding proteins *so* and *dac* to form a complex that is thought to regulate transcription of downstream genes (Chen et al., 1997; Pignoni et al., 1997). In addition, *eya*, *so* and *dac* can in turn induce the expression of *ey*, thus consisting a positive feedback loop (Bonini et al., 1997; Chen et al., 1997; Pignoni et al., 1997; Shen and Mardon, 1997). Mammals have homologs for the all four classes of “eye specification” genes, and some of them are implicated in eye development. For example, reduction of *Pax6* function cause small eye in mice, and aniridia in human (Quiring et al., 1994); three of the four mammalian Eya genes are expressed in the developing eye in a manner dependent on *Pax6* (Duncan et al., 1997; Xu et al., 1997; Zimmerman et al., 1997). Thus, at least some aspects of
the “eye specification” gene regulatory network seem to be conserved between *Drosophila* and mammals.

In addition to the well-characterized function of Eya proteins in the *Drosophila* eye development, they are involved in the development of various other tissues and organs as well. For instance, *Drosophila* *eya* is expressed in wide range of tissues, and strong loss-of-function alleles cause embryonic lethality and sterility (Bonini et al., 1998). Haploinsufficiency for the human EYA1 locus results in the dominantly inherited disorders brachio-oto-renal (BOR) syndrome and brachio-oto (BO) syndrome that is characterized by craniofacial defects, sensorineural deafness and kidney defects (Abdelhak et al., 1999). Similarly, *Eya1* knockout mice show lack of ear and kidney, and hypoplasia of organs derived from the pharyngeal region such as thymus, thyroid and parathyroid (Johnson et al., 1999; Xu et al., 1999). Zebrafish *eya1* was identified as a mutation *dog eared*, which results in inner ear developmental abnormalities (Kozlowski et al., 2005). Thus, Eya proteins appear to play roles in various tissue developments by regulating transcription of distinct sets of genes in a context-specific manner.

The developmental context-specific function of Eya proteins might be accomplished by differential interactions with other “eye specification” gene families (Rebay et al., 2005). For instance, in vertebrate muscle development, one of the four Eya proteins Eya2 acts with Dach2 and Six1 in an analogous manner with *Drosophila* eye specification network, but Pax3 replaces Pax6 as the upstream regulator (Heanue et al., 1999; Laclef et al., 2003; Ridgeway and Skerjanc, 2001). In the *Drosophila* spermatocyte development, *eya* and *so* are necessary, but no *ey* expression is observed (Fabrizio et al., 2003). Thus, the gene networks that contain the “eye specification” genes appear to vary in each tissue, but their details are yet to be understood.

The genome of the nematode *Caenorhabditis elegans* contains homologs for all the “eye specification” gene members, although this animal does not have eyes. The single *pax-6* ortholog, *pax-6* encodes multiple isoforms with distinct functions. *pax-6* mutations affecting paired domain containing isoforms cause defects in epidermal morphology and were previously known as *vab-3* mutations (Chisholm and Horvitz, 1995). Mutations affecting non-paired domain containing *PAX-6* isoforms affect male tail development and were known as *mab-18* (Zhang and Emmons, 1995). The *vab-3* mutant class causes defects in epidermal cell fates, anterior morphogenesis and gonadal cell migration (Chisholm and Horvitz, 1995). The *mab-18* mutant class causes cell fate defects in the male tail (Zhang and Emmons, 1995). The *pax-6* mutant class that affects all isoforms exhibits severe defects in epidermal morphogenesis (Cinar and Chisholm, 2004), that are qualitatively different from those seen in *vab-3* mutants. Based on these allelic differences, *PAX-6* isoforms are thought to interact in a complex manner depending on the cellular context (Chisholm and Horvitz, 1995; Cinar and Chisholm, 2004; Zhang and Emmons, 1995). Four SIX family genes (*ceh-32, ceh-33, ceh-34, and unc-39/ceh-35*) are found in the *C. elegans* genome (Dozier et al., 2001). Reduction of *ceh-32* function by RNAi causes head morphological defects similar to those of *pax-6* mutants, and expression of *ceh-32* was shown to be regulated by *pax-6* within the anterior epidermis (Dozier et al., 2001). In *unc-39/ceh-35* mutants, migration and differentiation defects in a subset of mesodermal and ectodermal cells are observed (Yanowitz et al., 2004). No phenotype is detected by RNAi of *ceh-33* and *ceh-34* (Kamath et al., 2003; Maeda et al., 2001; Rual et al., 2004). The mutant of the Dac homolog, *dac-1*, shows anterior thermosensory neuron defects, but no developmental abnormality is detected (Colosimo et al., 2004). Large-scale protein-protein interaction analysis reported that the Eya homolog (C49A1.4 = *eya-1*) shares many binding partners with PAX-6 (Li et al., 2004), but functional analysis of the Eya homolog has not been reported. Thus, although at least some of the “eye specification” gene homologs appear to play developmental roles in *C. elegans*, how they interact each other in each developmental process has not been clear.

To understand the developmental role of the Eya homolog in *C. elegans*, we performed in this paper the first functional characterization of this gene, *eya-1*. Loss of *eya-1* function caused larval lethality with incomplete penetrance, and affected differentiation and morphogenesis of several tissues during both embryogenesis and post-embryonic development. In developing embryos, EYA-1 was expressed in the nuclei of several anterior cells prior to and during morphogenesis. Finally, we found that *eya-1* and *pax-6/Pax6* show a strong genetic interaction for larval viability and head morphogenesis. Thus, *eya-1* is involved in differentiation of several tissues, and some of its roles seem to be partially redundant with *pax-6*.

### Materials and methods

#### C. elegans strains

*C. elegans* strains were cultured using standard methods (Brenner, 1974) at 20°C unless otherwise noted. The wild-type strain was N2. Mutations used were: LGI, *eya-1(tm759)* (isolated by National Bioresource Project) and *eya-1(ok554)* (isolated by the C. elegans Gene Knockout Consortium), *hh1[unc-54(h1040)]* (a suppressor for the right end of LGI), *mls13* (integration of GFP-marker transgenes); LGII, *ced-9(n1950)*; LGIV, *ced-3(n717);* LGV, *egl-1(n1084);* LGX, *pax-6[509];* The *eya-1* alleles were out-crossed at least six times prior to analysis.

#### RNAi experiments

The cDNA clone yk601a6 that corresponds to the *eya-1* gene was originally identified in the RNAi screen. For further experiments presented in this study, PCR products amplified from the full-length cDNA clone yk1195a07 (a gift from Y. Kohara) were used as a template for *eya-1* dsRNA synthesis. As a control, dsRNA corresponds to GFP (green fluorescent protein) was used. Microinjection (Fire et al., 1998) or the soaking method (Maeda et al., 2001) was used for dsRNA delivery. dsRNA solution at the concentration of 0.5–1.2 μg/μl was microinjected into wild-type young adult hermaphrodites, and incubated at 25°C. In the case of RNAi-by-soaking, L4 worms were soaked in the dsRNA solution for 24 h at 20°C, recovered on NGM plates, and incubated at 25°C. Phenotypes were characterized in F1 generation laid by the soaked or injected worms. To examine the post-embryonic role of *eya-1*, RNAi-by-L1-soaking was performed (Kuroyanagi et al., 2000). L1 worms were soaked in the dsRNA solution for 24 h at 20°C, recovered on NGM plates, and incubated at 25°C. Post-embryonic development of the soaked worms were characterized after recovery. To describe the RNAi treated worms by various methods, we use the following abbreviation in this paper: “irRNA” for RNAi by injection, “sRNAi” for RNAi-by-L4-soaking, and “L1-sRNAi” for RNAi-by-L1-soaking.
**Phenotype analysis ofeya-1 mutants**

Synchronized embryos were collected and their growth/lethality was scored at the time when wild-type worms become adults (i.e., about 36 h at 25°C, 48 h at 18 and 20°C and 60 h at 15°C, after removal of P0 worms). Morphological abnormalities of gonads and vulvae in the worms escaped to be adults, and the head region in newly hatched L1 larvae were scored with Nomarski optics.

**Expression analysis ofeya-1:**

Spatiotemporal expression pattern ofeya-1::gfp was examined in tjEx25, tJEx26, tJEx27, tJEx28, and tJEx29 animals. To examine the co-localization ofeya-1::GFP with DNA and body wall muscle, transgenic embryos were fixed and stained with diamidino-phenylindole (DAPI) and the monoclonal antibody 5–6 that recognizes myosin heavy chain, as described previously (Miller et al., 1983; Miller and Shakes, 1995). In brief, embryos permeabilized by the freeze-crack method were fixed with methanol and acetone, incubated with 5–6 at a 1:100 dilution followed by Alexa-Fluor 568 goat anti-mouse antiserum (Molecular Probes) at a 1:100 dilution, then mounted with DAPI (1 μg/ml) in DABCO/glycerol mounting medium.

**Microscopy**

Nomarski images were captured by a cooled CCD camera, Cool Snap HQ (Photometrics) attached to a Zeiss AxioPlan2 microscope or by Olympus BX61. For the 3D time-lapse analysis, images were taken every 2 min with 1 μm Z steps (25 slices that covered the whole depth of an embryo) per each time point. Images were analyzed by Metamorph software (Universal imaging corporation). Fluorescent images were taken by Zeiss LSM 510 attached to Zeiss AxioPlan2 microscope or by Olympus BX61-DSU system.

**Results**

**Identification ofeya-1, the C. elegans homolog of eyes absent/Eya**

The C. elegans cDNA clone yk601a6 was identified through our large-scale RNAi analyses using a non-redundant CDNA library (Maeda et al., 2001, and data not shown), as one of the rare clones whose RNAi caused uncoordinated phenotype and morphological abnormality in larvae without affecting embryonic viability. This cDNA clone corresponded to C49A1.4 that encodes a protein showing similarity to the eyes absent/Eya family. C49A1.4 is the only gene in the C. elegans genome that encodes a protein with significant similarity to the Eya family genes, and we named this geneeya-1.

Theeya-1 locus consists of nine exons that span 10 kb (Fig. 1A), and encodes a predicted polypeptide of 503 amino acids. The carboxyl-terminal 273 amino acids of EYA-1 have homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Figs. 1B and C) and the rest homology to the conserved Eya domain (ED) (Xu et al., 1997; Zimmerman et al., 1997)(Fig. 1C).

For further analyses of theeya-1 function in C. elegans, two deletion alleles,eya-1(ok654)(isolated by the International C. elegans Gene Knockout Consortium) andeya-1(tm759)(isolated by the National Bioresource Project) were obtained. The ok654 allele deleted 1315 bp that would result in a truncated protein product without the carboxyl-terminus of ED including the HAD motif I, and the tm759 allele removed 541 bp that would result in a truncated protein product without the middle of ED including the HAD motif II and III (Figs. 1A and C). Thus, both alleles are likely to be defective in the phosphatase activity.
Loss of EYA-1 function results in early larval arrest with an incomplete penetrance

The *eya-1* phenotypes originally detected in the RNAi screen were larval developmental abnormalities (growth arrest, morphological abnormality and uncoordinated movement). For further phenotypic characterization, RNAi (soaking and injection) using a full-length cDNA clone *yk1195a07* as the template for dsRNA synthesis, and the two deletion alleles (*ok654* and *tm759*), were used. In all cases, loss or reduction of *eya-1* function resulted in L1 or L2 larval arrest with incomplete penetrance (Table 1), and no embryonic lethality was detected. The penetrance of the larval lethality of *ok654* and *tm759* homozygotes was temperature dependent: Both at high temperature (25°C) and low temperature (15°C), a greater number of the *eya-1* homozygotes arrested as early larvae (48–77% in *ok654* and 47–62% in *tm759*, respectively) compared to 18°C (34–35%) (Table 1). RNAi of *eya-1* in the *eya-1(tm759)* background did not increase the penetrance of larval lethality (Table 1). These results and the molecular lesions of *ok654* and *tm759* found in ED region (Figs. 1A and C) imply that both deletion mutations are likely to be null alleles. Thus, the temperature dependent larval lethality of the deletion alleles is probably not due to the residual *eya-1* protein activity, rather, it indicates the functional redundancy with some other factors. Because *ok654* and *tm759* showed indistinguishable phenotypes, *tm759* was mainly used for further analysis. Genomic DNA that covered only the *eya-1* locus rescued all the phenotypes of *tm759* (Table 1), confirming the defects observed in this strain were caused by the loss of *eya-1* function.

**eya-1** affects anterior morphogenesis

*eya-1* larvae showed aberrant anterior morphology (Figs. 2A and B). The anterior tip of the newly hatched *eya-1* L1 worms was thinner and often twisted. The defect of anterior
body morphology was first detected during the elongation stage in embryogenesis (from comma to 2-fold stage), as slightly expanded anterior region (data not shown). The pharynx of 

eya-1 mutant was thinner and distorted, and metacorpus and terminal bulb were asymmetric, unlike the typical globular shape seen in the wild type (Figs. 2A and B, arrowheads). No defect was detected in the posterior half of the body. Thus, EYA-1 appears to be involved in the development of anterior tissues during late embryogenesis, which leads to the morphological abnormalities in newly hatched L1 larvae.

In addition to the embryonic and early larval morphological defects, eya-1 worms that escaped to undergo postembryonic development showed several tissue differentiation defects (Table 2, Fig. 3). In the eya-1 larvae, pharyngeal cell nuclei often showed altered appearance; for example, two nucleoli were present in the m3 cell that normally has a single nucleolus (Table 2, Figs. 3A and B). The pharyngeal pumping was weaker than wild type and the pharynx was often stuffed with ingested bacteria, implying defective differentiation of pharyngeal cells. Body morphology defects (small body size and/or dumpy morphology) and uncoordinated movement were observed in eya-1 late larvae and adults with high penetrance (Table 2).

Gonad morphogenesis defects (distal tip cell migration defect and other morphological abnormality), egg-laying defects and multivulva phenotypes were also observed with low penetrance (Table 2, Figs. 2C–E, and data not shown). Postembryonic development specific gene knock-down by RNAi-by-L1-soaking (L1-sRNAi) also caused the adult tissue defect (Table 2), suggesting that these larval and adult phenotypes were not merely the secondary effect of the embryonic defects, rather, results of the loss of post-embryonic EYA-1 function. Thus, eya-1 appears to function in multiple tissues in both embryogenesis and postembryonic development.

**Table 1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temperature (°C)</th>
<th>% Early larval arrest (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>25</td>
<td>0 (911)</td>
</tr>
<tr>
<td>eya-1(tm759)</td>
<td>25</td>
<td>62 (507)</td>
</tr>
<tr>
<td>eya-1(ok654); eya-1(tm759); ced-3(n717)</td>
<td>25</td>
<td>22 (549)</td>
</tr>
<tr>
<td>eya-1(tm759); gEx21[eya-1(+)]</td>
<td>25</td>
<td>5.8 (633)</td>
</tr>
<tr>
<td>eya-1(tm759); gEx25[eya-1::gfp]</td>
<td>25</td>
<td>77 (446)</td>
</tr>
<tr>
<td>eya-1(long)</td>
<td>25</td>
<td>30 (488)</td>
</tr>
<tr>
<td>eya-1(2xRNAi)</td>
<td>25</td>
<td>35 (484)</td>
</tr>
<tr>
<td>eya-1(RNAi)</td>
<td>25</td>
<td>48 (283)</td>
</tr>
<tr>
<td>eya-1(sRNAi)</td>
<td>25</td>
<td>58 (492)</td>
</tr>
<tr>
<td>eya-1(2xRNAi); eya-1(RNAi)</td>
<td>25</td>
<td>89 (955)</td>
</tr>
</tbody>
</table>

sRNAi: RNAi-by-soaking, iRNAi: RNAi-by-injection.

*a A representative transgenic line is shown. Other transgenic lines also rescued tm759.

**Loss of eya-1 function causes the increase of cell deaths during embryogenesis**

During embryogenesis of *C. elegans*, 113 out of 671 cells produced undergo programmed cell death (Sulston et al., 1983), most of which occurs between 250–450 min after the first cell division. We noticed that eya-1 mutant embryos grown at 25°C had excess cell corpses in the anterior region in late embryogenesis (Figs. 2D–F). Excess cell corpses could be caused by an increase of cell deaths, or by accumulation of undegraded corpses due to the defect in engulfment process. To distinguish these two possibilities, all detectable cell corpses from the first cell division to 1.5-fold stage (until when scoring became difficult due to the twitching of embryos) were traced from 3-dimensional time-lapse recording images, and the total number of cell deaths and their duration time were quantified (Sugimoto et al., 2001). Most cell corpses in eya-1(tm759) embryos were engulfed within 10 min as in wild-type embryos, suggesting that the engulfment mechanism in eya-1 was normal (Fig. 2G). On the other hand, total number of dead cells detected in the eya-1 embryo was 60% higher than the wild type (132 in eya-1 vs. 82 in the wild type; note that 113 cell deaths occurs in wild-type embryogenesis, but not all corpses are detectable in this recording condition). Total cell numbers scored at the beginning of the morphogenesis stage by DAPI staining were not significantly different between the wild type and eya-1 (data not shown). Taking these results together, we concluded that loss of the eya-1 function induced abnormal death of cells that are normally not programmed to die.

Programmed cell deaths in the wild type are regulated by an evolutionarily conserved genetic pathway consisting of egl-1, ced-3, ced-4 and ced-9 (Metzstein et al., 1998). We asked whether excess cell deaths in the eya-1 mutants were induced by the activation of this programmed cell death pathway, by constructing double mutants of eya-1(tm759) with the cell death defective mutants (ced-3(n717), ced-9(n1950gf) and egl-1 (n1084n3082)) (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986; Hengartner et al., 1992). In all the double mutants constructed (eya-1(tm759);ced-3(n717), eya-1(tm759);ced-9 (n1950gf) and egl-1 (n1084n3082)), no cell corpses were observed (data not shown), suggesting that the increased cell deaths in eya-1 mutants were caused by the activation of the programmed cell death pathway. These double mutants showed the morphological abnormalities equivalent to eya-1 single mutants (Fig. 2C). Thus, we conclude that morphological abnormalities of eya-1 were not directly caused by the excessive cell deaths, but by aberrant cell specification or differentiation.

eya-1 and pax-6 mutations have a strong genetic interaction for larval viability and anterior morphogenesis

The genetic analysis of *Drosophila* compound eye formation revealed the feedback regulation between eyes absent/Eya and eyeless/Pax6: expression of *eyes absent* is dependent on eyeless (Halder et al., 1998), and ectopic expression of *eyes absent* induces eyeless expression (Bonini et al., 1997). *C.
The *elegans* genome has a single Pax6 locus, *pax-6/vab-3/mab-18* (Chisholm and Horvitz, 1995; Cinar and Chisholm, 2004; Zhang and Emmons, 1995), that produces multiple isoforms. The strong loss-of-function alleles of *pax-6* exhibit abnormal anterior morphology ("notched head" phenotype) that is distinct from *eya-1* mutants (Chisholm and Horvitz, 1995; Cinar and Chisholm, 2004). We examined whether *eya-1* and *pax-6* have genetic interactions as in other organisms.

Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenoypes % (N)</th>
<th>L4 animals with abnormal pharyngeal nuclei</th>
<th>Unc</th>
<th>Dpy</th>
<th>Muv</th>
<th>Egl</th>
<th>Mig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td>1</td>
<td>(102)</td>
<td>0</td>
<td>(300)</td>
<td>0</td>
</tr>
<tr>
<td><em>eya-1</em>(tm759)</td>
<td></td>
<td></td>
<td>28</td>
<td>(90)</td>
<td>93</td>
<td>(365)</td>
<td>66</td>
</tr>
<tr>
<td><em>eya-1</em>(ok654)</td>
<td></td>
<td></td>
<td>n.d.</td>
<td></td>
<td>88</td>
<td>(347)</td>
<td>53</td>
</tr>
<tr>
<td><em>eya-1</em>(L1-sRNAi)</td>
<td></td>
<td></td>
<td>n.d.</td>
<td></td>
<td>18</td>
<td>(489)</td>
<td>5</td>
</tr>
</tbody>
</table>


* Number of gonad arms scored.

Fig. 2. The *eya-1* mutations cause anterior morphological abnormalities and increased cell deaths during embryogenesis. (A–C) Anterior region of L1 larvae. (A) Wild type. (B) *eya-1*(tm759). Anterior tip of the *eya-1*(tm759) larva is twisted and the pharynx is thinner. Pharyngeal bulb structure is asymmetrical and distorted (indicated by arrowheads). (C) *eya-1*(tm759);*ced-3*(n717). The morphological defects were not suppressed by repression of cell death. m: metacorpus, tb: terminal bulb. (D, E) Embryos at 2-fold stage (25°C). (D) Wild type. (E) *eya-1*(tm759). Excess cell corpses are observed (indicated by white arrowheads). Scale bar, 10 μm. (F) Number of cell corpses observed at each stage in embryogenesis. The vertical axis indicates the average number of cell corpses detected per embryo at each embryonic stage. Asterisks indicate significant difference between the wild type and *eya-1*(tm759) or *eya-1*(ok654) (*P* < 0.05, **P** < 0.01). Embryonic stages are defined as follows: I, 200–230 min; II, 240–270 min; III, 280–310 min; IV, 320–350 min; V, 360–390 min after the first cleavage. (G) Duration time of cell corpses. Every cell corpse detectable in one embryo was traced from the 4D images, and its duration time was scored. Total number of cell corpses detected from the first division to 2-fold stage is indicated in each graph. In the wild type, 82 of the 113 expected cell deaths are detected.
Larval lethality of *eya-1(tm759)* and *pax-6(ju468)* single mutants (both are putative null mutants) at 18°C was ~30% and ~50%. In our attempt to construct a double mutant (see Materials and methods), we noted that worms with the genotypes *eya-1; pax-6/+* and *eya-1/+; pax-6* mostly arrested as early larvae. Rare fertile adults of these genotypes were generally sick and laid very few progeny. No *eya-1; pax-6* double mutant adults were obtained. These results suggest that the larval lethality of *eya-1* and *pax-6* was significantly enhanced by reducing the dosage of the other gene. This effect was confirmed by partial reduction of the *eya-1* function by RNAi-by-soaking (sRNAi) in the *pax-6(ju468)* background. The penetrance of the early larval (L1 or L2) lethality was significantly enhanced in *eya-1(sRNAi); pax-6(ju468)* worms to over 80%, compared to the *pax-6(ju468)* (~50%) or *eya-1(sRNAi)* (~20%) (Fig. 4A).

To examine the genetic interaction of these two genes on morphogenesis, newly hatched *eya-1(RNAi-by-injection[iRNAi]), pax-6(ju468)* and *eya-1(iRNAi); pax-6(ju468)* animals were compared. As seen in the *eya-1* deletion mutants, *eya-1(iRNAi)* worms exhibited a thinner anterior tip (without a bulge) and a disorganized pharyngeal structure with a complete penetrance ("Eya" phenotype, Figs. 4B and C). *pax-6(ju468)* worms show wide range of morphological abnormalities in the anterior region (Cinar and Chisholm, 2004), which we classified into four classes by their severity (class I: a small bulge, class II: multiple small bulges, class III: large bulges, and class IV: anterior expansion). In the *eya-1(iRNAi); pax-6(ju468)* animals, the ratio of the worms showing severe abnormalities (classes II–IV) was increased, and no class I nor Eya phenotype was observed (Figs. 4B and C).
Thus, *eya-1* and *pax-6* have synergistic effect on anterior morphology, suggesting that *eya-1* and *pax-6* might play partially redundant roles for anterior morphogenesis during embryogenesis.

**EYA-1 is expressed in a subset of anterior nuclei during embryonic morphogenesis**

Expression of *eya-1* during embryogenesis was analyzed using transgenic *eya-1(tm759)* animals rescued by a functional full-length translational *gfp* fusion construct, in which *gfp* is inserted in the exon 2 (Fig. 1A, Table 1). EYA-1::GFP expression was first detected in the nuclei of several anterior cells at the bean stage when embryo starts morphogenesis (Fig. 5A), which corresponds to the stage right before anterior morphological abnormality is first detected in the *eya-1* deletion mutants. The nuclear localization of EYA-1::GFP is consistent with the predicted EYA-1 function as a transcriptional regulator. As embryogenesis progressed, the number of EYA-1 expressing cells in the anterior region and the intensity of the signal increased. From 1.5-fold to pretzel stage when embryos elongate and pharyngeal structure is organized, expression of EYA-1::GFP was prominent in the nuclei of a subset of pharyngeal cells (Figs. 5B–E) as well as several anterior body wall muscle cells (Figs. 5F–H). The EYA-1::GFP expression continued until hatch. Thus, the spatiotemporal expression pattern of EYA-1::GFP coincided with the observed abnormalities in the *eya-1* embryos.

**Discussion**

*eya-1* is involved in differentiation of various tissues

In this paper, we described the genetic and molecular analysis of the only Eya ortholog in *C. elegans*, *eya-1*. The conserved C-terminal region called Eya domain (ED) was originally identified as a protein–protein interaction motif (Chen et al., 1997; Pignoni et al., 1997), but more recently, it was also shown to belong to the haloacid dehalogenase (HAD) superfamily of protein tyrosine phosphatase (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). Although the similarity of ED domain of the *C. elegans* EYA-1 to those in other Eya family members are relatively low, all the HAD motifs required for the protein phosphatase activity were conserved. In *Drosophila* and mice, the phosphatase activity of Eya proteins was shown to contribute to their ability as transcriptional regulators (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). Because the two alleles delete regions within ED including the HAD motifs, they are likely to be null.

Loss of *eya-1* function caused defects in several tissues at different developmental stages. In embryogenesis, anterior body morphology and pharyngeal structure were affected; in post-embryonic development, gonads, vulvae and overall body shape were affected. Consistent with the anterior abnormalities in embryogenesis, functional *eya-1::gfp* transgenes were expressed in the nuclei of anterior cells including a subset of pharyngeal and body wall muscle cells. The expression and
mutant defects were first detected at morphogenesis stage of embryogenesis, after most of the embryonic cell proliferation is completed. For example, EYA-1 expression in pharyngeal cells increased after the comma stage after the majority of pharyngeal cell is formed, and the morphological abnormality of pharynx of the \textit{eya-1} mutant became obvious later in the pretzel stage. In addition, some pharyngeal cell in \textit{eya-1} mutants displayed aberrant nuclear morphology, indicating defective differentiation. From these results, we speculate that, at least for the pharynx, EYA-1 is probably not involved in cell fate determination, rather, is involved in tissue differentiation after the cell fate is determined.

We also found that excess cells underwent programmed cell deaths in the anterior region of \textit{eya-1} mutant embryos. Because inhibition of cell deaths in \textit{eya-1} does not suppress morphogenesis defects, excess deaths are not the direct cause of aberrant tissue morphology. Excess apoptotic cell deaths are also reported in other Eya family mutants. In the \textit{Drosophila eya} mutant, progenitor cells in the eye disc undergo excessive apoptotic cell deaths, resulting in the eyeless phenotype (Bonini et al., 1993). The vertebrate Eya1 mutants also display excess cell deaths (Kozlowski et al., 2005; Xu et al., 1999). In vertebrates, failure in activating normal differentiation program is thought to induce abnormal apoptosis (Raff, 1992). In the core cell deaths genetic pathway in \textit{C. elegans}, transcription of the \textit{egl-1} gene is regarded as the most upstream event (Conradt and Horvitz, 1998). Since \textit{egl-1(lf)} mutant suppressed the excess cell deaths phenotype in \textit{eya-1}, we speculate that transcription of \textit{egl-1} is ectopically turned on in the excess dying cells in the \textit{eya-1} embryos. The ectopic induction of cell deaths in \textit{eya-1} mutants might be the secondary effect of failure of differentiation, as in vertebrates. Alternatively, repression of cell deaths might be one of the direct roles of EYA-1, and excess deaths in \textit{eya-1} mutants might be caused by the release from the repression. Currently, we cannot distinguish these possibilities.

**Genetic interaction of \textit{eya-1} and \textit{pax-6}**

\textit{eya}/Eya and \textit{ey/Pax6} in the \textit{Drosophila} eye primordia are components of the “eye specification” network, making a
feedback circuit (Hanson, 2001). To examine whether this genetic network is conserved in *C. elegans*, we tested the genetic interaction between *eya-1* and *pax-6*. Whereas the single mutations of *eya-1* and *pax-6* show distinct morphological defects and larval lethality with incomplete penetrance (Cinar and Chisholm, 2004), we found that *pax-6* enhanced the larval lethality of *eya-1*, and has a strong synergistic effect with *eya-1* on anterior morphogenesis. These results suggest that *eya-1* and *pax-6* play partially redundant functions, but they also have distinct developmental roles. Interestingly, large-scale yeast two-hybrid analysis of *C. elegans* genes identified many EYA-1 and PAX-6-interacting proteins, and 16 of them bind to both EYA-1 and PAX-6 (Li et al., 2004). One possibility is that EYA-1 and PAX-6 might cooperatively act on the common interactors, whereas each of them plays distinct roles by binding to the independent interactors. Previously, PAX-6 was shown to be expressed in anterior hypodermal and neuronal cells during embryogenesis (Chisholm and Horvitz, 1995), but whether it is co-expressed with EYA-1 in a subset of cells needs to be examined for further understanding of the mode of action of EYA-1 and PAX-6.

A synergistic effect of *eya/Eya* and *ey/Pax6* was also reported in *Drosophila* eye formation. While overexpression of either *eya* or *ey* can induce ectopic eye formation in several tested tissues with variable penetrance, when both are co-overexpressed in the same tissues, extent of the ectopic eye formation is dramatically enhanced (Bonini et al., 1997); the ectopic eyes are larger and formed with higher penetrance than with overexpression of *eya* or *ey* alone (Bonini et al., 1997). Thus, functional synergy of Eya and Pax6 in the co-expressing cells might be an evolutionarily conserved phenomenon.

**Does the “Eye specification” gene network exist in *C. elegans***?

The “eye specification” gene network originally identified in *Drosophila* eye formation consists of Pax6, Eya, Six and Dach proteins (Hanson, 2001). In *Drosophila* eye, *eya/Pax6* direct expression of *eya/Eya* and *so/Six* (Halder et al., 1998), which make a protein complex to induce *dac/Dach* (Pignoni et al., 1997). *dac* can also bind to *eya* (Chen et al., 1997), and the *eya-so-dac* complex is thought to regulate transcription of downstream genes. This network is also utilized in other developmental contexts in other organisms (Heanue et al., 1999; Li et al., 2003).

Is this genetic network also used in the development of *C. elegans*? The *C. elegans* genome has corresponding genes for all four families: *pax-6, eya-1*, four *Six* genes (ceh-32, ceh-33, ceh-34 and *unc-39/ceh-35*) (Dozier et al., 2001) and *dac-1* (Colosimo et al., 2004). Among these, CEH-33 and UNC-39/CEH-35 was shown to bind to EYA-1 by a large-scale yeast two-hybrid analysis (Li et al., 2004). Notably, *unc-39* mutants exhibit similar phenotypes with *eya-1* mutants for pharyngeal morphology and some aspects of post-embryonic defects, such as uncoordinated locomotion, egg-laying defects and multiple vulvae (Yanowitz et al., 2004). Thus, UNC-39/CEH-35 is a good candidate for the EYA-1 binding partner in some developmental contexts in *C. elegans*. On the other hand, *ceh-32* shows abnormal head morphology similar to the *pax-6* mutants (Dozier et al., 2001), and transcription of *ceh-32* in some cells is directly regulated by *pax-6* (Dozier et al., 2001). *dac-1* mutants show only a subtle behavioral defect, but does not display significant developmental abnormalities (Colosimo et al., 2004). No detectable phenotype was reported for *ceh-33* and *ceh-34* by high-throughput RNAi analyses (Kamath et al., 2003; Maeda et al., 2001; Rual et al., 2004). Thus, although the combinations of *eya-1–pax-6, eya-1–unc-39* and *pax-6–ceh-32* may interact in particular developmental contexts, there is no evidence for the presence of the “eye specification” (*Pax6-Eya-Six-Dach*) network in *C. elegans*. Rather, currently available protein–protein interaction data (Li et al., 2004) raise the possibility that *eya-1* and *pax-6* might act as the core of a large genetic network that is dynamically modulated in different developmental contexts; *eya-1* and *pax-6* have many common (as well as independent) interactors that include not only SIX family genes, but also other DNA binding proteins and transcriptional regulators. Thus, *eya-1* and *pax-6* are likely to be involved in the transcriptional regulation for the various aspects of development by alternating their binding partners.

**Acknowledgments**

We thank the National Bioresource Project in Japan lead by S. Mitani and the *C. elegans* Gene Knockout Project at OMRF for providing *eya-1* deletion mutants, Y. Kohara for cDNA clones, Y. Iida for technical assistance, and K. L. Chow and members of the Sugimoto lab for critically reading the manuscript. Some of the worm strains used in this study were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. Work in A.D.C.’s laboratory was supported by the NIH (GM54657). This work was supported by Grant-in-Aid for Scientific Research on Priority Areas to A.S. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**References**


