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

Manon L. Guillermin, Michelle L. Castelletto, and Elissa A. Hallem¹

Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, California 90095

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.

NEARLY 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

Copyright © 2011 by the Genetics Society of America
doi: 10.1534/genetics.111.133835

Manuscript received August 12, 2011; accepted for publication September 21, 2011
Supporting information is available online at <http://www.genetics.org/content/suppl/2011/09/27/genetics.111.133835.DC1>.

¹Corresponding author: UCLA MIMG, 237 BSRB, 615 Charles E. Young Dr. E., Los Angeles, CA 90095-7364. E-mail: ehallem@microbio.ucla.edu

et al. 2011; Hallem *et al.* 2011b). The ASE and AFD neurons also contribute to CO₂ response (Bretscher *et al.* 2011). O₂ detection requires a family of soluble guanylate cyclases that act as receptors for O₂ as well as the cGMP-gated cation channel TAX-2/TAX-4 (Gray *et al.* 2004; Chang *et al.* 2006; Zimmer *et al.* 2009). By contrast, CO₂ detection requires a different guanylate cyclase, the receptor guanylate cyclase GCY-9, as well as TAX-2/TAX-4 (Bretscher *et al.* 2008; Hallem and Sternberg 2008; Hallem *et al.* 2011b).

To investigate the molecular mechanisms underlying development of gas-sensing neurons, we focused on development of the BAG neurons. The BAG neurons comprise a single pair of sensory neurons located in the head (White *et al.* 1986) and are unique in their ability to detect both O₂ and CO₂ and to mediate behavioral responses to both gases (Hallem and Sternberg 2008; Zimmer *et al.* 2009; Bretscher *et al.* 2011; Hallem *et al.* 2011b). BAG neurons are also the only neurons known to be required for acute behavioral avoidance of CO₂ (Hallem and Sternberg 2008). Whereas wild-type animals exposed to CO₂ abruptly cease forward locomotion and initiate backward locomotion, BAG-ablated animals continue forward locomotion in the presence of CO₂ (Hallem and Sternberg 2008). Acute CO₂ avoidance behavior thus provides a useful behavioral assay for identifying candidate genes that might be required for the development and function of the BAG neurons.

To identify transcription factors required for BAG neuron development, we used the acute CO₂ avoidance assay to screen animals with mutations in transcription factor genes that show enriched expression in BAG neurons (Hallem *et al.* 2011b). Here we show that one of these transcription factors, the ETS (E-twenty six)-domain winged helix-turn-helix protein ETS-5, is required for the normal differentiation and function of the BAG neurons. We demonstrate that *ets-5* is required for CO₂-avoidance behavior, as *ets-5* mutants are completely defective in their ability to respond to CO₂. *ets-5* is expressed specifically or nearly specifically in BAG neurons at all developmental stages and is required for the normal expression of all of the genes known to play a role in the CO₂- and O₂-sensing functions of the BAG neurons. *ets-5* may also autoregulate its expression in BAG neurons. Thus, *ets-5* functions specifically in the BAG neurons to promote and maintain expression of the BAG neuron gene battery. Finally, we show that mutation of a putative ETS-5 binding site in the promoter region of one of the BAG-expressed genes results in greatly reduced expression in BAG neurons, suggesting that ETS-5 may directly regulate expression of at least some members of the BAG neuron gene battery. ETS-5 is one member of a large family of ETS transcription factors defined by an ~85 amino acid winged helix-turn-helix DNA binding domain that is conserved in mammals, *Drosophila*, and *C. elegans* (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 acTACGTAtgg to eliminate the core GGAA sequence and introduce a unique *Sna*BI 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 PfuUltra High-Fidelity (Agilent Technologies, Santa Clara, CA) and digested with *Dpn*I 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 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;

A BAG neuron transcriptional profile

B Intron-exon structure of the *ets-5* gene

C *ets-5::mCherry*

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.

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 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.

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.

A BAG neurons are present in *ets-5* mutants

B *ets-5* does not affect BAG neuron morphology

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.

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 both the wild-type and *ets-5* mutant backgrounds. This set of genes consists of the receptor guanylate cyclase gene *gcy-9*, which is required for the CO₂-evoked activity of BAG neurons and is a candidate receptor for CO₂ (Hallem *et al.* 2011b); the soluble guanylate cyclase genes *gcy-31* and *gcy-33*, which are required for the O₂-evoked activity of BAG

neurons (Zimmer *et al.* 2009); and the cGMP-gated cation channel genes *tax-2* and *tax-4*, which are required for both the CO₂- and the O₂-sensing functions of the BAG neurons (Hallem and Sternberg 2008; Zimmer *et al.* 2009; Hallem *et al.* 2011b). We also examined expression of the BAG-expressed FMRFamide-like neuropeptide genes *flp-10* and *flp-17*, which are involved in the regulation of egg-laying behavior (Li and Kim 2008; Ringstad and Horvitz 2008).

To examine expression of *gcy-9*, we first generated a *gcy-9::GFP* transcriptional fusion construct. Although we previously showed that *gcy-9* expression is highly enriched in embryonic BAG neurons relative to all other embryonic cells and is required in BAG neurons for CO₂ response (Hallem *et al.* 2011b), expression of *gcy-9* had not been directly examined. We found that *gcy-9* is expressed specifically in the BAG neurons (Figure S1). These results support a specific role for *gcy-9* in CO₂ detection by BAG neurons.

We then analyzed expression of all seven of the genes described above in both the wild-type and *ets-5* mutant backgrounds. We found that *ets-5* is required for the normal expression of all of these genes: in the absence of *ets-5*, expression is significantly reduced (Figure 4). Thus, *ets-5* regulates the expression of genes required for all known aspects of BAG neuron function. To quantify the reduction in gene expression, we assayed both the number of animals that express each gene and the intensity of expression, in the wild-type and *ets-5* mutant backgrounds. We found that many *ets-5*

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'etole 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 (Wagmaister *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

Reduced expression of BAG genes in the *ets-5* mutant

Figure 4 Gene expression is reduced in the BAG neurons of *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 *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 *flp-10::GFP; ets-5* because no BAG neuron expression was visible. Anterior is to the left. The 10 μ m scale bar applies to the entire figure.

characterized. Like *ets-5*, the *ast-1* gene is a regulator of neuronal differentiation: *ast-1* is required for the terminal differentiation of all dopaminergic neurons (Flames and Hobert 2009). *AST-1* also regulates axon guidance in some classes of interneurons (Schmid *et al.* 2006). By contrast, the *ets-4* and *lin-1* genes are not known to affect neuronal

differentiation. *ETS-4* is a transcriptional regulator of life span and *ets-4* mutants have significantly extended life spans relative to wild-type animals (Thyagarajan *et al.* 2010). *LIN-1* acts in the MAP kinase pathway to control vulval development (Sternberg 2005) and some *lin-1* mutants have a multi-vulva or an egg-laying defective phenotype (Jacobs *et al.*

Expression of BAG genes in *ets-5* mutants across developmental 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.

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 *flp-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

A *ets-5::mCherry* is expressed in the *ets-5* mutant background

B Expression of *ets-5::mCherry* is decreased in the *ets-5* mutant background

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.

C *ets-5* expression across developmental stages

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

A The Fli-1 and Pet-1 consensus binding motifs

B Mutation of the *gcy-31* promoter (*gcy-31Mut*)

C Reduced expression of *gcy-31Mut::GFP* in BAG neurons

Figure 7 Mutation of a putative ETS-5 binding site in the *gcy-31* promoter results in reduced expression of *gcy-31*. (A) Consensus binding motifs for the mammalian ETS proteins Fli-1 and Pet-1 (Hendricks *et al.* 1999; Mao *et al.* 1994). (B) The *gcy-31* promoter was mutated to eliminate the putative Fli-1 binding motif, which is located ~120 bp upstream of the start of the *gcy-31* coding region. The mutated promoter is designated *gcy-31Mut*. (C) Expression of *gcy-31Mut::GFP* is greatly reduced relative to expression of *gcy-31::GFP*. Left, epifluorescence images of GFP expression in BAG neurons driven by the *gcy-31* and *gcy-31Mut* promoters. Both epifluorescence images were taken at the same exposure. Arrowheads indicate BAG neurons; arrows indicate the nerve ring. Right, the mean relative intensity of expression of *gcy-31::GFP* and *gcy-31Mut::GFP*. Mean values were obtained by quantifying BAG neuron fluorescence from four to five independent lines for each transgene (Figure S5). ***, $P < 0.001$, 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.

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

We thank Shohei Mitani, Cori Bargmann, and the *Caenorhabditis* Genetics Center (CGC) for *C. elegans* strains, Jagan Srinivasan for critical reading of the manuscript, and members of the Hallem lab for helpful comments and discussion. This work was supported by a National Institutes of Health Pathway to Independence award to E.A.H.

Literature Cited

- Bensafi, M., J. Frasnelli, J. Reden, and T. Hummel, 2007 The neural representation of odor is modulated by the presence of a trigeminal stimulus during odor encoding. *Clin. Neurophysiol.* 118: 696–701.
- Bonora, M., and M. Boule, 1994 Effects of hypercapnia and hypoxia on inspiratory and expiratory diaphragmatic activity in conscious cats. *J. Appl. Physiol.* 77: 1644–1652.
- Bowen, M. F., 1991 The sensory physiology of host-seeking behavior in mosquitoes. *Annu. Rev. Entomol.* 36: 139–158.
- Bretscher, A. J., K. E. Busch, and M. de Bono, 2008 A carbon dioxide avoidance behavior is integrated with responses to ambient oxygen and food in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 105: 8044–8049.
- Bretscher, A. J., E. Kodama-Namba, K. E. Busch, R. J. Murphy, Z. Soltesz *et al.*, 2011 Temperature, oxygen, and salt-sensing neurons in *C. elegans* are carbon dioxide sensors that control avoidance behavior. *Neuron* 69: 1099–1113.
- Chang, A. J., N. Chronis, D. S. Karow, M. A. Marletta, and C. I. Bargmann, 2006 A distributed chemosensory circuit for oxygen preference in *C. elegans*. *PLoS Biol.* 4: e274.
- Cheung, B. H., M. Cohen, C. Rogers, O. Albayram, and M. de Bono, 2005 Experience-dependent modulation of *C. elegans* behavior by ambient oxygen. *Curr. Biol.* 15: 905–917.
- Etchberger, J. F., A. Lorch, M. C. Sleumer, R. Zapf, S. J. Jones *et al.*, 2007 The molecular signature and *cis*-regulatory architecture of a *C. elegans* gustatory neuron. *Genes Dev.* 21: 1653–1674.
- Flames, N., and O. Hobert, 2009 Gene regulatory logic of dopamine neuron differentiation. *Nature* 458: 885–889.
- Gordon, C. J., and L. Fogelson, 1991 Comparative effects of hypoxia on behavioral thermoregulation in rats, hamsters, and mice. *Am. J. Physiol.* 260: R120–R125.
- Gray, J. M., D. S. Karow, H. Lu, A. J. Chang, J. S. Chang *et al.*, 2004 Oxygen sensation and social feeding mediated by a *C. elegans* guanylate cyclase homologue. *Nature* 430: 317–322.
- Hallem, E. A., and P. W. Sternberg, 2008 Acute carbon dioxide avoidance in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 105: 8038–8043.
- Hallem, E. A., A. R. Dillman, A. V. Hong, Y. Zhang, J. M. Yano *et al.*, 2011a A sensory code for host seeking in parasitic nematodes. *Curr. Biol.* 21: 377–383.
- Hallem, E. A., W. C. Spencer, R. D. McWhirter, G. Zeller, S. R. Henz *et al.*, 2011b Receptor-type guanylate cyclase is required for carbon dioxide sensation by *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 108: 254–259.
- Hart, A. H., R. Reventar, and A. Bernstein, 2000 Genetic analysis of *ETS* genes in *C. elegans*. *Oncogene* 19: 6400–6408.
- Hendricks, T., N. Francis, D. Fyodorov, and E. S. Deneris, 1999 The ETS domain factor Pet-1 is an early and precise marker of central serotonin neurons and interacts with a conserved element in serotonergic genes. *J. Neurosci.* 19: 10348–10356.
- Hollenhorst, P. C., L. P. McIntosh, and B. J. Graves, 2011 Genomic and biochemical insights into the specificity of ETS transcription factors. *Annu. Rev. Biochem.* 80: 437–471.
- Hu, J., C. Zhong, C. Ding, Q. Chi, A. Walz *et al.*, 2007 Detection of near-atmospheric concentrations of CO₂ by an olfactory subsystem in the mouse. *Science* 317: 953–957.
- Jacobs, D., G. J. Beitel, S. G. Clark, H. R. Horvitz, and K. Kornfeld, 1998 Gain-of-function mutations in the *Caenorhabditis elegans lin-1 ETS* gene identify a C-terminal regulatory domain phosphorylated by ERK MAP kinase. *Genetics* 149: 1809–1822.
- L'Etoile, N. D., and C. I. Bargmann, 2000 Olfaction and odor discrimination are mediated by the *C. elegans* guanylyl cyclase ODR-1. *Neuron* 25: 575–586.
- Langford, N. J., 2005 Carbon dioxide poisoning. *Toxicol. Rev.* 24: 229–235.
- Laudet, V., C. Hanni, D. Stehelin, and M. Duterque-Coquillaud, 1999 Molecular phylogeny of the ETS gene family. *Oncogene* 18: 1351–1359.
- Li, C., and K. Kim, 2008 Neuropeptides. WormBook, <http://www.WormBook.org>.
- Liu, C., T. Maejima, S. C. Wyler, G. Casadesus, S. Herlitze *et al.*, 2010 Pet-1 is required across different stages of life to regulate serotonergic function. *Nat. Neurosci.* 13: 1190–1198.
- Macleod, K., D. LePrince, and D. Stehelin, 1992 The *ets* gene family. *Trends Biochem. Sci.* 17: 251–256.
- Mann, R. S., and S. B. Carroll, 2002 Molecular mechanisms of selector gene function and evolution. *Curr. Opin. Genet. Dev.* 12: 592–600.
- Mao, X., S. Miesfeldt, H. Yang, J. M. Leiden, and C. B. Thompson, 1994 The FLI-1 and chimeric EWS-FLI-1 oncoproteins display similar DNA binding specificities. *J. Biol. Chem.* 269: 18216–18222.
- Mortola, J. P., and C. Lanthier, 1996 The ventilatory and metabolic response to hypercapnia in newborn mammalian species. *Respir. Physiol.* 103: 263–270.
- Pereira, R., C. T. Quang, I. Lesault, H. Dolznig, H. Beug *et al.*, 1999 FLI-1 inhibits differentiation and induces proliferation of primary erythroblasts. *Oncogene* 18: 1597–1608.
- Reece-Hoyes, J. S., J. Shingles, D. Dupuy, C. A. Grove, A. J. Walhout *et al.*, 2007 Insight into transcription factor gene duplication from *Caenorhabditis elegans* promoterome-driven expression patterns. *BMC Genomics* 8: 27.
- Ringstad, N., and H. R. Horvitz, 2008 FMRFamide neuropeptides and acetylcholine synergistically inhibit egg-laying by *C. elegans*. *Nat. Neurosci.* 11: 1168–1176.
- Rogers, C., A. Persson, B. Cheung, and M. de Bono, 2006 Behavioral motifs and neural pathways coordinating

- O₂ responses and aggregation in *C. elegans*. *Curr. Biol.* 16: 649–659.
- Schmid, C., V. Schwarz, and H. Hutter, 2006 AST-1, a novel ETS-box transcription factor, controls axon guidance and pharynx development in *C. elegans*. *Dev. Biol.* 293: 403–413.
- Scott, M. M., K. C. Krueger, and E. S. Deneris, 2005 A differentially autoregulated Pet-1 enhancer region is a critical target of the transcriptional cascade that governs serotonin neuron development. *J. Neurosci.* 25: 2628–2636.
- Seth, A., and D. K. Watson, 2005 ETS transcription factors and their emerging roles in human cancer. *Eur. J. Cancer* 41: 2462–2478.
- Sharrocks, A. D., 2001 The ETS-domain transcription factor family. *Nat. Rev. Mol. Cell Biol.* 2: 827–837.
- Sternberg, P. W., 2005 Vulval development. *WormBook*, <http://www.WormBook.org>.
- Suh, G. S., A. M. Wong, A. C. Hergarden, J. W. Wang, A. F. Simon *et al.*, 2004 A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*. *Nature* 431: 854–859.
- Swiers, G., R. Patient, and M. Loose, 2006 Genetic regulatory networks programming hematopoietic stem cells and erythroid lineage specification. *Dev. Biol.* 294: 525–540.
- Thyagarajan, B., A. G. Blaszcak, K. J. Chandler, J. L. Watts, W. E. Johnson *et al.*, 2010 ETS-4 is a transcriptional regulator of life span in *Caenorhabditis elegans*. *PLoS Genet.* 6: e1001125.
- Uchida, O., H. Nakano, M. Koga, and Y. Ohshima, 2003 The *C. elegans che-1* gene encodes a zinc finger transcription factor required for specification of the ASE chemosensory neurons. *Development* 130: 1215–1224.
- Van Buskirk, C., and P. W. Sternberg, 2010 Paired and LIM class homeodomain proteins coordinate differentiation of the *C. elegans* ALA neuron. *Development* 137: 2065–2074.
- Vovk, A., D. A. Cunningham, J. M. Kowalchuk, D. H. Paterson, and J. Duffin, 2002 Cerebral blood flow responses to changes in oxygen and carbon dioxide in humans. *Can. J. Physiol. Pharmacol.* 80: 819–827.
- Wagmaister, J. A., G. R. Miley, C. A. Morris, J. E. Gleason, L. M. Miller *et al.*, 2006 Identification of cis-regulatory elements from the *C. elegans* Hox gene *lin-39* required for embryonic expression and for regulation by the transcription factors LIN-1, LIN-31 and LIN-39. *Dev. Biol.* 297: 550–565.
- West, J. B., 2004 The physiologic basis of high-altitude diseases. *Ann. Intern. Med.* 141: 789–800.
- White, J. G., E. Southgate, J. N. Thomson, and S. Brenner, 1986 The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B* 314: 1–340.
- Wingrove, J. A., and P. H. O'Farrell, 1999 Nitric oxide contributes to behavioral, cellular, and developmental responses to low oxygen in *Drosophila*. *Cell* 98: 105–114.
- Zimmer, M., J. M. Gray, N. Pokala, A. J. Chang, D. S. Karow *et al.*, 2009 Neurons detect increases and decreases in oxygen levels using distinct guanylate cyclases. *Neuron* 61: 865–879.

Communicating editor: L. Harshman

GENETICS

Supporting Information

<http://www.genetics.org/content/suppl/2011/09/27/genetics.111.133835.DC1>

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 *ynIs64[flp-17::GFP]*; *him-5(e1490)*; EAH20 *ynIs64[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 *ynIs64[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

- HART, A. H., R. REVENTAR and A. BERNSTEIN, 2000 Genetic analysis of *ETS* genes in *C. elegans*. *Oncogene* **19**: 6400-6408.
- HOLLENHORST, P. C., L. P. MCINTOSH and B. J. GRAVES, 2011 Genomic and biochemical insights into the specificity of ETS transcription factors. *Annu Rev Biochem* **80**: 437-471.

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.

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.

A Expression of *odr-1* in the *ets-5* mutant

B *odr-1* is expressed normally in the *ets-5* mutant

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.

A The *C. elegans* ETS family

B ETS proteins in humans and *C. elegans*

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°C. n = 50-120 animals for each genotype. B. *ets-5* mutants have normal brood sizes. Animals were grown at 20°C. n = 8-21 animals for each genotype. Error bars represent SEM.