INVESTIGATION

Differentiation of Carbon Dioxide-Sensing Neurons in *Caenorhabditis elegans* Requires the ETS-5 Transcription Factor

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ABSTRACT

Many animals sense environmental gases such as carbon dioxide and oxygen using specialized populations of gas-sensing neurons. The proper development and function of these neurons is critical for survival, as the inability to respond to changes in ambient carbon dioxide and oxygen levels can result in reduced neural activity and ultimately death. Despite the importance of gas-sensing neurons for survival, little is known about the developmental programs that underlie their formation. Here we identify the ETS-family transcription factor ETS-5 as critical for the normal differentiation of the carbon dioxide-sensing BAG neurons in *Caenorhabditis elegans*. Whereas wild-type animals show acute behavioral avoidance of carbon dioxide, *ets-5* mutant animals do not respond to carbon dioxide. The *ets-5* gene is expressed in BAG neurons and is required for the normal expression of the BAG neuron gene battery. *ets-5* may also autoregulate its expression in BAG neurons. *ets-5* is not required for BAG neuron formation, indicating that it is specifically involved in BAG neuron differentiation and the maintenance of BAG neuron cell fate. Our results demonstrate a novel role for ETS genes in the development and function of gas-detecting sensory neurons.

Near all animals are capable of sensing changes in ambient levels of gases such as oxygen (O₂) and carbon dioxide (CO₂). Animals respond to fluctuating levels of O₂ and CO₂ with rapid physiological responses that include altered respiration rate, metabolic rate, and cardiovascular activity (Bonora and Boule 1994; Mortola and Lanthier 1996; Vovk et al. 2002). Many animals also display behavioral responses to O₂ and CO₂. For example, exposure to hypoxia alters thermotaxis behavior in rodents (Gordon and Fogelson 1991) and foraging behavior in flies and nematodes (Wingrove and O’farrell 1999; Cheung et al. 2005; Rogers et al. 2006), while exposure to environmental CO₂ triggers an avoidance response in many free-living animals and an attractive response in many parasites and disease vectors (Bowen 1991; Suh et al. 2004; Hu et al. 2007; Bretscher et al. 2008; Hallem and Sternberg 2008; Hallem et al. 2011a). Humans are also capable of detecting CO₂ as an aversive trigeminal stimulus (Bensafi et al. 2007). The inability to respond appropriately to changes in environmental O₂ and CO₂ in mammals can result in lethargy, reduced neural activity, unconsciousness, and ultimately death (West 2004; Langford 2005). Thus, the proper development and function of gas-sensing neurons is critical for survival. However, remarkably little is known about the molecular mechanisms that specify the development of these neurons in any species.

The free-living soil nematode *Caenorhabditis elegans* is a powerful model system for dissecting the molecular programs that specify the development of gas-sensing neurons. *C. elegans* senses environmental O₂ and CO₂ and displays robust behavioral responses to both gases (Gray et al. 2004; Chang et al. 2006; Bretscher et al. 2008; Hallem and Sternberg 2008; Bretscher et al. 2011; Hallem et al. 2011b). In addition, *C. elegans* has a relatively simple nervous system consisting of only 302 neurons (White et al. 1986), which facilitates identification of the specific factors that confer neuronal cell fates. Adult *C. elegans* respond to changes in ambient O₂ concentration by migrating toward their preferred concentration of 10% O₂ and to changes in ambient CO₂ concentration by moving away from the source of CO₂ (Gray et al. 2004; Cheung et al. 2005; Bretscher et al. 2008; Hallem et al. 2011b). O₂ is detected primarily by the AQR, URX, PQR, and BAG neurons, while CO₂ is detected primarily by the BAG neurons (Gray et al. 2004; Chang et al. 2006; Hallem and Sternberg 2008; Zimmer et al. 2009; Bretscher...
expression of at least some members of the BAG neuron (Hollenhorst et al. 1992; Hollenhorst et al. 2011). ETS-5 have been shown to regulate cellular differentiation and proliferation in mammals (Pereira et al. 1994; Macleod et al. 1992; Hollenhorst et al. 2011). Close orthologs of ETS-5 have been shown to regulate cellular differentiation and proliferation in mammals (Pereira et al. 1999; Scott et al. 2005; Liu et al. 2010). Our results demonstrate a novel role for ETS-domain transcription factors in the development and function of gas-detecting sensory neurons in C. elegans and raise the possibility that ETS proteins might also regulate gas sensing in mammals.

Materials and Methods

Strains and genetics

C. elegans strains are listed in Supporting Information, File S1. All worms were grown at room temperature (~22°) or 20°. To generate strains for comparison of transgene expression in the wild-type and ets-5 mutant backgrounds, all transgenes were crossed into the ets-5 mutant background to enable a direct comparison of expression levels. To stage animals for quantification of fluorescence, 10 young adult worms were placed onto NGM agar plates seeded with Escherichia coli OP50 bacteria and allowed to lay eggs for 2 hr. Animals collected 19–21 hr later were scored as L1’s. Animals collected 28–31 hr later were scored as L2’s. Animals collected 38–40 hr later were scored as L3’s. L4 animals were picked on the basis of their characteristic morphological appearance. Young adult worms were picked as L4’s and scored the following day.

Generation of reporter constructs

To generate the ets-5::mCherry translational fusion construct, the entire ets-5 coding region (1.8 kb) and a 3.2-kb region upstream of the start codon was amplified from N2 genomic DNA. It was then cloned into a modified pPD95.77 vector in which the GFP coding sequence was replaced by an mCherry coding sequence. The ets-5::mCherry construct was injected into N2 animals at a concentration of 50 ng/μl along with 50 ng/μl of pax-2::GFP as a co-injection marker. To generate the gcy-9::GFP transcriptional fusion construct, a 1.9 kb region upstream of the start codon was cloned into the pPD95.75 vector. The gcy-9::GFP construct was then injected into N2 animals at a concentration of 50 ng/μl along with 50 ng/μl of pax-2::GFP as a co-injection marker.

Mutagenesis of the gcy-31 promoter

A putative binding site for ETS-5 that matches the Fli-1 consensus sequence (Mao et al. 1994), accggaagtgg, is located ~120 bp upstream of the gcy-31 coding region. Using site-directed mutagenesis, this sequence was mutated to acTAGTArgg to eliminate the core GGAA sequence and introduce a unique SnaBI restriction site. Mutagenesis was performed on a plasmid containing a 3.7-kb region directly 5’ of the gcy-31 ATG start site essentially as described (Quik-Change site-directed mutagenesis kit, Agilent Technologies, Santa Clara, CA). Briefly, primers were designed to create the desired mutation and to incorporate at least 20 bases on either side of the mutation matching the original sequence. The mutated plasmid was PCR-amplified using PfUltra High-Fidelity (Agilent Technologies, Santa Clara, CA) and digested with DpnI to remove the original methylated plasmid.
Both the wild-type *gcy-31* promoter region and the promoter region containing the mutated binding site (*gcy-31Mut*) were cloned into the pPD95.75 vector and sequenced. Constructs were injected into N2 animals at a concentration of 50 ng/µl along with 50 ng/µl of *pax-2::GFP* as a co-injection marker.

**Microscopy**

Nematodes were anesthetized with either 5 mM sodium azide or 3 mM levamisole and mounted on 5% Noble agar pads. DIC and epifluorescence images were captured using a Zeiss AxioImager A2 microscope with an attached Zeiss AxioCam camera and analyzed with Zeiss AxioVision software. Confocal images were captured using a Zeiss LSM 5 PASCAL microscope and AxioVision software. For quantification of epifluorescence, images of transgene expression were taken at the same exposure in the wild-type and *ets-5* mutant backgrounds. Average pixel intensities in the region containing the BAG neuron cell body were quantified using either AxioVision or ImageJ software. In cases where no BAG neuron expression was visible through the microscope eyepieces, average pixel intensity was reported as zero. The graphs of relative intensity in Figures 5, 6C, and 7C were normalized by setting the highest mean intensity value for each graph to one.

For quantifying the number of animals showing transgene expression in the wild-type and *ets-5* mutant backgrounds in Figure S2, animals were selected as follows: for the extrachromosomal *gcy-33::GFP* transgene as well as all integrated transgenes, approximately three to five GFP+ hermaphrodites were placed onto plates and F1 progeny were selected randomly for scoring without regard for GFP expression. For all other extrachromosomal transgenes, approximately three to five GFP+ hermaphrodites were placed onto plates and F1 progeny were selected randomly for scoring without regard for GFP expression. For all other extrachromosomal transgenes, approximately three to five GFP+ hermaphrodites were placed onto plates and F1 progeny were selected on the basis of either expression of the co-injection marker (for BAG-specific transgenes) or expression of GFP in cells other than BAG. For quantifying expression in Figure 5, animals were selected the same way except that *gcy-33::GFP* animals were scored only if they had visible transgene expression in BAG neurons because *gcy-33* is specific to BAG and animals did not contain a fluorescent co-injection marker.

For comparing BAG neuron fluorescence in animals with either a wild-type or mutated *gcy-31* transcriptional fusion construct in Figures 7 and Figure S5, transgenic animals containing either *gcy-31::GFP* or *gcy-31Mut::GFP* were generated in parallel and four to five independent lines were scored for each transgene. Animals were selected for scoring on the basis of the presence of the *pax-2::GFP* co-injection marker.

**Acute CO2 avoidance assays**

CO2 avoidance assays were performed as previously described (Hallem and Sternberg 2008; Hallem et al. 2011b). Briefly, animals were tested as young adults unless otherwise indicated. For each trial, ~10–15 animals were placed onto 5-cm assay plates consisting of NGM agar seeded with a thin lawn of *E. coli* OP50 bacteria. Gases were certified mixtures consisting of the indicated CO2 concentration, 10% O2, and the rest N2. A concentration of 10% CO2 was used unless otherwise indicated. Two 50-ml gas-tight syringes were filled with gas, one with and one without CO2. The mouths of the syringes were connected to flexible PVC tubing (1/8-in. thickness) attached to Pasteur pipets, and gases were pumped through the Pasteur pipets using a syringe pump at a rate of 1.5 ml/min. Worms were exposed to gases by placing the tip of the Pasteur pipet near the head of a forward-moving worm, and a response was scored if the worm reversed within 4 sec. Gases were delivered blindly and worms were scored blindly. An avoidance index was then calculated by subtracting the fraction of animals that reversed in response to the air control from the fraction of animals that reversed in response to the CO2 (Figure 2A).

**Data analysis**

Statistical analysis was performed using GraphPad InStat. For all graphs, statistical significance is shown relative to the wild-type control. The exon–intron graphic of *ets-5* was generated using Exon–Intron Graphic Maker by Nikhil Bhatla (http://www.wormweb.org/exonintron). Sequence logos were generated using WebLogo by Crooks, Hon, Chandonia, and Brenner (University of California, Berkeley; http://weblogo.berkeley.edu).

**Results**

**ets-5 is expressed in BAG sensory neurons**

We previously transcriptionally profiled embryonic BAG neurons and identified 850 genes that show enriched expression in BAG neurons (Figure 1A). One of the genes that was most highly enriched in BAG neurons was the ETS-domain transcription factor gene *ets-5* (Figure 1B). *ets-5* was previously shown to be expressed in a single pair of head neurons, but these cells had not been identified (Hart et al. 2000; Reece-Hoyes et al. 2007). Moreover, nothing was known about the function of *ets-5*. To confirm that *ets-5* is expressed in BAG neurons, we generated a translational reporter containing the promoter and coding region of *ets-5* fused to mCherry. We found that *ets-5* was strongly and consistently expressed in the BAG neurons (Figure 1C). In some animals, *ets-5* was also expressed at low levels in a few other unidentified cells in the head; however, the only consistent expression we observed was in BAG neurons. These results suggest that *ets-5* functions primarily, if not exclusively, in BAG sensory neurons.

**ets-5 is required for CO2-avoidance behavior**

The BAG neurons are paired sensory neurons located in the head anterior to the nerve ring that are required for CO2 detection (White et al. 1986; Hallem and Sternberg 2008;
Hallem et al. 2011b). To determine whether ets-5 is required for the ability of BAG neurons to respond to CO₂, we first tested whether ets-5 mutants display behavioral responses to CO₂. We used an acute CO₂ avoidance assay in which the head of a forward-moving worm is exposed to CO₂ and the worm is given 4 sec to reverse in response to the CO₂. An avoidance index (a.i.) is then calculated as: a.i. = fraction of animals that reverse in response to CO₂ – fraction of animals that reverse in response to an air control (Figure 2A) (Hallem and Sternberg 2008). Whereas wild-type worms displayed an a.i. of 0.7 in response to 10% CO₂, ets-5 mutants displayed an a.i. of <0.1 in response to 10% CO₂ (Figure 2B). Furthermore, ets-5 mutants were defective in their ability to respond to all tested concentrations of CO₂ (Figure 2C), and this CO₂ response defect was present at all developmental stages (Figure 2D). These results demonstrate that ets-5 is required for acute behavioral avoidance of environmental CO₂.

Figure 1 ets-5 is expressed in the BAG sensory neurons. (A) The transcriptional profile of BAG sensory neurons. Previous transcriptional profiling of BAG neurons identified 850 genes that showed enriched expression in BAG neurons relative to the aggregate of all embryonic cells (Hallem et al. 2011b). The 10 genes whose mRNA transcripts were most highly enriched in BAG neurons are shown on the right. ets-5 mRNA transcripts showed an enrichment of ~14-fold in BAG neurons. The pie chart is adapted from Hallem et al. (2011b). (B) The intron–exon structure of the ets-5 gene. Brackets indicate tm1734, tm1755, and tm866 deletion alleles. (C) An ets-5::mCherry translational reporter shows expression in BAG sensory neurons. Left, DIC image; center, epifluorescence image; right, overlay. Anterior is left; dorsal is up. Arrowheads indicate BAG neurons.
To gain further evidence that ets-5 acts in BAG neurons and to confirm the functionality of our ets-5::mCherry translational reporter, we tested whether the ets-5::mCherry transgene was capable of rescuing the ets-5 mutant phenotype. We found that the ets-5::mCherry transgene provides substantial rescue of the behavioral response of ets-5 mutants to CO2 (Figure 2E). These results further support the conclusion that ets-5 functions in BAG neurons to regulate CO2-avoidance behavior.

Figure 2 ets-5 mutants do not show behavioral responses to CO2. (A) An acute assay for behavioral avoidance of CO2. An airstream containing CO2 is delivered to the head of a forward-moving animal through the end of a Pasteur pipet, and the animal is given 4 sec to reverse in response to the CO2 (Hallem and Sternberg 2008; Hallem et al. 2011b). (B) ets-5 mutants do not respond to CO2. All three deletion mutants of ets-5 do not respond to 10% CO2. *n* = 11–15 trials for each genotype. ***P < 0.001, one-way ANOVA with Bonferroni post-test. (C) ets-5 mutants are defective in CO2 response across concentrations. *n* = 6–12 trials. (D) ets-5 mutants are defective in CO2-avoidance behavior at all developmental stages. Wild-type animals show acute CO2 avoidance at the L2 to young adult stages. ets-5(tm866) mutant animals do not avoid CO2 at any developmental stage. ets-5(tm866) animals were not tested at the L1 stage because wild-type animals do not avoid CO2 at the L1 stage. *n* = 6–11 trials for each condition. ***P < 0.001, unpaired t-test. (E) An ets-5::mCherry transgene containing full-length ets-5 genomic DNA partly rescues the ets-5(tm866) mutant phenotype, confirming appropriate expression of the transgene. *n* = 15–31 trials. ***P < 0.001 and **P < 0.01 (both relative to wild type), one-way ANOVA with Bonferroni post-test. Error bars represent SEM.
BAG neurons adopt a normal neuronal morphology in ets-5 mutants

We then investigated the mechanism by which ets-5 affects BAG neuron function. One possibility is that ets-5 could be required for the initial formation of BAG neurons; alternatively, ets-5 could be required later in development for the proper differentiation of BAG neurons. To test the first possibility, we examined ets-5 mutants by DIC microscopy and found that BAG neuron cell bodies are present in ets-5 mutants (Figure 3A). Moreover, the BAG neurons appear morphologically normal in ets-5 mutants, with an anterior process extending toward the tip of the nose and a posterior process that enters the nerve ring (Figure 3B). These results demonstrate that ets-5 is not required for the initial development of the BAG neurons, nor is it required for them to adopt a grossly normal neuronal morphology.

ets-5 is required for normal BAG neuron differentiation

To test whether ets-5 is required for the normal differentiation of BAG neurons, we examined expression of the set of genes known to mediate CO2 and O2 detection in BAG neurons in wild-type and ets-5(tm866) mutant animals. The bilaterally symmetric BAG neurons are labeled with GFP using a gcy-33::GFP reporter, which is expressed specifically in the BAG neurons. BAG neurons extend dendrites toward the tip of the nose and a posterior process into the nerve ring. Arrowheads indicate cell bodies, small arrows indicate dendrites, and large arrows indicate posterior processes. Anterior is to the left; dorsal is up. Although each image captures only one of the BAG neurons, the dendritic tips of the other BAG neuron are visible. Images of wild-type and ets-5 mutant animals are not shown at the same exposure; the exposure time for each image was optimized independently for maximum BAG neuron visibility.

ets-5 is required for normal BAG neuron differentiation

Figure 3 BAG neurons appear morphologically normal in ets-5 mutants. (A) The BAG neurons are present in ets-5 mutant animals. DIC image of an ets-5(tm866) mutant animal. Anterior is toward the left; dorsal is toward the top. BAG neuron cell bodies are located in the head (arrowhead). (B) The BAG neurons appear morphologically normal in ets-5(tm866) mutants. Maximum projection images of a series of confocal micrographs of BAG neurons in wild-type and ets-5(tm866) mutant animals. The bilaterally symmetric BAG neurons are labeled with GFP using a gcy-33::GFP reporter, which is expressed specifically in the BAG neurons. BAG neurons extend dendrites toward the tip of the nose and a posterior process into the nerve ring. Arrowheads indicate cell bodies, small arrows indicate dendrites, and large arrows indicate posterior processes. Anterior is to the left; dorsal is up. Although each image captures only one of the BAG neurons, the dendritic tips of the other BAG neuron are visible. Images of wild-type and ets-5 mutant animals are not shown at the same exposure; the exposure time for each image was optimized independently for maximum BAG neuron visibility.
mutant animals still show low levels of expression of the BAG genes (Figure S2). Moreover, inappropriate expression of these genes in other cells was not observed (Figure 4). However, the intensity of expression of all of the BAG genes was reduced (Figure 5). Thus, ets-5 is required for normal levels of gene expression in BAG neurons, but other transcription factors are also likely to contribute to BAG neuron differentiation.

The extent to which expression was reduced differed for the different BAG genes. The genes that are known to function specifically or nearly specifically in BAG neurons, such as flp-17, gcy-9, gcy-31, and gcy-33, showed greatly reduced expression across developmental stages in ets-5 mutants (Figure 5). By contrast, genes that function in multiple sensory neurons, such as tax-2 and tax-4, showed less severely reduced expression in the BAG neurons of ets-5 mutants. Both tax-2 and tax-4 were expressed at normal levels in young larvae but showed significantly but moderately reduced expression at later developmental stages (Figure 5).

These results suggest that ets-5 may be the primary transcription factor that regulates expression of the BAG-specific gene battery, whereas more broadly expressed genes may rely on a combinatorial code of transcription factors for expression in BAG neurons. An exception is the flp-10 gene, which is expressed in multiple neurons, functions in multiple neurons besides BAG (Li and Kim 2008; Ringstad and Horvitz 2008), and yet shows greatly reduced expression in the BAG neurons of ets-5 mutants (Figures 4 and 5).

To confirm that the role of ets-5 in BAG neuron differentiation is specific to BAG neurons, we quantified the expression levels of the receptor guanylate cyclase gene odr-1, which is expressed in AWC chemosensory neurons (L’etoile and Bargmann 2000), in the wild-type and ets-5 mutant backgrounds. We found that ets-5 mutants show normal levels of odr-1 expression across developmental stages (Figure S3). Consistent with the relatively specific expression of ets-5 in BAG neurons, these results suggest that ets-5 is not required for the differentiation of chemosensory neurons in general but rather plays a specific role in differentiation of the BAG neurons.

**Autoregulation of ets-5 expression in BAG neurons**

The fact that ets-5 is required for normal expression of all genes known to function in CO₂ and O₂ detection by BAG neurons raised the possibility that ets-5 might also regulate its own expression in BAG. To test this possibility, we compared the expression levels of the ets-5::mCherry transgene in wild-type and ets-5 mutant animals. We found that ets-5 mutants show reduced levels of ets-5::mCherry expression at multiple developmental stages (Figure 6). These results suggest that ets-5 expression is subject to autoregulation and that tagged ETS-5 shows reduced autoregulation relative to wild-type ETS-5. Many of the mammalian ETS genes, including a closely related mammalian ortholog of ets-5, Pet-1 (human ortholog, FEV), are also subject to autoregulation (Scott et al. 2005; Swiers et al. 2006; Liu et al. 2010), suggesting that the mechanisms underlying ETS gene function may be at least partly conserved across phyla. However, it is also possible that the reduced expression of ets-5::mCherry in the ets-5 mutant background instead results from reduced stability or functionality of the tagged ETS-5 protein in the absence of wild-type ETS-5.

**ETS-5 is likely to directly regulate expression of the gcy-31 gene**

ETS-5 could regulate expression of the BAG neuron gene battery by binding directly to the promoters of BAG-expressed genes, or it could regulate expression indirectly via other transcription factors. The binding sites for mammalian ETS proteins are well characterized, and at least some of the C. elegans ETS proteins bind to cis-regulatory motifs that are similar to mammalian ETS binding sites (Wagman-er et al. 2006; Flames and Hobert 2009; Thyagarajan et al. 2010). All mammalian ETS family members bind to the conserved core recognition sequence 5’-GGA(A/T)-3’, and the bases surrounding this core recognition sequence determine binding specificity for individual ETS proteins (Macleod et al. 1992; Hollenhorst et al. 2011). Consensus binding sites have been identified for two of the closely related mammalian orthologs of ETS-5, Fli-1, and Pet-1 (Mao et al. 1994; Hendricks et al. 1999) (Figure S4B). We hypothesized that ETS-5 might use binding sites similar to those of the Fli-1 and Pet-1 binding sites. We identified putative ETS-5 binding sites similar to both Fli-1 and Pet-1 binding sites upstream of many of the BAG-expressed genes (data not shown), raising the possibility that ETS-5 directly regulates expression of BAG genes using these binding sites.

To test whether ETS-5 regulation of BAG-expressed genes is likely to be direct, we focused on expression of the gcy-31 gene for the following reasons. First, gcy-31 expression is severely reduced in ets-5 mutants, indicating that ETS-5 is essential for gcy-31 expression (Figure 5). Second, the gcy-31 promoter contains cis-regulatory motifs that closely resemble Fli-1 and Pet-1 consensus binding sites (Figure 7, A and B). The presence of these putative binding sites raised the possibility that ETS-5 binds to one or both of these sites to directly regulate expression of gcy-31.

We used site-directed mutagenesis to eliminate the core recognition sequence of a putative ETS-5 binding site in the gcy-31 promoter (Figure 7B). This putative binding site completely matches the Fli-1 consensus binding site (Mao et al. 1994). We then compared expression levels of GFP driven by the wild-type (gcy-31) and mutated (gcy-31Mut) promoters. We found that expression of gcy-31Mut::GFP in BAG neurons was greatly reduced relative to expression of gcy-31::GFP (Figure 7C and Figure S5). These results suggest that ETS-5 may use the same binding motif as Fli-1 and may directly regulate expression of gcy-31 using a conserved Fli-1 binding motif in the gcy-31 promoter.

**Different ETS genes play distinct roles in C. elegans development and physiology**

The C. elegans genome encodes 10 ETS genes (Figure S4A), of which only 3—ast-1, ets-4, and lin-1—have been previously
characterized. Like \textit{ets-5}, the \textit{ast-1} gene is a regulator of neuronal differentiation: \textit{ast-1} is required for the terminal differentiation of all dopaminergic neurons (Flames and Hobert 2009). \textit{AST-1} also regulates axon guidance in some classes of interneurons (Schmid et al. 2006). By contrast, the \textit{ets-4} and \textit{lin-1} genes are not known to affect neuronal differentiation. \textit{ETS-4} is a transcriptional regulator of life span and \textit{ets-4} mutants have significantly extended life spans relative to wild-type animals (Thyagarajan et al. 2010). \textit{LIN-1} acts in the MAP kinase pathway to control vulval development (Sternberg 2005) and some \textit{lin-1} mutants have a multi-vulva or an egg-laying defective phenotype (Jacobs et al.)

\textbf{Figure 4} Gene expression is reduced in the BAG neurons of \textit{ets-5} mutants. In each row, the left image is an epifluorescence image of a wild-type L4 animal with the indicated reporter transgene, while the right image is an epifluorescence image of an \textit{ets-5} (tm866) mutant L4 animal shown at the same exposure. In cases where BAG neuron expression was visible but not at that exposure, a third image is shown with a longer exposure in which the BAG neurons are visible. Arrows indicate the location of the nerve ring; arrowheads indicate the location of the BAG neuron cell body. No arrowhead is shown for \textit{flp-10::GFP; ets-5} because no BAG neuron expression was visible. Anterior is to the left. The 10 µm scale bar applies to the entire figure.
1998; Sternberg 2005). To test whether ets-5 also regulates life span and vulval development, we examined the life span and brood size of ets-5 mutant adults. We found that ets-5 mutants have normal life spans and brood sizes, suggesting that ets-5 does not regulate aging or vulval development (Figure S6). Thus, different ETS family members play distinct roles in the regulation of C. elegans development, physiology, and neuronal function.

**Discussion**

**Differentiation of a gas-sensing neuron**

Here we show that the ETS-domain transcription factor ETS-5 is expressed in the BAG neurons throughout development and is required for normal behavioral responses to CO₂. In addition, we show that ETS-5 is required for the proper expression of a BAG-specific gene battery that includes genes required for CO₂ response, O₂ response, and egg laying (Figures 4 and 5). ets-5 may also regulate its own expression in BAG neurons (Figure 6). These results suggest that ets-5 functions both to initiate and maintain the normal program of gene expression in the BAG sensory neurons.

Although ets-5 is expressed at a relatively constant level throughout development (Figure 6C), we found that for a number of genes the requirement for ets-5 differs at different developmental stages. For example, expression of flip-17 and gcy-31 is severely reduced at all developmental stages, whereas expression of tax-2 is normal at the L1–L3 larval stages but is significantly reduced at the L4 and young adult stages.

**Figure 5** ets-5 is required for the normal program of BAG neuron gene expression across developmental stages. Graphs show the relative intensity of expression of each transgene in the wild-type, ets-5(tm1734), and ets-5(tm866) mutant backgrounds across developmental stages. n = 7–40 animals for each condition. *, P < 0.05; **, P < 0.01; ***, P < 0.001, one-way ANOVA with Dunnett’s post-test or Kruskal-Wallis test with Dunn’s post-test. Error bars represent SEM.
adult stages (Figure 5). These results raise the possibility that other transcription factors act with ETS-5 in a temporally dynamic manner to control gene expression in BAG neurons, but that the importance of these factors varies for different genes in the BAG neuron gene battery. Transcriptional profiling of BAG neurons identified over 40 transcription factor genes that showed enriched expression in BAG neurons (Hallem et al. 2011b), all of which are candidates for genes that might act with ets-5 to regulate BAG neuron differentiation.

Taken together, our results suggest a model for ets-5 function in which ets-5 regulates expression of the BAG neuron gene battery, but different BAG-expressed genes are regulated by ets-5 to different degrees (Figure 8). In general, ets-5 more strongly regulates expression of BAG-specific genes than genes that are expressed in other sensory neurons as well as BAG. The more broadly expressed genes are likely to be regulated by multiple transcription factors capable of driving expression in BAG neurons. Identification of these additional factors will provide further insight into the regulatory mechanisms that control the development of gas-sensing neurons.

**ets-5 functions as a selector gene in BAG neurons**

ets-5 is required to initiate and maintain the normal program of gene expression in BAG neurons (Figures 4–6). Thus, ets-5 is a selector gene (Mann and Carroll 2002) that is specifically required for BAG neurons to adopt their normal neuronal identity. The role of ets-5 in BAG neuron differentiation resembles that of a number of other transcription factors that are required for the differentiation of specific classes of neurons in C. elegans. For example, the C2H2 zinc

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**Figure 6** ets-5 may show autoregulation in BAG neurons. (A) The percentage of young adult animals that express ets-5::mCherry in the wild-type and ets-5(tm866) mutant backgrounds. n = 20 for each genotype. (B) DIC and epifluorescence images of ets-5::mCherry in wild-type and ets-5(tm866) mutant L4 animals. Both epifluorescence images were taken at the same exposure. Arrowheads indicate BAG neurons. (C) The relative intensity of expression of ets-5::mCherry in the wild-type and ets-5(tm866) mutant backgrounds across developmental stages. n = 9–23 animals for each condition. *, P < 0.05; ***, P < 0.001, unpaired t-test or Mann–Whitney test. Error bars represent SEM.
finger transcription factor che-1 is required for differentiation of a different set of chemosensory neurons, the salt-sensing ASE neurons (Uchida et al. 2003). che-1 reporter constructs show relatively specific expression in ASE neurons, and che-1 mutants show salt-sensing defects (Uchida et al. 2003; Etchberger et al. 2007). Like ets-5, che-1 is not required for the generation of ASE neurons or for the ASE neurons to adopt their normal neuronal morphology but is required for expression of an ASE-specific gene battery (Uchida et al. 2003; Etchberger et al. 2007). Similarly, the homeodomain genes ceh-14 and ceh-17 are required for normal gene expression in the ALA neuron, which controls sleep-like behavior in C. elegans (Van Buskirk and Sternberg 2010). Thus, ETS-5 is one of a number of different selector genes that act to control the differentiation of specific neuronal subtypes in C. elegans.

**ETS-5 is likely to directly regulate expression of at least some BAG neuron genes**

We found that mutation of a Fli-1 consensus binding motif in the gcy-31 promoter results in greatly reduced expression in BAG neurons (Figures 7 and Figure S5). These results suggest that ETS-5 directly regulates expression of at least some of the BAG neuron genes by binding to conserved Fli-1 cis-regulatory motifs. The fact that expression of gcy-31 was reduced by mutation of the Fli-1 motif, but not to the level observed in ets-5 mutants, suggests that ETS-5 may also bind to other cis-regulatory elements within the gcy-31 promoter. At least one other putative ETS-5 binding site, an 11-bp element that resembles the Pet-1 binding site, is present in the gcy-31 promoter (Figure 7, A and B). Thus, ETS-5 may induce low levels of gcy-31 expression in the absence of the Fli-1 consensus site by binding to other sites such as the Pet-1 cis-regulatory motif.

**ETS-5 Regulates BAG Neuron Development**

We found that mutation of a Fli-1 consensus binding motif in the gcy-31 promoter results in greatly reduced expression in BAG neurons (Figures 7 and Figure S5). These results suggest that ETS-5 directly regulates expression of at least some of the BAG neuron genes by binding to conserved Fli-1 cis-regulatory motifs. The fact that expression of gcy-31 was reduced by mutation of the Fli-1 motif, but not to the level observed in ets-5 mutants, suggests that ETS-5 may also bind to other cis-regulatory elements within the gcy-31 promoter. At least one other putative ETS-5 binding site, an 11-bp element that resembles the Pet-1 binding site, is present in the gcy-31 promoter (Figure 7, A and B). Thus, ETS-5 may induce low levels of gcy-31 expression in the absence of the Fli-1 consensus site by binding to other sites such as the Pet-1 cis-regulatory motif.

Figure 8 shows weaker regulation of the more broadly expressed tax-2 and tax-4 genes. ets-5 may also show autoregulation in BAG neurons.

**Figure 8** A model for ets-5 regulation of the BAG neuron gene battery. The ets-5 gene strongly regulates expression of genes that are expressed specifically or nearly specifically in BAG neurons. ets-5 shows weaker regulation of the more broadly expressed tax-2 and tax-4 genes. ets-5 may also show autoregulation in BAG neurons.
A novel role for ETS proteins in gas sensing

ETS proteins comprise an evolutionarily conserved family of transcription factors that play critical roles in cell differentiation, proliferation, and development in mammals (Figure S4B) (Seth and Watson 2005; Sharrocks 2001). Misregulation of ETS proteins can lead to developmental abnormalities of the nervous and immune systems as well as cancer (Sharrocks 2001; Seth and Watson 2005; Flames and Hobert 2009). Here we demonstrate a novel role for ETS proteins in the development and function of gas-sensing neurons by identifying one of these proteins, ETS-5, as a critical regulator of BAG neuron function. Closely related orthologs of ETS-5 are present in mammals, including humans (Figure S4B) (Laudet et al. 1999; Hollenhorst et al. 2011). Our results raise the possibility that ETS transcription factors might also play a previously unrecognized role in the differentiation of gas-sensing neurons in mammals.

Acknowledgments

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Differentiation of Carbon Dioxide-Sensing Neurons in *Caenorhabditis elegans* Requires the ETS-5 Transcription Factor

Manon L. Guillermin, Michelle L. Castelletto, and Elissa A. Hallem
**File S1**

**Supporting Methods**

**Strains:**  
*C. elegans* strains are listed in the order in which they appear in the figures. The following strains were used: EAH14 bruEx12[ets-5::mCherry, pax-2::GFP]; N2; FX1734 ets-5(tm1734); FX0866 ets-5(tm866); FX1755 ets-5(tm1755); EAH41 ets-5(tm866); bruEx12[ets-5::mCherry, pax-2::GFP]; DA1290 lin-15B(n765) X; adEx1290[gcy-33::GFP, lin-15(+)]; EAH43 ets-5(tm866); adEx1290[gcy-33::GFP, lin-15(+)]; OH4841 otis92[flp-10::GFP]; EAH18 otis92[flp-10::GFP]; ets-5(tm866); NY2064 ynsls64[flp-17::GFP]; him-5(e1490); EAH20 ynsls64[flp-17::GFP]; ets-5(tm866); EAH65 bruEx47[gcy-9::GFP, pax-2::GFP]; EAH77 ets-5(tm866); bruEx59[gcy-9::GFP, pax-2::GFP]; EAH26 kyEx2116[gcy-31::SL2::GFP, odr-1::DsRed2]; EAH27 ets-5(tm866); kyEx2116[gcy-31::SL2::GFP, odr-1::DsRed2]; CX3584 kyls111[tax-2::GFP]; lin-15(n765ts); EAH22 kyls111[tax-2::GFP]; ets-5(tm866); BC13862 dpy-5(e907); sEx13862 [tax-4::GFP, pCeh361]; EAH24 ets-5(tm866); sEx13862[tax-4::GFP, pCeh361]; EAH19 otis92[flp-10::GFP]; ets-5(tm1734); EAH21 ynsls64[flp-17::GFP]; ets-5(tm1734); EAH78 ets-5(tm1734); bruEx60[gcy-9::GFP, pax-2::GFP]; EAH28 ets-5(tm1734); kyEx2116[gcy-31::SL2::GFP, odr-1::DsRed2]; EAH48 ets-5(tm1734); adEx1290[gcy-33::GFP, lin-15(+)]; EAH23 kyls111[tax-2::GFP]; ets-5(tm1734); EAH25 ets-5(tm1734); sEx13862[tax-4::GFP, pCeh361]; EAH68 bruEx50[gcy-31::GFP, pax-2::GFP]; EAH69 bruEx51[gcy-31::GFP, pax-2::GFP]; EAH70 bruEx52[gcy-31::GFP, pax-2::GFP]; EAH71 bruEx53[gcy-31::GFP, pax-2::GFP]; EAH72 bruEx54[gcy-31Mut::GFP, pax-2::GFP]; EAH73 bruEx55[gcy-31Mut::GFP, pax-2::GFP]; EAH74 bruEx56[gcy-31Mut::GFP, pax-2::GFP]; EAH75 bruEx57[gcy-31Mut::GFP, pax-2::GFP]; EAH76 bruEx58[gcy-31Mut::GFP, pax-2::GFP].

**Phylogenetic analysis:**  
Phylogenetic trees of the ETS proteins were generated from an alignment of the ETS domains. *C. elegans* sequences were obtained from WormBase (www.WormBase.org) and *H. sapiens* sequences were obtained from the NCBI protein database. Protein sequences of the ETS domains were aligned with ClustalW using the default settings in Lasergene (DNASTAR, Madison, WI). An unrooted phylogenetic tree was then constructed from the aligned ETS domains with PHYLIP 3.65 (Joseph Felsenstein, University of Washington, Seattle, WA). Bootstrapping was performed using 100 replicates.

**Lifespan assays:**  
For lifespan assays, 10 L4 hermaphrodites were placed onto each NGM agar plate seeded with OP50. Animals were transferred to fresh plates every day for the first 8 days. The number of live, dead, or censored (missing or bagged) animals was recorded every day for the first 8 days. After 8 days, animals were transferred to fresh plates every 2 days for the remainder of the assay. The number of live, dead, or censored animals was recorded every 2 days until all animals were dead or censored. The percentage of surviving worms was then calculated for each of the recorded days.

**Brood size assays:**  
To assay brood sizes, individual L4 hermaphrodites were placed onto NGM agar plates seeded with OP50. Animals were transferred to a fresh plate every 24 hours for 4 days. The total number of progeny on all of the plates was then scored.

**SUPPORTING REFERENCES**


Expression of *gcy-9::GFP* in the BAG neuron

**Figure S1** A *gcy-9::GFP* reporter is expressed specifically in BAG sensory neurons. Left, DIC image; center, epifluorescence image; right, overlay. Anterior is left; dorsal is up. Arrowheads indicate BAG neurons.
Expression of BAG genes in the ets-5 mutant

**Figure S2** The percentage of young adult animals that express each transgene in the wild-type and ets-5(tm866) mutant backgrounds. n = 7-31 animals for each genotype.
**Figure S3**  The odr-1 gene is expressed at normal levels in the AWC neurons of ets-5 mutants. A. Epifluorescence images of the odr-1::DsRed transgene in the AWC neurons of wild-type and ets-5(tm1734) mutant animals shown at the same exposure. Arrows indicate the AWC neurons; arrowheads indicate the nerve ring. B. The relative intensity of expression of the odr-1::DsRed transgene in the wild-type and ets-5(tm1734) mutant backgrounds across developmental stages. No differences in expression levels were observed between wild-type and ets-5 mutant animals. n = 8-22 animals for each condition. Error bars represent SEM.
Figure S4  Phylogenetic tree of ETS proteins. A. The ETS family of C. elegans. B. ETS proteins of C. elegans and Homo sapiens (Hart et al. 2000; Hollenhorst et al. 2011). “C.e.” indicates C. elegans ETS proteins; all other proteins are H. sapiens ETS proteins. The ETS-5 protein is highlighted in red. The closest human ortholog of ETS-5 is FEV (rodent ortholog, Pet-1).
Figure S5  Mutation of a putative ETS-5 binding site reduces expression of gcy-31::GFP in BAG neurons. A conserved Fli-1 consensus binding motif in the gcy-31 promoter was mutated as shown in Figure 7B. Graph depicts the relative intensity of expression of either gcy-31::GFP or gcy-31Mut::GFP. Four (gcy-31::GFP) or five (gcy-31Mut::GFP) independent lines were quantified for each transgene. The mean intensities across all independent lines for each transgene are shown in Figure 7. n = 10-13 animals for each transgenic line. Error bars represent SEM.
Figure S6  ets-5 mutants have normal lifespans and brood sizes. A. Survival plots of wild-type and ets-5 mutant animals. Animals were grown at $20^\circ$C. $n = 50$-120 animals for each genotype. B. ets-5 mutants have normal brood sizes. Animals were grown at $20^\circ$C. $n = 8$-21 animals for each genotype. Error bars represent SEM.