# Modular Organization of *Cis*-regulatory Control Information of Neurotransmitter Pathway Genes in *Caenorhabditis elegans*

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**ABSTRACT** We explore here the *cis*-regulatory logic that dictates gene expression in specific cell types in the nervous system. We focus on a set of eight genes involved in the synthesis, transport, and breakdown of three neurotransmitter systems: acetylcholine (*unc-17*/*VAChT*, *cha-1*/*ChAT*, *cho-1*/*ChT*, and *ace-2*/*AChE*), glutamate (*eat-4*/*VGluT*), and γ-aminobutyric acid (*unc-25*/*GAD*, *unc-46*/*LAMP*, and *unc-47*/*VGAT*). These genes are specifically expressed in defined subsets of cells in the nervous system. Through transgenic reporter gene assays, we find that the cellular specificity of expression of all of these genes is controlled in a modular manner through distinct *cis*-regulatory elements, corroborating the previously inferred piecemeal nature of specification of neurotransmitter identity. This modularity provides the mechanistic basis for the phenomenon of "phenotypic convergence," in which distinct regulatory pathways can generate similar phenotypic outcomes (*i.e.*, the acquisition of a specific neurotransmitter identity) in different neuron classes. We also identify cases of enhancer pleiotropy, in which the same *cis*-regulatory element is utilized to control gene expression in distinct neuron types. We engineered a *cis*-regulatory allele of the vesicular acetylcholine transporter, *unc-17*/*VAChT*, to assess the functional contribution of a "shadowed" enhancer. We observed a selective loss of *unc-17*/*VAChT* expression in one cholinergic pharyngeal pacemaker motor neuron class and a behavioral phenotype that matches microsurgical removal of this neuron. Our analysis illustrates the value of understanding *cis*-regulatory information to manipulate gene expression and control animal behavior.

**KEYWORDS** *C. elegans*; *cis*-regulatory control; neurotransmitter; transcription factors

ONLY a small fraction of an animal's genome is transcribed into messenger, regulatory, or structural RNAs. Much of the remainder of the genome carries *cis*-regulatory information in the form of binding sites for *trans*-acting regulatory factors that dictates patterns of gene expression (Davidson 2006). Genomic binding profiles have been determined for many transcriptional regulators, but it is far from clear how reliably such binding profiles predict the presence of functionally relevant *cis*-regulatory elements (Ecker *et al.* 2012; Doolittle 2013; Eddy 2013; Brown and Celniker 2015). *De novo* prediction of functional *cis*-regulatory elements by bioinformatic analysis has also proven to be challenging, at least in part due to the fact that binding sites of *trans*-acting regulatory factors are often small and degenerate. An alternative approach to define and decode *cis*-regulatory control elements employs transgenic reporter gene analysis, which can reveal the functionality of *cis*-regulatory elements based


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on their ability to direct reporter expression in specific cell types (Yáñez-Cuna *et al.* 2013).

The identification of *cis*-regulatory elements by reporter gene analysis has the potential to reveal insights into the regulatory logic of cell type-specific gene expression. Genes are often expressed in multiple distinct cell types. This could indicate that distinct cell types may utilize the same transcription factor that operates through a singular *cis*-regulatory element to turn on expression of a given target gene in distinct cell types. Alternatively, a gene could be expressed in different cell types by harboring multiple *cis*-regulatory elements that are responsive to distinct, cell type-specific transcription factors. This problem is particularly evident in the nervous system, in which distinct neuron types choose to express usually one of a discrete number of different neurotransmitter systems, thereby acquiring a specific neurotransmitter identity. Neurotransmitter identity is determined by specific enzymes and transporters that synthesize and synaptically package a given neurotransmitter ("neurotransmitter pathway genes") (Figure 1A). The neurotransmitter identity of a specific neuron can be considered a phenotypic trait. This trait (*e.g.*, the usage of glutamate as neurotransmitter) is shared by many different neuron types. Hence, the question arises as to whether such a shared trait is (1) induced by the same transcriptional regulatory mechanism that operates through the same *cis*-regulatory elements in all neuron types that share this neurotransmitter phenotype ("singular control"), or (2) is independently controlled by a different transcription factor or transcription factor combinations in distinct neuron types through distinct *cis*-regulatory elements ("modular control") (Figure 1B). The analysis of *cis*-regulatory control regions of neurotransmitter pathway genes has the potential to distinguish between these two possibilities. If the phenotypic trait of a specific neurotransmitter identity is regulated separately in distinct neuron types, *cis*-regulatory information should be organized in a modular manner. That is, discrete *cis*-regulatory elements will be responsible for driving expression of the same neurotransmitter pathway gene in different neuron types.

There is presently ample evidence to support such modular *cis*-regulatory organization, particularly in the nematode *Caenorhabditis elegans* (Eastman *et al.* 1999; Wenick and Hobert 2004; Flames and Hobert 2009; Kratsios *et al.* 2011; Serrano-Saiz *et al.* 2013; Lloret-Fernandez *et al.* 2018). We further extend this concept here via analysis of the *cis*-regulatory control regions of several neurotransmitter pathway genes. However, we also uncover additional complexities. For a few neuron classes we have not been able to define independent *cis*-regulatory elements, indicating that the same transcription factor may act in different cell types, but is assisted by different coregulators in distinct cell types. We discuss our findings in the context of phenotypic convergence, a concept aiming to explain how a phenotypic trait shared by distinct neuron types (neurotransmitter identity) can be specified by distinct regulatory means (Konstantinides *et al.* 2018; Kratsios and Hobert 2018). We also report on evidence for the existence of enhancer pleiotropy, in which similar *cis*-regulatory elements can drive expression in distinct neuron classes.

Besides providing mechanistic insights into gene regulation, substantial methodological benefits can be gained from the dissection of *cis*-regulatory control regions. *Cis*-regulatory elements that drive expression in a small subset of cell types provide "genetic access" to these cell types. That is, these elements can be used as drivers to express genetic tools that allow the visualization or isolation of these cells or, in the context of the nervous system, the optogenetic activation or inhibition of these cells.

## Materials and Methods

### *Transgenes*

A list of all transgenic strains used in this study is shown in Supplemental Material, Table S1. Strains were generated by injecting the transgene mix as simple or complex arrays. Briefly, complex arrays contained the fosmid (for *otIs388*, *otIs518*, *otIs354*, *otEx6066*, and *otEx4431*) linearized by restriction enzyme digestion or obtained as a PCR product (for *unc-17*, *cho-1*, *ace-2*, *unc-25*, *unc-46*, and *unc-47* reporters; Table S2). The reporters were mixed with bacterial genomic DNA from *Escherichia coli* OP50 (previously sonicated and purified with a Gentra Puregene kit; QIAGEN, Valencia, CA) and a co-injection marker plasmid, also linearized by restriction enzymes. For simple arrays, plasmids were mixed directly with the co-injection marker plasmid. For each construct, two to three transgenic lines and at least 10 worms per line were analyzed.

### *DNA constructs*

A list of all reporter constructs and sequences can be found in Table S2. Reporters were generated by different methods: (1) cloning into pDP95.75, pOH569, or pPD95.67 using standard molecular biology cloning techniques; (2) by PCR fusion, as described in Hobert (2002); or (3) fosmid recombineering, as described in Tursun *et al.* (2009).

### *Clustered regularly interspaced short palindromic repeats/Cas9 genome engineering*

The *unc-17* regulatory allele *ot1025* was generated using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome engineering, as described previously (Dokshin *et al.* 2018). The E3 motif was replaced with a PmeI restriction site with the following oligo repair template: tttttctgtctcaaaacggagctcgagcccatgacgtgtGTTTAAACtccgcc caattccttca (capital letters indicate the introduced PmeI site replacing the E3 motif). The engineering was done in *unc-17-(ot907[unc-17*::*mKate2*::*3xFLAG]IV*) animals (Pereira *et al.* 2019) (strain OH15568), generating strain OH16363 *unc-17(ot907ot1025)*.

### Neuron identification

Neuronal identification was performed by colocalization of the reporter constructs with landmark strains. Specifically, individual reporter transgene-expressing *rfp* or *mChopti* strains were crossed with a green transgenic landmark strain, *otIs354* [*cho-1$^{fosmid}$::sl2::yfp::h2b*] (Pereira *et al.* 2015) or *otIs388* [*eat-4$^{fosmid}$::sl2::yfp::h2b*] (Serrano-Saiz *et al.* 2013), and *gfp* or *yfp* transgene-expressing lines were crossed with either *otIs544* [*cho-1$^{fosmid}$::sl2::mChopti::h2b*], *otIs518* [*eat-4$^{fosmid}$::sl2::mChopti::h2b*], or *otIs564* [*unc-47$^{fosmid}$::sl2::h2b::mChopti* (Gendrel *et al.* 2016).

Worms were anesthetized using 100 mM of sodium azide ($NaN_3$) and mounted on 5% agarose on glass slides. All images were acquired using a Zeiss ([Carl Zeiss], Thornwood, NY) confocal microscope (LSM880). Representative images are shown following orthogonal projection of 2–10 z-stacks. Images shown in Figure 8 were taken using an automated fluorescence microscope (AXIO Imager Z1 Stand; Zeiss). Acquisition of several z-stack images was performed with the Micro-Manager software. Representative images are shown using the maximum intensity projection type. Image reconstruction was performed using ImageJ software (Schneider *et al.* 2012).

### Scoring of pharyngeal pumping

Pumping assays were performed at room temperature. Household bleach-mediated egg preparations were used to synchronize *C. elegans*, and animals were then transferred from an uncrowded nonstarved plate to a fresh NGM plate with a uniform thin layer of OP50. After 5 min recovery, they were observed under a Nikon (Garden City, NY) Eclipse E400 upright microscope equipped with DIC optics using a 50× objective lens for scoring L1-stage animals and a 20× objective for adult animals. The number of pumps in a 20-sec period was measured by focusing on a single worm and then this number was multiplied by three to obtain pumps per minute (ppm). For each stage, ppm was counted for 30 worms.

### Phylogenetic analysis

Genomic sequences spanning the *cho-1* and *unc-17* loci of *C. elegans* and 12 closely related *Caenorhabditis* species (*C. brenneri*, *C. briggsae*, *C. doughertyi*, *C. inopinata*, *C. latens*, *C. nigoni*, *C. remanei*, *C. sinica*, *C. tribulationis*, *C. tropicalis*, *C. wallacei*, and *C. zanzibari*) were identified by Basic Local Alignment Search Tool search, and then downloaded from WormBase.org or Caenorhabditis.org. Sequences were aligned using MAFFT or ClustalW and, if necessary, adjusted by inspection; sequence logos were generated by LogOddsLogo (https://www.ncbi.nlm.nih.gov/CBBresearch/Yu/logoddslogo/).

### Data availability

The authors state that all data necessary for confirming the conclusions presented in the manuscript are represented fully within the manuscript. Supplemental material available at figshare: https://doi.org/10.25386/genetics.12354695.

## Results

In previous work, we used fosmid (large genomic clone)-based reporters and/or CRISPR/Cas9-engineered reporter alleles to comprehensively assign neurotransmitter identities throughout the entire *C. elegans* nervous system (Serrano-Saiz *et al.* 2013; Pereira *et al.* 2015; Gendrel *et al.* 2016) (Figure 1C). Cholinergic neurons are marked by two fosmid-based reporters: (1) the *unc-17* locus, which encodes the *C. elegans* ortholog of the vesicular acetylcholine (ACh) transporter (VAChT) (Alfonso *et al.* 1993) and reports on transcription of both *unc-17/VAChT* and *cha-1/ChAT*, which shares its first noncoding exon with *unc-17* (Alfonso *et al.* 1994); and (2) *cho-1*, the choline reuptake transporter (Okuda *et al.* 2000; Mullen *et al.* 2007), which marks most, but not quite all, cholinergic neurons (Pereira *et al.* 2015). The expression pattern of the fosmid-based *unc-17* reporter is mimicked by a CRISPR/Cas9-engineered reporter insertion into the *unc-17* locus (Pereira *et al.* 2015). A fosmid-based reporter for *ace-2/AChE*, one of several *C. elegans* acetylcholinesterase-encoding genes, is expressed in a subset of cholinergic neurons (Pereira *et al.* 2015). All glutamatergic neurons are marked by a fosmid-based reporter of the vesicular transporter for glutamate, *eat-4*/VGLUT (Lee *et al.* 1999; Serrano-Saiz *et al.* 2013). Lastly, γ-aminobutyric acid (GABA)-ergic neurons are marked by CRISPR/Cas9-engineered reporter alleles of the *unc-25* locus, which encode glutamic acid decarboxylase (GAD; *unc-25*) (Jin *et al.* 1999; Gendrel *et al.* 2016), and by fosmid-based reporters for the *unc-47* and *unc-46* loci (Gendrel *et al.* 2016), which encode the vesicular GABA transporter *unc-47/VGAT* (McIntire *et al.* 1997) and an associated cofactor (Schuske *et al.* 2007), respectively. We collectively refer to this cohort of genes as neurotransmitter pathway genes (Figure 1A).

Armed with the knowledge of the precise expression pattern of those fosmid-based and/or CRISPR/Cas9-engineered reporters, we generated a series of reporter constructs that contained distinct DNA fragments of the respective loci. We generated transgenic *C. elegans* animals carrying these reporters and analyzed their expression patterns in the nervous system with single-cell resolution. This analysis was motivated by a specific hypothesis. Based on previous studies of *cis*-regulatory control regions of neurotransmitter pathway genes, as well as extensive analysis of genetic mechanisms that control neurotransmitter specification, a theme of modular control of neurotransmitter identity has been emerging (Eastman *et al.* 1999; Wenick and Hobert 2004; Kratsios *et al.* 2011; Serrano-Saiz *et al.* 2013; Pereira *et al.* 2015; Gendrel *et al.* 2016; Lloret-Fernandez *et al.* 2018). To test whether the theme of modularity broadly applies to multiple neurotransmitter pathway genes, we carried out an extensive dissection of their *cis*-regulatory control regions.

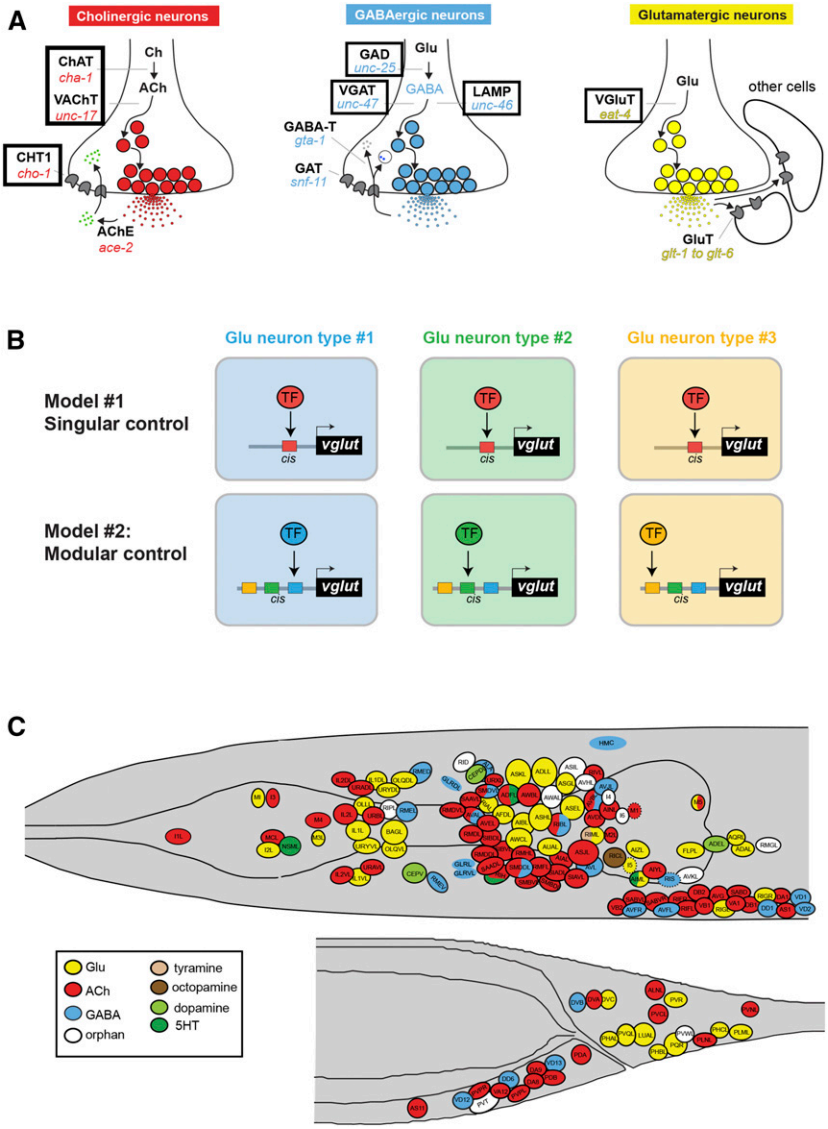


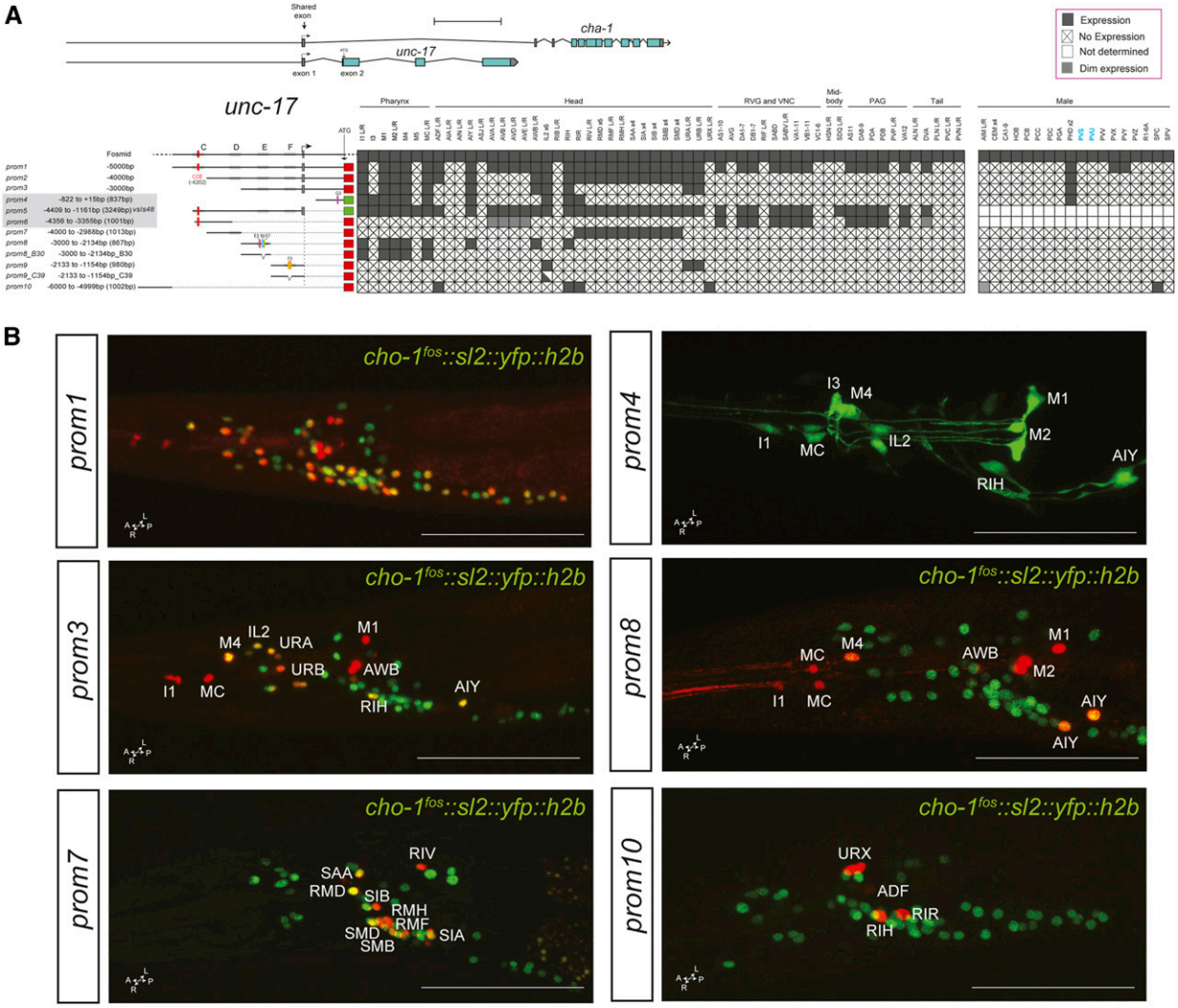

**Figure 1** Introduction to the problem of neurotransmitter identity specification. (A) Neurotransmitter pathway genes. In this paper, fluorescence reporters were generated for *cis*-regulatory elements contained in the regulatory regions of the genes highlighted in boxes. (B) Conceptual models for how the shared phenotypic trait of neurotransmitter identity could be regulated in different neuron types. Previous studies as well as this paper corroborate the modular control model. However, as we will show here in this paper, there are cases where the same transcription factor (TF) is used in multiple different neuron types (seemingly in support of the singular model), but this is usually because a given TF usually operates in distinct combinations in different cell types, as discussed in the text. For simplicity, these two models just focus on the question of whether a specific neurotransmitter identity is controlled by single TFs. (C) Neurotransmitter atlas of the *C. elegans* adult hermaphrodite head and tail. Adapted from Gendrel *et al.* (2016).; ChAT, choline acetyltransferase; VAChT, vesicular acetylcholine (ACh) transporter; CHT1: choline reuptake transporter, AChE: acetylcholinesterase, GAD: glutamic acid decarboxylase; VGAT, vesicular GABA transporter; GABA-T, GABA transaminase GAT, GABA transporter; LAMP, lysosomal associated membrane protein; GluT, Glutamate transporter; 5HT, 5-hydroxytryptamine; ACh, acetylcholine; Ch, choline; GABA, γ-aminobutyric acid; Glu, glutamate; TF, transcription factor.

### *Cholinergic pathway genes: regulation of the cholinergic locus (unc-17/VAChT and cha-1/ChAT)*

In animal species ranging from worms to vertebrates, the enzymes that synthesize (choline acetyltransferase; *cha-1*) and package ACh into synaptic vesicles (VAChT; *unc-17*) are organized into an operon-like conserved structure, termed the cholinergic locus (Alfonso *et al.* 1994; Eiden 1998). 5 kb upstream of the first protein-coding exon of *unc-17* drive reporter gene expression in the majority, but clearly not all, cholinergic neurons (*prom1* in Figure 2A,B). Within the male-specific nervous system, only a very small fraction of neurons express this reporter. A 1-kb fragment preceding this 5-kb fragment captures a number of additional cholinergic neurons, in both the sex-shared as well as male-specific nervous system (*unc-17prom10*, Figure 2A,B).

Reporter expression is eliminated in cholinergic motor neurons of the ventral nerve cord (VNC), the flanking retrovesicular ganglion (RVG), and the preanal ganglion (PAG), as well as the command interneurons by removal of distal sequences within the 5-kb fragment (compare *unc-17prom1 vs. unc-17prom2*). These distal sequences contain a binding site for the UNC-3 transcription factor ("COE motif"), the terminal selector of ventral cord motor neuron and command interneuron identity (Kratsios *et al.* 2011; Pereira *et al.* 2015). Conversely, a construct containing only distal sequences and the COE motif (*unc-17prom6*) is sufficient to drive reporter gene expression in cholinergic motor neurons in the VNC, RVG, and PAG (Kratsios *et al.* 2011). Further shortening of the 5 kb fragment (*unc-17prom3*) leads to consecutive loss of expression in more neuron classes (Figure 2A).

Reporter animals carrying 3249 bp of *cis*-regulatory information upstream of the noncoding first exon shared by *unc-17* and *cha-1* (*unc-17prom5*) yielded similar but not identical expression as the 5-kb construct that contained the first




C

| Length (bp) | COE motif | Expression intensity | No. of neurons in RVG, VNC, PAG | No. of lines |
|---|---|---|---|---|
| 250 | | +++ | ~42±3 | 3/3 |
| 200 | | ++ | ~41±3 | 3/3 |
| 150 | | +++ | ~42±4 | 3/3 |
| 125 | | +++ | ~40±4 | 3/3 |
| 250_A47_B30 | Δ47 Δ30 | +++ / ++ | ~48±3 | 3/3 |
| 250_C72_B30 | Δ72 Δ30 | ++ / + | ~43±7 | 2/4 |

**Figure 2** Analysis of *cis*-regulatory elements of the *unc-17*/*cha-1* locus. (A) Overview of the *unc-17*/*cha-1* locus, reporter constructs, and summary of sites of expression. The constructs are referred to the *unc-17*/*cha-1* locus to scale. Expression of the fosmid reporter is from Pereira *et al.* (2015). *unc-17prom4*, *prom5* worms were shown in previous papers (Wenick and Hobert 2004; Kratsios *et al.* 2011) but expressing cells were not identified. Within the matrix, the gray color indicates expression, "X" indicates no expression, and white color indicates "not determined." Scale bar = 2 kb. (B) Representative images of several constructs in the adult head (lateral views). In the images, the *unc-17* reporters are shown in red and the *cho-1* fosmid

intron of the locus (*unc-17prom1*). We broke this 3249-bp upstream region into several nonoverlapping pieces. One small piece, which contains the COE motif, is sufficient to drive expression in VNC motor neurons (*unc-17prom6*), as previously described (Kratsios *et al.* 2011). Three additional, nonoverlapping ~1-kb fragments (*unc-17prom7* to *prom9*) show distinct patterns of expression in largely (but not completely) nonoverlapping neuron classes. One fragment (*unc-17prom7*) drives expression exclusively in head motor neuron classes (RMD, SMD, SMB, SIA, SIB, RMF, and RMH) as well as two ring interneuron classes (RIR and RIV). Another, distinct ~900-bp element captures expression in pharyngeal inter- and motor neuron classes (*unc-17prom8*). Notably, very similar expression is observed from a different regulatory element in the first intron of the *unc-17* locus (*unc-17prom4*). The ~900-bp upstream pharyngeal element is also expressed in a few extrapharyngeal neuron classes (AIY, AWB, and RIH). Lastly, the third ~1-kb fragment is exclusively expressed in three neuron classes of the anterior ganglion (IL2, URA, and URB) (*unc-17prom9*).

We sought to increase the granularity of our *cis*-regulatory analysis of the *unc-17/cha-1* cholinergic locus by phylogenetic footprinting. Examining 10 different nematode species, we identified a host of small conserved elements (Figure 3A). We examined the functional relevance of two small clusters of these elements in the context of two separate ~1-kb fragments (Figure 2, A and B). One is the ~900-bp element (*unc-17prom8*) that drives expression in a few pharyngeal and extrapharyngeal neuron classes (AIY, AWB, and RIH). This element contains a small motif ("E6") that we previously identified to be a binding site for the terminal selector-type TTX-3/CEH-10 homeodomain complex ("AIY motif"). We found this motif to be required for *unc-17* expression and hence the acquisition of cholinergic identity of the AIY interneuron (Wenick and Hobert 2004). An adjacent element ("E7") matches the consensus binding site for the UNC-86 POU homeodomain transcription factor and we previously found UNC-86 to be required for cholinergic identity acquisition of the RIH neuron (Pereira *et al.* 2015). We find that combined deletion of E6 and E7 results in the expected loss of expression of this element, both in AIY and RIH (*unc-17prom8_B30*; Figure 2A). Expression in AWB, which expresses neither *ttx-3*, *ceh-10*, nor *unc-86*, is also lost, suggesting that a related homeodomain transcription factor that recognizes the E6 and/or E7 motifs may control AWB cholinergic identity.

The ~1-kb element, that is exclusively expressed in the IL2, URA, and URB neuron classes (*unc-17prom9*), contains a stretch of conserved sequence motifs (termed F2 in Figure 3B) including another predicted binding site for the UNC-86 POU homeodomain transcription factor, previously shown to be required for cholinergic identity acquisition of the IL2, URA, and URB neuron classes (Zhang *et al.* 2014). Deletion of the F2 motif affects expression in all these neuron classes (*unc-17prom9_C39*; Figure 2A). In summary, this *cis*-regulatory analysis indicates that: (1) the terminal selector UNC-86 exerts its effect on cholinergic identity specification in distinct neuron classes, likely via direct binding and activation of the *unc-17/cha-1* cholinergic locus, and (2) that UNC-86 operates through distinct regulatory elements in different cholinergic neuron cell types. These points are further supported by a distal *cis*-regulatory element that is expressed in four different neuron classes: ADF, RIH, RIR, and URX (*unc-17prom10*; Figure 2, A and B). Three of these neuron classes express UNC-86 and at least two of them (RIH and URX) have been shown to require UNC-86 for proper *unc-17* expression (Pereira *et al.* 2015).

Lastly, we sought to define the minimal *cis*-regulatory element upstream of *unc-17* that is sufficient to drive *unc-3*-dependent expression in cholinergic motor neuron classes in the RVG, VNC, and PAG. We focused on *unc-17prom6* (1001 bp) (Figure 2A), which contains the UNC-3-binding site (COE motif) previously shown to be required for reporter gene expression in *unc-3*-dependent neuron classes in the RVG, VNC, and PAG (Kratsios *et al.* 2011). Shorter reporter versions of 250, 200, 150, and 125 bp that contain the COE motif are sufficient to drive expression in motor neuron classes of the RVG, VNC, and PAG (Figure 2C). Through the introduction of a number of deletions surrounding the COE motif, we found no evidence for the existence of additional required *cis*-regulatory elements (Figure 2C). While this may indicate that UNC-3 is the sole factor required for expression of *unc-17* in motor neuron classes of the RVG, VNC, and PAG, we note that there are highly conserved sequences directly adjacent to the COE motif. Whether these sequences are covered by the UNC-3 protein (which contacts DNA beyond the COE motif (Treiber *et al.* 2010) or may represent a binding site for a cofactor is presently not clear.

### *Cholinergic pathway genes: regulation of the cho-1 locus*

Dissection of the *cho-1/ChT* locus reveals a similar modular organization of *cis*-regulatory elements. A ~5-kb fragment upstream of the *cho-1* locus recapitulates almost the entire expression pattern of the fosmid-based reporter (*cho-1prom1*; Figure 4A). Successive deletions from the 5′ end result in sequentially increasing losses of expression in multiple cholinergic neuron types. The 2.7-kb fragment

(*otIs354*) is also shown in green as a landmark. Cells in the *unc-17*$^{prom1}$ worm are not labeled because there is complete colocalization with the *cho-1* fosmid. Usually two or three lines were scored for each reporter and > 10 animals/line. Bar, 50 μm. (C) Analysis of an *unc-3* responsive element from the *unc-17* locus required for expression in the RVG, VNC, and PAG. This small 250-bp element is also expressed in a few unidentified head neurons, but we only consider here the expression in the RVG, VNC, and PAG, which has previously been shown to require the indicated COE motif and *unc-3* (Kratsios *et al.* 2011). No., number; PAG, preanal ganglion; RVG, retrovesicular ganglion; VNC, ventral nerve cord.

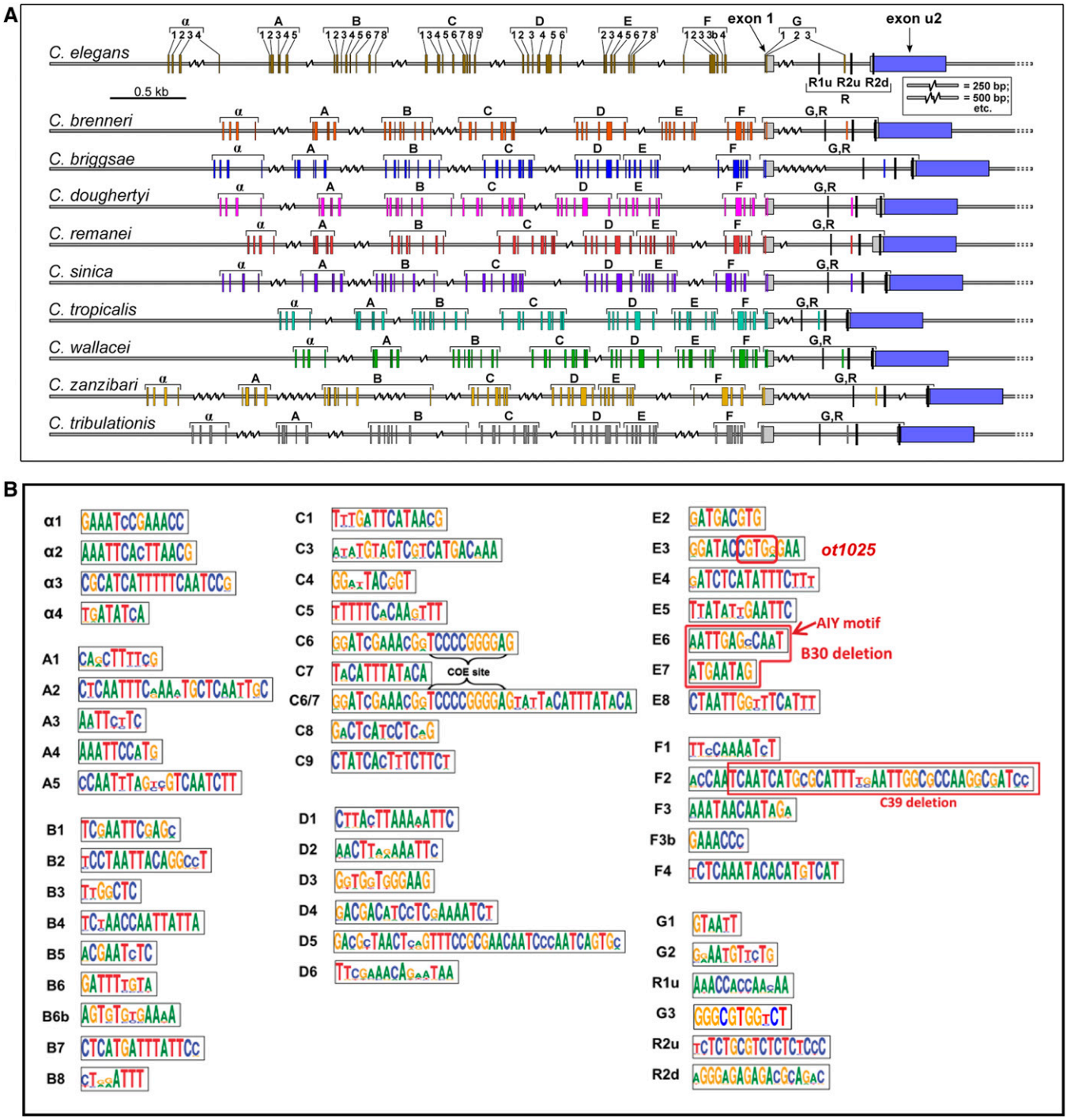



**Figure 3** Identification of conserved motifs upstream of the cholinergic locus (*unc-17*/*cha-1*) of 10 nematode species. (A) Locations of 51 conserved motifs in *unc-17* promoters derived from sequence alignments, as described in the *Materials and Methods*. The R1 and R2 motifs have been described previously, and regulate the alternate splicing of *unc-17* and *cha-1* transcripts (Mathews *et al.* 2015). (B) Sequence logos of the conserved promoter motifs. The small element designated E6 = AIY motif (TTX-3/CEH-10-binding site) has been functionally validated in *C. elegans* to be required to drive *unc-17* expression in AIY by Wenick and Hobert (2004). The element designated E7 contains the consensus binding site for the UNC-86 transcription factor. The combined deletion of E6 and E7 (designated *unc-17prom8_B30* in Figure 2) results in the loss of expression of the reporter in AIY and RIH. The red box in E3 indicates the nucleotides that were replaced in *ot1025* (Figure 8). The red box in F2 indicates the deleted nucleotides in *unc-17prom9_C39* (Figure 2).

(*cho-1prom3*) directly upstream of the start site of the gene only contains regulatory information for six classes of head and tail neurons, including the only two pharyngeal cholinergic neuron classes that express *cho-1*. Expression in the pharyngeal neurons can be separated from the remaining few head and tail neurons through a deletion from the 3′ end of this construct (compare *cho-1prom3* with *cho-1prom11*, *prom12*, and *prom16*). As is the case for the *unc-17* locus,

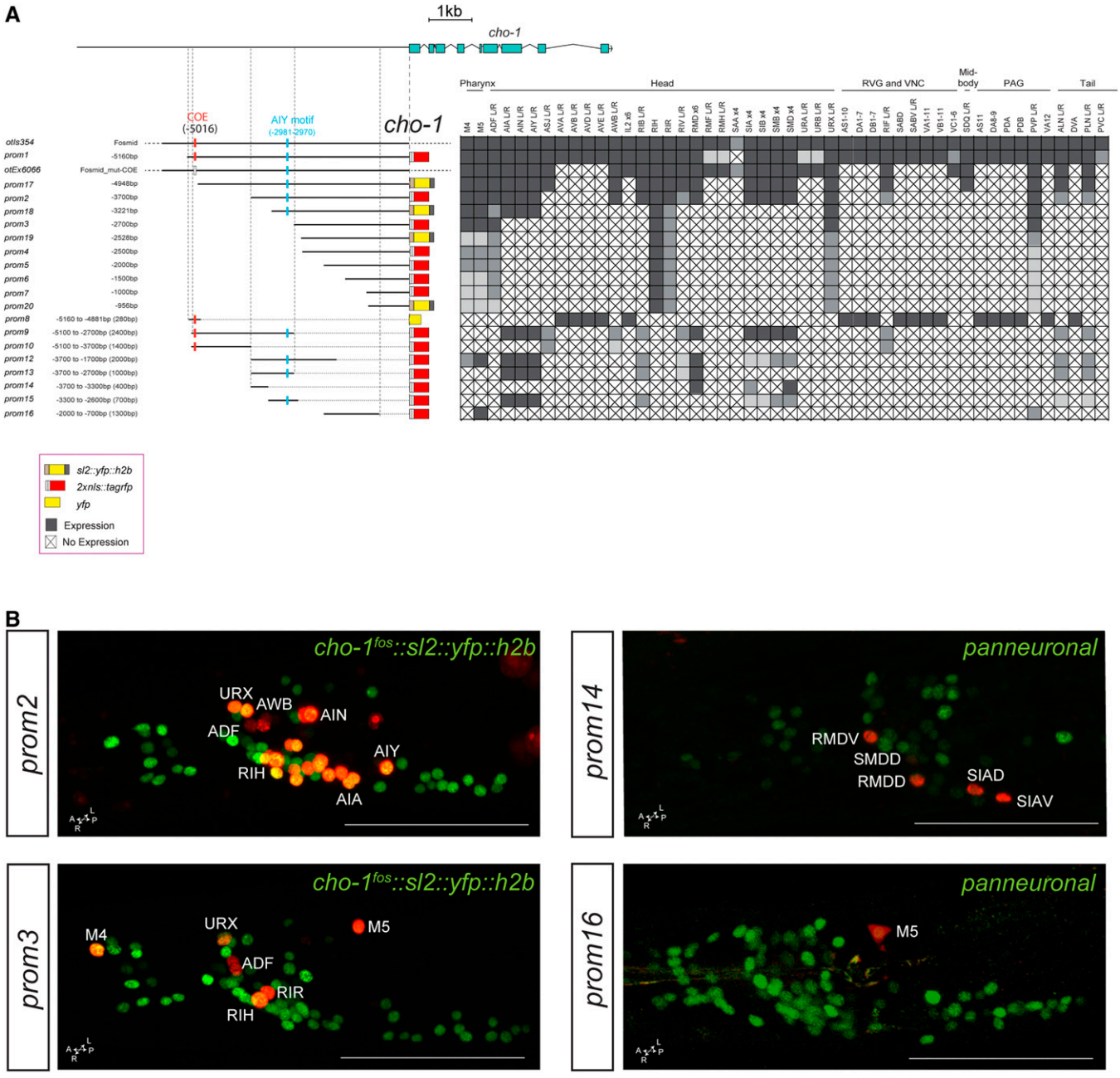



**Figure 4** Analysis of *cis*-regulatory elements of the *cho-1* locus. (A) Overview of the *cho-1* locus. Schematic of the *cho-1* reporters and their expression in hermaphrodite. The constructs are referred to the *cho-1* locus to scale. *otIs354* (fosmid reporter) and *otEx6066* reporters were shown in previous papers (Pereira *et al.* 2015; Stefanakis *et al.* 2015), but expressing cells were not identified. Within the matrix, gray color indicates expression, "X" indicates no expression, and white indicates not determined. (B) Representative images of several constructs in the adult head (lateral views). In the images the *cho-1* reporters are shown in red and the *cho-1* fosmid (*otIs354*) is also shown in green as a landmark. Usually two or three lines were scored for each reporter and > 10 animals/line. Bar, 50 μm. PAG, preanal ganglion; RVG, retrovesicular ganglion; VNC, ventral nerve cord; *yfp*, yellow fluorescent protein.

there appear to be two distinct regions of the *cho-1* locus that can drive expression in pharyngeal neurons (Figure 4). Another striking similarity to *unc-17* regulation is that we isolated an element from both *cho-1* and *unc-17* that is active in the same set of extrapharyngeal neuron classes: ADF, RIH, RIR, and URX. As mentioned above, three of these neuron classes express UNC-86 and two have been shown to require UNC-86 for both *unc-17* and *cho-1* expression (Pereira *et al.* 2015).

Successive deletions of constructs from the 3′ end also separate expression into distinct neuron classes (Figure 4A). Most notable is the previously reported 280-bp distal element that contains the *unc-3*-binding COE motif (*cho-1prom8*). The expression of this element precisely matches all neurons

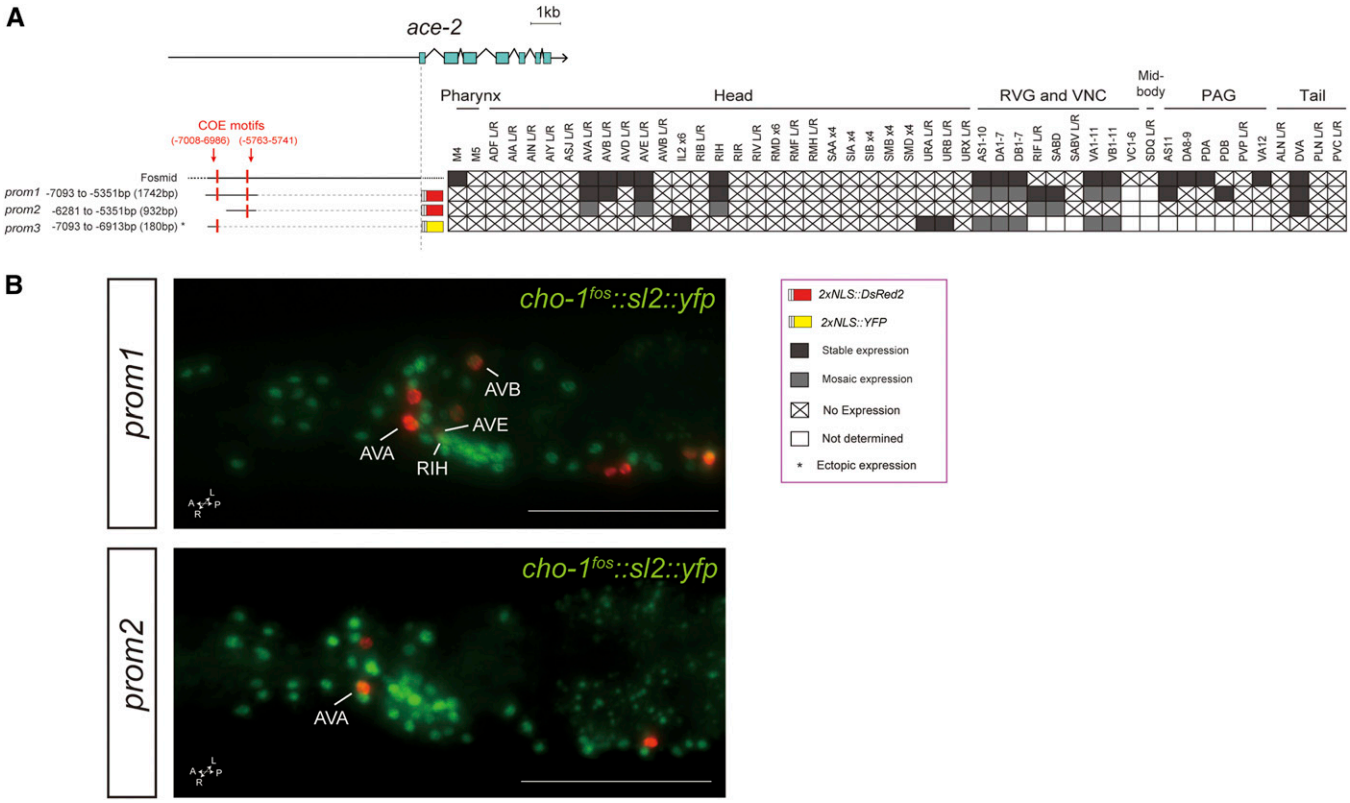



**Figure 5** Analysis of *cis*-regulatory elements of the *ace-2* locus. (A) Overview of the *ace-2* locus. Schematic of *ace-2* reporters and their expression in hermaphrodites. The constructs are referred to the *ace-2* locus to scale. (B) In the images two *ace-2* reporters are shown in red and the *cho-1* fosmid (*otIs354*) is also shown in green as a landmark. Expression of the *ace-2* fosmid was characterized in Kratsios *et al.* (2011) and Pereira *et al.* (2015). Constructs *prom1* and *prom2* were described in Kratsios *et al.* (2011), but cell identification in the head and tail neurons was not performed. Within the matrix, gray color indicates expression, "X" indicates no expression, and white indicates not determined. Some representative images of two reporter constructs are shown (also containing a *cho-1* fosmid landmark reporter). Usually two or three lines were scored for each reporter and > 10 animals/line. Bar, 50 μm. PAG, preanal ganglion; RVG, retrovesicular ganglion; VNC, ventral nerve cord.

throughout the *C. elegans* nervous system that require UNC-3 for their cholinergic identity specification. Removal of 60 bp from the 5′ end of this element, leaving the COE motif intact, eliminates expression in the UNC-3(+) neurons (compare prom8 and prom9), suggesting that these 60 bp contain a binding site for another factor that may collaborate with UNC-3. Such a factor is expected to exist because UNC-3 alone is not sufficient to induce cholinergic fate [*e.g.*, UNC-3 is expressed in the noncholinergic ASI neurons (Prasad *et al.* 1998)].

### *Cholinergic pathway genes: regulation of ace-2.*

The *ace-2* gene is an ortholog of human acetylcholinesterase (*ACHE*), an enzyme that catalyzes the breakdown of ACh (Culotti *et al.* 1981). Using a fosmid-based reporter, we previously determined that *ace-2* is expressed in some, but not all, cholinergic neuron classes (Pereira *et al.* 2015) (Figure 5). A distal element of 1.8 kb (*prom1*) contains two COE motifs and is sufficient for expression in cholinergic VNC motor neurons, as previously described (Kratsios *et al.* 2011). We find this element to also be expressed in command interneuron classes (AVA, AVB, and AVE), as well as the RIH and DVA neuron classes, almost completely recapitulating the *ace-2* fosmid expression pattern; Figure 5). Supporting the notion of modularity, a shorter 1-kb version of this 1.8-kb element (*prom2*) drives reporter expression only in AVA, AVB, and DVA, and a limited number of VNC motor neuron classes (Figure 5). A small 180-bp element (*prom3*) at the distal end of the 1.8-kb fragment drives expression in VNC motor neuron classes, as well as a dozen head (IL2D/V, URA, and URB) and tail (PLM and PHC) neuron classes that normally do not express *ace-2* (based on a fosmid-based reporter). This is one of the rare cases where an isolated *cis*-regulatory element showed derepression in ectopic neuron types.

### *Glutamatergic identity: regulation of eat-4/VGluT expression*

In previous work, we had already mapped *cis*-regulatory elements that drive expression of *eat-4* in distinct *eat-4(+)* neuron classes in the hermaphrodite nervous system (Serrano-Saiz *et al.* 2013). While we had examined *eat-4* expression in the male nervous system as well (Serrano-Saiz *et al.* 2017b), we had not examined *cis*-regulatory elements that drive expression in the male nervous system. We now find that such elements are located in distinct regions of

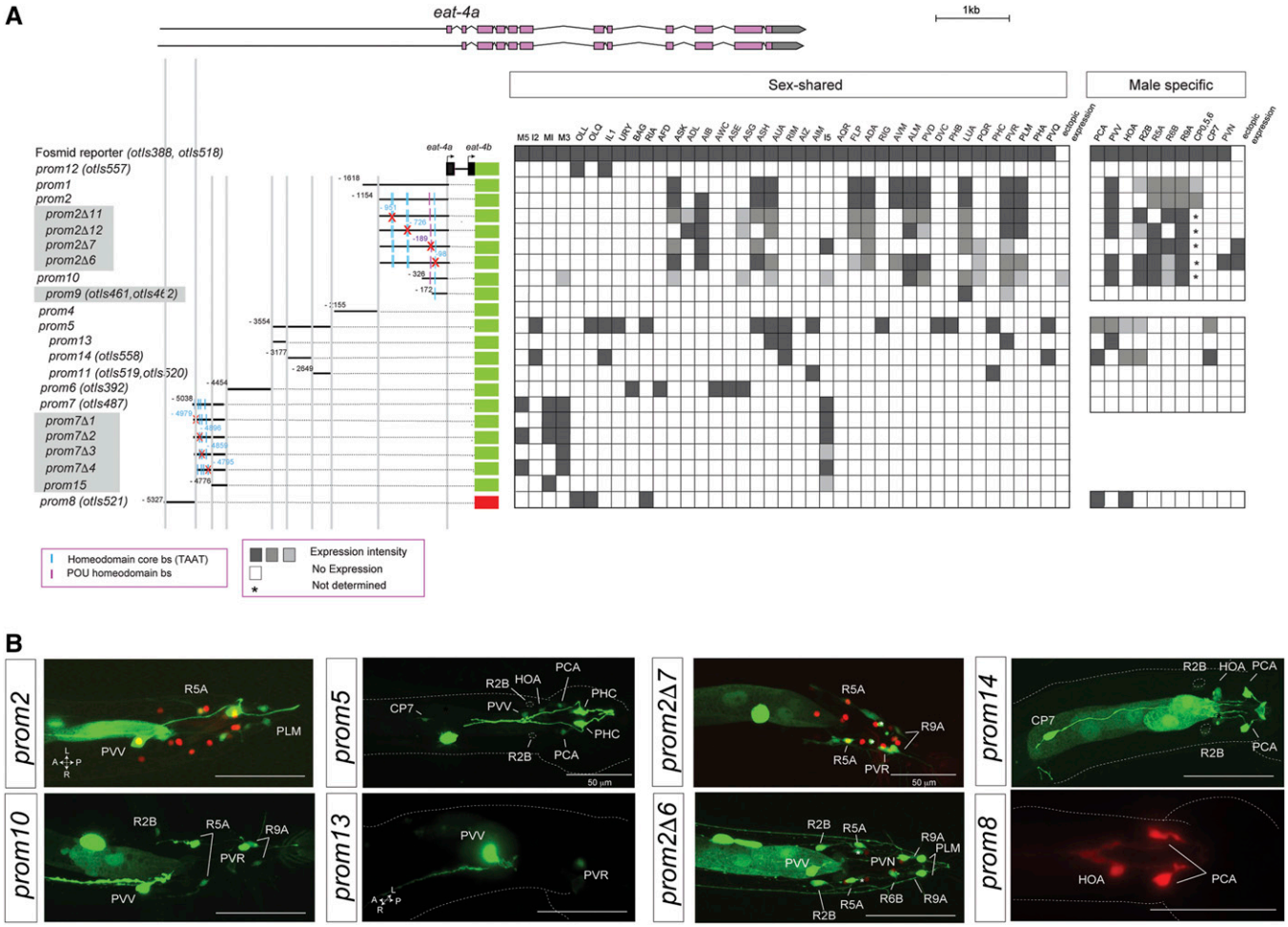



**Figure 6** Analysis of *cis*-regulatory elements of the *eat-4* locus. (A) Overview of the *eat-4* locus. Schematic of *eat-4 gfp* reporter constructs and their expression in hermaphrodite and male nervous systems. The constructs are referred to the *eat-4* locus to scale. Gray boxed reporters have not been published before, while the information for the other constructs had been partially shown in Serrano-Saiz *et al.* (2013), but only in the context of the hermaphrodite and not in the male nervous system (Table S1). The red cross indicates the position of the deletion in each construct. Within the matrix, the gray intensity of each box indicates relative GFP intensity; white boxes indicate no expression. (*) CP1-6 expression was not determined. (B) Representative images of several constructs in the adult male tail. In the images for *eat-4*$^{prom2}$ and *eat-4*$^{prom2\Delta7}$, the *eat-4* fosmid (*otIs518*) is also shown in red as a landmark. Usually two or three lines were scored for each reporter and > 10 animals/line. Bar, 50 µm.

the intergenic sequence upstream of the *eat-4* locus (Figure 6A). One element, the 377-bp *eat-4prom13* element, drives reporter gene expression exclusively in the male-specific PVV neuron (Figure 6B). This element will be a useful driver to visualize and manipulate PVV neuron anatomy and function. Some of the elements of the *eat-4* locus appear to act redundantly, *i.e.*, separate regions drive expression in the same male-specific neuron classes. For example, two nonoverlapping elements are expressed in PVV (*eat-4prom1* and *eat-4prom13*) (Figure 6).

We also undertook a more fine-grained analysis of the previously identified regulatory elements that drive expression in multiple glutamatergic neuron classes with the goal of identifying putative transcription factor-binding sites. Given the preponderance of homeobox genes in controlling neurotransmitter identity (Jin *et al.* 1994; Serrano-Saiz *et al.* 2013; Zhang *et al.* 2014; Pereira *et al.* 2015; Gendrel *et al.* 2016), we mutated a number of predicted homeodomain-binding sites (TAAT) as well as a predicted and highly conserved POU homeodomain site in various *cis*-regulatory elements of the *eat-4* locus. We found that several of these deletions lead to losses of expression of the respective *eat-4* reporter in very specific neuron classes. For example, deletion of distinct, predicted homeodomain-binding sites eliminates expression in individual neuron classes of the pharynx within the *eat-4prom7* element (Figure 6A). Similarly, deletion of distinct homeodomain-binding sites eliminates expression in distinct ray sensory neurons and deletion of a conserved POU homeodomain-binding site specifically eliminates expression in the male-specific PVV neuron (*eat-4prom2Δ7*; Figure 6). Taken together, this *cis*-regulatory analysis confirms and extends our previous conclusion that the *eat-4* locus, and hence glutamatergic neurotransmitter identity, is controlled in a modular manner through distinct *cis*-regulatory elements that likely respond to neuron type-specific homeodomain transcription factors (Serrano-Saiz *et al.* 2013).

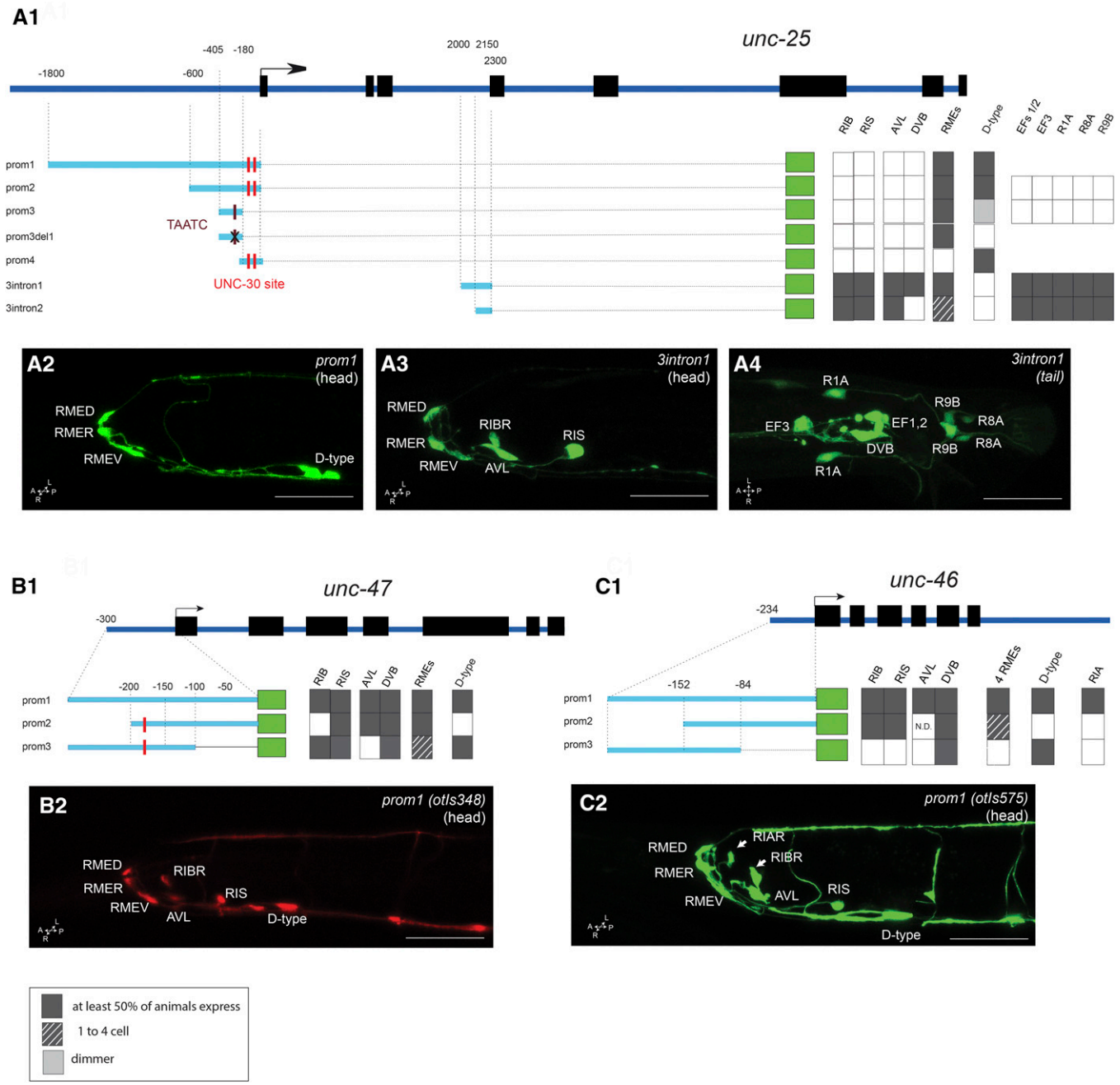



**Figure 7** Analysis of *cis*-regulatory elements of GABA pathway genes. Analysis of the *unc-25*/*GAD* (A1), *unc-47*/*VGAT* (B1), and *unc-46*/*LAMPG* (C1) loci. (A1, B1, and C1) Schematic of the *unc-47*, *unc-25*, and *unc-46* GABA pathway genes. The constructs are referred to their respective loci to scale. Dark gray boxes indicate that in at least two lines, > 50% of the worms show *gfp* expression in the cell. Dashed lined color indicates that, in at least two lines, > 50% of the worms had *gfp* expression in at least one RME (if there are no dashed lines, *gfp* expression is always present in the four RMEs). The black cross indicates the position of the deletion in *unc-25prom3del1* (TAATC). The red line indicates the position of the UNC-30-binding site sequence (TAATCC). Note that there is no TAATCC sequence in the *unc-46* promoter. (A2, A3, A4, B2, and C2) Representative confocal images of adult animals for *unc-25prom1* head (A2), *unc-25_3intron1* [head (A3) and tail (A4)], *unc-47prom1* head (B2), and *unc-46prom1* head (C2). Usually two or three lines were scored for each reporter and > 10 animals/line. Bar, 50 μm. GABA, γ-aminobutyric acid.

### *GABAergic pathway*

The *cis*-regulatory region of the *unc-25/GAD* locus that directs expression in all GABAergic neuron classes, except the RIB neuron class whose GABAergic identity was discovered recently (Gendrel *et al.* 2016), had been previously localized to < 1 kb upstream of the first exon of *unc-25* (Eastman *et al.* 1999; Jin *et al.* 1999). We sought to locate the *cis*-regulatory elements for the previously unrecognized RIB neurons, as well as for expression in the male-specific nervous system (Figure 7). We found that 600-bp upstream of the *unc-25*

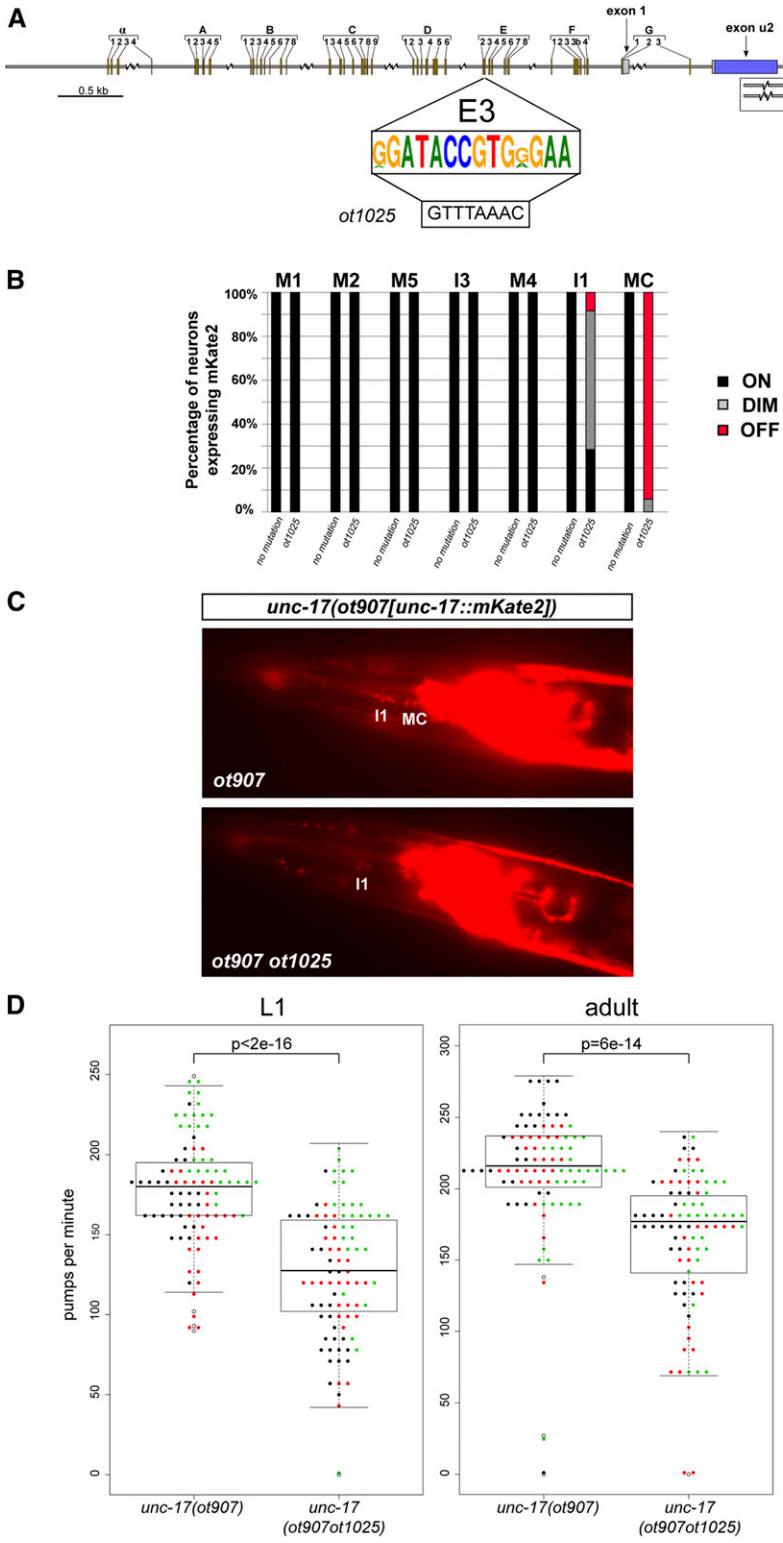



**Figure 8** *Cis*-regulatory alleles of *unc-17*, generated by clustered regularly interspaced short palindromic repeats/Cas9 genome engineering. (A) Box E3 is the consensus sequence of multiple *Caenorhabditis* species, as shown in Figure 3. The E3 box was replaced with a PmeI site (*ot1025* allele) in the background of the *ot907* allele, which designates the *mKate2* tag on the *unc-17* locus. (B) Scoring of fluorescent reporter expression of *unc-17(ot907)* and *unc-17-(ot907ot1025)* animals in cholinergic pharyngeal neurons. The E3 box deletion affects *unc-17* expression in the bilaterally symmetric MC neuron class and, mildly, the I1 neuron class. The percentages of neurons that show the indicated phenotypes are indicated in the bar chart. Representative images are shown in panel C Animals were scored at the L4 or young adult stage, $n > 50$. (D) Pumping rates in worms of different genotypes were compared at the L1 and adult stages. Three different cohorts of worms were tested on different days (each dot color represents a worm from a different assay day). The ANOVA test was used to make a model to fit a linear mixed-effects model to the obtained data. Then, pairwise comparisons of genotypes were done with Tukey's correction. Statistical significance indicated for L1 comparison $P <$ 2e–16, and for adult comparison $P$ = 6e–14.

locus drove reporter gene expression exclusively in the RME motor neurons and the D-type motor neurons (*unc-25prom2*; Figure 7), consistent with this element containing a previously characterized binding site for the *unc-30* transcription factor, a terminal selector that controls *unc-25* expression (Eastman *et al.* 1999). However, this element did not show expression in other GABAergic neuron classes in the sex-shared or sex-specific nervous system. Rather, we found those elements to

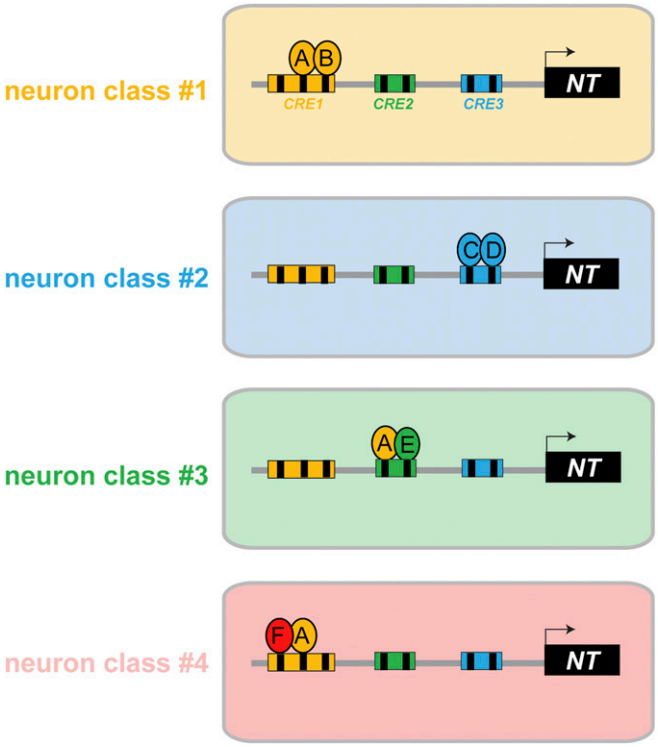



**Figure 9** Illustrations of the organization of *cis*-regulatory elements for neuron type-specific gene expression. A neurotransmitter pathway gene (NT) is controlled by different sets of transcription factors (A–F) in four different neuron classes. Reporter gene analysis ("promoter bashing") has revealed distinct *cis*-regulatory elements in the NT locus that drive expression in four different neuron classes. The indicated transcription factors act through specific binding sites (black boxes) within a given *cis*-regulatory element. The following scenarios are illustrated: comparing neuron class 1 and class 2 illustrates the simplest scenario of modularity where different combinations of neuron type-specific transcription factors (A + B or C + D) control NT expression in different neuron classes. Comparing neuron class 1 and 3 illustrates that the same transcription factor A can be used in different neuron types, but it operates through distinct, separable *cis*-regulatory elements that each contain a cognate binding site for this transcription factor. One example for this are the two distinct elements from the *unc-17/VAChT* locus that drive expression in distinct sets of UNC-86-dependent neurons (RIH *vs.* IL2/URA/URB), with each of these elements containing validated UNC-86-binding sites (see *Results*). Comparing neuron class 1 and 4 illustrates a *cis*-regulatory element that drives expression in multiple distinct neuron classes, and for both neuron classes the same transcription factor-binding site and the same cognate transcription factor are required (*e.g.*, *unc-17* element driving UNC-86-dependent expression in IL2, URA, and URB). In such a case, UNC-86 may interact with different transcription factors in neuron 1 *vs.* neuron 4, but it will not be possible to generate reporters that separate expression in neuron 1 and neuron 4. Hence, the modularity of *cis*-regulatory control is not manifested by distinct separable elements, but by distinct transcription factor-binding sites that are used in a modular and combinatorial manner.

reside within an intron of the *unc-25* locus. Specifically, we found that 300 bp (*3intron1*) revealed expression in RIS, AVL, and DVB, as well as the newly identified GABAergic RIB neuron and the GABAergic neuron classes of the male-specific nervous system (Figure 7A, 3 and 4). Similar to the upstream 600-bp element (*prom3*), the intronic 300-bp element also drives expression in the RME neurons. Further deletion of this 300-bp element (*3intron2*) eliminated reporter gene expression in the DVB motor neuron, but retained expression in the RME neuron class (Figure 7A1).

A previous analysis of the *unc-47/VGAT* locus revealed that *cis*-regulatory elements required for expression in GABAergic neurons also reside in a few hundred base pairs upstream of the start of the gene (Eastman *et al.* 1999). We corroborated these findings with a smaller 300-bp element (*prom1*) that also reveals expression in the RIB neurons, which were not identified in the original *unc-47* studies as GABAergic (McIntire *et al.* 1993; Eastman *et al.* 1999) (Figure 7B). Further dissection of these 300 bp separated expression in D-type motor neurons from the remainder of the GABAergic neuron classes (Figure 7B1).

The *cis*-regulatory regions for *unc-46* expression in GABA neuron classes were previously found to reside somewhere upstream of the *unc-46* start site (Schuske *et al.* 2007). We narrowed down this region to a 246-bp element (*unc-46prom1*), which recapitulates the expression in the entire GABAergic nervous system, including the RIB interneurons (Figure 7C), as well as in the RIA non-GABAergic neuron. As with *unc-47*, two overlapping subfragments again separate expression in D-type motor neurons from the remainder of the GABAergic neuron classes.

In conclusion, in all GABAergic pathway genes, the *unc-30*-dependent regulatory element that drives expression in VNC GABAergic (D-type) motor neurons can generally be separated easily from elements driving expression in other GABAergic neuron classes, but expression in other neurons is harder to separate from one another. This is not surprising because the RME, AVL, RIS, and DVB neuron classes appear to share several common *trans*-acting factors for their identity specification, namely the orphan nuclear hormone receptor *nhr-67* and the LIM homeobox gene *lim-6* (Hobert *et al.* 1999; Sarin *et al.* 2009; Gendrel *et al.* 2016).

### *Assessment of potentially redundant activity of distinct cis-regulatory modules in the native genome context*

In most neurotransmitter pathway genes, we noted some incidences of distinct, nonoverlapping *cis*-regulatory regions driving expression in the same neuron type. For example, in the *unc-17* locus, two distinct *cis*-regulatory elements (*unc-17prom4* and *unc-17prom8* in Figure 2) produce the exact same expression in five pharyngeal neuron classes (I1, M1, M2, M4, and MC). At first sight, a sufficiency of two distinct elements to direct expression in these neurons would argue for redundancy, *i.e.*, one would predict that mutations in either element in the context of the genomic locus should not result in expression defects because the other element should be able to compensate for the mutated one. To test whether such redundancy does indeed exist, we used CRISPR/Cas9 genome engineering to mutate a highly conserved motif within the *unc-17prom8* element (Figure 2A and Figure 8A). This motif, the E3 box, is completely conserved in 10 nematode species (Figure 3A). The E3 box mutation (Figure 8A) was engineered in a

strain in which the endogenous *unc-17* locus had been tagged with *mKate2* [the *ot907* allele (Pereira *et al.* 2019)]. We found that this mutation *unc-17(ot907ot1025)* resulted in an almost fully penetrant loss of *unc-17/VAChT* expression in the MC neuron class and a much milder effect on expression in I1 (Figure 8, B and C). This observation argues that, at least for expression in the MC neuron, the existence of the regulatory elements present in the *unc-17prom4* fragment cannot compensate for disruption of the *unc-17prom8* element.

The *unc-17/VAChT* and *cha-1/ChaT* genes share a common first exon, and are thought to be jointly regulated (Alfonso *et al.* 1994) (Figure 2A). Therefore, *unc-17(ot907ot1025)* animals are predicted to be unable to synthesize ACh (due to loss of the CHA-1 protein) and unable to package ACh into synaptic vesicles (due to loss of the UNC-17 protein) specifically in the MC neurons. The two MC neurons are the key pacemaker neurons of the pharynx (Avery and Horvitz 1989; Raizen *et al.* 1995; Trojanowski *et al.* 2014). Hence, we tested whether animals carrying the regulatory allele *unc-17(ot907-ot1025)* display pharyngeal pumping defects akin to those observed upon laser ablation or genetic silencing of the MC neurons. We indeed find that *unc-17(ot907ot1025)* animals, which appear otherwise behaviorally indistinguishable from wild-type animals, indeed pump less (Figure 8D). Reductions in pumping are also observed in animals treated with nicotinic ACh receptor (nAChR) antagonist or in animals that lack the AChR subunit EAT-2 that is expressed in MC target tissue (Raizen *et al.* 1995; McKay *et al.* 2004). Our finding of neuron type-specific removal of the *unc-17/VAChT* therefore provides further evidence for a cholinergic signal from MC neurons to target tissues being essential for pharyngeal pumping behavior.

## Discussion

Past work has defined the modular nature of neurotransmitter identity specification in *C. elegans* from two different perspectives. First, distinct terminal selector-type transcription factors were found to be required to specify the same neurotransmitter identity in different neuron types [reviewed in Hobert (2016)]. In several cases, it was explicitly shown that this specificity is achieved through the binding of the respective transcription factor to defined *cis*-regulatory elements within neurotransmitter pathway genes (Eastman *et al.* 1999; Wenick and Hobert 2004; Kratsios *et al.* 2011). Second, reporter constructs that capture distinct regions of the loci that encode for neurotransmitter pathway genes were shown to be selectively expressed in distinct subsets of ACh, glutamate, GABA, or monoaminergic neurons, respectively (Eastman *et al.* 1999; Wenick and Hobert 2004; Flames and Hobert 2009; Kratsios *et al.* 2011; Serrano-Saiz *et al.* 2013; Lloret-Fernandez *et al.* 2018). We have expanded here the analysis of *cis*-regulatory control regions of neurotransmitter pathway genes, thereby corroborating the largely modular control of neurotransmitter identity (Figure 1B). However, not every neuron type is controlled by an independent, dedicated module, since the same *cis*-regulatory element can be employed by the same transcription factor in different cell types, likely by acting in the context of a distinct combinatorial code of transcription factors. Hence, cell type-specific gene expression profiles are controlled by combinations of different types of regulatory constellations, as illustrated in Figure 9. We discuss these points in more detail below.

This and past analysis clearly shows that distinct, modular regulatory inputs control the expression of neurotransmitter pathway systems in distinct neuron types (Eastman *et al.* 1999; Jin *et al.* 1999; Wenick and Hobert 2004; Flames and Hobert 2009; Kratsios *et al.* 2011; Serrano-Saiz *et al.* 2013; Stefanakis *et al.* 2015; Lloret-Fernandez *et al.* 2018) (illustrated in Figure 9). Apart from using transgenic reporter gene assays, we provide here a striking example of this modularity by engineering a *cis*-regulatory allele of the *unc-17/VAChT* locus in which we mutated a small, phylogenetically conserved motif. We find that this manipulation not only leads to a selective loss of *unc-17/VAChT* expression in a single neuron class, but also causes the phenotype that one would expect from dysfunctionality of this neuron.

Nevertheless, past work, as well as the work presented here, also demonstrates that regulatory elements that drive expression in distinct neuron types can be difficult to disentangle, *i.e.*, even small *cis*-regulatory elements still drive expression in multiple neuron classes. Does this observation contrast with the modular control model and support the singular control model shown in Figure 1B? Theoretically, a more finely grained mutational analysis may still separate distinct elements for distinct cell types. However, there are already clear cases where we know that the same transcription factor acts in distinct cells to specify, for example, their cholinergic identity, seemingly in support of the singular model. However, in many if not all cases, this singular transcription factor may act in combination with different sets of cell type-specific partner proteins (illustrated in Figure 9, compare neurons 1 and 4). For example, the COE-type transcription factor *unc-3* controls *unc-17/VAChT* and *cho-1/ChT* expression in VNC motor neurons and their presynaptic command interneurons, which are all cholinergic and specified by *unc-3* (Kratsios *et al.* 2011; Pereira *et al.* 2015). Mutation of the *unc-3*-binding site leads to loss of expression in all these neuron classes. Similarly, a regulatory element from the *unc-17/VAChT* locus shows expression in the IL2, URA, and URB neuron classes, all of which require *unc-86* for their proper neurotransmitter identity acquisition (Zhang *et al.* 2014). Likewise, a regulatory element from the *eat-4* locus is expressed in the AIM and PHC neuron classes, both of which require *unc-86* for their glutamatergic identity specification. *Cis*-regulatory elements that control expression of GABA pathway genes in the RIS and AVL neuron classes (both controlled by the *nhr-67* and *lim-6* transcription factors) can also not be easily separated, while *cis*-regulatory elements for GABAergic VNC motor neurons (which are dependent on the *unc-30* transcription factor) can be separated from RIS and AVL. These examples

demonstrate that the modularity of *cis*-regulatory control does not need to manifest itself by the existence of distinct, completely separable *cis*-regulatory elements that drive expression in distinct neuron types. Rather, the same *cis*-regulatory element and its cognate transcription factor may be deployed in different cell types because transcription factors operate in distinct, cell type-specific combinations.

Enhancers that are employed to drive expression in different cell types have been termed "pleiotropic enhancers" (Sabarís *et al.* 2019). Such pleiotropy has been mostly inferred from genome-wide binding profiles of histone modification marks that indicate (but do not prove) enhancer activity at a relatively coarse level. Specifically, marks of enhancer activity can be found in the same location in distinct tissue types (McKay and Lieb 2013). The underlying mechanistic basis for enhancer pleiotropy is often not clear. The *cis*-regulatory analysis conducted in this and previous papers (Kratsios *et al.* 2011; Serrano-Saiz *et al.* 2013, 2017a) not only supports the concept of enhancer pleiotropy in an orthogonal and more granular manner, but also strongly hints at its mechanistic basis. Different neuron types utilize the same *cis*-regulatory element because this regulatory element contains binding sites for the same *trans*-acting factor (Figure 9; compare neurons 1 and 4).

While the *unc-3* case provides the clearest evidence for the same *cis*-regulatory motif to be employed in different neuron types, our analysis also reveals that a transcription factor required for acquisition of the same neurotransmitter identity in distinct neuron types does not necessarily always act through the same *cis*-regulatory element. For example, UNC-86 operates in more than a dozen different neuron types to specify neurotransmitter identity (Sze *et al.* 2002; Serrano-Saiz *et al.* 2013; Zhang *et al.* 2014; Pereira *et al.* 2015). It appears that UNC-86 functions through separable *cis*-regulatory elements in the *unc-17* locus to enable cholinergic gene expression in different cell types. These distinctive types of engagement of UNC-86 with its target promoters are a likely reflection of distinct molecular partnerships that UNC-86 may engage in (Figure 9, compare neurons 1 and 3).

In several neurotransmitter pathway genes, we noted the existence of separable elements that drive expression in the same neuron types. For example, RME motor neuron expression is controlled by separable *cis*-regulatory elements in the *unc-25/GAD* locus. This is akin to the "shadow enhancers" that have been observed to drive the expression of developmental control genes in *Drosophila* (Hong *et al.* 2008; Lagha *et al.* 2012). One immediate question that arises from such a constellation is whether this is an indication of true enhancer redundancies. Complete redundancy would predict that deletion of one of these elements would result in no expression defect because of the existence of the other element. Alternatively, the sufficiency of isolated elements to drive expression could be a result of the multicopy nature of concatamerized *C. elegans* transgenes (Mello *et al.* 1991). In the natural genomic context, several separable *cis*-regulatory elements may need to cooperate to turn on expression in a specific cell type. We tested this notion here explicitly through mutating a small conserved motif that is located in one of two separate *cis*-regulatory elements of the *unc-17* locus, which each drive expression in the same set of pharyngeal neurons. We find completely penetrant defects in *unc-17* expression in a single neuron class, thereby indicating that, in their endogenous context, a *cis*-regulatory element may be required even if another element produces, in the context of a multicopy array, the same pattern of expression. This finding is not without precedent. We had previously defined two separate *cis*-regulatory elements in the *cog-1* homeobox gene locus that are alone sufficient to drive expression in the ASE neuron (O'Meara *et al.* 2009). Nevertheless, we isolated a *cis*-regulatory allele of *cog-1* from a mutant screen in which only one of those two elements is mutated. These examples illustrate an important limitation for the interpretation of reporter gene analysis: the expression of isolated elements may demonstrate that an element is sufficient to produce an expression pattern, but they do not prove that such an element is required in its normal genomic context.

Pioneering work by Eric Davidson and colleagues in the sea urchin embryo revealed that "nuts and bolts" differentiation genes, *i.e.*, genes that do not encode regulatory factors, but rather "function" proteins that endow a cell with specific anatomical or functional properties, are largely controlled by positive regulatory inputs, *i.e.*, by transcriptional activators (Davidson 2006). Our *cis*-regulatory analysis of neurotransmitter pathway genes provides further support for this notion. If the expression patterns of neurotransmitter pathway genes were frequently shaped by repressive mechanisms, our successive deletion of regulatory elements should have revealed ectopic expression of reporter genes. While we observed such derepression in some rare cases, repression does not appear to be a major mechanism in shaping the expression of neurotransmitter pathway genes. The *cis*-regulatory analysis of other neuronal differentiation genes, undertaken by our laboratory over the years (Wenick and Hobert 2004; Etchberger *et al.* 2007, 2009; Kratsios *et al.* 2011; Doitsidou *et al.* 2013; Zhang *et al.* 2014; Serrano-Saiz *et al.* 2018), also provides only limited evidence in favor of repression being an important mechanism to shape neuron type-specific gene expression profiles. This is not to say that transcriptional repression plays no role in shaping the terminal differentiation program. Several studies have revealed that transcriptional repression is used in a number of distinct cellular contexts (sensory neurons and motor neurons) to establish subtle, but important molecular differences between members of the same neuron class [*e.g.*, differences between left *vs.* right ASE neurons (Etchberger *et al.* 2009) or differences between different VNC motor neuron classes (Kerk *et al.* 2017)].

Lastly, the dissection of *cis*-regulatory elements of genes that define specific phenotypic traits of distinct neuron types (*i.e.*, neurotransmitter identities) highlights the underlying mechanistic basis of phenotypic convergence. In evolutionary theory, phenotypic convergence refers to independent evolution of

similar traits in different organisms (Rosenblum *et al.* 2014). This terminology has recently been applied to the acquisition of the phenotypic trait of neurotransmitter identity (Konstantinides *et al.* 2018). In both *C. elegans* and *Drosophila*, distinct transcription factors control the same phenotypic trait in distinct neuron types (Serrano-Saiz *et al.* 2013; Konstantinides *et al.* 2018; Kratsios and Hobert 2018). *Cis*-regulatory analyses like the ones described here demonstrate that it is the *cis*-regulatory control regions of neurotransmitter pathway genes that integrate distinct regulatory inputs to turn on a common neurotransmitter trait in distinct neuron types.

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