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The homeodomain protein hmbx-1 maintains asymmetric gene expression in adult *C. elegans* olfactory neurons

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Differentiated neurons balance the need to maintain a stable identity with their flexible responses to dynamic environmental inputs. Here we characterize these opposing influences on gene expression in *Caenorhabditis elegans* olfactory neurons. Using transcriptional reporters that are expressed differentially in two olfactory neurons, AWCON and AWCOFF, we identify mutations that affect the long-term maintenance of appropriate chemoreceptor expression. A newly identified gene from this screen, the conserved transcription factor hmbx-1, stabilizes AWC gene expression in adult animals through dosage-sensitive interactions with its transcriptional targets. The late action of hmbx-1 complements the early role of the transcriptional repressor gene nsy-7: Both repress expression of multiple AWCOFF genes in AWCON neurons, but they act at different developmental stages. Environmental signals are superimposed onto this stable cell identity through at least two different transcriptional pathways that regulate individual chemoreceptor genes: a cGMP pathway regulated by sensory activity, and a daf-7 (TGF-β)/daf-3 (SMAD repressor) pathway regulated by specific components of the density-dependent *C. elegans* dauer pheromone.

**Keywords:** Chemoreceptor, olfactory receptor; homeostasis; neuronal development; olfactory neuron

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Neurons and other long-lived cells are subject to ongoing modification throughout life, but use transcriptional strategies to maintain stable cell fates [Shirasaki and Pfaff 2002; Ringrose and Paro 2004]. In some cells, transcription factors required for the establishment of cell fate remain active throughout life. In the mammalian immune system, the transcription factor Pax5 is required both for initial commitment to the B-cell lineage and for continued expression of B-cell identity: Deletion of Pax5 from committed pro-B cells resulted in their reversion to a multipotential state [Nutt et al. 1999; Mikkola et al. 2002]. In other cases, dedicated transcriptional regulators maintain cell identity. For example, the *Caenorhabditis elegans* aristaless homolog alr-1 is required to maintain, but not establish, functions of sensory glia [Tucker et al. 2005]. In addition to cell-specific transcription factors, general chromatin remodeling factors can stabilize cell fates. The Polycomb group (PcG) genes in *Drosophila* maintain a precise Hox gene expression pattern after the disappearance of early developmental regulators [Busturia and Morata 1988; Cao et al. 2002; Muller and Kassis 2006], and the histone acetyltransferase system regulated by BET-1 and MYST maintains cell type-specific gene expression in *C. elegans* neuronal lineages [Shibata et al. 2010].

Sensory neurons are faced with the special challenge of maintaining a stable state while responding to a changing environment. In the senses of taste and smell, heterogeneous populations of sensory neurons express different chemoreceptor genes to detect different environmental chemicals [Buck and Axel 1991; Troemel et al. 1995; Vosshall et al. 1999; Vosshall et al. 1999; Clyne et al. 2000; Etchberger et al. 2007]. The expression of chemoreceptor genes is initiated by innate developmental programs. For example, in *C. elegans*, chemoreceptor expression in the two ASE taste neurons is initiated by a general transcriptional regulator for ASE, che-1, and refined by a double negative feedback loop that distinguishes right and left ASE fates [Chang et al. 2003, 2004; Johnston and Hobert 2003; Johnston et al. 2005; Etchberger et al. 2007, 2009]. che-1 maintains its own expression in the ASE chemosensory neurons, and also acts on target genes throughout life [Etchberger et al. 2009]; this combination of initiation and differentiation roles defines che-1 as a “terminal selector gene” [Hobert 2008]. Analogous genetic studies of other chemosensory cell types—including the AWA, AWB, and AWC neurons—suggest that the genetic circuitry of chemosensory systems is conserved across phyla.
olfactory neurons—have generated a sophisticated un-
derstanding of the transcription factors that initiate unique neuronal identities (Sengupta et al. 1994; Sagasti et al. 1999; Sarafi-Reinach and Sengupta 2000; Sarafi-Reinach et al. 2001; Colosimo et al. 2003; Lanjuijn et al. 2003; Nokes et al. 2009; Kim et al. 2010).

Superimposed on stable chemosensory neuron fates are environmental factors that modify gene expression. The C. elegans dauer pheromone, a mixture of compounds containing the sugar ascorylrose, represses the expression of chemoreceptor genes in ASH and ASI neurons by regulating intercellular signaling through a TGF-β signaling pathway (Peckol et al. 2001; Nolan et al. 2002; Kim et al. 2009). A salt-inducible kinase affects chemoreceptor expression in AWB olfactory neurons by regulating the transcription factor MEF2 (Lanjuijn and Sengupta 2002; van der Linden et al. 2007, 2008). The relationship between these environmental regulators and stable cell fates raises intriguing questions about the relative roles of fixed and variable aspects of neuronal function.

The two C. elegans AWC olfactory neurons provide a system in which the acquisition and maintenance of cell fates are distinct. The Otx transcription factor CEH-36 and the HMX/NLX homeodomain protein MLS-2 initiate a general AWC identity, which is subsequently maintained by ceb-36 (Lanjuijn et al. 2003; Kim et al. 2010). In addition to promoting the expression of an AWC-specific transcriptional program, ceb-36 maintains its own expression, suggesting that ceb-36 is the terminal selector gene in AWC (Kim et al. 2010). Later in embryogenesis, a stochastic cell fate decision causes the right and left AWC olfactory neurons to take on asymmetric fates, such that one AWC becomes AWC\textsuperscript{ON}, defined as a neuron that expresses the G protein-coupled receptor (GPCR) str-2 and senses the odor butanone, and the other AWC becomes AWC\textsuperscript{OFF}, which expresses the GPCR srxs-3 and senses the odor 2,3-pentanedione (Troemel et al. 1999; Wes and Bargmann 2001). The decision to become AWC\textsuperscript{ON} or AWC\textsuperscript{OFF} is made through a signaling pathway that generates the initial asymmetry of chemoreceptor gene expression and also drives asymmetric expression of the transcription factor NSY-7 in AWC\textsuperscript{ON} (Troemel et al. 1999; Lesch et al. 2009). After hatching, the initial signaling pathway becomes inactive, and NSY-7 maintains appropriate chemoreceptor expression in AWC\textsuperscript{ON}.

In addition, a cGMP pathway regulated by olfactory signal transduction maintains post-embryonic expression of both str-2 and srxs-3 using two receptor-type guanylate cyclases [encoded by odr-1 and daf-11], a cyclic-nucleotide gated cation channel [encoded by tax-2 and tax-4], and a cGMP-responsive protein kinase [encoded by egl-4] (Troemel et al. 1999; Lesch et al. 2009). To better understand the factors responsible for the stability of AWC\textsuperscript{ON} and AWC\textsuperscript{OFF} fates, we performed a screen for mutants that failed to maintain expression of one or both of the asymmetric AWC markers str-2 and srxs-3. Here, we describe the mutants isolated from this screen. We identify the transcription factor hmbx-1, a homolog of mammalian HMBOX1, as a regulator of AWC receptor gene expression that acts primarily in adult animals. Using newly identified GPCR genes expressed in AWC neurons, we show that the maintenance of asymmetric receptor gene expression involves at least three repressor pathways: nsy-7, an AWC\textsuperscript{ON}-specific cell identity gene; hmbx-1, a dosage-sensitive repressor of AWC\textsuperscript{OFF} genes in AWC\textsuperscript{ON} neurons; and daf-3, a pheno-
mone-regulated repressor that affects chemoreceptors on a gene-by-gene basis.

**Results**

**Genetic pathways required for maintenance of GPCR expression in AWC**

We sought mutants that expressed the AWC\textsuperscript{ON}-specific receptor str-2 and the AWC\textsuperscript{OFF}-specific receptor srxs-3 appropriately as early larvae, but failed to maintain expression of one or both of these receptors in adulthood. A strain with an integrated transgene containing str-2::dsRed2 and srxs-3::GFP reporters was mutagenized, and the adult F2 progeny were examined for defects in expression of str-2, srxs-3, or both genes (Fig. 1A). After verifying the adult phenotype in subsequent generations, expression of str-2 and srxs-3 was evaluated in early larvae, 14 h after hatching (L1 stage), and mutants with a wild-type phenotype at this stage were retained. The screen yielded 19 mutants with defects in the mainte-

ance of AWC markers (Fig. 1B). Genetic mapping and complementation testing indicated that 13 mutations fell in previously characterized genes and pathways, although only a subset had been known to affect AWC gene expression. These genes are described briefly below.

**Olfactory transduction**

Six of the new mutations affect the olfactory cGMP transduction pathway that maintains str-2 and srxs-3 expression, including three alleles of odr-1, one allele of daf-11, one allele of tax-2, and one allele of tax-4 (Fig. 1B; Troemel et al. 1999; Lesch et al. 2009). Previous studies have revealed both cell-autonomous and nonautonomous effects of sensory signaling proteins on AWC gene expression (Lans and Jansen 2006). Moreover, tax-4 promotes expression of daf-7 (Coburn et al. 1998), which acts in ASI neurons to promote srxs-3 expression in AWC neurons (see below). To ask where tax-4 acts to regulate srxs-3 expression, we expressed the tax-4 cDNA in AWC or ASI in a tax-4[ky791] mutant background. AWC-selective expression of TAX-4 rescued the srxs-3 expression defect of tax-4[ky791] mutants, but ASI expression did not (Supplemental Fig. S1). Therefore, tax-4 acts in AWC to promote srxs-3 expression.

The screen also yielded a dominant mutation in the Gα subunit odr-3, odr-3[ky879], which encodes a G → S missense mutation at position 185. The affected glycine is a conserved residue in the region that changes confor-
mation upon GTP binding, stabilization of the GTP
-bound conformation should result in a constitutively active protein (Rens-Domiano and Hamm 1995). The nature of the odr-3[ky879] allele suggests that increased olfactory G protein activity disrupts maintenance of
GPCR expression, perhaps by reducing cGMP levels (Chalasani et al. 2007). Loss-of-function mutations in odr-3 have little effect on str-2 expression, although str-2 expression is reduced when odr-3(lf) alleles are combined with mutations in other Ga subunits (Lans and Jansen 2006).

Transcriptional regulation Two alleles of nsy-7 isolated in the screen had defective maintenance of str-2 expression, accompanied by bilateral srsx-3 expression—the same phenotype observed in previously characterized nsy-7 alleles (Lesch et al. 2009).

Another mutation affected the tam-1 gene, which encodes a transcriptional regulator that inhibits silencing of repetitive transgenes (Hsieh et al. 1999). A null allele of tam-1 also affects str-2 expression, but had a weaker defect than the new missense allele from the screen (Fig. 1B). The stronger phenotype of the missense allele could result from an altered function of the mutant protein, or from modifying effects of background mutations. tam-1

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was not known previously to affect gene expression in AWC, but diminished expression of the AWC reporter genes is consistent with the general reduction in transgene expression reported in \textit{tam-1} mutants.

\textbf{DAF-7/TGF\textsubscript{\beta} signaling} Several mutations from this screen primarily affected \textit{srsx-3} and not \textit{str-2} expression. These included an allele of \textit{daf-7} [the TGF\textsubscript{\beta} ligand that regulates the dauer developmental decision and chemoreceptor gene expression in ASH and ASI neurons] and an allele of \textit{daf-1} [a TGF\textsubscript{\beta} type I receptor] \cite{Figs. 1B, 2A,B; Georgi et al. 1990, Ren et al. 1996, Schackwitz et al. 1996, Peckol et al. 2001, Nolan et al. 2002}. A mutation in \textit{unc-3}, a transcription factor required for expression of \textit{daf-7} in the ASI neurons, was also isolated in the screen, and had a similar phenotype \cite{Prasad et al. 1998, Kim et al. 2005}. Existing \textit{daf-4} [the type II TGF\textsubscript{\beta} receptor], \textit{daf-8} [a Smad-like protein], and \textit{daf-14} [a Smad-like protein] mutants displayed similar adult phenotypes to \textit{daf-7} and \textit{daf-1} \cite{Fig. 2C}. These results indicate that TGF\textsubscript{\beta} signaling maintains \textit{srsx-3} expression in AWC\textsuperscript{OFF}.

\textit{daf-3}, which encodes a co-Smad that binds DNA, and \textit{daf-5}, which encodes a proline-rich transcriptional regulator, act downstream from and antagonistically to \textit{daf-1} to promote dauer formation \cite{Patterson et al. 1997, da Graca et al. 2004}. Expression of \textit{str-2} and \textit{srsx-3} reporters was normal in \textit{daf-3} and \textit{daf-5} single mutants, and in \textit{daf-3}, \textit{daf-1} or \textit{daf-5}; \textit{daf-1} double mutants, recapitulating the regulatory relationships seen in dauer formation \cite{Fig. 2B,C}. Expression of DAF-1 in AWC rescued the \textit{srsx-3} expression defect of \textit{daf-1(m40)} mutants, suggesting that TGF-\beta signals directly to AWC to maintain receptor gene expression \cite{Fig. 2C}.

\textit{daf-7} expression is inhibited by the dauer pheromone, a mixture of structurally related chemicals termed ascarosides \cite{Jeong et al. 2005, Butcher et al. 2007, 2008}. To ask whether \textit{srsx-3} expression responded acutely to pheromones, we exposed adult worms to the ascarosides C3, C6, and C9 for 4 h, and monitored expression of \textit{srsx-3} using a destabilized GFP protein that has a half-life of \textit{1 h} in \textit{C. elegans} \cite{Gaudet and Mango 2002, Frand et al. 2005}. An equal mixture of C3, C6, and C9 suppressed \textit{srsx-3} expression in a dose-dependent manner \cite{Fig. 2D}. The ascarosides C3 and C6 each suppressed \textit{srsx-3} expression about as well as the mixture, while C9 was less effective. All effects of ascarosides were blocked in \textit{daf-3} mutants, suggesting that C3 and C6 pheromones regulate \textit{srsx-3} expression through the TGF\textsubscript{\beta} pathway \cite{Fig. 2D}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{TGF\textsubscript{\beta} and dauer pheromone signals regulate \textit{srsx-3} expression. (A) Images of L1 larvae and adults in wild-type, \textit{daf-7(e1372)}, and \textit{daf-1(m40)} animals. AWC neurons are labeled, and asterisks mark AWB neurons. (B) Schematic illustration of the \textit{daf-7} TGF\textsubscript{\beta} pathway. (C) \textit{srsx-3} and \textit{str-2} expression phenotypes for \textit{daf-7(TGF-\beta pathway mutants and rescue of daf-1(m40)} under the AWC-selective promoter \textit{odr-3}. Also shown is the phenotype of the \textit{hmbx-1(ky777), daf-3(e1376)} double mutant. \textit{n > 25} for all genotypes. (D) Regulation of \textit{srsx-3} by a mixture of C3, C6, and C9 ascarosides [left] and by each ascaroside alone [two middle and right]. Graphs show the fraction of adults expressing a destabilized GFP in AWC under the control of the \textit{srsx-3} promoter. [*] \textit{P < 0.01}; [**] \textit{P < 0.001} compared with no pheromone control (one-way ANOVA with Dunnett’s post-test).}
\end{figure}
A missense allele of the transcription factor hmbx-1 suppresses srsx-3 expression

In the mutant ky777, srsx-3 expression in AWC <sup>OFF</sup> was lost after L1, but str-2 expression in AWC <sup>ON</sup> was retained (Figs. 1, 3A). Although this phenotype resembled that of daf-7/TGFβ mutants, ky777 mutants were normal for dauer formation, and the srsx-3 expression defect in these mutants was not suppressed by daf-3 [Fig. 2C]. Therefore, ky777 appeared to act separately from the TGFβ pathway. ky777 was mapped to an interval of ~440 kb on the left arm of chromosome I, but no rescue was observed after injection of fosmids or PCR products covering the interval. Therefore, a Solexa-Illumina whole-genome sequencing approach was used to identify mutations in the interval. Unique alignments of ky777 sequence reads to the C. elegans genome accounted for 84% of sequence in the interval, with an average coverage depth of 7.9 ×. The gigaBayes program identified 19 high-probability point mutations and three single-base indels between ky777 and the reference genome. Two missense mutations and one silent mutation were present in coding exons, one mutation was in a 3′ untranslated region (UTR), and one mutation was in a 5′ UTR. PCR and conventional sequencing determined that one of the coding mutations and both UTR mutations were present both in the mutant strain and in the original, unmutagenized strain, but that the remaining coding mutation was present in the ky777 mutant but not in the original strain. This mutation represents a C → T transition in the predicted gene F54A5.1 that results in a missense H → Y mutation at the C-terminal end of the protein (Fig. 3B, red box), and was considered the most likely candidate for ky777. Transgenic introduction of a wild-type genomic copy of F54A5.1 partially restored srsx-3 expression in ky777 [Fig. 3C; see below], supporting further analysis of this gene.

F54A5.1 encodes a predicted conserved homeodomain transcription factor that contains an HNF-1 N-terminal-like domain and a serine-rich region at its C terminus; it is closely related to the mammalian gene HMBOX1 [Fig. 3C]. The homeodomain of F54A5.1 and its homologs includes an unusual 17-amino-acid insertion not present in other homeodomains [Fig. 3B, black box]; the entire protein is highly conserved in multiple species, including zebrafish, mice, and humans [Fig. 3C]. Because of this high degree of conservation with HMBOX1 genes, we named F54A5.1 hmbx-1.

A transgene in which GFP was expressed under the control of 7 kb of the hmbx-1 upstream region drove expression in both AWCs, in the chemosensory neurons ASI, AFD, ASH, and URX; in the mechanosensory neurons ALM, PLM, PVD, and FLP; in a few additional head and tail neurons; and in the seam cells of the hypodermis [Fig. 3D, data not shown]. The expression of hmbx-1 in the AWCs supports the hypothesis that it regulates srsx-3 expression in these neurons. An HMBX-1 cDNA tagged with GFP localized to the nucleus of AWC, consistent with its predicted function as a transcription factor [Fig. 3E]. A GFP-tagged HMBX-1[H404Y] protein corresponding to the ky777 missense mutant also localized to the nucleus.

ky777 is an altered-function allele of hmbx-1

A deletion allele of hmbx-1, tm1274, was kindly provided by the National BioResource Project in Japan. The
HMBX-1 maintains neuronal identity

Deletion eliminates the HMBX-1 homeodomain, and results in a frameshift and early stop codon, and is therefore likely to be a null allele. The hmbx-1(tm1274) mutant was healthy and fertile, and had mild defects in chemotaxis to odors sensed by AWC neurons (Supplemental Fig. S2). Surprisingly, srsx-3 expression was normal in the hmbx-1(tm1274) mutants [Fig. 4A]. The different phenotypes of the hmbx-1(ky777) and hmbx-1(tm1274) mutants indicate that ky777 is not a null allele of hmbx-1.

A set of genetic experiments suggested that the ky777 allele results in altered, dosage-sensitive activity of the hmbx-1 gene. hmbx-1(ky777) animals were largely normal, indicating that ky777 is recessive to the wild-type allele [Fig. 4A]. hmbx-1(ky777)/hmbx-1(tm1274null) animals had an intermediate phenotype compared with either starting strain, a result suggesting that tm1274 eliminates the wild-type gene activity that suppresses ky777, and supporting the hypothesis that the two mutations affect the same gene [Fig. 4A]. Reducing hmbx-1 expression using RNAi in wild-type animals had little effect on srsx-3 expression, but RNAi against hmbx-1 in hmbx-1(ky777) mutants restored srsx-3 expression to many animals [Fig. 4B]. These results suggest that RNAi is reducing an altered hmbx-1 activity to generate an hmbx-1-null [wild-type-like] phenotype.

A moderate increase in hmbx-1 activity was attained by injecting wild-type hmbx-1 and hmbx-1(ky777) genomic DNAs into wild-type, hmbx-1(ky777), and hmbx-1(tm1274) animals. In a wild-type background, neither the wild-type nor the mutant hmbx-1 gene had a significant effect on srsx-3 expression, in agreement with the observation that hmbx-1(ky777) is a recessive allele [Fig. 4C]. In a null background, expression of the hmbx-1(ky777) mutant DNA, but not wild-type hmbx-1, partly repressed srsx-3 expression, confirming that hmbx-1(ky777) represses srsx-3 under conditions in which the wild-type hmbx-1 gene does not. Finally, in a hmbx-1(ky777) mutant background, wild-type hmbx-1 partly restored srsx-3 expression, indicating that it antagonized hmbx-1(ky777) [Fig. 4C].

The results described above indicate that wild-type hmbx-1 antagonizes hmbx-1(ky777), and are consistent with two additional possibilities: (1) On its own, the hmbx-1(ky777) allele has a high or unregulated level of hmbx-1 activity, or (2) the hmbx-1(ky777) allele has an abnormal activity unrelated to normal hmbx-1 function. To distinguish between these alternatives, wild-type hmbx-1 cDNA was expressed at high levels in the AWC neurons using the odr-3 promoter, and the effects were examined in wild-type, hmbx-1(tm1274), and hmbx-1(ky777) animals. High-copy odr-3::hmbx-1 transgenes repressed srsx-3 in wild-type and tm1274 backgrounds, like the recessive ky777 mutant [Fig. 4D]. These results suggest that hmbx-1(ky777) mutants resemble animals with increased hmbx-1 repressor activity in AWC. However, a few complications suggest that the effects of

Figure 4. ky777 is an altered-function allele of hmbx-1. (A) srsx-3 and str-2 expression phenotypes of wild-type, hmbx-1(tm1274), and hmbx-1(ky777) homozygotes, and ky777+/+ and ky777/tm1274 heterozygotes. n > 50 for all genotypes. (B) RNAi against hmbx-1 in wild-type and ky777 backgrounds. All strains contained the RNAi-sensitizing era-1(mg366) and lin-15B(n744) mutations. (*** Different at P < 0.001 (Fisher's exact test). n > 40 for all conditions. (C) Moderate overexpression of hmbx-1 under endogenous regulatory elements. Expression of str-2 and srsx-3 in wild-type, ky777, or tm1274 mutant animals expressing a genomic fragment covering wild-type or ky777 mutant genomic coding sequence with 7 kb of upstream sequence. (*** Different from transgene-negative control at P < 0.001 (χ² test), n > 30 for all conditions. (D) Overexpression of hmbx-1 from a strong AWC promoter. Expression of str-2 and srsx-3 in wild-type, hmbx-1(tm1274), and hmbx-1(ky777) animals overexpressing a wild-type HMBX-1 cDNA under the odr-3 promoter. (*** Different from transgene-negative control at P < 0.001 (χ² test). n > 60 for all genotypes.
hmbx-1 overexpression may not be entirely straightforward. First, str-2 expression was sometimes misregulated in odr-3::hmbx-1 animals, but not in hmbx-1(ky777) animals (Fig. 4D). Second, expression of hmbx-1 under the osm-3 promoter, which drives expression in 26 chemosensory neurons but not in AWC ([Tabish et al. 1995], resulted in ectopic expression of srsx-3 in AWC\textsuperscript{ON} in some animals [Supplemental Fig. S3]. hmbx-1 may therefore have cell-nonautonomous as well as cell-autonomous effects on srsx-3.

Bearing these potential complications in mind, the genetic results suggest that hmbx-1(ky777) has unregulated or increased hmbx-1 activity that inappropriately represses srsx-3 activity in AWC\textsuperscript{OFF}. A GFP-tagged HMBX-1(ky777) protein was expressed at similar levels to a tagged wild-type protein, suggesting that there were no major effects on protein stability. It is possible that the mutation affects an autoregulatory activity of hmbx-1, as expression of an hmbx-1::GFP transcriptional reporter was reduced in the AWC neurons of hmbx-1(ky777) mutants [Supplemental Fig. S3].

Single-copy srsx-3::GFP transgenes are regulated by hmbx-1 and its predicted binding site

During the gene dosage studies, we were struck by the variation in the mutant phenotype, depending on whether the copy number of hmbx-1 was low [endogenous hmbx-1], intermediate [genomic hmbx-1 DNA injection], or high [odr-3::hmbx-1 cDNA injection]. In all of these experiments, str-2::dsRed2 and srsx-3::GFP reporter genes were present at high-copy number in the kyIs408 transgene, which could distort their interactions with a dosage-sensitive hmbx-1 transcription factor. Therefore, to provide a more natural context for examining gene regulation effects, we generated single-copy srsx-3::GFP transgene reporters at a defined site on chromosome II using the Mos Single-Copy Insertion [MosSCI] technique [Frokjaer-Jensen et al. 2008].

In wild-type animals, the single-copy srsx-3::GFP reporter recapitulated the expression pattern of a high-copy srsx-3::GFP array, albeit with a weaker GFP signal [Fig. 5A]. Young larvae expressed GFP in both AWC neurons, but expression was restricted to a single AWC\textsuperscript{OFF} neuron in adults [Fig. 5A; data not shown]. Expression of the single-copy srsx-3::GFP transgene was lost in hmbx-1(ky777) mutants, and genetic interactions with hmbx-1(ky777) mutations were similar to those with a high-copy srsx-3::GFP array [Fig. 5A, B; Supplemental Fig. S4]. Remarkably, the single-copy srsx-3::GFP reporter uncovered an opposite phenotype for the hmbx-1(tm1274)-null allele: The 3′-specific srsx-3::GFP reporter was misexpressed in both AWCs in a fraction of hmbx-1(tm1274) adults [Fig. 5A, B]. Thus, in an hmbx-1-null mutant, srsx-3 is derepressed in AWC\textsuperscript{ON}, whereas, in the hmbx-1(ky777) mutant, srsx-3 is inappropriately repressed in AWC\textsuperscript{OFF}. These straightforward results with single-copy transgenes support and extend the conclusions from high-copy arrays. They suggest that hmbx-1 represses srsx-3 expression in AWC\textsuperscript{ON} neurons in the adult stage, that its effect is partly redundant with other repressors, as it is only partially penetrant, and that hmbx-1(ky777) is a recessive gain-of-function allele of hmbx-1 that inappropriately represses srsx-3 in AWC\textsuperscript{OFF} neurons.

The binding site of the mouse homolog of HMBX-1, HMBOX1, has been identified using an in vitro binding assay [Berger et al. 2008]. A similar site is present in the srsx-3 promoter, suggesting a potential site for regulation by HMBX-1 [Fig. 5C]. The significance of this site was examined by deleting it from the srsx-3 promoter [srsx-3(ΔH)] and introducing a single-copy insertion of the srsx-3(ΔH)::GFP sequence into the same MosSCI site used for the wild-type srsx-3::GFP reporter. Unlike the wild-type reporter, the mutated reporter was expressed in both AWCs in a fraction of wild-type animals, suggesting that

Figure 5. Regulation of single-copy srsx-3::GFP lines by hmbx-1. [A] Confocal images of the single-copy srsx-3::GFP reporter in wild-type, hmbx-1(tm1274), and hmbx-1(ky777) animals. [Arrowsheads] AWC, [asterisks] AWB. [B] Expression of singly integrated srsx-3::GFP in wild-type and mutant adults. n > 40 for all conditions. [C, top] A diagram of the srsx-3 promoter with the positions of predicted transcription factor-binding sites. [Bottom] Binding site for HMBOX1, the mouse homolog of HMBX-1, shown above the sequence in the srsx-3 promoter that was deleted in the srsx-3(ΔH)::GFP reporter. [D] Phenotypes of wild-type, hmbx-1(tm1274), and hmbx-1(ky777) animals expressing the singly integrated srsx-3(ΔH)::GFP reporter. n > 40 for all genotypes. [E] Expression of singly integrated srsx-3::GFP in young [1-d-old; 12 h after the L4 stage] and older (2-, 4-, 7-, or 10-d-old) adults in wild-type and mutant backgrounds. For hmbx-1(tm1274), one asterisk (*) indicates difference from young [1-d-old] adults at P < 0.05 and two asterisks (**) indicate difference from young adults at P < 0.01 (Fisher’s exact test). For hmbx-1(ky777), three asterisks (****) indicate difference from young adults at P < 0.001.
the sequence normally represses srsx-3 in AWCON [Fig. 5D]. The expression of single-copy srsx-3 reporters with and without the predicted HMBX-1-binding site was then compared in wild-type, hmbx-1(tm1274), and hmbx-1(ky777) backgrounds. The single-copy srsx-3 reporter lacking the binding site behaved identically in wild-type and hmbx-1(1tm1274) mutants [Fig. 5D], as predicted if HMBX-1 regulates srsx-3 expression by binding to this site. Deletion of the predicted binding site partly suppressed the effects of the hmbx-1(ky777) mutation, suggesting that the altered-function protein also interacts with this site [Fig. 5D]. Thus, the use of single-copy srsx-3::GFP reporters identified a loss-of-function phenotype for hmbx-1, and a likely site for HMBX-1 binding and regulation in vivo.

Multiple repressors interact to regulate asymmetric AWC-specific genes

Because str-2 is the only known AWCON-specific gene and srsx-3 is the only known AWCOFF-specific gene, it was not clear whether transcription factors such as hmbx-1, nsy-7, and daf-3 regulate individual receptor genes or the entire asymmetric AWC identity. Therefore, we sought and characterized additional markers that distinguished the AWCON and AWCOFF neurons. The C. elegans gene expression project at the University of British Columbia has reported expression of several dozen predicted chemoreceptor genes in head sensory neurons [Dupuy et al. 2007], by examining 20 of these strains, we found that the srt-26 and srt-28 reporter genes were expressed strongly in a single AWC neuron, and the srt-29 gene was expressed weakly in a single AWC. These three genes were coexpressed with str-2 but not srsx-3, indicating that their expression was specific to AWCON.

AWCOFF-specific reporters were sought using the binding site for the transcription factor NSY-7, which was defined previously by direct DNA-binding experiments [Lesch et al. 2009]. Fourteen predicted chemoreceptor genes in the C. elegans genome contain the CCTTAAC NSY-7-binding sequence within 300 base pairs (bp) of the coding start site. Fluorescent reporters for these 14 genes were generated by fusing 2 kb upstream of the start site to GFP; two of these 14 genes, srt-45 and srt-47, were expressed strongly in a single AWC neuron and weakly in an additional pair of head neurons. In both cases, expression was present in AWCOFF but not AWCON, based on coexpression with the srsx-3::mCherry reporter [Fig. 6A]. It is interesting that five genes of the srt family are expressed in AWC neurons, but four other tested srt genes were expressed in different neurons, so it is not a universal pattern [data not shown].

The newly identified AWCOFF markers srt-45 and srt-47 and the AWCON markers srt-26 and srt-28 were examined in mutants that affect srsx-3 and str-2 expression. In nsy-7 mutant backgrounds, two CEH-36-binding sites involved in driving the AWCON reporter srsx-3::GFP, which also labels AWB (asterisks), [B] Expression of the AWCOFF markers [srsx-3, srt-45, and srt-47] and the AWCON markers [str-2, srt-26, and srt-28] in wild-type and mutant backgrounds. n > 30 for all conditions; (*) difference from wild-type at P < 0.01 (Fisher’s exact test). [C] Schematic of AWCON and AWCOFF promoters showing predicted binding sites for the transcription factors CEH-36, HMBX-1, NSY-7, and DAF-3. [D, top] Schematic of odr-1::GFP constructs inserted as single copies. [odr-1(a)] Wild-type 2.4-kb odr-1 promoter fragment; [odr-1(b)] NSY-7-binding site added to the 5’ end of the odr-1 promoter; [odr-1(c)] NSY-7-binding site added 200 bp upstream of the ATG in the odr-1 promoter. For odr-1(c), the NSY-7-binding site is inserted close to, but does not disrupt, two CEH-36-binding sites involved in driving odr-1 expression [Kim et al. 2010]. [Bottom] Expression of integrated single-copy odr-1 plasmids in wild-type and nsy-7(tm3080) backgrounds. Multiple bars within the same condition represent independently integrated lines. n > 25 for all genotypes.
mutants, all three AWC\textsuperscript{OFF} markers were expressed in both AWC\textsubscript{s}, and all three AWC\textsuperscript{ON} markers were reduced or absent [Fig. 6B]. As predicted by earlier studies, this result suggests that, in \textit{nsy-7} mutants, AWC\textsuperscript{ON} is transformed into AWC\textsuperscript{OFF}.

\textit{hmbx-1(ky777)} regulated AWC\textsuperscript{OFF} chemoreceptor expression in a cell-wide manner, reducing expression of all three AWC\textsuperscript{OFF}-specific markers, but sparing the three AWC\textsuperscript{ON} markers [Fig. 6B]. The symmetric AWC signaling genes \textit{odr-3} and \textit{odr-1} were expressed normally in AWC\textsuperscript{OFF} neurons in \textit{hmbx-1(ky777)} mutants, and AWC\textsuperscript{OFF} morphology appeared normal, indicating that the AWC\textsuperscript{OFF} neurons lost their asymmetric identity, but did not die or degenerate (Supplemental Fig. S5; data not shown).

Both \textit{daf-1} TGF\textbeta\ mutations and \textit{odr-1} cGMP olfactory transduction mutants showed a distinct, gene-specific pattern of regulation of AWC\textsuperscript{OFF} and AWC\textsuperscript{ON} chemoreceptors. In \textit{daf-1} mutants, expression of the AWC\textsuperscript{OFF} markers \textit{srsx-3} and \textit{srt-47} was reduced, but the AWC\textsuperscript{OFF} marker \textit{srt-45} and all AWC\textsuperscript{ON} markers were expressed at wild-type levels [Fig. 6B]. \textit{odr-1} mutants were defective for expression of all three AWC\textsuperscript{OFF} markers and the AWC\textsuperscript{ON} marker \textit{str-2}, but expression of the AWC\textsuperscript{ON} markers \textit{srt-26} and \textit{srt-28} was largely intact [Fig. 6B]. Thus, the TGF\textbeta\ and cGMP-dependent pathways regulate subsets of chemoreceptor genes in both AWC\textsuperscript{OFF} and AWC\textsuperscript{ON} neurons.

The relationships between the different transcriptional pathways were probed by examining double mutants, using the multicopy \textit{str-2} and \textit{srsx-3} reporters and the \textit{srsx-3} single-copy reporter. In general, double mutants recapitulated single mutant phenotypes in simple patterns, suggesting that \textit{hmbx-1}, \textit{nsy-7}, and \textit{daf-1} act independently of each other [Supplemental Fig. S6]. \textit{odr-1} defects were partly suppressed by \textit{hmbx-1(tm1274)}, suggesting that these two pathways are additive and independent; \textit{hmbx-1(ky777)} and \textit{nsy-7} effects were also additive.

The seven asymmetric AWC promoters had many potential binding sites for AWC-regulating proteins [Fig. 6C]. All seven promoter fragments had predicted binding sites for CEH-36, the AWC terminal selector gene, and potential binding sites for DAF-3; six of seven promoters had matches to the consensus binding site for HMBX-1. NSY-7 sites were only present in the three AWC\textsuperscript{OFF} promoters. In previous studies, analysis of the \textit{srsx-3} promoter suggested that NSY-7 binding was necessary for asymmetric expression [Lesch et al. 2009]. The identification of additional transcriptional repressors raised the question of whether NSY-7 binding was also sufficient for asymmetric expression. To address this question, a single copy of the CCTTAAC sequence was inserted into an \textit{odr-1}::\textit{GFP} reporter, which is ordinarily expressed in both AWC neurons [and also in AWB neurons]. Single-copy \textit{odr-1}::\textit{GFP} reporters with and without CCTTAAC sequences were inserted at a defined site on chromosome II using the MosSCI method. The wild-type single-copy \textit{odr-1}::\textit{GFP} fusion was expressed in both AWC neurons, but an \textit{odr-1} plasmid with a CCTTAAC site 200 bp upstream of the ATG was expressed asymmetrically in a single AWC neuron [Fig. 6D]. Coexpression of this transgene with \textit{srsx-3} but not \textit{str-2} reporters indicated that the CCTTAAC site repressed expression in AWC\textsuperscript{ON}. When crossed into a \textit{nsy-7} mutant, the modified transgene was again expressed in both AWC neurons [Fig. 6D]. A more distal insertion of the NSY-7 site was expressed bilaterally. These results support the hypothesis that NSY-7 is a transcriptional repressor, and demonstrate that a single promoter-proximal NSY-7-binding site is sufficient to repress gene expression in AWC\textsuperscript{ON}.

**Discussion**

Multiple environmental and cell-intrinsic influences converge at the transcriptional level to regulate chemoreceptors in AWC neurons. Together with the symmetric AWC terminal selector gene \textit{ceh-36} [Kim et al. 2010], at least four different systems for transcriptional regulation contribute to adult AWC\textsuperscript{OFF}-specific expression of the chemoreceptor gene \textit{srsx-3}. First, the previously identified transcriptional repressor NSY-7, which is expressed preferentially in AWC\textsuperscript{ON}, can repress \textit{srsx-3}-expression by direct binding to a consensus site. Second, cGMP signaling promotes \textit{srsx-3} expression via a cGMP-dependent protein kinase, a cell-autonomous cGMP-gated channel, and unknown transcriptional regulators [Lesch et al. 2009]. Third, density-dependent dauer pheromones repress expression of the secreted TGF\textbeta\ homolog DAF-7, which otherwise acts continuously to maintain \textit{srsx-3} expression. The TGF\textbeta\ pathway regulates multiple chemoreceptor genes\textsubscript{i}, in agreement with this observation, binding sites for SMAD transcription factors such as DAF-3 are among the most common sequences found upstream of chemoreceptor start sites [McCarroll et al. 2005]. Finally, the conserved transcription factor \textit{hmbx-1} represses \textit{srsx-3} expression preferentially in adult AWC\textsuperscript{ON} neurons, although it is expressed in both AWC neurons and can act in AWC\textsuperscript{OFF} when bearing the \textit{ky777} point mutation or when overexpressed.

Genetic analysis of \textit{hmbx-1} suggests that it is involved in long-term maintenance of a specific cell identity, not the developmental establishment of that identity or the regulation of specific genes. The original \textit{hmbx-1(ky777)} mutation is a recessive gain-of-function allele with effects that resemble those of \textit{hmbx-1} overexpression. Because of its nature and its dosage sensitivity, it was identified only by whole-genome sequencing. Although recessive gain-of-function alleles are relatively rare, they have been described in genes encoding potassium channels and their regulators, tyrosine kinase receptors, and the ETS domain transcription factor LIN-1 [Klingler et al. 1988; Jacobs et al. 1998; Perez de la Cruz et al. 2003]. LIN-1-recessive gain-of-function mutations disrupt a negative regulatory domain; it is possible that \textit{ky777} does the same to HMBX-1.

The analysis of a single-copy insertion of the \textit{srsx-3}::\textit{GFP} reporter uncovered a phenotype for the \textit{hmbx-1} null mutant that was not evident with a multicopy reporter, demonstrating that \textit{hmbx-1} normally represses
AWC^OFF chemoreceptor genes in AWC^ON neurons. Single-copy reporter genes also identified a predicted HMBX-1-binding site required for repression of srsx-3 by hmbx-1. Multicopy integrated and extrachromosomal arrays are commonly and successfully used to analyze gene expression in *C. elegans.* However, high-copy transgenes are subject to repeat-induced silencing in the germline and, to a lesser extent, somatic tissues [Hsieh and Fire 2000], and this leads to altered genetic requirements for their expression. For example, specific genes including *tam-1,* a gene isolated in our screen, are required for efficient expression of high-copy transgenes, but not of the corresponding endogenous genes [Hsieh et al. 1999]. Moreover, high-copy transgenes have a high propensity to form heterochromatin, which is not observed with low-copy transgenes of the same sequence [Meister et al. 2010]. We suggest that the heterochromatic state of the high-copy transgene may bypass the normal requirement for *hmbx-1* and perhaps other repressors that maintain gene silencing. The ability to introduce single-copy transgenes into defined genomic locations by MosSCI represents a significant advance for controlling copy number and genomic context effects on gene expression [Frokjaer-Jensen et al. 2008].

*hmbx-1* has effects that are temporally distinct from those of *nsy-7,* a transcription factor that acts in AWC^ON beginning in the L1 larval stage. The relative importance of wild-type *hmbx-1* is greater in older compared with younger adults, and, likewise, the elevated repressive activity of the *hmbx-1*(ky777) mutant increases in adults over time. It may be that NSY-7 loses activity in older animals, or that other changes in gene expression alter the relative importance of the two transcription factors as the animal ages.

The results described here point to a significant role for single transcription factor-binding sites in chemoreceptor expression. The presence of a single binding site for NSY-7 is sufficient to repress expression of an *odr-1* promoter ordinarily expressed in both AWC neurons. Likewise, deletion or mutation of the HMBX-1-binding site in the *srsx-3* promoter permitted misexpression of *srsx-3* in AWC^ON. The relative ease with which sensory receptor expression can be altered suggests genetic malleability and potentially evolutionary flexibility in the specificity of GPCR expression in chemosensory neurons [Jovelin 2009]. Particularly in *C. elegans,* where ectopic expression of a gene in a single additional neuron can dramatically change behaviors, altered expression due to new mutations might have significant consequences for the animal’s behavior and fitness [Troemel et al. 1997].

Despite the potent effects of single binding sites, many additional candidate binding sites were identified in each asymmetric AWC gene. Functional studies of asymmetrically expressed promoters in ASE neurons have uncovered multiple repressor and activator elements in each promoter [Etchberger et al. 2009]. In ASE, *che-1*-binding sites with different affinities contribute to left–right asymmetry of gene expression as well as general ASE identity [Etchberger et al. 2009], it may be informative that the AWC^ON-specific promoters contain more predicted CEH-36-binding sites than the AWC^OFF-specific promoters.

*hmbx-1* is expressed in *C. elegans* sensory neurons, and its human and mouse homologs also display high levels of nervous system expression [Chen et al. 2006]. The human homolog exhibits repressor activity in vitro, and associates with telomeric sequences in several human cell lines [Chen et al. 2006; Dejardin and Kingston 2009]. Telomeres are zones of strong transcriptional repression, suggesting a possible repressor function for HMBX-1 at these locations [Gottschling et al. 1990]. The sequence conservation of HMBX-1 genes between worm and mammalian homologs, their conserved repressor activity, the apparently conserved binding site specificity, and their neuronal expression pattern all hint at possible conserved neuronal functions. With this in mind, it will be interesting to determine the identities of *hmbx-1* target genes, particularly those that are important in older adult animals, and to explore the association of human HMBX1 with telomeric repeats during normal growth and aging.

**Materials and methods**

Genetics and strains

*C. elegans* strains were cultured using standard methods [Brenner 1974]. All strains were grown at 20°C unless otherwise specified. Mutants were isolated by direct inspection of GFP and dsRed fluorescence following mutagenesis of the strain CX7894 *klys408* [srsx-3::GFP,str-2::DsRed2,elt-2::GFP] II [Lesch et al. 2009]. Mutagenesis was performed with ethyl methane sulfonate (EMS) using standard protocols [Brenner 1974]. Among the new mutations, *tax-4* and *tax-2* alleles differed in phenotype from *tax-4* and *tax-2* mutants characterized previously using the *kyIs140* [str-2::GFP] transgene [Troemel et al. 1999; Lans and Jansen 2006]. Whereas expression of *str-2::GFP* from *klys140* was lost in most *tax-2* and *tax-4* mutants, the effect of *tax-2* and *tax-4* on *str-2::DsRed* from *klys408* was milder. Analysis of the canonical tax(dip678)-null mutant indicated that the difference was due to the transgene and not to the nature of the mutant alleles [Fig. 1B]. *klys140* fluorescence is much dimmer than that of *klys408,* so it may be more sensitive to small reductions in expression level.

A strain list appears as Supplemental Table S1.

Molecular biology

Standard molecular biology techniques were used. The *gfp-pest* reporter was made by overlap extension PCR between the GFP vector (d1EGFP-N1, Clontech). The product was then amplified from the pSM-GFP expression vector and the PEST reporter was made by overlap extension PCR between the GFP domain template from a commercially available destabilized GFP reporter with telomeric repeats during normal growth and aging.
MCS2 of a modified pSM expression vector (containing a NotI site in MCS2 instead of MCS1) using the restriction sites SalI and NotI.

The *hmbx-1*(7kb)::*GFP*, *srt-45::GFP*, and *srt-47::GFP* reporters were created by PCR fusion of the promoter, amplified from N2 lysate, with GFP template amplified from pHPD95.75. Reactions were carried out as described in Hobert [2002] with the following primers: *hmbx-1*(7kb): A, 5'-TGGCGATTGCGACAAA ACTCC-3, and A*, 5'-GTAGACCTGCAGCAGTGCT-3', and B, 5'-AGTCGACCTGCAGCAGTGCT-3'; *srt-45*: A, 5'-CTTTTA AGATTGCTACCTGCAGG-3', and A*, 5'-TTGGACATGTAAT CAGCAGTGCT-3'; and B, 5'-AGTCGACCTGCAGCAGTGCT-3', CTTGCGGAAATCTGATTTTCG-3'; *srt-47*: A, 5'-GCCGTA AAAACAGACAGAAA-3', and A*, 5'-ATCTTTTAAAGGTGCT ATTTATTGTTG-3', and B, 5'-AGTCGACCTGCAGCAGTGCT AAGCTTTGTGAATTCAGATG-3'.

To create the *srt-26::GFP*, *srt-28::GFP*, and *srt-29::GFP* reporters, the *srt-26*, *srt-28*, and *srt-29* promoter sequences were amplified by PCR and cloned into MCS1 of the pSM-GFP expression vector using the FseI and AscI restriction enzymes.

For MosSCI experiments, NSY-7-binding sites were added to the *odr-1::*GFP expression vector, and the *ssrx-3::GFP* plasmid was modified to generate the *ssrx-3(AH)::GFP* reporter by site-directed mutagenesis using the the QuikChange II Site-Directed Mutagenesis Kit [Stratagene]. *odr-1::GFP* or *ssrx-3::GFP* sequences were cut out of pSM using the FseI and SpeI restriction sites, and were cloned into a pCFJ151 MosSCI insertion vector (Frokjaer-Jensen et al. 2008) that had been modified to include a FseI site in the MCS.

**Whole-genome sequencing**

Whole-genome sequencing of the *ky777* mutant was performed by the Rockefeller Genomics Resource Center using Solexa-Illumina Genome Analyzer technology. SNP mapping was used to determine a genetic interval of ~440 kb for the mutation. Purified genomic DNA was prepared from 1000–2000 worms, and was used for library preparation. fastq sequences were aligned to the cce6 genome assembly at the University of California at Santa Cruz using the Mosaik alignment program [http://bioinformatics.ucdavis.edu/marshlab/Mosaik#Current_Documentation], and single-base changes, insertions, or deletions were verified using GigaBases polymorphism detection software [http://bioinformatics.ucdavis.edu/marshlab/GigaBases]. High-probability single-base changes that were predicted to fall within an exon, a 3' UTR, or a 5' UTR were checked by PCR and conventional [Sanger] sequencing.

**Sequence alignment**

The *hmbx-1* homeodomain sequence was aligned with HMBOX1 homeodomain sequences using ClustalW at Pôle BioInformatique Lyonnais [http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html]. UniProt ID numbers are as follows: for *C. elegans*, Q9YTY0; for *Brugia malayi*, A8QCW9; for *Danio rerio*, Q4V904; for *Mus musculus*, Q8BJA3; for *Homo sapiens*, A8K5R8.

**RNAi**

RNAi was performed by injection of dsRNA. All assays were performed in an *eri-1*(mg366),*lin-15B*(n744)-sensitized background [Sieburth et al. 2005]. For injection, a dsDNA template corresponding to an exon of the target ORF was amplified from N2 lysate with a T7 sequence [TAATACGACTCACTATAGGG AGA] added at the 5' ends. The following gene-specific sequences were used for these primers: *nsy-7* (exon 2), 5'-GTTGCG AAAAGATATTTCAGATG-3' and 5'-CTTACGAAAACAGCTTGCTTCG-3'; *hmbx-1* (exon 3), 5'-CAAGCTTCCGGTAAAC AAC-3' and 5'-TCACTACGACTCTAAAATAGAAGCC-3'.

Transcription was performed using the T7 Ribomax Express RNAi System [Promega] according to instructions, and the unpurified reaction mix was injected into the body cavity, gut, or gonad of adult hermaphrodites. F1 progeny from eggs laid at least 24 h after the injection were scored after 3–4 d for *ssrx-3* expression. Control experiments established the normal RNAi response of *hmbx-1*(ky777) animals: RNAi against *nsy-7* in *ky777* mutants resulted in a 100% loss of *ssrx-3* expression [44 out of 44 RNAi+ vs. zero out of 90 RNAi− animals, P < 0.0001 [Fisher's exact test]], consistent with the phenotype of *hmbx-1*(ky777):*nsy-7*(tm3080) double mutants.

**MosSCI integrations**

Mos single-copy integrants were generated using the direct insertion protocol described in Frokjaer-Jensen et al. [2008]. Thirty to 50 EG4322, tr[l5605, unc-119(ed3)] worms were injected with *rab-3::mCherry, myo-2::mCherry, myo-3::mCherry, pL43.1* [a vector containing the Mos1 transposase under the control of the germline promoter glh-2], and a vector containing the specific promoter::GFP sequence to be inserted flanked by sequences homologous to the insertion site. Animals that were rescued for the *unc-119* phenotype [array-positive] were allowed to starve out twice, and then *unc-119* rescued animals that lacked the three mCherry coincidence markers [integrant-positive, array-negative] were cloned out from separate plates to find independent integrated lines. These lines were outcrossed twice to wild-type animals, and the presence of the intact insertion was verified by PCR and sequencing.

**Microscopy**

For all microscopy, live animals were immobilized on an agarose pad containing 5 mM NaNO3. Fluorescence microscopy was carried out on a Zeiss Axioplan2 imaging system with a Hamamatsu Photonics C2400 CCD camera, or a Zeiss Axioscope Z1 with ApoTome with a Zeiss AxioCam MRm CCD camera, or a Zeiss Axio Cam MR CCD camera. Most animals were scored under a 20X or 40X Plan-Neofluor objective, where “bright” and “faint” fluorescence were scored qualitatively, and photographs were taken under a 40X Plan-Neofluor or 63X Plan-APOCHROMAT objective. Confocal microscopy [Fig. 5A] was done under a 40X/1.2 W C-Apochromat water immersion objective on a Zeiss LSM 510 confocal imaging system using the Zeiss LSM 510 version 3.2 confocal software.

**Developmental timing**

To evaluate marker expression in the L1 larval stage, larvae were staged by hatch-off. Late embryos were picked to an NGM plate seeded with the *Escherichia coli* strain OP50. After 30 min, just-hatched L1s were transferred to a fresh plate and grown for 14 h at 20°C (for L1s) or ~70 h at 20°C for adults. To compare young and old adults, 25 L4 animals were picked per plate and grown for 12 h (1 d) at 20°C, 36 h (2 d) at 20°C, 84 h (4 d) at 20°C, 156 h (7 d) at 20°C, or 228 h (10 d) at 20°C before scoring. Animals were transferred to new plates every 24 h to prevent crowding and starvation.
Pheromone assays
Ascarosides C3, C6, and C9 (generously provided by R. Butcher and J. Clardy, Harvard Medical School) were added to liquid agar at the concentrations indicated. For negative controls, the same volume of solvent (ethanol) was added to the agar. Ten milliliters of agar was poured into 6-cm culture dishes and allowed to cool. Plates were then seeded with 100 μL of OP50 bacteria and dried in a hood for 1.5–2 h. These plates were either used immediately or stored overnight at 4°C. Twenty srx:3::gfp-pest array-positive young adults (older than L4, with no eggs yet visible in the gonad) were picked to each plate and incubated for 4 h at 25°C. Animals were scored for presence or absence of GFP in AWC neurons under a 40× objective.

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References

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