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.

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olfactory neurons—have generated a sophisticated understanding 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; Lanjuin 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 ascaroside, 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 MEK2 (Lanjuin 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 CEBH-36 and the HMX/NLX homeodomain protein MLS-2 initiate a general AWC identity, which is subsequently maintained by ceh-36 (Lanjuin et al. 2003; Kim et al. 2010). In addition to promoting the expression of an AWC-specific transcriptional program, ceh-36 maintains its own expression, suggesting that ceh-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*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*OFF, which expresses the GPCR *srsx-3* and senses the odor 2,3-pentanedione (Troemel et al. 1999; Wes and Bargmann 2001). The decision to become *AWC*ON or *AWC*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*ON (Troemel et al. 1999; Wes and Bargmann 2001). This asymmetric expression is dependent on the NSY-7 transcription factor (Lans and Jansen 2006). More-
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 Gα 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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**Figure 1.** Expression phenotypes of mutants defective for str-2::dsRed and srsx-3::GFP maintenance. [A] DIC image of the head of an adult worm overlaid with a fluorescence image of the str-2::dsRed2 [AWC(ON)] and srsx-3::GFP (AWC(OFF)) reporters. [B] Mutants from the screen. (Group I) srsx-3 expression affected more strongly than str-2 expression. (Group II) str-2 expression affected more strongly than srsx-3 expression. (Group III) Both markers affected. Other alleles of genes identified in the screen are shown for reference. Bright and faint fluorescence were scored qualitatively; in other figures, these two categories are combined into a single “1 AWC” class.

| genotype       | str-2::dsRed | srsx-3::GFP |
|----------------|--------------|-------------|
|                | 2 on | 1 on | 1 on | 2 off | 1 on | 1 on | 2 off | n    |
| wild type      |      |      |      |      |      |      |      |      |
|                | 100  | 0    | 0    | 100  | 0    | 0    | 100  | 343  |
| Group I:       |      |      |      |      |      |      |      |      |
| srsx-3::GFP    |      |      |      |      |      |      |      |      |
| def-1(ly632)   | 1    | 65   | 4    | 10   | 0    | 0    | 4    | 90   | 210  |
| def-1(ky791)   | 1    | 70   | 13   | 16   | 0    | 0    | 10   | 90   | 286  |
| Q51STOP, after tm domain | 0 | 97 | 0 | 3 | 0 | 0 | 5 | 95 | 66 |
| Q165STOP       | 0 | 73 | 0 | 27 | 0 | 0 | 0 | 100 | 44 |
| Q6256, kinase  | 0 | 98 | 2 | 0 | 0 | 0 | 0 | 100 | 65 |
| unc-94(ky793)  | 0 | 100 | 0 | 0 | 0 | 0 | 1 | 99 | 199 |
| H2404F         | 0 | 100 | 0 | 0 | 0 | 0 | 7 | 93 | 144 |
| Group II:      |      |      |      |      |      |      |      |      |
| str-2::dsRed   |      |      |      |      |      |      |      |      |
| nsy-7(ky873)   | 0 | 0 | 0 | 100 | 98 | 0 | 0 | 2 | 53 |
| tam-1(ky98)    | 0 | 0 | 0 | 27 | 73 | 100 | 0 | 0 | 0 | 64 |
| G765, zinc finger | 0 | 8 | 24 | 68 | 0 | 95 | 5 | 0 | 0 | 264 |
| ky789          | 0 | 0 | 11 | 89 | 3 | 97 | 0 | 0 | 0 | 38 |
| ky802          | 0 | 0 | 0 | 100 | 92 | 8 | 0 | 0 | 0 | 52 |
| Group III:     |      |      |      |      |      |      |      |      |
| both markers   |      |      |      |      |      |      |      |      |
| def-1(ky793)   | 0 | 0 | 0 | 64 | 0 | 0 | 0 | 100 | 51 |
| def-1(ky807)   | 0 | 0 | 0 | 91 | 0 | 0 | 1 | 1 | 99 | 69 |
| def-1(ky807)   | 0 | 0 | 0 | 66 | 34 | 0 | 0 | 2 | 98 | 99 |
| def-1(ky819)   | 0 | 1 | 36 | 63 | 0 | 21 | 42 | 36 | 184 |
| D685V, cyclase domain | 0 | 9 | 16 | 74 | 0 | 61 | 23 | 17 | 150 |
| def-1(ky793)   | 0 | 2 | 58 | 40 | 0 | 2 | 2 | 98 | 43 |
| def-1(ky807)   | 0 | 0 | 12 | 88 | 0 | 0 | 100 | 0 | 52 |
| known alleles  |      |      |      |      |      |      |      |      |
| def-1(ky873)   | 0 | 0 | 18 | 82 | 0 | 0 | 0 | 91 | 34 |
| def-1(ky807)   | 0 | 0 | 4 | 96 | 0 | 74 | 4 | 22 | 54 |
| tax-2(ky31)    | 0 | 94 | 3 | 3 | 0 | 57 | 31 | 12 | 72 |
| tax-4(ky78)    | 0 | 99 | 10 | 0 | 0 | 13 | 87 | 39 |
| nsy-7(ky802)   | 0 | 0 | 0 | 100 | 59 | 0 | 0 | 0 | 86 |
| lam-1(ky57)    | 0 | 86 | 12 | 2 | 2 | 98 | 0 | 0 | 0 | 43 |
| def-7(ky877)   | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 100 | 31 |
| def-7(ky40)    | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 100 | 95 |
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\textbeta 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\textbeta 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\textbeta type I receptor] (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 [Prasad et al. 1998; Kim et al. 2005].

Existing \textit{daf-4} (the type II TGF\textbeta 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} (Fig. 2C). These results indicate that TGF\textbeta 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 [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; daf-1} or \textit{daf-5; daf-1} double mutants, recapitulating the regulatory relationships seen in dauer formation [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 (Fig. 2C).

\textit{daf-7} expression is inhibited by the dauer pheromone, a mixture of structurally related chemicals termed ascarosides [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} [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 (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\textbeta pathway (Fig. 2D).
A missense allele of the transcription factor hmbx-1 suppresses srsx-3 expression

In the mutant ky777, srsx-3 expression in AWCoff was lost after L1, but str-2 expression in AWCOn 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
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
**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 AWCOn 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 AWCOff. 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** cDNA injection), or high (**odr-3::hmbx-1** DNA 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 AWCOff 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 **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 AWCOn, whereas, in the **hmbx-1(ky777)** mutant, **srsx-3** is inappropriately repressed in AWCOff. 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 AWCOn 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 AWCOff 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 tested by deleting it from the **srsx-3::GFP** reporter (Fig. 5C). 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 tested by deleting it from the **srsx-3::GFP** reporter.

**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. (Arrowheads) 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 AWC\textsuperscript{ON} (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(tm1274) 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 altered-function protein also interacts with this site (Fig. hmbx-1(tm1274) lacking the binding site behaved identically in wild-type

Because str-2 is the only known AWC\textsuperscript{ON}-specific gene and srsx-3 is the only known AWC\textsuperscript{OFF}-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 AWC\textsuperscript{ON} and AWC\textsuperscript{OFF} 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 AWC\textsuperscript{ON}.

AWC\textsuperscript{OFF}-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 AWC\textsuperscript{OFF} but not AWC\textsuperscript{ON}, 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 AWC\textsuperscript{OFF} markers srt-45 and srt-47 and the AWC\textsuperscript{ON} markers srt-26 and srt-28 were examined in mutants that affect srsx-3 and str-2 expression. In nsy-7

![Figure 6](image-url)
mutants, all three AWC\textsuperscript{OFF} markers were expressed in both AWCs, and all three AWC\textsuperscript{ON} markers were reduced or absent [Fig. 6B]. As predicted by earlier studies, this result suggests that, in 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\beta mutants 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{srt-2}, but expression of the AWC\textsuperscript{ON} markers \textit{srt-26} and \textit{srt-28} was largely intact [Fig. 6B]. Thus, the TGF\beta 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-3} 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-::GFP} reporter, which is ordinarily expressed in both AWC neurons (and also in AWB neurons). Single-copy \textit{odr-1-::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-::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 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\beta homolog DAF-7, which otherwise acts continuously to maintain \textit{srsx-3} expression. The TGF\beta pathway regulates multiple chemoreceptor genes, 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 [McCarron 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-::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<sup>OFF</sup> chemoreceptor genes in AWC<sup>ON</sup> 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 <i>C. elegans</i>. 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 <i>tam-1</i>, 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 <i>hmbx-1</i> 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].

<i>hmbx-1</i> has effects that are temporally distinct from those of <i>nsy-7</i>, a transcription factor that acts in AWC<sup>ON</sup> beginning in the L1 larval stage. The relative importance of wild-type <i>hmbx-1</i> is greater in older compared with younger adults, and, likewise, the elevated repressive activity of the <i>hmbx-1</i>(<i>ky777</i>) 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 <i>odr-1</i> promoter ordinarily expressed in both AWC neurons. Likewise, deletion or mutation of the HMBX-1-binding site in the <i>srsx-3</i> promoter permitted misexpression of <i>srsx-3</i> in AWC<sup>ON</sup>. 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 <i>C. elegans</i>, where ectopic expression of a gene in a single additional neuron can dramatically change behaviors, altered expression due to expression of a gene in a single additional neuron can dramatically change behaviors, altered expression due to 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 <i>hmbx-1</i> 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].

<i>hmbx-1</i> is expressed in <i>C. elegans</i> 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 HMOBOX-1 at these locations [Gottschling et al. 1990]. The sequence conservation of HMOBOX-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 <i>hmbx-1</i> target genes, particularly those that are important in older adult animals, and to explore the association of human HMOBX1 with telomeric repeats during normal growth and aging.

**Materials and methods**

**Genetics and strains**

<i>C. elegans</i> 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 <i>kyIs408 [srsx-3::GFP, str-2::dsRed2, elt-2::GFP] II</i> [Lesch et al. 2009]. Mutagenesis was performed with ethyl methane sulfonate [EMS] using standard protocols [Brenner 1974]. Among the new mutations, <i>tax-4</i> and <i>tax-2</i> alleles differed in phenotype from <i>tax-4</i> and <i>tax-2</i> mutants characterized previously using the <i>kyIs140 [str-2::GFP] I</i> transgene [Troemel et al. 1999; Lans and Jansen 2006]. Whereas expression of <i>str-2::GFP</i> from <i>kyIs140</i> was lost in most <i>tax-2</i> and <i>tax-4</i> mutants, the effect of <i>tax-2</i> and <i>tax-4</i> on <i>str-2::dsRed</i> from <i>kyIs408</i> was milder. Analysis of the canonical <i>tax-4(p678)</i>-null mutant indicated that the difference was due to the transgene and not to the nature of the mutant alleles [Fig. 1B]. <i>kyIs140</i> fluorescence is much dimmer than that of <i>kyIs408</i>, 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 <i>gfp-pest</i> reporter was made by overlap extension PCR between the <i>GFP</i> and <i>pest</i> regions and 7 kb of upstream sequence was amplified from N2 lysate using the primers 5′-CATATCATACTCCTCTTGGTTTTC AAG-3′ and 5′-CCAACAAAATTTCAGGGCAAGC-3′. The primer was then cloned into MCS2 of pSM with the <i>srsx-3</i> promoter in MCS1 to create the <i>srsx-3::gfp-pest</i> vector.

A PCR fragment containing the <i>hmbx-1</i> genomic coding region and 7 kb of upstream sequence was amplified from N2 lysate using the primers 5′-ATGCTATTCACACATCTGCTTTTC AAG-3′ and 5′-CCAACAAAATTTCAGGGCAAGC-3′. The PCR product was cloned into pCS2 of pSM with the <i>srsx-3</i> promoter in MCS1 to create the <i>srsx-3::gfp-pest</i> vector.

A PCR fragment containing the <i>hmbx-1</i> genomic coding region was amplified from N2 lysate using the primers 5′-ATGCTATTCACACATCTGCTTTTC AAG-3′ and 5′-CCAACAAAATTTCAGGGCAAGC-3′. The PCR product was cloned into pCS2 of pSM with the <i>srsx-3</i> promoter in MCS1 to create the <i>srsx-3::gfp-pest</i> vector.

HMBX-1 maintains neuronal identity
MCS2 of a modified pSM expression vector (containing a Not1 site in MCS2 instead of MCS1) using the restriction sites Sall 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 pPD95.75. Reactions were carried out as described in Hobert [2002] with the following primers: hmbx-1(7kb): A, 5'-TGGCAGTGCACAGCTGAACTCC-3', and A*, 5'-CCATACATCTTCTCTGTCGCCA-3'; hmbx-1 exon 3, B, 5'-TTGGACATGTAATTCTCCCTTGG-3', and A*, 5'-TCTTTAAAGGTGCCTTCTTTA-3'; hmbx-1 exon 2, C, 5'-AGTCGACCTGCAGGCATGCAAATTACATAGGTTTCGAATAGTTTTTGTTGATAAAGAGAGGTTATAG-3', and A*, 5'-GGTAACGCACTGAGGATAGTCTGAGTGAGT-3'; hmbx-1 exon 1, D, 5'-TACCTCCATTTGG-3', and A*, 5'-CTTTTA-3'.

For injection, a dsDNA template was used.

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RNAi

The 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 str-2 and srsx-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 str-2 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);nysy-7(tm3080) double mutants.

MossCl integrations

MossCl experiments, Nsy-7-binding sites were added to the odr-1::GFP expression vector, and the srsx-3::GFP plasmid was modified to generate the srsx-3(AH)::GFP reporter by site-directed mutagenesis using the the QuikChange II Site-Directed Mutagenesis Kit [Stratagene]. odr-1::GFP or srsx-3::GFP sequences were cut out of pSM using the FseI and SpeI restriction sites, and were cloned into a pCFJ151 MosSCI insertion vector. MosSCI integrations were performed using the FseI and AscI restriction sites. The specific promoter sequences were homologous to the insertion site. Animals that were rescued for the odr-1 phenotype (array-positive) were allowed to starve out twice, and then unc-119 rescued animals that lacked the three mCherry connotation 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 NaN₃. Fluorescence microscopy was carried out on a Zeiss Axioplan2 imaging system with a Hamamatsu Photonics C2400 CCD camera, or a Zeiss Axiolmager.Z1 with ApoTome with a Zeiss AxioCam MRm CCD camera, or a Zeiss Axio Imager.Z1 with ApeToM with a Zeiss AxioCam MRm CCD camera. Most animals were scored under a 20× or 40× Plan-Neofluar objective, where “bright” and “faint” fluorescence were scored qualitatively, and photographs were taken under a 40× Plan-Neofluar or 63× Plan-Apochromat objective. Confocal microscopy [Fig. 5A] was done under a 40×/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 12–14 h 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.
Phosphomine 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 srx3:3::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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